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# Oncolytic Therapy with Vaccinia Virus GLV-1h68

## - Comparative Microarray Analysis of Infected Xenografts and Human Tumor Cell Lines-

**Dissertation**

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Würzburg, Januar 2010

Andrea Worschech

*Die Neugier steht immer am Anfang eines Problems, das gelöst werden will.*

Galileo Galilei

# 1 Summary

It is commonly accepted that chronic infections and inflammations may lead to mutagenesis of normal tissue and eventually facilitate development of cancerous lesions. A well-described example is cervical cancer which often is associated with an infection with human papilloma virus 16 or 18. On the contrary, newly gained insight into tumor biology has shown that the host's immune system can be a helpful tool for tumor therapy when triggered and stimulated accordingly. In this case, the chronic fostering inflammation in the tumor microenvironment is changed into an acute one and tissue rejection can occur. One such stimulus are oncolytic viruses that can be utilized to target manifested cancers and promote tumor regression due to their selective ability to infect and kill human tumor cells.

Aim of this thesis was to study the contribution of the host`s immune system during tumor regression. A wild-type rejection model was studied in which tumor regression is mediated through an adaptive, T cell host response (Research article 1). Additionally, the relationship between VACV infection and cancer rejection was assessed by applying organism-specific microarray platforms to infected and non-infected xenografts. It could be shown that tumor rejection in this nude mouse model was orchestrated solely by the host`s innate immune system without help of the adaptive immunity. In a third study the inflammatory baseline status of 75 human cancer cell lines was tested *in vitro* which was correlated with the susceptibility to VACV and Adenovirus 5 (Ad5) replication of the respective cell line (Manuscript for Research article 3).

Research article 1

HER2/neu (also known as ErbB-2) stands for "Human Epidermal growth factor Receptor 2" and is a proto-oncogene whose overexpression is associated with worse prognosis and increased relapse rate in breast cancer patients. Wild-type FVB mice are capable of rejecting neu-overexpressing mammary carcinomas (MMC) within three weeks because of specific recognition of rat neu protein by their T cells as opposed to their transgenic counterparts, FVBN202, which tolerate rat neu protein and fail to reject MMC. After subcutaneous MMC inoculation all mice rejected MMC

within three weeks after the challenge. However, a fraction of these animals (eight out of 15 mice) developed recurrent tumors at the site of inoculation and these relapsed tumors had lost neu expression under immune pressure. Total RNA from both FVB and FVBN202 carrier mice as well as total RNA from spontaneous tumor in FVBN202 mice was hybridized to 36k whole genome mouse arrays and statistical analysis was performed. The top categories of genes that were up-regulated in primary rejected MMC tumors were cytokine-cytokine interaction, mitogen-activated protein kinase signaling, cell adhesion–related transcripts and axon guidance, T-cell receptor, STAT and TLR signaling pathways. NK cell–mediated cytotoxicity and calcium signaling pathways were also enriched in up-regulated genes. In contrast, very little evidence of immune activation could be observed in either category of nonregressing tumors, suggesting that lack of immune rejection is due to absent or severely hampered immune responses in the tumor microenvironment independent of the mechanisms leading to this resistance. From this analysis it became clear that T-cell infiltration into tumors was associated with activation of various pathways leading to the expression of IFN-α, IFN-γ, and several ISGs, including IRF4, IRF6, and STAT2. In addition, several cytotoxic molecules were overexpressed, including calgranulin A, calgranulin B, and granzyme B, all of them representing classic markers of effector T-cell activation. Although the transcriptional patterns differentiating regressing from nonregressing tumors were striking and in many ways representative of previous observations in humans differences among MMC tumors nonregressing in FVBN202 mice and those relapsing after regression in FVB mice were subtle. The paradoxical relationship between adaptive immune responses against cancer antigens and rejection or persistence of antigen bearing cancers was studied. Gene profiling confirmed that immune rejection is primarily mediated through activation of IFN stimulated genes and T cell effector mechanisms.

Research article 2

Some xenografts continued to grow after infection with the oncolytic VACV GLV-1h68 and did not respond to the therapy while growth of others slowed down and the tumors got rejected. Of particular interest was a pair of cell lines: While HT-29 tumors did not respond to the oncolytic therapy with VACV GLV-1h68 and continued to grow (and will be called non-responder here and therefore), GI-101A

xenografts growth stagnated after one dose of GLV-1h68 administration and eventually got rejected (i.e. responder). 21 days post GLV-1h68 administration viral titers were lower in non-responding xenografts but the difference was less pronounced after 42 days, suggesting that the lack of responsiveness to oncolytic therapy may be associated with delayed but not completely absent VACV replication. VACV gene expression was assessed by a custom-made VACV array platform to compare the expression of VACV transcripts. This microarray platform consists of customized probes for GLV-1h68-encoded viral genes and additional house keeping and intrinsic control genes. A high-stringency Student *t* test comparing the number of VACV genes differentially expressed at day 21 or 42 from infected animals with those from uninfected ones identified significant differences only in GI-101A xenografts at day 21 and day 42. As to be expected, an almost complete overlap of VACV probes or genes expressed at day 21 and 42 was observed in the GI-101A xenografts. Notably, a reverse behavior was observed in the expression pattern of human house keeping genes represented in the VACV array platform. The expression of these genes was profoundly down-regulated in permissive cell lines suggesting a shut off of cellular metabolism in infected cells that correlated inversely with viral transcription.

To better characterize the transcriptional program of VACV-infected cancer cell lines, we compared responding (GI-101A) and non-responding (HT-29) xenografts using a 36k whole genome, human oligo array platform at day 21 and 42. In summary, analysis of human transcripts demonstrated that differences among xenografts from infected and non-infected mice are non-existent in non-responding tumors and limited to a small set of up-regulated genes in responding tumors several of them representing over-expression of host's genes cross-hybridizing to the human platform. In case of GI-101A we identified 1,073 genes differentially expressed between infected and non-infected xenografts and the large majority was down-regulated, suggesting that viral replication depresses cellular metabolism consistent with the down-regulation of house keeping genes observed in the VACV chip. Little evidence instead pointed towards apoptotic or necrotic induction by the oncolytic process at this early time point suggesting that at day 42 cells are starting to be strongly altered in their metabolism but are still alive. On the contrary, only nine genes were found to be differentially expressed by HT-29 xenografts in a similar analysis.

To define the host's involvement in the early phases of the oncolytic process HT-29 and GI-101A xenografts were analyzed using a custom-made, whole genome 36k mouse array platform. A statistical overview of gene expression modulation of GI-101A xenografts from GLV-1h68-infected grafts gave a completely opposite picture compared to the human arrays. In particular, most mouse genes were up-regulated in xenografts excised from infected animals suggesting that, while the metabolism of cancer cell was declining the host response was enhanced. At day 21, Ingenuity Pathway Analysis (IPA) revealed that the 2 canonical pathways predominantly affected in GI-101A xenografts from VACV-infected mice reflected chemokine and IFN signaling. At day 42, additional canonical pathways became affected including those associated with cellular stress. In general, immunologic differences between the early (day 21 from VACV injection) and the later (day 42) time points were quantitative rather than qualitative. Furthermore, Interleukin (IL)-18, the IL-18 binding protein and CCR2, CCR3 and CCR5 ligand chemokines played a prominent role early in the course of infection, while later IL-15 and CXCR3 and CXCR4 ligand chemokines became increasingly up-regulated. ISGs and other genes associated with the IFN signaling were among the most up-regulated at either time point studied; these included IFN-γ induced GTPase, whose expression was increased 48-fold at day 42 in GI-101A tumors from GLV-1h68-infected animals compared to controls. Additionally, macrophage presence/function also played an important role and was associated with over-expression of MHC class II genes supporting the presence of activated macrophages in infected GI-101A xenografts. Furthermore, this prominent and specific infiltration could be substantiated by immunohistochemical analyses that demonstrated a strong peri- and intra-tumoral infiltration of MHC class II-expressing host's cells surrounding virally-infected cancer cells. Finally, as expected no genes associated with B or T cell signaling or function in the grafts were significantly up-regulated at this phase of the immune-response against infected GI-101A xenografts, in accordance with the biology of the host's model system. This data suggest that at least in this model, adaptive immunity is not necessary for tissue-specific destruction (TSD).

Manuscript for Research article 3

75 human cancer cell lines were screened regarding their inflammatory baseline activation and a possible correlation with their susceptibility to Ad 5 and the oncolytic VACV GLV-1h68 infection. There is evolving evidence that two phenotypes of human cancers exist which can be characterized based on their different levels of interferon- and chemokine-related gene expression activation. This suggests that the existence of inflammatory and “quiescent” cancers is a common phenomenon among different cancers and that the intrinsic activation of ISGs is due to two independent taxonomies of cancer cells and not due to the individual host’s reaction as observed in xenograft studies.

A perfect correlation between the expression of ruc-gfp with and the IMV-surface protein, interferon resistance protein and DNA polymerase processivity factor was oberserved in infected tumor cells. Furthermore, FACS analysis of GFP protein expression post infection with Ad5 and VACV was performed and the frequency of $GFP^+$ cells and the geometric mean in the respective population was measured. All cell lines were ranked according to the infectivity indices post infection with either virus and grouped into cells with high, intermediate and low permissivity. Direct comparison of individual cell lines from both VACV and Ad5 infection showed that there is no correlation between the quantitative susceptibility of the two viruses that we examined. However, half (38 out of 75) of the cancers cells showed high, intermediate and low permissivity to both viruses.

Secondarily amplified RNA from 75 untreated cell lines was hybridized to whole genome human arrays in order to assess the endogenous transcript level based on the classification derived from the FACS analysis. The analysis revealed 335 differentially expressed genes between high and low VACV replicators with 168 down-regulated and 167 up-regulated transcripts in the low replicator cell lines. IPA displayed up-regulated pathways in low replicators such as DNA methylation and transcriptional repression signaling. Interestingly, one of the most involved networks is centered around the up-regulated NfκB complex. Among the down-regulated genes involved in the same network is IFN-α and –β, Il-12 complex and Il-12B, CCL1 and GDF-15. Interestingly, two probes of the Finkel-Reilly-Biskis murine sarcoma virus-associated ubiquitously expressed gene (Fau) were consistently up-regulated in

the low replicator cell lines which we have already observed in a previous smaller study with VACV infected cancer cells.

In parallel to the VACV analysis we also studied baseline differences in cancer cells which led to different levels of permissivity to Ad5. Interestingly, the most significant down-regulated pathway among cancer cell lines with low susceptibility to Ad5 is tight junction signaling which is related to the coxsackievirus and adenovirus receptor (CAR). We then analyzed molecule networks which were created based on the input of 722 differentially expressed genes between high and low Ad5 replicator cell lines and Mx1 was among up-regulated genes in low replicators and has previously been identified to be associated with impaired adenovirus replication in both, pancreatic cancer cell lines and primary tumors *in vivo*. Furthermore, the highest up-regulated gene in low replicators (15fold compared to high replicators) was immune-responsive gene 1 (IRG1).

Although xenografts by themselves lack the ability to signal danger and do not provide sufficient proinflammatory signals to induce acute inflammation, the presence of viral replication in the oncolytic xenograft model provides the "tissue-specific trigger" that activates the immune response and in concordance with the hypothesis, the ICR is activated when chronic inflammation is switched into an acute one. Thus, in conditions in which a switch from a chronic to an acute inflammatory process can be induced by other factors like the immune-stimulation induced by the presence of a virus in the target tissue, adaptive immune responses may not be necessary and immune-mediated rejection can occur without the assistance of T or B cells. However, in the regression study using neu expressing MMC in absence of a stimulus such as a virus and infected cancer cells thereafter, adaptive immunity is needed to provoke the switch into an acute inflammation and initiate tissue rejection.

Taken together, this work is supportive of the hypothesis that the mechanisms prompting TSD differ among immune pathologies but the effect phase converges and central molecules can be detected over and over every time TSD occurs. It could be shown that in presence of a trigger such as infection with VACV and functional danger signaling pathways of the infected tumor cells, innate immunity is sufficient to orchestrate rejection of manifested tumors.

# 2 Zusammenfassung

Die bisher verbreitete Vorstellung ist, dass chronische Entzündungen und Infektionen im Körper über einen längeren Zeitraum die Mutagenese der normalen Zellen fördern und möglicherweise zu kanzerogenen Malignomen führen. Ein weitgehend akzeptiertes Beispiel hierfür ist eine Infektion mit dem humanen Papillomavirus 16 oder 18, die in Einzelfällen zur Entstehung von Gebärmutterhalskrebs führt. Im Gegensatz hierfür zeigen neuere Befunde, dass das körpereigene Immunsystem durchaus auch positive, synergistische Efekte mit konventionellen Therapien haben kann. In diesem Fall wird angenommen, dass das immunologische Umfeld im Tumor stimuliert und die chronische Entzündung in eine akute verwandelt wird. Diese Stimulation kann durch onkolytische Viren erfolgen, da diese manifestierte Tumore befallen können, sich selektiv in diesen vermehren und dadurch Tod der malignen Zellen und Tumorregression vermitteln.

Ziel dieser Arbeit war, die Beteiligung des Wirts-eigenen Immunsystems bei der Tumoregression zu analysieren. Mittels eines Wildtyp-Regressionsmodells, wurde der Anteil des adaptiven Immunsystems studiert (Research-Artikel 1). Mit Hilfe von Organismus-spezifischen Mikroarrays und Genexpressionsanalysen konnte in einem Nacktmausmodell gezeigt werden, dass erfolgreiche, durch onkolytische VACV-vermittelte Tumortherapie auch ohne Beteiligung des adaptiven Immunsystems möglich ist (Research Artikel 2). In einer dritten Studie wurden 75 humane Tumorzelllinien auf ihren intrinsischen Entzündungsstatus hin getestet und bezüglich eines Zusammenhanges von diesem mit der Replikationsfähigkeit von VACV und Adenovirus 5 (Ad5) analysiert (Manuskript für den Research-Artikel 3).

Research-Artikel 1

HER2/neu, auch „human epidermal growth factor receptor 2“ (ErbB-2) genannt, ist ein Proto-Onkogen, dessen Expression mit schlechter Prognose und erhöhter Rückfallrate in Brustkrebspatienten assoziiert wird. In Wildtyp-FVB-Mäusen regredieren neu-überexprimierende „mammary carcinomas“ (MMC) innerhalb von

drei Wochen, da T-Zellen das Neu-Protein spezifisch erkennen; im Gegensatz dazu tolerieren transgene FVBN202-Mäuse das Neu-Protein und MMCs werden nicht eliminiert. Nach subkutaner MMC-Inokulation regredieren die Tumore in beiden Mausgruppen innerhalb von drei Wochen, allerdings war in acht von 15 Mäusen ein Rückfall durch Verlust des Tumorantigens zu beobachten. RNA von Tumoren von FVB- und FVBN202-Mäusen sowie von spontan regredierenden Tumoren in FVBN202-Mäusen wurde nach Amplifikation mit Maus-spezifischen Mikroarrays hybridisiert und die Resultate mit statistischen Tests ausgewertet. Hierbei zeigte sich, dass folgende zelluläre Prozesse und Komponenten die primäre MMC-Regression durch Hochregulation charakterisieren: Zytokin-Zytokin-Interaktion, Mitogen-aktivierter Proteinkinase-Signalweg, Zelladhäsion, Axon-Führung, T-Zellrezeptoren, STAT- und TLR-Signalwege, NK-Zell-vermittelte Zytotoxizität und Calcium-Signalweg. Allerdings wurden nur wenige immunologische Signalwege in nicht-regredierenden, tolerierten Tumoren gefunden. Dies deutet darauf hin, dass die Immunreaktion in diesen Tumoren beeinträchtigt und nicht in der Lage ist, zur Tumorrückbildung zu führen. Die T-Zell-Infiltration in sich zurückbildenden Tumoren war mit Aktivierung von Signalwegen assoziiert, die zur Expression von IFN-α, IFN-γ und „interferon stimulated genes“ (ISGs), wie IRF4, IRF6 und STAT2, führten. Weiterhin waren zytotoxische Moleküle, wie „calgranulin A and B und granzyme B“ hochreguliert, die alle klassische Marker einer T-Zell-Aktivierung sind. Obwohl diese transkriptionellen Unterschiede zwischen regredierenden und nicht-regredierenden Tumoren deutlich ausgeprägt waren, konnten kaum Unterschiede zwischen nicht-regredierenden MMCs in FVBN202-Mäusen und Rückfalltumoren in FVB-Mäusen feststellgestellt werden. Zusammenfassend lässt sich sagen, dass die auf den Tumorzellen exprimierten neu- von den T-Zellen erkannt wurden, die darauf in die Tumore einwanderten. Diese Infiltration in die regredierenden MMCs führte zur Expression von IFN-α und –γ und nachfolgend zur Hocvon ISGs. Expression von zytotoxischen Molekülen wie „calgranulin A and B“ und „granzyme B“, spricht dabei für einen CD8+-vermittelten T-Zelleffektormechanismus, der zur immun-vermittelten Regression der neu-exprimierenden MMCs führte.

Research-Artikel 2

Während einige Xenograft-Tumore nach Infektion mit dem onkokytischen VACV GLV-1h68 weiterwuchsen und nicht auf eine Therapie ansprachen, war bei anderen ein verlangsamtes Wachstum gefolgt von einem Wachstumsstop und anschließender kompletter Regression zu beobachten. Von besonderem Interesse war das kolorektales Karzinom HT-29 und das Brustkrebsadenokarzinom GI-101A, da GI-101A-Tumore, im Gegensatz zu HT-29, auf die Therapie mit GLV-1h68 ansprachen und regredierten. 21 Tage nach der GLV-1h68-Injektion waren die Virustiter in HT-29-Xenografts im Vergleich zu GI-101A-Tumoren reduziert. Diese Differenz war nach 42 Tagen nicht mehr so stark ausgeprägt, so dass anzunehmen ist, dass sich die verzögerte Virusreplikation nachteilig auf den Therapieerfolg auswirkt. Mit Hilfe von speziellen VACV-Microarrays konnten differentiell exprimierte VACV-Gene in infizierten und uninfizierten Kontrolltumoren an Tag 21 und 42 post Infektion miteinander verglichen werden. Diese Mikroarray-Plattform beinhaltet sowohl Probensets für alle von GLV-1h68 codierten viralen Gene als auch eine Gruppe von humanen „house keeping“-Genen und weiteren internen Kontrollen. Bei der statistischen Auswertung wurden signifikante Unterschiede lediglich beim Vergleich infizierter vs. nichtinfizierter GI-101A-Xenografts festgestellt. Eine weitgehend gleiche Gruppe von VACV-Genen war zu beiden getesteten Zeitpunkten exprimiert. Im Gegensatz dazu waren die „house keeping“-Gene in den permissiven GI-101A-Tumoren signifikant herunterreguliert. Dies deutet darauf hin, dass die Virusinfektion den Zellmetabolismus beeinträchtigt und die Zellvitalität mindert.

Weiterhin wurden GI-101A- und HT-29-Tumore mittels eines humanen 36k „whole genome microarray“ auf Infektions-induzierte Veränderungen hin untersucht. Es waren lediglich geringfügige Unterschiede zwischen infizierten und uninfizierten Tumoren festzustellen. Wir identifizierten 1073 Gene, die in infizierten GI-101A-Tumoren, verglichen mit uninfizierten, zum Großteil herunterreguliert waren und, im Einklang mit den VACV-Array-Daten, zeigt sich, dass der Zellmetabolismus durch die VACV-Infektion beeinträchtigt ist. Allerdings wiesen keine der Transkripte auf aktive Apoptose- oder Nekrose-Signalwege hin, so dass anzunehmen ist, dass am Tag 42 post Infektion die Tumorzellen noch vital sind. Im Gegensatz dazu waren nur 9 Gene im Fall von HT-29 durch die VACV-Infektion beeinflusst.

In einem weiteren Schritt wurden HT-29- und GI-101A-Tumore mittels Maus-spezifischen Mikroarrays untersucht, um den Anteil der Wirts-vermittelten Tumorinfiltration zu bestimmen. Durch Trankriptionsanalyse von regredierenden GI-101A-Tumoren konnte deutlich eine akute Entzündungsreaktion festgestellt werden, die durch das wirtseigene Immunsystem vermittelt wurde. So war an Tag 21 der Chemokine- und IFN- Signalweg aktiviert. Zusätzlich dazu waren nach 42 Tagen auch zelluläre Stress-Signalwege aktiviert, wie mit der Ingenuity-Pathway-Analyse Software (IPA) gezeigt werden konnte. Allgemein war zu beobachten, dass immunologische Differenzen zu beiden Zeitpunkten eher quantitativer als qualitativer Natur waren. Während zum früheren Zeitpunkt der Tumorregression noch Interleukin (IL)-18, „IL-18 binding protein“ sowie CCR2, CCR3 und CCR5 Chemokin-Liganden exprimiert wurden, waren im weiteren Verlauf v.a. IL-15 und CXCR3 und CXCR4 Chemokin-Liganden hochreguliert. ISGs und weitere Mitglieder des IFN-Signalweges waren zu beiden Zeitpunkten gleichermaßen aktiviert. So war z.B. die IFN-γ induzierte GTPase in GI-101A-Tumoren durch die Infektion an Tag 42 48fach induziert. Zusätzlich dazu waren Gene, die auf Makrophagen-Aktivierung und –Infiltration hindeuten wie z.B. „MHC class II“ in GI-101A-Tumoren hochreguliert. Diese peri- und intra-tumorale Infiltration konnte auch durch immunhistochemische Färbungen bestätigt werden. Allerdings wurden keine Transkripte mit Verbindung zu T- und B-Zellaktivierung gefunden, was mit dem biologischen Hintergrund der Nacktmäuse in Einklang steht. Daher ist zu folgern, dass zumindest in dem untersuchten Modell, die adaptive Immunantwort für „tissue-specific destruction“ (TSD) nicht erforderlich ist.

Manuskript für Research Artikel 3

Ziel dieser Studie war es, den endogenen inflammatorischen Status von 75 humanen Tumorzelllinien zu bestimmen und zu prüfen, ob dieser möglicherweise mit der Suszeptibilität für VACV und Adenovirus 5 assoziiert ist. Nach Literaturangaben ist es ist wahrscheinlich, dass die Existenz von „entzündeten“ und nicht aktivierten Tumoren ein allgemeines Phänomen verschiedener Tumorarten ist. Diese intrinsische immunologische Aktivierung ist charakterisiert durch erhöhte Interferon- und Chemokinexpression und ist vermutlich auf unterschiedliche Ätiologie der Tumorzellen zurückzuführen und nicht auf die Wirtsinteraktion.

Es konnte eine strikte Korrelation zwischen der gfp-Expression und dem „IMV-surface protein“, dem „Interferon-resistance protein“ und dem „DNA polymerase processivity factor“ in infizierten Tumorzellen nachgewiesen werden. FACS-Analyse von Ad5- und VACV-infizierten Zellen wurde ausgewertet, indem die Frequenz von GFP+-Zellen mit dem geometrischen Mittel der Intensität der GFP+-Zellen multipliziert und so der Infektivitätsindex bestimmt wurde. Alle 75 Zelllinien wurden nach dem Infektivitätsindex geordnet und jeweils für beide Viren in die Kategorien hoch-, mittel- und niedrig-permissiv eingeordnet. Während keine strikte Korrelation zwischen der VACV- und Ad5-Anfälligkeit der individuellen Zelllinien hergestellt werden konnte, ist aber von Bedeutung, dass insgesamt die Hälfte (38 von 75) aller getesteten Zelllinien hoch-, mittel- bzw. niedrig-permissiv für beide Viren waren.

Das endogene Transkriptions-Level von 75 uninfizierten Zelllinien wurde mittels humaner Mikroarrays getestet. Dabei wurde die Klassifizierung der FACS-Analyse herangezogen, um die einzelnen Zelllinien zu vergleichen. 335 differentiell exprimierte Gene unterschieden Zellen mit hoher und niedriger Permissvität für VACV-Infektion und konnten jeweils zur Hälfte als hoch- bzw. herunterreguliert beschrieben werden. Mittels IPA wurden DNA-Methylierung und transkriptionelle Repression als die beiden Signalwege identifiziert, die in den hoch-suszeptiblen Zelllinien aktiviert sind. Auf Molekülebene sind die Nachbarmoleküle des NfκB-Komplexes in nicht-permissiven Zelllinien signifikant hochreguliert, während IFN-α und –β, der IL-12-Komplex, IL-12B, CCL1 und GDF-15 herunterreguliert sind. Interessanterweise waren zwei Proben-Sets des „Finkel.Reilly-Biskis murine sarcoma virus-associated ubiquitously expressed genes“ (Fau) in den nicht permissiven Zelllinien herunterreguliert.

Simultane Analyse der Ad5-Permissivität und assoziierter Transkriptionsmuster zeigte, dass der „tight-junction“-Signalweg in Zelllinien, die weniger permissiv für die Ad5-Infektion waren, herunterreguliert ist. Interessanterweise ist der bekannte „coxsackie und adenovirus receptor“ (CAR) selbst Bestandteil dieser Zell-Zellkontakte. Von den 722 differentiell exprimierten Genen von Zelllinien, die hoch- bzw nicht-permissiv für die Ad5-Infektion waren, wurde auch Mx-1 als hochreguliert in den nicht-permissiven Zelllinien identifiziert. Zusätzlich dazu, war das „immune-responsive gene 1“ (IRG-1) 15fach in nicht-permissiven Zelllinien hochreguliert.

Obwohl Xenografts allein kein ausreichendes „Gefahrsignal“ geben und durch das Fehlen einer pro-inflammatorischen Stimulierung keine akute Entzündung verursachen können, ist die Infektion mit onkolytischem VACV ausreichend, um den Gewebe-spezifischen „Trigger“ darzustellen. In diesem Fall wird die Immunantwort aktiviert und nach der Hypothese des „Immunologic Constant of Rejection“ (ICR) geschieht dies, wenn eine chronische in eine akute Inflammation verändert wird. In dem beschriebenen onkolytischen Regressionsmodell ist die Präsenz des Virus ausreichend, um das Immunsystem zu aktivieren, d.h. die chronische Entzündung im Tumor in eine akute umzuwandeln. Dabei ist die adaptive Immunität mit T- und B-Zell-Aktivierung nicht notwendig für die Rückbildung des Tumors. In Abwesenheit eines solchen Stimulus, wie in der ersten Studie mit neu-exprimierenden MMCs, wird die Spezifität der adaptiven Immunantwort benötigt, um die akute Inflammation anzustoßen und die Tumorregression voranzutreiben.

Zusammengefasst unterstützt diese Arbeit die Hypothese, dass die Mechanismen, die zu „tissue specific destruction“ (TSD) führen, in verschiedenen immunologischen Erkrankungen zwar divergieren, der Effektor-Mechanismus aber stets der Gleiche ist. Es zeigte sich, dass in Anwesenheit eines „triggers“, wie z.B. der VACV-Infektion und intakten „danger signaling pathways“ der Tumorzellen, die angeborene Immunität allein ausreicht, um die Tumorrückbildung zu vermitteln.

# 3 Introduction

## 3.1 The complexity of cancer biology

Even with continued education, developing technologies and high standard research and clinical trials, cancer related deaths are still accountable for more than 20% of all fatalities in the United States over the past years (1). The hetererogeneity and huge variety of different malignancies and clinical courses translates into a tremendous amount of research data in individual settings and mixed outcome in clinical trials. One parameter though, links the biology of all cancers and their tumor microenvironment and even goes back to the 19$^{th}$ century. Virchow first noticed that there was indeed a common phenomenon of the relationship between cancerogenesis and inflammation and “lymphoreticular infiltrates” reflected the origin of cancers at sites of chronic inflammation (2). Over the past decade newly gained insight and knowledge about tumor growth, treatment and prognosis has been supportive of Virchow’s hypothesis and has shown that the immunologic microenvironment is a key player in promoting cancer growth but more importantly, can be a helpful tool for tumor therapy when triggered and stimulated accordingly.

One such stimulus is known to be oncolytic therapy in which oncolytic viruses are utilized to target manifested cancers and promote tumor regression. The selective ability of oncolytic viruses to infect and kill human tumor cells is a powerful tool which goes back to discoveries in the early twentieth century when cancer patients where noted to undergo tumor regression after systemic viral infections (3), for instance a leukemia patient who underwent complete remission after an acute infection with influenza virus (4).

Interestingly, not only cancer regression but also other immune-mediated tissue destruction models seem to employ similar immunological functions and eventually converge into one final effector pathway which we called “immunologic constant of rejection (ICR)”. Tissue-specific destruction is associated with the activation of type I proinflammatory modulators, in particular the combination of IFN-α and IFN-γ (Interferon), which results in the activation of Intereron-stimulated genes (ISGs) in turn. Additionally, infiltration of cytotoxic T-lymphocytes (CTLs) and natural killer cells

(NKs) also participate in the activation of cytotoxic mechanisms that lead to tissue destruction as described for autoimmunity diseases, spontaneous pathogen clearance, e.g. in chronic hepatic C patients, allograft rejection and tumor rejection (5).

Further studies revealed that not only clinical characteristic of cancers and responsiveness to treatment is highly divers but also the cancer cells themselves can be classified into two different phenotypical subgroups. There is evolving evidence that various cancers including melanoma, glioma, breast, head and neck, prostate and lung carcinomas display different levels of interferon- and chemokine-related gene expression activation (6-11).

This suggests that the existence of inflammatory and “quiescent” cancer phenotypes is a common phenomenon among different cancers and that the intrinsic activation of ISGs is due to two independent taxonomies of cancer cells and not due to the individual host’s reaction (12) as observed in xenograft studies.

These seemingly unrelated phenomena share the importance for the understanding of the biology of cancers which is a necessary configuration if attempting to treat cancers in human. The heterogeneity of tumors regarding their inflammation = anti-viral state, the pathways and factors that lead to cancer regression and the in-debt understanding of the principals of oncolytic therapy with Vaccinia Virus (VACV) for instance, are all equally important and our interest arose to extensively study the biology of different tumor cells, the tumor microenvironment and especially the involvement of the host’s innate immune system during oncolytic therapy with VACV.

## 3.2 Tissue rejection requires a switch from a chronic to an acute inflammation – Review 1

# The immunologic constant of rejection

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# The immunologic constant of rejection

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**The complexity underlying a pathologic process does not necessarily require a complex explanation. The biology determining allograft or cancer rejection, autoimmunity or tissue damage during pathogen infections is complex; however, common patterns are emerging that lead to a common final outcome. For instance, tissue destruction occurs with resolution of the pathogenic process (cancer, infection) or tissue damage and organ failure (autoimmunity, allograft rejection). Observations in humans based on transcriptional profiling converge into what we call an 'immunologic constant of rejection' that characterizes such occurrences. This constant includes the coordinate activation of interferon-stimulated genes (ISGs) and immune effector functions (IEFs). Understanding this final effector pathway may suggest novel strategies for the induction or inhibition of tissue-specific destruction with therapeutic intent in cancer and other immune pathologies.**

## The 'delayed allergy reaction'

At the dawn of cancer, neoplastic cells need to merely acquire the capacity for unlimited division to retain a growth advantage over their normal progenitors. Gradually, as the population of cancer cells expands, they can rely less and less on the refined structure of normal tissues for the maintenance of homeostatic function. Thus, in a cancer cell's progress to independence, a new nonphysiologic 'organ' is formed. With the expedients of a rapidly evolving process, cancer cells initiate angiogenesis and the production of growth factors. Furthermore, they produce chemokines that recruit normal cells whose physiologic role is to respond to injury [1]. As suggested by Virchow, discussed by Balkwill and Mantovani [2], and others [3], these cells, with their tissue remodeling properties, nurse cancer as a healing wound and so sustain a chronic inflammatory process.

The same inflammation that fosters cancer growth and other pathogenic processes such as chronic infection and autoimmunity sets this disease apart from the rest of the host's tissues that are not inflamed. In other circumstances, well-controlled allograft rejection results in a similar tissue-specific chronic inflammatory process. Thus, as suggested by Salk [4] many years ago, cancer, nonresolving chronic infections, autoimmunity and chronic allograft rejection all represent facets of a similar process (i.e. so-called 'delayed allergy'). This reaction challenges the immune system with the continual predicament of tolerance versus destruction. Identifying and targeting the differences between chronic inflammation and inflammation that causes tissue destruction in acute allograft rejection or flares of autoimmunity might provide a selective therapeutic treatment for cancer and chronic infections. An example of how this difference may serve therapeutic purposes is the relative selectivity of anti-CTLA-4 (cytotoxic T lymphocyte antigen 4) antibody therapy of cancer patients. These antibodies target activated T cells populating chronically inflamed tissues with a predominant effect on T regulatory cells (Tregs), therefore boosting anti-cancer immune responses. The same effects are induced as collateral tissue damage but only in chronically inflamed tissues such as the gut, whereas normal tissues are not affected because no such infiltrate is constitutively present. This can explain why the side effects of anti-CTLA-4 therapy are strictly tissue specific and predominantly directed against the gut [5].

## Different immune pathologies are triggered by different mechanisms

Autoimmunity consists of a reaction of the host against self. This clearly demonstrates that the cognate arm of the immune response does not require non-self discrimination to initiate tissue-specific destruction [6,7]. Human cancers are similar to mild autoimmunity; most tumor-associated antigens (TAAs) are nonmutated proteins also expressed by normal cells [8]. However, cancers can also broaden their antigenicity by expressing self proteins not expressed by most normal cells [9], overexpressing proteins expressed by normal cells [10] and expressing mutated proteins that could be recognized as non-self (quasi-self) [11].

Chronic infections also result in the expression of non-self products; however, contrary to infections resulting in acute inflammation and clearance, they last indeterminately. This is well exemplified by hepatitis C virus (HCV) infection [12]; a disease caused by the same infectious agent can take two radically distinct courses in different individuals, with acute hepatitis resulting in clearance of infection and chronic hepatitis remaining unresolved. It is likely that factors other than the immunogenic potential of the pathogen modulate the quality of the immune response. Thus, self–non-self discrimination does not necessarily lead to tissue destruction and clear the pathogenic insult.

Allografting triggers one of the strongest immune responses, necessitating continual immunosuppression to maintain graft survival. Although it is thought that

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the potency of this immune response is dictated by broad antigenic disparities between graft and host, we suggest that it is the pattern in which allo-antigens are expressed that primarily distinguishes allograft reactions from other immune pathologies. However, in some cases, such as in antitumor immune responses, the immunogenicity of the antigens is also likely to be an important variable because TAAs expressed on the cancer cell membrane are not as immunogenic as the histocompatiblity antigens responsible for allograft rejection. In most cases, the previously described immune pathologies do not display conformational antigenic epitopes on the target cell surface that can be directly recognized by antibodies or B-cell receptors (BCRs), but rather express linear epitopes that can be recognized only by T cells in association with self-major histocompatibility complexes (MHCs). Allografts express conformational epitopes as non-self antigens (for instance the MHC molecules themselves) that are readily accessible to antibodies and BCRs on B cells. Thus, allografts naturally induce activation of both B and T cells in the target organ, resulting in chemotaxis and *in situ* activation of immune mechanisms (see later). Sarwal *et al*. [13] observed that acute allograft rejection, a T cell–mediated phenomenon, requires the infiltration of CD20-expressing B cells. This association has never been observed in other varieties of immune-mediated tissue destruction. What these graft infiltrated and activated B cells are doing is unclear, but it is possible that they are powerful stimulators of T-cell responses through the production of CXCL chemokines that attract activated cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells [14]. This powerful combined immune reaction may set allograft rejection apart from other immune pathologies with the exception of some autoimmune diseases. In cancer, most humoral responses are directed against intracellular TAAs that cannot be recognized by antibodies [15]. Humoral and cellular immune responses likely play different roles in chronic infections; neutralizing antibodies are directed against free pathogens, but in most cases, they cannot alter the course of a disease predominantly caused by intracellular pathogens [12].

## Four postulates support an immunologic constant of rejection

Although the prompt may vary, we propose that distinct immune processes ultimately converge into what we term an 'immunologic constant of rejection' (Figure 1). This hypothesis is based on four axioms (Box 1).

### *Tissue-specific destruction does not necessarily only occur after non-self recognition but can also occur against self- or quasi-self*

Autoimmunity and the recognition of human cancers occurs in the context of self- or quasi-self recognition.

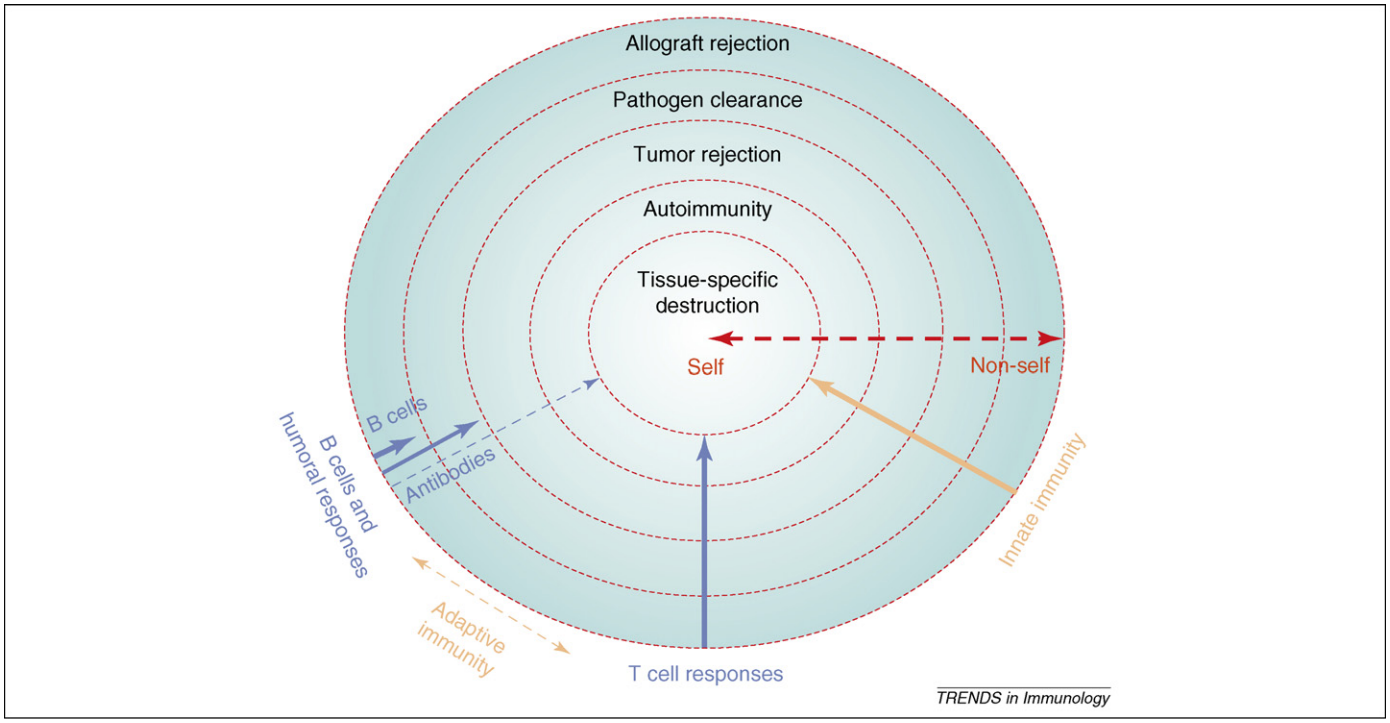


**Figure 1**. A mechanism common to most immune pathologies is at the basis of tissue-specific destruction. Distinct immune phenomena leading to tissue destruction are probably prompted by different mechanisms across the boundaries of self–non-self discrimination as suggested by the concentric circles. Allografts, by expressing surface antigens could trigger activation of B cells directly through specific B-cell receptor signaling and in the absence of T-cell help, therefore stimulating the promotion of tertiary lymph nodes. Chronic infections present non-self products to T cells after antigen processing and binding to major histocompatiblity molecules. The role of antibodies is more likely relegated to neutralization of extracellular pathogen. Inflammation in cancer (tumor rejection) and autoimmunity are triggered independently of self–non-self discrimination but they can induce humoral and T-cells responses that occasionally lead to tissue-specific destruction. In all cases, we postulate that innate immune effector mechanisms play a role not only at initiating but finishing the immune response. Thus, although non-self discrimination may decrease the requirements for endogenous or exogenous inflammatory stimuli, when a threshold is reached that turns a chronic inflammatory process into an acute one, the final pathways to tissue destruction converge into a constant mechanism. The thickness of the arrows indicates that B cells have an important role in allograft rejection, less so in pathogen clearance and little involvement in self-tissue pathology, whereas T cells and innate immunity have roles in all stages of immunity.

**Box 1. Postulates on which the 'immunologic constant or rejection' hypothesis is formulated**

(i) Tissue-specific destruction does not necessarily occur because of non–self-recognition but also occurs against self or quasi-self.
(ii) The requirements for the induction of a cognate immune response differ from those necessary for the activation of an effector one.
(iii) Although the prompts leading to tissue-specific destruction vary in distinct pathologic states, the effector immune response converges into a single mechanism that includes the activation of adaptive and innate cytotoxic mechanisms.
(iv) Adaptive immunity participates as a tissue-specific trigger, but it is not always sufficient or necessary for tissue destruction.

Although for autoimmunity the postulate is obvious, cancer may require further elucidation. Immune responses directed against TAAs include nonmutated self proteins [8,9]. Moreover, effective immune responses against melanoma are associated with vitiligo, which results from destruction of normal melanocytes, suggesting a link between autoimmunity and cancer rejection [16,17]. Finally, active immunization against self TAAs [18] and adoptive transfer of CTLs recognizing self TAAs can induce cancer rejection [19]. Thus, cancer sits astride self–non-self discrimination, and its rejection could be considered as a variety of autoimmunity.

### *The requirements for the induction of a cognate immune response differ from those associated with the development of an effector one*

Tumor infiltrating lymphocytes that recognize melanoma cells can be readily expanded from melanoma metastases [20]; however, they co-exist *in vivo* with them. Thus, just because a T cell recognizes a target antigen (cognate response), it does not necessarily mean that the target is eliminated. Cellular and humoral immune responses against TAAs are primed systemically in cancer-bearing individuals, yet tumors continue to grow [21]. Similarly, immunization with TAA reproducibly increases the frequency of circulating TAA-specific CTLs, yet they cannot induce cancer regression [22]. TAA-specific T cells have a quiescent noncytotoxic phenotype *ex vivo* requiring antigen recall and co-stimulation to recover effector functions [23]. This could be partly caused by a reduced responsiveness of TAA-specific T cells [24] or a reduced recognition efficiency for the peptide target that leads to inability to lyse cancer cells [25]. Finally, vaccine-induced CTLs reach the tumor microenvironment, recognize tumor cells and produce interferon γ (IFN-γ), yet cannot limit tumor growth [26]. The same phenomena can be observed in chronic viral infections [12]. A variety of active regulatory mechanisms, and phenotypic changes of cancer cells or viral genomes have been proposed to explain this paradox [27–30]. In particular, in cancer, a gradual adjustment of tumor cell phenotypes in response to pressure from the immune system (immune editing) has been clearly demonstrated in animal models [31]. However, a simpler interpretation is that the requirements for the induction of a cognate immune response differ from those necessary to elicit an effector one.

### *Although the mechanisms prompting tissue-specific destruction differ among immune pathologies, the effector phase converges into a common activation of adaptive and innate cytotoxic mechanisms*

The same phenomenon may result from different causes, in the same way that a fever ensues for different reasons. Thus, independent of the prompting mechanism, tissue-specific destruction, whether with beneficial effects (tumor rejection, clearance of pathogen) or detrimental effects (autoimmunity, allograft rejection), occurs through the enactment of common effector pathways that include the activation of interferon stimulated genes (ISGs) and immune effector functions (IEFs) such as cell-mediated cytotoxicity [32].

### *Adaptive immunity triggers a tissue-specific reaction, but it is not always sufficient or necessary for tissue destruction*

Adaptive immunity can play a partial role in the effector phase of tissue destruction. Several cancer models demonstrate – at least in part – the redundancy of adaptive immunity. For instance, cutaneous lymphomas and basal cell carcinomas (BCCs) can be rejected by local injection of Toll-like receptor (TLR) agonists or adenoviral vectors, which predominantly stimulate innate immune responses [33,34]. Renal cell carcinoma is highly responsive to systemic interleukin 2 (IL-2) administration, even though there is little evidence that adaptive immune responses are responsible for resolution of this disease [35]. Similarly, experimental animal models suggest that immune-mediated cancer rejection can occur independently of adaptive mechanisms [36]. It is important to emphasize that this postulate does not propose that adaptive immune responses are irrelevant to tissue-specific rejection. Instead, we wish to emphasize that adaptive mechanisms might play a primary role in initiating a tissue-specific reaction that recruits innate effector cells to the target organ, but it is these latter cells that are primarily responsible for tissue destruction or cancer rejection.

## The 'immunologic constant of rejection'

Complex problems do not necessarily require complex solutions [37]. Identifying a mechanism shared by distinct immune pathologies that leads to tissue-specific destruction may facilitate the identification of novel treatments without requiring the understanding of individual phenomenologies. Admittedly, not all immune-mediated pathologies work through the same effector mechanism(s). Here, we focus on those predominantly delivering cytotoxic insults to the target tissues.

Sarwal *et al.* [13] studied kidney allografts identifying ISGs, IEFs (granzymes), B-cell (CD20) and T-cell (TCR) signatures as hallmarks of uncontrollable acute rejection. We observed that immune-mediated rejection of melanoma metastases during IL-2 therapy was associated with activation of ISGs, particularly interferon regulatory factor (IRF)-1, IEFs (granzymes) and other transcripts specific for activated CTLs or NK cells [38–40]. We concluded that broad activation of cytotoxic mechanisms by innate and/or adaptive immune cells was the final step leading to rejection [40]. To test this hypothesis, we studied the mechanisms

mediating the rejection of BCCs by the TLR-7 agonist Imiquimod in a double-blinded randomized study [33]. Biopsies were obtained before and early during treatment to catch the events triggering immune-mediated destruction. By having a control arm, we could distinguish changes caused by the application of the placebo or the wound healing after the adjacent pretreatment biopsy [41]. Rejection of BCCs was associated with the expression of IFN-α and IFN-γ, ISGs and IEFs (granzyme-A and -B) and accumulation of CTL and NK cells; no B cells were involved. With the exclusion of allografts, neither we nor others in subsequent studies described the involvement of B-cell signatures (as measured by CD20). We propose that this difference is because of the direct contact in allo-immunity by both T and B cells with foreign antigens expressed on the surface of grafted cells, whereas only T cells can directly recognize target cells in most other immune diseases. Thus, we postulate that B cells can be directly stimulated in a T-independent manner by transplants because their BCRs can be massively cross-linked on the surface of the allograft. This may explain the promising role of anti-CD20 therapy for the control of acute allograft rejection [42–44] and graft versus host disease [45].

Several models of immune-mediated tissue rejection include the expression of ISGs, infiltration of CTL and NK cells and activation of cytotoxic mechanisms (Figure 2). The significance of the tight association between tissue-specific rejection and expression of IFN-α, IFN-γ and ISGs is unclear; we previously postulated that their presence may be necessary but not sufficient to induce immune-mediated rejection [26]. Recently, several studies have characterized signatures of IFN-α activation *in vitro* and *ex vivo* in distinct immune pathologies including HCV infection [46] and metastatic melanoma [28,47–49]. Concurrently, He *et al.* [46] observed that *in vitro* stimulation with IFN-α of T cells from patients with chronic HCV led to patient-specific, reproducible patterns of ISG activation and STAT-1 phosphorylation, which were predictive of response to therapy. A related phenomenon was observed in T cells from patients with advanced melanoma that, compared with normal non–tumor-bearing individuals, demonstrated variable and predominantly decreased *ex vivo* responsiveness to IFN-α [49].

The chemokine receptor most consistently expressed during tissue rejection is CXCR3 and its ligands CXCL-9, -10 and -11, which promote localization of CTLs to inflamed tissues [50–52]. CXCR3 and CCR4 (the receptor for CCL17 and CCL22) determine CD4 T-cell polarization toward a Th1 or Th2 phenotype, respectively [53]. Moreover, CXCR3 modulates activated CTL physiology [54] and induces CTL infiltration in inflamed areas such as the cerebrospinal fluid in multiple sclerosis [55], atherosclerotic plaques [51], the lung of HIV patients with T-cell alveolitis [50] or allografts [56,57]. Although it is generally believed that CXCR3 ligand chemokines are produced by activated dendritic cells, they can be secreted by B cells [58,59] during formation of tertiary lymphoid organs [60]. We observed that the interaction between activated CTL and B cells leads to the release of CXCR3 ligands by B cells, which in turn amplify the inflammatory signal by further attracting immune cells [14]. As CTLs enter into contact with B cells, they are stimulated to proliferate. Thus, B

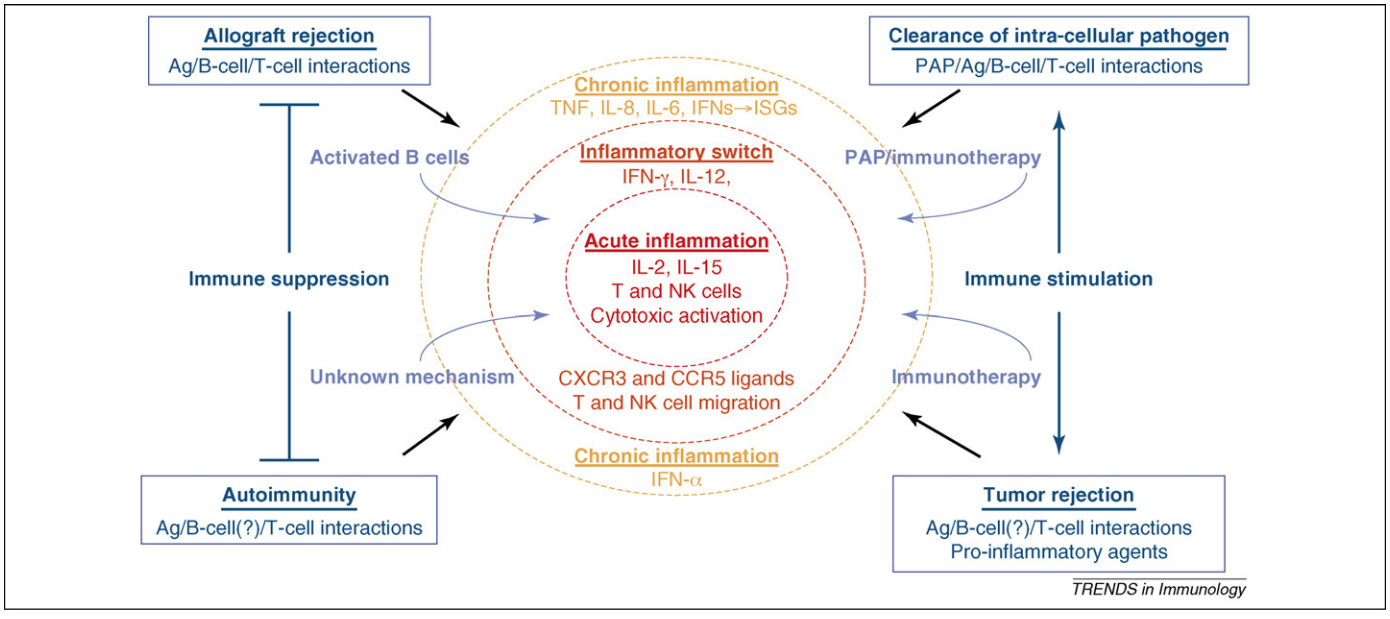


**Figure 2.** Although the mechanisms prompting tissue-specific destruction differ among immune pathologies, the effect phase converges. Chronic inflammatory processes are generally associated with the expression of interferon α (IFN-α) and related interferon-stimulated genes (ISGs). In addition, other early proinflammatory cytokines are often expressed such as tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and IL-8. These signatures are identified in chronically and acutely inflamed tissues. These processes can be prompted by variable interactions with antigen (Ag) with B or T cells or by non–Ag-specific interactions between pathogen-associated patterns (PAPs) and cells of the innate immune response through Toll-like receptor activation. In the case of allograft rejection and autoimmunity, the immune response is progressively destructive, and immune suppression is generally required. In chronic infections and in cancer, the immune response tends to be milder, and immune stimulation is required to induce tissue destruction that may lead to clearance of pathogen-infected cells or cancer cells. When acute inflammation leading to tissue destruction occurs, a subset of ISGs is consistently observed that includes CXCR3 ligands chemokines (CXCL-9, -10 and -11), most likely induced by IFN-γ, which could result from activation of several innate or adaptive immune mechanisms during immunotherapy of chronic infections or cancer or by the direct activation of B cells in allografts or autoimmune diseases. When this occurs, activated cytotoxic T cells (CTLs), Th1 cells and natural killer (NK) cells are attracted to the target organ initiating a powerful acute immune reaction characterized by the cytokines IL-2 and IL-15 and ultimately leading to tissue destruction.

cells might play a 'helper' role supporting CTL survival and expansion *in situ*. Moreover, this observation corroborates others' findings that B cells could function as central immune modulators through a reciprocal regulation of polarized cytokine production [61]. Paralleling the Th1 polarization of $CD4^+$ cells expressing CXCR3 [53], CXCR3 but not CCR4 is expressed by activated CTLs [14]. Because the expression of CXCR3 but not CCR4 ligands is most frequently observed during tissue-specific destruction, it is possible that this production might be key to the recruitment of activated effector cells. In the case of allograft rejection and some autoimmune pathologies, this process might be mediated directly by B cells activated in the target tissue through direct B-cell receptor signalling. In the case of other immune pathologies in which B cells are not directly stimulated by the presence of antigen on the surface of target cells, other immune modulators such as IL-12 or IFN-γ would be necessary. In the latter case, this activation might require immune manipulation of the host with immune-stimulatory agents.

The relevance of IEF activation is supported by Galon *et al.* [62], who observed that better prognosis in colon cancer is associated with infiltration of CTLs that express genes associated with their activation such as IFN-γ, IRF-1 and granzyme-B. Interestingly, several examples of immune rejection in humans report the enhanced expression of another IEF gene: natural killer protein-4 (IL-32). IL-32 is selectively expressed by activated CTLs and NK cells [23], potentiates the effect of IL-2 and IL-18, increases the expression of tumor necrosis factor-α (TNF-α) [63] and plays a central role in acute flares of inflammatory bowel disease [64], rheumatoid arthritis [65,66], and clearance of HCV infection during acute hepatitis, but it is not expressed in chronic unresolving hepatitis [67,68]. The reason for the strong association between IL-32 expression and immune rejection is unknown. The mouse genome does not include a sequence with close homology to IL-32; yet this species can readily reject tissues of various types, suggesting that IL-32 represents a marker of strong IEF activation rather than being an essential component.

The combined presence of NK and activated CTLs is observed often. Experimental animal models suggest that CTLs play a helper role in activating innate immune effector functions [69], whereas NK and other innate effector mechanisms actually destroy the affected tissue. We observed that antigen-activated CTLs release the CCR5 ligands CCL2 and CCL3 [70] that attract NK cells [71,72], therefore acting like sparks that initiate a broader tissue-specific effector immune response. Several clinical and experimental models suggest that tumor rejection does not necessarily require adaptive immune responses if alternative activation of innate effector mechanisms is present in the target tissue. Recently, we analyzed the mechanisms leading to disappearance of tumor xenografts in nude mice treated with oncolytic viruses. We observed that the regression of these tumors is predominantly mediated through the activation of ISGs (IRF-1), upregulation of CXCR3 and its ligand chemokines and the recruitment of NK cells with activation of IEFs (granzyme-B) (Worschech *et al.*, unpublished data). Because nude mice lack T- and B-cell functions, this immune-mediated tumor destruction is likely caused by innate immune mechanisms elicited at the tumor site by the tumor tropism of the oncolytic virus [73]. This is in line with the observation that tumor rejection in spontaneous regression models is mediated predominantly by innate immune effectors without requiring T and B cells [36]. Thus, the development of effector immune responses, whether adaptive or innate, follows a very specific pathway that differs from the pathway necessary for the induction of cognate immune responses; in either case, (adaptive versus innate) it is the activation of cytotoxic effector mechanisms that is primarily responsible for tissue-specific destruction. The final mediators of immune destruction is an 'NK-like' function that may be manifested by several members of the adaptive and innate immune system such as activated CTLs, NK cells, polymorpho nuclear granulocytes, macrophages, mast cells and interferon-producing killer dendritic cells (IK-DCs) [74]. We conclude that adaptive immune responses are not necessary to mediate tissue destruction but support its tissue specificity by directing the innate arm of the immune system to a specific site of the organism in which the expression of the particular target antigen is restricted [74].

In summary, tissue-specific destruction is associated with the activation of type I proinflammatory modulators; in particular the combination of IFN-α and IFN-γ, which results in the activation of ISGs. However, this alone is not necessarily sufficient to induce tissue rejection, because it is also observed in chronic inflammatory conditions such as unresolving HCV [68], wound healing [41] and nonregressing cancers [28,38]. Probably, a prevalence of expression of ISGs responsive to IFN-γ may provide the link to full activation of IEFs associated with tumor rejection. It remains unclear whether the threshold required to turn a chronic, unresolving inflammatory process into a destructive one is partially influenced by the genetic background of the host. We hypothesize that, when sufficient activation of ISGs occurs during the treatment of chronic HCV with IFN-α [46], the response of tumor to immunotherapy [33,38], or flares of autoimmunity and allograft rejection [13], the immune response broadens in the target organ producing chemokines of the CXCL-9 to -11 family that recruit CXCR3-bearing CTLs. These initiate a cascade that promotes the recruitment of other immune effector cells into the target tissue (Box 2). Other inflammatory

**Box 2. Propositions on the cascade that leads to immune-mediated tissue-specific destruction**

(i) CXCR3 ligand chemokines (CXCL-9, -10 and -11) are produced by contact activated B cells and proinflammatory stimuli-induced secretion of interleukin 12 and/or interferon γ by antigen-presenting cells.
(ii) CXCR3 expressing Th1-polarized CD4 T cells and cytotoxic T cells (CTLs) are recruited to the site of acute inflammation.
(iii) Antigen-activated T cells secrete CCR5 ligands (CCL2 and CCL3) to recruit natural killer (NK) cells and other innate immune effector cells to the site of acute inflammation.
(iv) Several cytotoxic mechanisms converge on the target tissue, and its complete destruction occurs through the activated effects of CTLs, NK cells, granulocytes, macrophages and interferon-producing killer dendritic cells.

mechanisms specific for individual immune pathologies may simultaneously participate, but because of this specificity, they are less commonly observed. The cascade outlined here may represent an oversimplification of the actual mechanism(s), leading to immune-mediated, tissue-specific destruction. Further studies should explore more extensively and validate in independent experimental settings the biological events deployed during rejection, independent of its primary trigger. Meanwhile, we could postulate that direct targeting of immune effector mechanisms associated with 'NK-like function' to control or stimulate tissue rejection may enhance the effectiveness of immunotherapy and ameliorate the side effects associated with the activation of other less relevant immunological pathways.

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## 3.3 The immunologic aspects of poxvirus oncolytic therapy – Review 2

# The immunologic aspects of poxvirus oncolytic therapy

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REVIEW

# The immunologic aspects of poxvirus oncolytic therapy

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**Abstract** The concept of using replicating oncolytic viruses in cancer therapy dates to the beginning of the twentieth century. However, in the last few years, an increasing number of pre-clinical and clinical trials have been carried out with promising preliminarily results. Novel, indeed, is the suggestion that viral oncolytic therapy might not operate exclusively through an oncolysis-mediated process but additionally requires the "assistance" of the host's immune system. Originally, the host's immune response was believed to play a predominant obstructive role against viral replication, hence limiting the anti-tumor efficacy of viral vectors. Recent data, however, suggest that the immune response may also play a key role in promoting tumor destruction in association with the oncolytic process. In fact, immune effector pathways activated during oncolytic virus-induced tumor rejection seem to follow a similar pattern to those observed when the broader phenomenon of immune-mediated tissue-specific rejection occurs in other immune-related pathologies. We recently formulated the "Immunologic Constant of Rejection" hypothesis, emphasizing commonalties in transcriptional patterns observed when tissue-destruction occurs: whether with a favorable outcome, such as in tumor rejection and pathogen clearance; or a destructive one, such as in allograft rejection or autoimmunity. Here, we propose that a similar mechanism induces clearance of virally infected tumors and that such a mechanism is primarily dependent on innate immune functions.

**Keywords** Vaccinia virus · Oncolytic therapy · Innate immunity · Tumor rejection

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## Historical synopsis of viral oncolytic treatment

Oncolytic viruses were first noticed in the early twentieth century when some cancer patients were noted to undergo tumor regression after systemic viral infections [1]. A leukemia patient, for instance, was reported to undergo remission after acute infection with influenza virus [2].

The realization that some viruses with lytic properties selectively propagate in and colonize tumors while leaving healthy tissues unharmed [3] originated the concept of "oncolytic viruses". These viruses were considerate attractive

candidates for the treatment of established tumors. Moreover, while a wide array of replication-deficient oncolytic viruses has been used in the past, recent studies have clearly shown that these constructs lack efficacy when compared to other replication-competent models [4].

The mutant adenovirus ONYX-015, which carries a deletion for E1B-55K, was the first replication-competent modified virus that displayed anti-cancer effects in humans [5], and was originally believed to target p53-deficient cancer cells. The use of this genetically engineered virus as a therapeutic agent has progressed to phase III clinical trials only 4 years after its first application in patients [6].

It is important to consider that the safe administration of oncolytic viruses in humans depends on an exclusive tropism for cancer cells. This has been achieved, among other methods, by the disruption of non-essential viral genes in viruses such as in HSV [7], adenovirus [8] and vaccinia [9] that, for unclear reasons, alters the replicative capacity of viruses in a tissue-specific fashion.

## Oncolytic therapy with vaccinia virus (VACV)

VACV is a promising candidate poxvirus for oncolytic therapy due to its extensive use in humans for vaccination worldwide against smallpox. This experience has clearly been demonstrated it to be safe. In addition, VACV displays several benefits when compared to other oncolytic viruses. One of the biggest advantages is that VACV's genome of about 200 kb is relatively permissive to the insertion of foreign genes up to 25 kb length [10], which have been used to modulate the in situ function of the virus, the infected cells and bystander host cells that are reacting to the intra-tumoral infectious process. This has been achieved through the expression of immune-modulatory genes, marker genes, therapeutic proteins or drug-converting enzymes. Another advantageous feature of VACV is the susceptibility of almost all cells within a tumor to infection with this virus, and VACV infection not only induces cell lysis but it also induces an associated, and in some cases preceded, activation of local cytotoxic T cells (CTLs) and natural killer cells (NK) responses. At the same time, with the exception of immune compromised patients or those with certain skin conditions such as eczema, VACV does not cause serious pathologies in humans and it has been shown to naturally and selectively propagate in tumor cells.

To enhance the natural tumor tropism of VACV, Kirn et al. [11] deleted the B18R gene, which encodes a protein that neutralizes type I Interferons (IFNs), producing a multi-functional and highly tumor-specific oncolytic vaccinia virus. Others [12] have designed a highly attenuated thymidine kinase- and vaccinia growth factor-depleted virus strain (vvDD-GFP) with enhanced anti-tumor efficacy. We recently introduced a novel VACV strain (GLV-1h68) derived from the LIVP progenitor strain that was modified by insertion of three expression cassettes (Renilla luciferase-Aequorea green fluorescent fusion protein, $\beta$-galactosidase and $\beta$-glucoronidase) into the F14.5L, J2R and A56R loci of the viral genome, respectively. Because of its light emitting properties, GLV-1h68 can be used simultaneously as an imaging tool to detect malignant cells in the body while exerting its oncolytic effects [13].

## Innate immune reactions to pathogenic vaccinia virus infection

Host cells possess the ability to sense viral infections through specific membrane bound or soluble intracellular pattern recognition receptors. Their ligands, pathogen-associated molecular patterns, trigger signaling cascades which ultimately lead to the production of type I IFNs and other cytokines. These innate immunity mechanisms protect the cells from uncontrolled virus spread while the adaptive immune response fully matures.

Recent studies have also demonstrated that the immune system may utilize ancestral autophagy mechanisms that have generally been adopted as a primary defense to battle microorganisms invading infected cells. The underlying mechanisms are still to be elucidated, however, it is clearly accepted that the anti-apoptotic function of autophagy provides protection against virus-induced pathologies [14].

Immune reactions against VACV infections have been studied and appear to differ according to the route of VACV administration. Intra-nasal infections of BALB/c mice with VACV strain Western Reserve induced early viral replication in the upper respiratory tract and lung that was associated with the infiltration of inflammatory cells into the lungs up to 15 days following infection. This infiltration included predominantly macrophages and T lymphocytes as well as the expression of several CCL chemokines (3, 2 and 11) and CXCL chemokines (1 and 2/3) [15]. Intra-dermal infection of the ears of BALB/c and C57BL/6 mice is followed by recruitment of macrophages, granulocytes and predominantly T cell receptor (TCR)-$\gamma\delta$-expressing T lymphocytes. This primary response is secondarily followed by a large infiltration of $CD4^+$ and $CD8^+$ T cells [15, 16]. This was consistent with findings observed with the intra-dermal and intra-nasal route of administration. Selin et al. [17] proposed a role for IFN-$\gamma$-producing $\gamma\delta$-T cells following VACV delivery in the peritoneal cavity of C57BL/6 mice. Interestingly, uninfected $\beta$-TCR knock out mice harbored a significant number of VACV-specific $\gamma\delta$-T cells, which rapidly expanded in response to VACV infection and secreted IFN-$\gamma$ while deploying increased cytotoxic activity.

Thus, IFN-$\gamma$ production is critical for the clearance of acute VACV infection [18, 19]. VACV, however, has evolved to protect itself from the host's anti-viral response. Like many other viruses, VACV encodes genes that can interfere with the host's innate immune defense [20]. VACV induces many intra- and extra-cellular proteins that can inhibit IFN-$\gamma$ anti-viral effects. A good example is the product of the viral gene B8R, a soluble-IFN-$\gamma$ receptor like-molecule, which binds specifically human and not mouse IFN-$\gamma$ [21, 22], and results in a species-specific immune evasion mechanisms in its natural host.

Whereas, a type II IFN-mediated immune response is responsible for the activation of the acute inflammatory response associated with oncolytic therapy and is central to the activation of cell-mediated immunity [23], both type I and type II IFNs are equally important in the containment of poxvirus infections [19, 24, 25]. In addition to its direct antiviral effects, IFN-$\alpha$ orchestrates a wide array of immune regulatory and other effects [26] including the regulation of NK cell activation [27, 28]. The direct signaling of NK cells, but not dendritic cells (DCs) through IL-15 secretion [29] seems to be required for the activation and exhibition of the effector functions which lead to VACV clearance both in vitro and in vivo [30].

## Cancer immunotherapy with vaccinia virus as an immunization agent

It is generally accepted that more than one genetic alteration is needed to transform normal cells into cancerous ones and to begin independent growth. It is also well accepted that the early evolution of cancer leads to the changing and adapting of phenotypes that allows tumor escape from immune surveillance through a mechanism referred to as immune editing [31–33]. Thus, tumors adapt to immune responses by down-modulating their antigenic properties and by producing factors that dampen the immune response. This creates a microenvironment in which inflammation predominantly fosters tumor growth [34–36]. Moreover, although cancer cells express tumor-associated-antigens (TAAs) that can be naturally recognized by the host's immune system [37, 38], their immunogenic potential is, in normal conditions, insufficient to induce effective cytotoxic cell activation due to lack of co-stimulatory signals generally provided in other immune-mediated phenomena such as by the expression of pathogen associated patterns [39]. In that context, the total loss or decrease in expression of HLA class I molecules has been reported for various cancer cells and this down-regulation eventually prevents tumor regression since this renders cancer cells insensitive to CTLs. In melanoma patients, low HLA class I antigen expression has been linked to poor clinical outcome and lesions that progress after immunotherapy [40, 41].

Thus, contrary to cells infected by lytic viruses, cancer cells survive in an immune quiescent microenvironment in which chronic inflammatory processes are not sufficient to induce their elimination. This is an important consideration when evaluating the immune effects that an oncolytic virus infection can produce and how it may alter the microenvironment.

Several clinical approaches have been tested to make a tumor "visible" to the immune system and thus to direct an immune response against it. Although cancer chemotherapy has usually been considered to be immunosuppressive, recent data suggest that some chemotherapeutic agents might trigger an anticancer immune response similar to that which can be achieved with oncolytic therapy [42, 43]. Viral vectors have been used to deliver and express tumor antigens with the aim of making the tumor cells more immunogenic [44, 45]. Virally infected cells provide the danger signals which are sensed by cells of the innate immune system. Once triggered by the presence of the pathogen, DCs, NK cells, macrophages, neutrophils, basophils, eosinophils and mast cells secrete cytokines and chemokines, which either lead to the direct killing of the pathogen-infected cell or lead to the recruitment of other inflammatory cells belonging to the adaptive immune response [46]. The concomitant activation of the immune system is one of the most important goals in the use of viral vectors as cancer vaccines. Thus, although these viruses have no oncolytic activity and have no specific cancer cell tropism, they can induce anti-cancer effects through the activation of the immune responses against TAAs that would be otherwise indolent due the lack of immune cell co-stimulation in the absence of a viral infection.

While the decision to select the optimal TAA is influenced by several factors beyond the scope of this review, the selection of the viral delivery system addresses more basic issues. Tumor-specific tropism, replication and induction of long-lasting, and potent cellular and humoral responses paired with safety for the patient are all considerations which places VACV among the most promising potential vectors [47]. A study conducted in the United Kingdom described the use of Modified Vaccinia Ankara (MVA) virus to deliver the tumor antigen 5T4 in colorectal cancer patients. 5T4 oncofetal antigen is a non-mutated self-antigen similar to carcinoembryonic antigen according to the classification system described by Amato et al. [47]. In a preliminary phase II study, it was observed that 6 out of 11 patients experienced a complete or partial response to the MVA-5T4 (TroVax®) administration [48]. More recently, the same group reported their experience in more than 200 patients who received over 700 doses of vaccine and immune responses were observed in approximately 95% of patients [47].

Kaufman et al. [49] reported the administration of two genetically engineered poxvirus family members: VACV and Fowlpox virus. Both constructs carried the TAAs carcinoembryonic antigen and mucin-1 and were administered to pancreatic cancer patients. Vaccination was well tolerated and five out of eight patients displayed antigen specific T-cell responses after VACV injection.

To stimulate strong T-cell responses against weak TAAs, co-stimulatory signals are needed [50, 51]. Stanford et al. [46] suggested that the achievement of immune-mediated tumor regression requires three criteria to be fulfilled: (a) the generation of a large frequency of high avidity tumor reactive-T-cells, (b) the trafficking of tumor-reactive T-cells to the tumor site, and (c) sustained T-cell activation within the tumor. Similarly, we have long argued that the localization and activation of T-cells at the tumor site is the key factor missing for successful immune-mediated tumor destruction [51–53] and that immunotherapy works primarily through the activation of potent innate immune responses within the tumor microenvironment, which could cause a switch from a lingering and chronic inflammatory process to an acute one [54–57]. Thus, poor clinical response rates in clinical trials with cancer vaccines [58] may be due to the failure to achieve the third criteria and secondary to tumor-evolved mechanisms that dampen adaptive responses and inhibit full activation of T-cells, the requirements of which have not been sufficiently characterized [53].

The expression of immunosuppressive factors like IL-10 or TGF-$\beta$ by tumor-associated immune cells, as well as the T-regulatory cell mediated suppression of CTL proliferation and effector function may prevent a successful outcome [46]. It is in this realm that oncolytic therapy may play an important role in facilitating the activation of vaccine-induced immune responses by providing powerful co-stimulation at the tumor site [53].

Recent data suggest that angiogenesis inhibitors may have a beneficial effect for clinical outcome. Tumor cells tend to produce angiogenic growth factors and factors that suppress the expression of endothelial cell adhesion molecules that are necessary for interaction with leukocytes. Agents such as anti-VEGF antibodies, endostatin and anginex share the ability to normalize the expression of adhesion molecules and thus stimulate leukocyte infiltration and make the tumor more vulnerable to the immune system [59].

Innate immune responses have been shown to induce tumor rejection without necessarily requiring the presence of adaptive immune responses [60]. Indeed, it has been argued that adaptive cytotoxic T cell responses may act primarily as "helpers" to promote powerful activation of innate immune effectors such as NK cells [61]. In humans, local injection of Toll-like receptor agonists or adenoviral vectors can induce the regression of basal cell carcinomas, cutaneous lymphomas, actinic keratosis and leukemias without evidence of B or T-cell participation [56, 62–64] although pre-clinical models suggest a potential role of Toll-like receptor signaling activation in expanding adaptive immune responses [65]. We have recently argued that adaptive immune responses provide specificity but are not necessarily sufficient to induce cancer rejection unless other components of the innate immune response are activated at the same time [57]. It is in this interface that VACV (and more broadly viral oncotropic viral vectors) may play a key role in tumor immunology as discussed in the next section.

## Oncolytic therapy with VACV and tumor rejection in the context of an innate immune response

The innate immune response initially stimulated by the virally infected cells and/or the VACV itself is directed automatically against the infected tumor cells and we suggest that this is part of the mechanism leading to tissue destruction by oncolytic therapy. A critical parameter is the likelihood of viral localization and replication at the tumor site before the pathogen can be neutralized by the host's immune system. Extra-cellular enveloped virus (EEV), a VACV form that naturally occurs early after infection, leads to rapid cell-to-cell spread before cell lysis occurs. EEV particles possess a host cell-derived lipid bi-layer which contains anti-complement proteins, hence providing protection against immune-mediated clearance. Infections in murine models with EEV-enhanced VACV strains result in improved anti-tumor effects due to their enhanced ability to spread and replicate in distant tumor parts and to be more resistant to neutralizing antibodies [66].

We have generated the efficiently replicating VACV GLV-1h68 strain, which leads to regression of GI-101A breast cancer xenografts in 95% of cases after intravenous delivery of $1 \times 10^7$ viral particles. Insertion of three foreign expression cassettes into the F14.5L, J2R, and A56R loci of the parental LIVP genome, expressing Renilla luciferase-Aequorea green fluorescent protein (RUC-GFP) fusion, $\beta$-galactosidase, and $\beta$-glucuronidase, respectively, resulted in a replicating, oncolytic VACV strain with increased tumor specificity and hence less systemic toxicity [13].

Transcriptional analysis of mouse xenografts using a mouse-specific platform to identify the host's response genes revealed the activation of innate immune mechanisms in regressing GI-101A tumors compared to non-infected control tumors [13]. Up-regulation of pro-inflammatory chemokine ligands such as CCL2, CCL17-19, CCL12, CXCL9, CXCL10 and CXCL12 was seen together with an increase in interleukin (IL), and chemokine receptors (IL13R, IL18, and CCR2) transcripts. Additionally, a

significant activation of Interferon-stimulated genes ISGs (Ifi202b, Ifi203, Ifi204, Ifi205, Ifi35, Ifi44, Ifi47, Ifih1, Ifit1, Ifit2, Ifit3, Ifitm3, Igtp, Iigp1, Isgf3g) was observed in association with increased STAT1 and Interferon-regulatory factor (IRF)-7. This strongly suggested that type I IFNs are critically involved in the process. Finally, immunohistochemistry of VACV-infected, regressing xenografts showed an intense peri- and intra-tumoral infiltration of mononuclear cells, which was confirmed by the up-regulation of CD69, CD48, CD52, and CD53 seen in the host's gene expression arrays. These markers are expressed on activated T-cells, NK cells, macrophages, granulocytes and DCs, and are associated with leukocyte activation and NK cytolytic function [13]. We concluded that tumor rejection induced by oncolytic viruses in this xenograft model is at least in part mediated through activation of innate immune mechanisms which correlate with the level of viral replication and precede tumor regression.

In a recent study, we compared GLV-1h68-infected GI-101A xenografts which were sensitive to oncolytic therapy to GI-101A xenografts from non-infected animals and to HT-29 colon cancer xenografts that do not respond to oncolytic therapy [67]. Moreover, we evaluated gene expression profiles of the oncolytic interaction by adopting organism-specific microarray platforms to simultaneously monitor gene expression changes in the tumor microenvironment. We applied 36k whole genome human arrays to test for alterations in the human cancer cells; 36k whole genome mouse arrays to examine the host's infiltrating stromal cells and lastly; custom-made 1K VACV arrays to characterize changes in viral transcription pattern.

Interestingly, human transcript analysis revealed no differences in non-responding, infected HT-29 tumors compared to control tumors. The expression of only a limited set of genes was altered after GLV-1h68 inoculation in regressing GI-101A xenografts. Most of the transcriptional changes that were observed in the infected responding tumors at a time when cell death had not yet occurred revealed a profound down-regulation of genes associated with cellular metabolic processes. These changes reflected the shut down of cancer cell metabolism due to VACV infection. Most of the few up-regulated transcripts in regressing GI-101A cells infected with VACV were associated with the activation of the innate immune mechanisms, but further sequence analysis showed that the majority represented the host's mouse cell transcripts that cross-hybridized onto human arrays.

The most interesting insights of this study were gained after analysis of the mouse expression arrays that represented the host's infiltrating cells. Infected, non-responsive HT-29 tumors did not show significant changes in gene expression compared to HT-29 tumors from non-infected control animals. On the contrary, a large number of genes were up-regulated in the GI-101A tumors after GLV-1h68 delivery compared to the non-infected GI-101A xenografts. Further analysis discovered a significant enrichment of immune-related genes. Among these immune-regulated genes, ISGs and other IFN signaling genes represented the most up-regulated canonical pathways both at an early time points when tumors were still continuing to grow in size (21 days post-infection) and later (42 days post-infection) when tumor rejection had started. This suggested that strong immune activation precedes tumor necrosis and, therefore, is unlikely secondary to the death of cancer cells, but rather is related to cancer cell infection. We also observed the up-regulation of IL-18 and IL-18 binding proteins, both of which played a dominant role early in infection, whereas IL-15 became the predominant cytokine expressed at later stages. Among CXCL chemokines, CXCL9, CXCL11 and CXCL12 were strongly expressed in regressing GI-101A xenografts together with CCL5 and CCL9 [67].

Based on these findings, we concluded that, in this immune deficient mouse model, the activation of innate immune responses might be sufficient to lead to tumor regression in cooperation with the viral oncolytic process. Further analysis of the genes activated upon tumor rejection in this mouse model leads to the integration of the results in the context of a much broader phenomenon that we recently described as the immunologic constant of rejection (ICR) [57].

## The immunologic constant of rejection

In 1969, Salk proposed the question of whether chronic infections, allograft rejection, autoimmune disorders and cancers belong to a common phenomenon that he termed the "delayed allergy reaction" [68]. The underlying mechanisms of these pathologies are clearly variable and distinct from each other. Infectious diseases like hepatitis C virus (HCV) infections become chronic if the pathogen is not cleared acutely; the latter process occurs rarely in humans [69]. Allograft transplant rejection can be controlled only through immune suppression because the broad antigenic differences between the allograft and host can trigger a strong immune response. Whereas, both allograft rejection and pathogen clearance represent an immune reaction against non-self structures, the immune system can also attack "self" tissues if the discrimination between self and non-self fails, as observed in autoimmunity. Immune responses against tumors fit self or non-self discrimination, as tumor cells are derived from normal progenitor cells and mostly express non-mutated TAAs [70]. However, some cancers display mutated antigens [71] that are unique to tumor cells and can be recognized as non-self by the immune system.

Even though the underlying triggering mechanisms differ among distinct immune pathologies, we postulated that the final outcome defined as tissue-specific destruction follows a common effector pathway which we called the "immunologic constant of rejection". We formulated four axioms that summarize the phenomenon: (1) tissue specific destruction does not necessarily occur because of non-self recognition but also occurs against self or quasi-self; (2) the requirements for the induction of a cognate immune response differ from those are necessary for the activation of an effector one; (3) although the prompts leading to tissue specific destruction vary in distinct pathologic states, the effector immune response converges into a single mechanism; and (4) adaptive immunity participates as a tissue-specific trigger, but it is not always sufficient or necessary [57].

The ICR theory was formulated based on studies conducted in humans but perfectly supports our findings in the xenograft model described above. Athymic mice harboring GI-101A xenografts mount an innate response and are able to reject the tumors after systemic delivery of the oncolytic VACV GLV-1h68 [13]. According to the ICR hypothesis, common effector pathways consisting of activation of ISGs and immune effector functions such as cell-mediated toxicity were all up-regulated in regressing GI-101A tumors in the mouse transcriptome suggesting a local innate immune response. No such immune reaction was seen in HT-29 tumors which corresponded with continued growth of the respective xenografts. Furthermore, responding tumors showed an activation of a distinct subset of macrophage-associated signatures and were highly infiltrated by MHC class II positive cells in the intra- and peri-tumoral compartment [67]. Interestingly, after being initially vaccinated with $5 \times 10^6$ pfu of GLV-1h68 responding GI-101A tumors showed much higher viral titers than persistent HT-29 tumors. It appears that in vivo viral titers are key players in determining the intensity of the immune response and, as a consequence, treatment outcome (Fig. 1).

While the weight and timing of the oncolytic effects compared to the activation of the innate immunity needs to be elucidated, it seems clear that adaptive immunity is not required to reject tumors during oncolytic therapy. This is not totally surprising as similar findings were observed in experimental tumor regression models [60]. During oncolytic therapy, the presence of the virus selectively in the tumor cells probably induces an immune-stimulation within the target organ that is sufficient to bypass the need for the specificity provided by TAA-specific T-cells. The potent pro-inflammatory viral infection in these cases is sufficient to induce a switch from a chronic inflammatory status to an acute one and leads to tissue specific destruction.

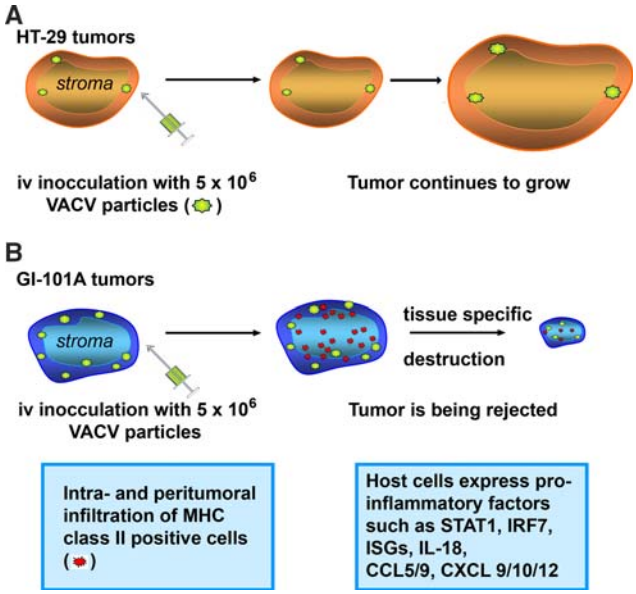


**Fig. 1** **a** HT-29 xenografts did not respond to oncolytic therapy with GLV-1h68, an attenuated vaccinia virus strain. No immune reaction was seen in HT-29 tumors which corresponded with continued growth of the respective xenografts. **b** After being initially vaccinated with the same dose of GLV-1h68 responding GI-101A tumors showed much higher viral titers than persistent HT-29 tumors. Responding tumors were highly infiltrated by MHC class II positive cells in the intra- and peri-tumoral compartment and presented a distinct activation of pro-inflammatory genes which lead to immune-mediated tissue destruction

## Conclusion

In summary, it appears that oncolytic therapy with VACV is associated in experimental models with powerful activation of immune responses within the tumor microenvironment. The ultimate role of the immune response in determining tumor rejection compared with the direct oncolytic process needs to be further evaluated by immune-depletion experiments. However, the strong association between viral replication in cancer cells and activation of immune responses suggests that oncolytic viruses characterized by specific tropism for cancer cells may be exploited not only as therapeutic tools in the context of classical oncolytic therapy but also as adjuvant in the context of other immunotherapy strategies, including the active specific immunization where, we believe, the limit to successful tumor eradication is due to the lack of stimulation of vaccine-induced T-cells in the target tissue [53]. This hypothesis needs to be further evaluated in future clinical trials that follow a systematic approach [72], but it is likely that oncolytic therapies will play a critical role in identifying important mechanisms leading to immune-mediated tissue-specific rejection.

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## 3.4 Constitutive activation of Interferon-Signaling Pathways in human cancers reveals two distinct taxonomies

### 3.4.1 Heterogeneous inflammatory phenotype in various cancer types

The explanation for the presence of two phenotypes of cancer cells has not been fully elucidated yet but the phenomenon is already described in the literature in various contexts. Fine needle aspiration samples from s.c. melanoma metastasis displayed a heterogeneous phenotype *in vivo* and could be segregated according to the coordinate expression of an inflammatory signature including cytokines, chemokines and angiogenic factors among them several with chemotactic properties (13). However, the data suggests that immune responsiveness may be predetermined by a tumor microenvironment conducive of immune recognition and not solely dependent upon the extent of the immune responses elicited by a given treatment.

Similarly, Weichselbaum et al. describe that a subset of human head and neck squamous cell carcinoma constitutively expresses STAT1 and other IFN-related genes (7). Cancers with high expression of Interferon-signaling pathways resisted ionizing radiation therapy and also restricted Herpes-simplex virus -1 replication *in vivo* (8).The same group recently reported that an IFN-related DNA damage resistance signature (IRDS) is a predictive marker for chemotherapy and radiation in breast cancer. The existence of IRDS (-) and IRDS (+) phenotypes in human cancers, such as breast, head and neck, prostate, lung and glioma has been clearly demonstrated and the authors propose that this chronically activated state might have selected for the failure to transmit a cytotoxic signal and therefore results in pro-survival signals mediated by STAT1 and other IRDS genes (7).

Additionally, studies of differences in the tumor microenvironment between African-American and European-American breast and prostate cancer patients revealed underlying immunological differences mainly related to angiogenesis and chemotactic functions (10, 11).

Lastly, the “anti-viral state” of some cancers might be also the result of a specific mutational profile which has been published recently for pancreatic cancers and needs to be further studied (14). Possible other control mechanisms at the

epigenetic level such as methylation might also play an important role since demethylation strictly correlates with an enhancement in STAT-1-phosphorylation and subsequent increased ISG expression (15).

### *3.4.2* Antiviral state in pancreatic cancers leads to various permissivity to Adenovirus replication *in vitro*

Pancreatic adenocarcinoma remains a leading cause of cancer mortality for which novel gene therapy approaches relying on tumor-tropic Ads are being tested. Applying global transcriptional profiling to samples derived from primary pancreatic adenocarcinomas, we observed two clearly distinguishable phenotypes according to the expression of ISGs. The two phenotypes could be readily recognized by immunohistochemical detection of the Myxovirus-resistance A protein (Mx-A/Mx-1), whose expression reflects the activation of interferon dependent pathways. The two molecular phenotypes discovered in primary carcinomas were also observed among established pancreatic adenocarcinoma cell lines, suggesting that these phenotypes are an intrinsic characteristic of cancer cells independent of their interaction with the host's microenvironment. Furthermore, the two phenotypes display diverse permissivity to adenoviral replication *in vitro* suggesting the practical implication that these signatures could facilitate the identification of patients likely to respond/resist viral vector-delivered gene therapy (12).

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# 4 Aim of the study

To better understand pivotal characteristics of cancer biology the presented work aimed to integrate the study of the heterogenic background of human tumors with basic principles of oncolytic therapy and gene expression changes during tumor rejection.

A wild-type rejection model in which tumor regression is mediated through a TAA-specific T-cell host response should be studied first to discover principal gene expression changes during tumor rejection in the target tissue (Research article 1).

Furthermore, based on the hyothesis that oncolytic treatment is at least partially mediated through an immune mechanism orchestrated by the host, the relationship between VACV infection and cancer regression should be assessed in innate immune system settings. This should be achieved by applying organism-specific microarray platforms to isolated RNA from both, infected and uninfected xenografts. Simultaneous gene expression patterns derived from human arrays (representative of tumor cell transcript), mouse arrays (representative of host infiltrating cells in the tumor microenvironment) and customized VACV arrays to monitor the viral transcription pattern should be applied to both regressing and non-regressing xenografts (Research article 2) to be able to distinguish between tumor cell-related and host-mediated transcriptional changes.

The relationship between the activation of ISGs which is indicative of the anti-viral phenotype of human cancers and the susceptibility to viral infections has been studied in a pancreatic carcinoma model after infection with Ad5 or Adeno-associated Virus (AAV) (12). Based on these previous findings it was hypothesized that high viral *in vivo* titers are necessary in oncolytic models but might not be sufficient to orchestrate cancer rejection. It seems very likely, that also the ability of cancer cells to launch a danger signal post infection and to trigger an immune response plays an important role (Figure 1A-C); this capacity in turn, might be hampered in non-responsive tumors (Figure 1D).

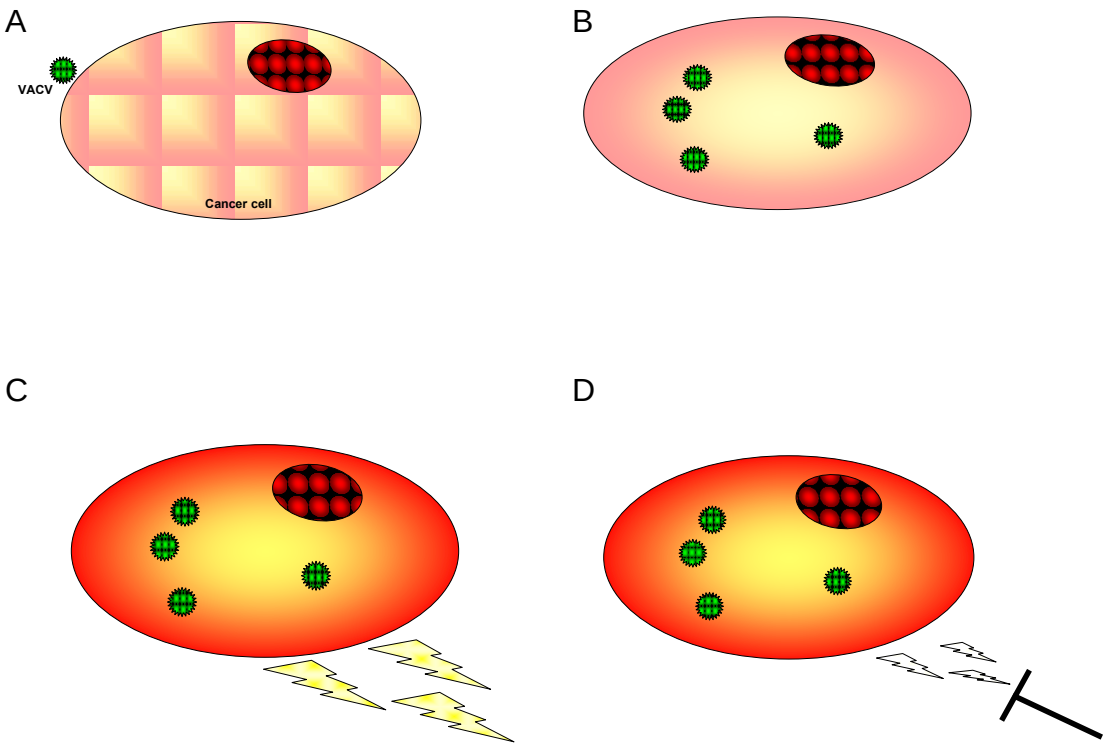


***Figure 1*** – ***(A)*** Initial infection of the cancer cell with VACV. ***(B)*** Cytoplasmic replication and assembly of new VACV particles. ***(C)*** Danger signal initiated by the infected cell triggers the host immune response. ***(D)*** Danger signal is interrupted and not sufficient to mount an immune response.

To address this question, 75 human cancer cells lines should be screened for their ability to allow replication of oncolytic VACV and an Ad-5 control virus (Manuscript for research article 3). Furthermore, investigation of a possible existence of pre-determined marker genes characteristic of sufficient viral replication should be done.

# 5 Results

## 5.1 Signatures associated with rejection or recurrence in HER-2/neu-positive mammary tumors - Research article 1

# Signatures Associated with Rejection or Recurrence in HER-2/neu–Positive Mammary Tumors

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## Signatures Associated with Rejection or Recurrence in HER-2/neu–Positive Mammary Tumors

RNA extraction, amplification and microarray hybridizations of tumor tissue, statistical data analyses and partial interpretation and formulation of the results were performed by the author of this thesis with help and supervision of the following co-authors who contributed to the manuscript:

The following co-authors participated in this study and contributed to the manuscript:

> Dr. M. Kmieciak, Dr. K. L. Knutson and Dr. H. D. Bear performed *in vivo* mouse experiments, FACS analysis and ELISA testing.
>
> Dr. E. Wang, Dr. F. M. Marincola, Dr. M. Manjili and Prof. Dr. A. A. Szalay supervised and designed the study and helped to write the paper.

Ms. A. Worschech, was a visitor at NIH, and is a graduate student in Dr. Szalay's laboratory in the Department of Biochemistry, University of Würzburg, Germany, and is supported by a graduate stipend and foreign travel grant from Genelux Corporation which provided free housing and daily allowance fellowship.

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# Signatures Associated with Rejection or Recurrence in HER-2/*neu*–Positive Mammary Tumors

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## Abstract

**We have previously shown T-cell–mediated rejection of the neu-overexpressing mammary carcinoma cells (MMC) in wild-type FVB mice. However, following rejection of primary tumors, a fraction of animals experienced a recurrence of a neu antigen-negative variant (ANV) of MMC (tumor evasion model) after a long latency period. In the present study, we determined that T cells derived from wild-type FVB mice can specifically recognize MMC by secreting IFN-γ and can induce apoptosis of MMC *in vitro*. Neu transgenic (FVBN202) mice develop spontaneous tumors and cannot reject it (tumor tolerance model). To dissect the mechanisms associated with rejection or tolerance of MMC tumors, we compared transcriptional patterns within the tumor microenvironment of MMC undergoing rejection with those that resisted it either because of tumor evasion/antigen loss recurrence (ANV tumors) or because of intrinsic tolerance mechanisms displayed by the transgenic mice. Gene profiling confirmed that immune rejection is primarily mediated through activation of IFN-stimulated genes and T-cell effector mechanisms. The tumor evasion model showed combined activation of Th1 and Th2 with a deviation toward Th2 and humoral immune responses that failed to achieve rejection likely because of lack of target antigen. Interestingly, the tumor tolerance model instead displayed immune suppression pathways through activation of regulatory mechanisms that included in particular the overexpression of interleukin-10 (IL-10), IL-10 receptor, and suppressor of cytokine signaling (SOCS)-1 and SOCS-3. These data provide a road map for the identification of novel biomarkers of immune responsiveness in clinical trials.** [Cancer Res 2008;68(7):2436–46]

## Introduction

Challenges in the immune therapy of cancers include a limited understanding of the requirements for tumor rejection and prevention of recurrences after successful therapy. Evaluation of T-cell responses in human tumors based predominantly on the metastatic melanoma model has clearly shown that the tumor-bearing status primes systemic immune responses against tumor-associated antigens, which, however, are insufficient to induce tumor rejection (1, 2). Moreover, the experience gathered through the induction of tumor antigen–specific T cells by vaccines has shown that the frequency of tumor antigen–specific T cells in the circulation (3, 4) or in the tumor microenvironment (5, 6) does not directly correlate with successful rejection or prevention of recurrence (7). Similarly, patients with preexisting immune responses against HER-2/*neu* are not protected from the development of HER-2/*neu*–expressing breast cancers (8). Although several and contrasting reasons have been proposed to explain this paradox, two lines of thoughts summarize these hypotheses: either tolerogenic and/or immune-suppressive properties of tumors may hamper T-cell function (9–11) or characteristics of the tumor microenvironment could induce tumor escape and evade the antitumor function of an otherwise effector T cells (12, 13).

In spite of this paradoxical coexistence of tumor-specific T cells and their target antigen-bearing cancer cells, recent observations in cancer patients suggest that T cells control tumor growth and mediate its rejection. Galon et al. and others (14–16) observed that T cells modulate the growth of human colon cancer and T-cell infiltration of primary lesions may forecast a better prognosis. In addition, these authors observed that tumor-infiltrating T cells in cancers with good prognosis displayed transcriptional signatures typical of activated T cells, such as the expression of IFN-stimulated genes (ISG), IFN-γ itself, and cytotoxic molecules, particularly granzyme B (15). Similar observations were reported by others in human ovarian carcinoma (17). These important observations derived from human tissues provide novel prognostic markers but cannot address the causality of the association between T-cell infiltration and natural history of cancer. Recent reports based on adoptive transfer of tumor-specific T cells suggest a cause-effect relationship between the administration of T cells and tumor rejection (18). However, the complexity of the therapy associated with adoptive transfer of T cells, which includes immune ablation and systemic administration of interleukin (IL)-2, prevents a clear interpretation of this causality.

We, therefore, adopted an experimental model that could address the paradoxical relationship between adaptive immune responses against cancer antigens and rejection or persistence of antigen-bearing cancers with the intent of comparing functional signatures between the experimental model and previous human observation that could shed mechanistic information on this relationship and potentially provide novel predictive or prognostic biomarkers to be tested in the clinical settings. In this study, we compared transcriptional patterns of mammary tumors undergoing rejection

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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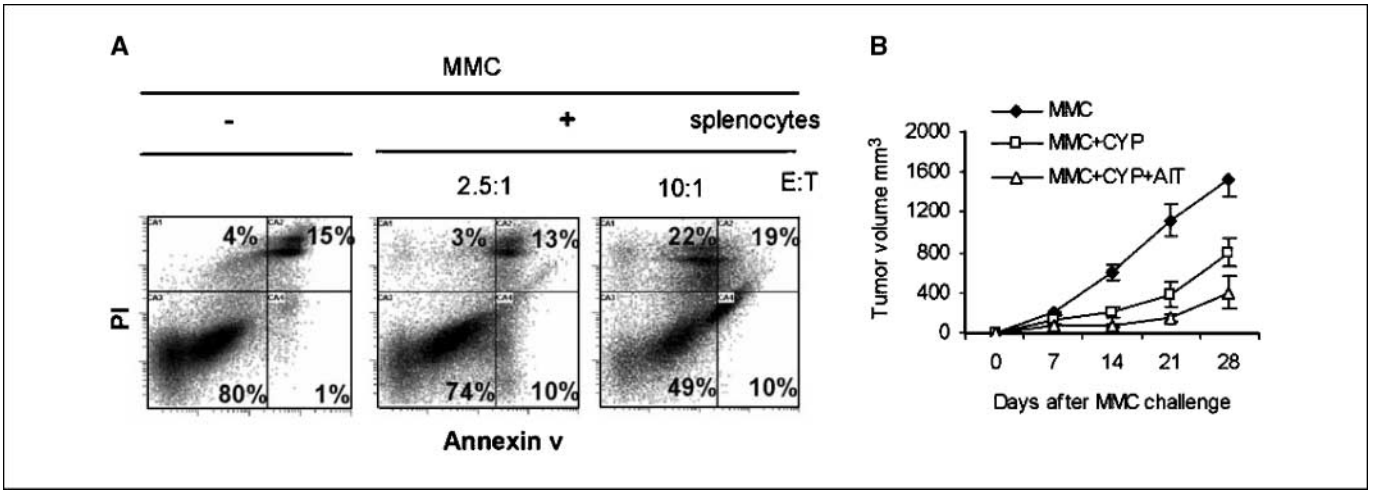


**Figure 1.** T cells derived from wild-type FVB mice will induce apoptosis in MMC *in vitro* but fail to reject MMC in FVBN202 mice following adoptive immunotherapy. *A,* flow cytometry analysis of MMC after 24 h of culture with splenocytes of FVB mice following three-color staining. Gated neu-positive cells were analyzed for the detection of Annexin $V^+$ and $PI^+$ apoptotic cells. Data are representative of quadruplicate experiments. *B,* donor T cells were enriched from the spleen of FVB mice using nylon wool column following the rejection of MMC. FVBN202 mice ($n = 4$) were injected with cyclophosphamide (*CYP*) followed by inoculation with MMC ($4 \times 10^6$ cells per mouse) and tail vein injection of donor T cells. Control groups were challenged with MMC in the presence or absence of cyclophosphamide treatment. Tumor growth was monitored twice weekly. *AIT,* adoptive immunotherapy.

with that of related tumors that evaded immune recognition through antigen loss (evasion model) or resided in tolerized transgenic mice (tolerogenic model). For this purpose, we used FVB mice that reject neu-overexpressing mammary carcinomas (MMC) because of the presence of a potent neu-specific T-cell response. Although MMCs are consistently rejected after a few weeks, occasionally MMCs recur and in such instance they resist further immune pressure by invariably loosing HER-2/neu expression (tumor evasion model; refs. 19, 20). Moreover, FVBN202 mice that constitutively express high levels of HER-2/neu fail to reject MMC because they cannot mount effective antitumor T-cell responses (tolerogenic model). Thus, we compared the tumor microenvironment at salient moments of immune response/evasion/tolerance to gain, in this previously well-characterized model (19, 20), insights about the immune mechanisms leading to tumor rejection and their failure in conditions of tumor evasion or systemic tolerance. Interestingly, the tolerance model, which was expected to show tolerance, displayed immune suppression pathways through activation of regulatory mechanisms that included in particular the overexpression of IL-10, IL-10 receptor, and suppressor of cytokine signaling (SOCS)-1 and SOCS-3.

## Materials and Methods

**Mice.** Wild-type FVB (The Jackson Laboratory) and FVBN202 female mice (Charles River Laboratories) were used throughout these studies. FVBN202 is the rat neu transgenic mouse model in which 100% of females develop spontaneous mammary tumors by 6 to 10 mo of age, with many features similar to human breast cancer. These mice express an unactivated rat *neu* transgene under the regulation of the mouse mammary tumor virus promoter (21). Because of the overexpression of rat neu protein, FVBN202 mice are expected to tolerate the neu antigen as self-protein and in cases where there might be a weak neu-specific immune response before the appearance of spontaneous mammary tumors are still well tolerated (22, 23). On the other hand, rat neu protein is seen as non–self-antigen by the immune system of wild-type FVB mice, resulting in aggressive rejection of primary MMC (19, 24). The studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

**Tumor cell lines.** The MMC cell line was established from a spontaneous tumor harvested from FVBN202 mice as previously described (11, 15). Tumors were sliced into pieces and treated with 0.25% trypsin at 4°C for 12 to 16 h. Cells were then incubated at 37°C for 30 min, washed, and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; refs. 19, 20). The cells were analyzed for the expression of rat neu protein before use. Expression of rat neu protein was also analyzed before each experiment and antigen-negative variants (ANV) were reported accordingly (see Results).

***In vivo* tumor challenge.** Female FVB or FVBN202 mice were inoculated s.c. with MMC ($4 \times 10^6$ to $5 \times 10^6$ cells per mouse). Animals were inspected twice every week for the development of tumors. Masses were measured with calipers along the two perpendicular diameters. Tumor volume was calculated by the following formula: $V = (L \times W^2) / 2$, where $L$ is the length and $W$ is the width. Mice were sacrificed before a tumor mass exceeded 2,000 $mm^3$.

**IFN-γ ELISA.** Secretion of MMC-specific IFN-γ by lymphocytes was detected by coculture of lymphocytes ($4 \times 10^6$ cells) with irradiated MMC or ANV (15,000 rads) at 10:1 E:T ratios in complete medium (RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin) for 24 h. Supernatants were then collected and subjected to IFN-γ ELISA assay using a Mouse IFN-γ ELISA Set (BD PharMingen) according to the manufacturer's protocol. Results were reported as the mean values of duplicate ELISA wells.

**Flow cytometry.** A three-color staining flow cytometry analysis of the mammary tumor cells ($10^6$ per tube) was carried out using mouse anti-neu (Ab-4) antibody (Calbiochem), control Ig, FITC-conjugated anti-mouse Ig (Biolegend), phycoerythrin (PE)-conjugated Annexin V, and propidium iodide (PI; BD PharMingen) at the concentrations recommended by the manufacturer. Cells were finally added with Annexin V buffer and analyzed at 50,000 counts with the Beckman Coulter EPICS XL within 30 min.

**Microarray performance and statistical analysis.** Total RNA from tumors was extracted after homogenization using Trizol reagent according to the manufacturer's instructions. The quality of secondarily amplified RNA was tested with the Agilent Bioanalyzer 2000 (Agilent Technologies) and amplified into antisense RNA (aRNA) as previously described (25, 26). Confidence about array quality was determined as previously described (27). Mouse reference RNA was prepared by homogenization of the following mouse tissues (lung, heart, muscle, kidneys, and spleen), and RNA was pooled from four mice. Pooled reference and test aRNA were isolated and amplified in identical conditions during the same amplification/hybridiza-

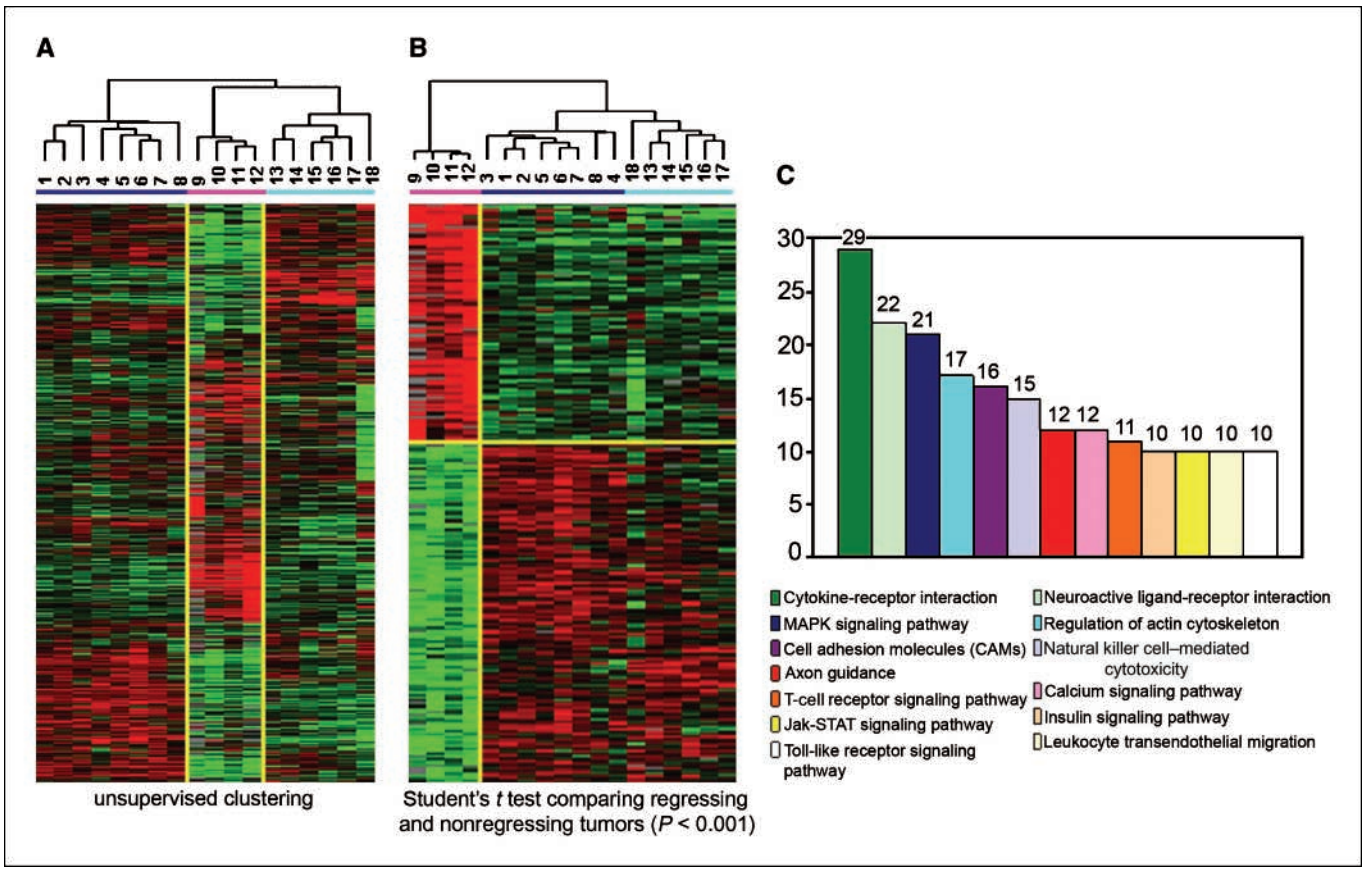


**Figure 2.** Gene expression profiling and gene oncology pathway analyses in tumor regressing and tumor nonregressing groups. *A,* unsupervised cluster visualization of genes differentially expressed among regressing tumors (*pink bar*) and nonregressing tumors (*blue bar*, evasion model; *turquoise bar*, tolerogenic model). MMC tumors were harvested 10 d after challenge and hybridized to 36K oligo mouse arrays. Genes (11,256) with at least 3-fold ratio change and 80% presence call among all samples were projected using log2 intensity. *B,* supervised cluster analysis ($P < 0.001$, Student's $t$ test; fold change, >3) comparing regressing tumors (*pink bar*) and nonregressing tumors (*blue bar*, evasion model; *turquoise bar*, tolerogenic model). Differentially expressed genes (2,449) have been selected for further analysis. *C,* Gene Ontology databank was queried to assign genes to functional categories and up-regulated genes within the tumor regression group to functional categories.

tion procedure to avoid possible interexperimental biases. Both reference and test aRNA were directly labeled using ULS aRNA Fluorescent Labeling kit (Kreatech) with Cy3 for reference and Cy5 for test samples.

Whole-genome mouse 36K oligo arrays were printed in the Infectious Disease and Immunogenetics Section of Transfusion Medicine, Clinical Center, NIH (Bethesda, MD) using oligos purchased from Operon. The Operon Array Ready Oligo Set version 4.0 contains 35,852 longmer probes representing 25,000 genes and ~38,000 gene transcripts and also includes 380 controls. The design is based on the Ensembl Mouse Database release 26.33b.1, Mouse Genome Sequencing Project, National Center for Biotechnology Information RefSeq, Riken full-length cDNA clone sequence, and other Genbank sequence. The microarray is composed of 48 blocks and one spot is printed per probe per slide. Hybridization was carried out in a water bath at 42°C for 18 to 24 h and the arrays were then washed and scanned on a GenePix 4000 scanner at variable photomultiplier tube to obtain optimized signal intensities with minimum (<1% spots) intensity saturation.

Resulting data files were uploaded to the mAdb databank[8] and further analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute (28)[9] and Cluster and TreeView software (29). The global gene expression profiling consisted of 18 experimental samples. Subsequent filtering (80% gene presence across all experiments and at least 3-fold ratio change) selected 11,256 genes for further analysis. Gene ratios were average corrected across experimental samples and displayed according to uncentered correlation algorithm (30).

**Statistical analysis.** Rate of tumor growth was compared statistically by unpaired Student's $t$ test. Unsupervised analysis was performed for class confirmation using the BRBArrayTools and Stanford Cluster program (30). Class comparison was performed using parametric unpaired Student's $t$ test or three-way ANOVA to identify differentially expressed genes among tumor-bearing, tumor rejection, and relapse groups using different significance cutoff levels as demanded by the statistical power of each comparison. Statistical significance and adjustments for multiple test comparisons were based on univariate and multivariate permutation test as previously described (31, 32).

## Results

**T-cell–mediated rejection of MMC and relapse of ANV in wild-type FVB mouse.** Wild-type FVB mice are capable of rejecting MMC within 3 weeks because of specific recognition of rat neu protein by their T cells as opposed to their transgenic counterparts, FVBN202, which tolerate rat neu protein and fail to reject MMC (19, 24). To determine whether aggressive rejection of primary MMC by T cells may lead to relapse-free survival in wild-type FVB mice, we performed follow-up studies. Animals ($n$ = 15) were challenged with MMC by s.c. inoculation at the right groin.

[8] http://nciarray.nci.nih.gov
[9] http://linus.nci.nih.gov/BRB-ArrayTools.html

Animals were then monitored for tumor growth twice weekly. All mice rejected MMC within 3 weeks after the challenge (Supplementary Fig. S1). However, a fraction of these animals (8 of 15 mice) developed recurrent tumors at the site of inoculation. These relapsed tumors had lost neu expression under immune pressure (19, 24). Relapse-free groups were maintained as breeding colonies and did not show any relapse during their life span. Splenocytes of FVB mice secreted IFN-γ in the presence of MMC only (2,200 pg/mL), whereas no appreciable IFN-γ was detected when lymphocytes were stimulated with ANV (110 pg/mL). No IFN-γ was secreted by splenocytes or tumor cells alone (data not shown).

**T cells derived from wild-type FVB mice will induce apoptosis in MMC.** To determine whether neu-specific recognition of MMC by T cells may induce apoptosis in MMC, *in vitro* studies were performed. Splenocytes of naive FVB mice were stimulated with irradiated MMC for 24 h followed by 3-day expansion in the presence of IL-2 (20 units/mL). Lymphocytes were then cocultured with MMC (E:T ratio of 2.5:1 and 10:1) for 48 h in the presence of IL-2 (20 units/mL). Control wells were seeded with MMC or splenocytes alone in the presence of IL-2. Cells (floaters and adherents) were collected and subjected to a three-color flow cytometry analysis using mouse anti-rat neu antibody (Ab-4), PE-conjugated anti-mouse Ig, control Ig, Annexin V, and PI. Gated neu-positive cells were analyzed for the detection of Annexin $V^+$ and $PI^+$ apoptotic cells. As shown in Fig. 1*A*, 80% of MMC were Annexin $V^-$ and $PI^-$ in the absence of lymphocytes, whereas only 49% of MMC were Annexin $V^-$ and $PI^-$ in the presence of lymphocytes at 10:1 E:T ratio. At a lower E:T ratio (2.5:1), there was a slight dropping in the number of viable MMC (from 80% to 74%) but marked increase in the number of early apoptotic cells (Annexin $V^+/PI^-$) from 1% to 10%. At a higher E:T ratio (10:1), early (Annexin $V^+/PI^-$) or late (Annexin $V^+/PI^+$) apoptotic cells and necrotic cells (Annexin $V^-/PI^+$) were markedly increased.

**Adoptive immunotherapy of FVBN202 mice using T cells derived from wild-type FVB donors failed to reject MMC.** To determine whether T cells of FVB mice with neu-specific and antitumor activity may protect FVBN202 mice against MMC challenge, adoptive immunotherapy was performed. Using nylon wool column, T cells were enriched from the spleen of FVB donor mice following the rejection of MMC. FVBN202 recipient mice were injected i.p. with cyclophosphamide (100 μg/g) to deplete endogenous T cells. After 24 h, animals were challenged with MMC tumors ($4 \times 10^6$ cells per mouse). Four to 5 h after tumor challenge, donor T cells were transferred into FVBN202 mice ($6 \times 10^7$ cells per mouse) by tail vein injections. Control FVBN202 mice were challenged with MMC in the presence or absence of cyclophosphamide treatment. Animals were then monitored for tumor

**Table 1.** Differentially expressed ISGs, chemokines, and their receptors

| | | Mouse | Human | | Rejection | Controls |
|---|---|---|---|---|---|---|
| CXC chemokines and receptors | | | | | | |
| *Cxcl2* | Chemokine (C-X-C motif) ligand 2 | *GRO; MIP-2;KC?* | *GROξ;MGSA-ξ* | CXCR2 | 14.54 | 0.44 |
| *Cxcl1* | Chemokine (C-X-C motif) ligand 1 | *GRO; MIP-2;KC?* | *GROa;MGSA-ξ* | CXCR2>1 | 5.40 | 0.70 |
| *Cxcl11* | Chemokine (C-X-C motif) ligand 11 | *I-TAC* | *I-TAC* | CXCR3 | 3.93 | 0.68 |
| CC chemokines and receptors | | | | | | |
| *Ccl1* | Chemokine (C-C motif) ligand 1 | *TCA-2;P500* | *I-309* | CCR8 | 2.15 | 0.80 |
| *Ccl4* | Chemokine (C-C motif) ligand 4 | *MIP-1ξ* | *MIP-1ξ* | CCR5 | 5.06 | 0.63 |
| *Ccl5* | Chemokine (C-C motif) ligand 5 | *RANTES* | *RANTES* | CCR1;3,5 | 5.42 | 0.62 |
| *Ccl5* | Chemokine (C-C motif) ligand 5 | *RANTES* | *RANTES* | CCR1;3,5 | 3.96 | 0.67 |
| *Ccl6* | Chemokine (C-C motif) ligand 6 | *C10;MRP-1* | Unknown | Unknown | 3.79 | 0.68 |
| *Ccl8* | Chemokine (C-C motif) ligand 8 | *MCP-2* | *MCP-2* | CCR3;5 | 5.94 | 0.60 |
| *Ccl9* | Chemokine (C-C motif) ligand 9 | *MRP-2;CCF18;MIP-1ξ* | Unknown | CCR1 | 6.72 | 0.58 |
| *Ccl11* | Small chemokine (C-C motif) ligand 11 | *Eotaxin* | *Eotaxin* | CCR3 | 4.34 | 0.64 |
| *Ccl22* | Chemokine (C-C motif) ligand 22 | *ABCD-1* | *MDC/STCP-1* | CCR4 | 4.08 | 0.74 |
| *Ccrl2* | Chemokine (C-C motif) receptor-like 2 | | | CCL2, 7, 12, 13, 16 | 6.58 | 0.62 |
| *Ccr10* | Chemokine (C-C motif) receptor 10 | | | CCL27, 28 | 2.22 | 0.83 |
| *Cklf* | Chemokine-like factor | | | | 2.86 | 0.74 |
| *Darc* | Duffy blood group, chemokine receptor | | | | 2.83 | 0.86 |
| *Cklf* | Chemokine-like factor, transcript variant 1 | | | | 2.82 | 0.74 |
| ISGs | | | | | | |
| *Ifna2* | IFN-α2 | | | | 2.91 | 0.80 |
| *Ifng* | IFN-γ | | | | 2.89 | 0.72 |
| *Ifi202b* | IFN-activated gene 202B | | | | 6.05 | 0.60 |
| *Ifi27* | IFN, α-inducible protein 27 | | | | 4.68 | 0.64 |
| *Ifi204* | IFN-activated gene 204 | | | | 4.14 | 0.67 |
| *Ifitm1* | IFN-induced transmembrane protein 1 | | | | 3.73 | 0.75 |
| *Irf6* | IRF6 | | | | 3.59 | 0.76 |
| *Ifit1* | IFN-induced protein with tetratricopeptide repeats 1 | | | | 3.33 | 0.77 |
| *Irf4* | IRF4 | | | | 2.97 | 0.73 |
| *Mx1* | Myxovirus (influenza virus) resistance 1 | | | | 2.63 | 0.76 |
| *Stat2* | STAT2 | | | | 2.35 | 0.78 |
| *Stat6* | STAT6 | | | | 2.14 | 0.80 |
| *Irf2bp1* | IRF2 binding protein 1 | | | | 0.29 | 1.42 |

**Table 2.** Differentially expressed cytokines and signaling molecules

| Genes up-regulated in the rejection model | | | | Genes down-regulated in the rejection model | | | |
|---|---|---|---|---|---|---|---|
| Symbol | Description | Reject | Control | Symbol | Description | Reject | Control |
| ILs and receptors | | | | | | | |
| *Il1a* | IL-1α | 2.71 | 0.81 | | | | |
| *Il1b* | IL-1β | 8.91 | 0.54 | | | | |
| *Il1f9* | IL-1 family, member 9 | 1.97 | 0.82 | | | | |
| *Il5* | IL-5 | 1.64 | 0.87 | | | | |
| *Il7* | IL-7 | 3.11 | 0.84 | | | | |
| *Il17f* | IL-17F | 3.86 | 0.68 | | | | |
| *Il31* | IL-31 | 2.16 | 0.80 | | | | |
| | | 1.00 | 1.00 | | | | |
| *Il1rap* | IL-1 receptor accessory protein, transcript variant 2 | 3.56 | 0.70 | | | | |
| *Il2rg* | IL-2 receptor, γ chain | 1.75 | 0.85 | | | | |
| *Il7r* | IL-7 receptor | 2.46 | 0.88 | | | | |
| *Il23r* | IL-23 receptor | 7.38 | 0.63 | | | | |
| *Il17rb* | IL-17 receptor B | 4.46 | 0.65 | | | | |
| | | 1.00 | 1.00 | | | | |
| Cytotoxic and proapoptotic molecules | | 1.00 | 1.00 | | | | |
| *Gzmb* | Granzyme B | 1.90 | 0.83 | | | | |
| *Gzmb* | Granzyme B | 1.57 | 0.88 | | | | |
| *Ctla2a* | CTL-associated protein 2α | 3.60 | 0.69 | | | | |
| *Klra9* | Killer cell lectin-like receptor subfamily A, member 9 | 1.95 | 0.87 | | | | |
| *Klrd1* | Killer cell lectin-like receptor, subfamily D, member 1 | 7.36 | 0.57 | | | | |
| *Fasl* | Fas ligand [tumor necrosis factor (TNF) superfamily, member 6] | 2.78 | 0.75 | *Tnfaip1* | TNF, α-induced protein 1 | 0.51 | 1.21 |
| *Tnfsf11* | TNF (ligand) superfamily, member 11 | 2.56 | 0.80 | | | | |
| *Tnfrsf1b* | TNF receptor superfamily, member 1b | 2.21 | 0.83 | *Tnfrsf12a* | TNF receptor superfamily, member 12a | 0.14 | 1.52 |
| *Tnfrsf4* | TNF receptor superfamily, member 4 | 2.77 | 0.73 | *Ngfrap1* | Nerve growth factor receptor (TNFRSF16)-associated protein 1 | 0.38 | 1.15 |
| TLRs and lymphocyte signaling | | | | | | | |
| *Tlr4* | TLR4 | 2.14 | 0.80 | | | | |
| *Tlr6* | TLR6 | 2.98 | 0.86 | | | | |
| *Il4i1* | IL-4 induced 1 | 3.34 | 0.77 | | | | |
| *Alcam* | Activated leukocyte cell adhesion molecule | 2.12 | 0.81 | | | | |
| *Bcl2a1c* | B-cell leukemia/lymphoma 2–related protein A1c | 3.41 | 0.70 | | | | |
| *Itk* | IL2-inducible T-cell kinase | 2.63 | 0.76 | *Ilf3* | IL enhancer binding factor 3, transcript variant 2 | 0.44 | 1.27 |
| *Ebf4* | Early B-cell factor 4 | 6.32 | 0.75 | | | | |
| *Ly6a* | Lymphocyte antigen 6 complex, locus A | 2.83 | 0.74 | | | | |
| *Ly6c* | Lymphocyte antigen 6 complex, locus C | 3.18 | 0.72 | *Ly6e* | Lymphocyte antigen 6 complex, locus E | 0.22 | 1.53 |
| *Ly6f* | Lymphocyte antigen 6 complex, locus F | 3.62 | 0.67 | *Ly6e* | lymphocyte antigen 6 complex, locus E | 0.47 | 1.18 |
| *Ly6f* | Lymphocyte antigen 6 complex, locus F | 2.41 | 0.78 | *Btla* | B and T lymphocyte-associated, transcript variant 2 | 0.38 | 1.32 |
| *Lck* | Lymphocyte protein tyrosine kinase | 2.26 | 0.79 | *Bcap31* | B-cell receptor–associated protein 31 | 0.34 | 1.36 |
| *Tagap* | T-cell activation Rho GTPase-activating protein | 2.36 | 0.78 | *Tiam2* | T-cell lymphoma invasion and metastasis 2 | 0.47 | 1.24 |
| *Tlx1* | T-cell leukemia, homeobox 1 | 7.36 | 0.65 | *Ikbkap* | Inhibitor of κ light polypeptide enhancer in B cells | 0.44 | 1.27 |
| *Tcl1b1* | T-cell leukemia/lymphoma 1B, 1 | 2.37 | 0.78 | | | | |
| *Nkrf* | NF-κB repressing factor | 3.90 | 0.75 | | | | |
| *Nkiras2* | NF-κB inhibitor interacting Ras-like protein 2 | 2.48 | 0.82 | *Irak1bp1* | IL-1 receptor–associated kinase 1 binding protein 1 | 0.33 | 1.26 |

(*Continued on the following page*)

**Table 2.** Differentially expressed cytokines and signaling molecules (Cont'd)

| Genes up-regulated in the rejection model | | | | Genes down-regulated in the rejection model | | | |
|---|---|---|---|---|---|---|---|
| Symbol | Description | Reject | Control | Symbol | Description | Reject | Control |
| *Nfkbiz* | NF-κB light polypeptide gene enhancer in B-cell inhibitor, ζ | 3.83 | 0.68 | *Irak1* | IL-1 receptor–associated kinase 1 | 0.31 | 1.40 |
| *Nfat5* | Nuclear factor of activated T cells 5, transcript variant b | 3.34 | 0.71 | | | | |
| FC-type receptors | | | | | | | |
| *Lilrb4* | Leukocyte immunoglobulin-like receptor, subfamily B, member 4 | 3.60 | 0.74 | | | | |
| *Mgl1* | Macrophage galactose *N*-acetyl-galactosamine–specific lectin 1 | 6.23 | 0.59 | | | | |
| *Mgl2* | Macrophage galactose *N*-acetyl-galactosamine–specific lectin 2 | 2.25 | 0.79 | | | | |
| *Msr1* | Macrophage scavenger receptor 1 | 2.78 | 0.80 | | | | |
| Immunoglobulins | | | | | | | |
| *Igh-6* | Immunoglobulin heavy chain 6 | 7.43 | 0.56 | | | | |
| *Igh-6* | Immunoglobulin heavy chain 6 (heavy chain of IgM) | 2.39 | 0.78 | | | | |
| *Igj* | Immunoglobulin joining chain | 2.86 | 0.74 | | | | |
| *Igk-V28* | Immunoglobulin κ chain variable 28 | 2.82 | 0.79 | | | | |
| *Igl-V1* | Immunoglobulin λ chain, variable 1 | 3.69 | 0.76 | | | | |
| *Igl-V1* | Immunoglobulin λ chain, variable 1 | 2.03 | 0.82 | | | | |
| *Igkv4-90* | Immunoglobulin light chain variable region | 1.82 | 0.84 | | | | |

growth. As shown in Fig. 1*B*, cyclophosphamide treatment of animals resulted in retardation of tumor growth in FVBN202 mice as expected. Student's *t* test analysis on days 14, 21, and 28 after challenge showed significant differences between these two groups ($P = 0.005$, 0.007, and 0.01, respectively). Adoptive transfer of neu-specific effector T cells from MMC-sensitized FVB mice into cyclophosphamide-treated FVBN202 groups did not significantly inhibit tumor growth compared with cyclophosphamide-treated control groups ($P > 0.05$). Adoptive transfer of neu-specific effector T cells from untreated FVB mice into cyclophosphamide-treated FVBN202 groups showed similar trend of tumor growth (data not shown). These experiments suggest that T-cell responses associated with MMC rejection in wild-type FVB mice (19) may represent an epiphenomenon with no true cause-effect relationship or that FVBN202 mice retain tolerogenic properties in spite of cyclophosphamide treatment that can hamper the function of potentially effective anticancer T-cell responses. We favor the second hypothesis based on our previous depletion experiments that showed the requirement of endogenous effector T cells of FVB mice for rejection of MMC tumors (19).

**Genetic signatures defining rejection or tolerance of MMC tumors.** To ascertain whether the presence of neu-specific effector T cells may trigger a cascade of events that may determine success or failure in tumor rejection, wild-type FVB and FVBN202 mice were inoculated with MMC. Historically, all FVB mice reject MMC; however, a fraction of mice develop a latent tumor relapse. In contrast, FVBN202 mice fail to reject transplanted MMC. Ten days after the tumor challenge, transplanted MMC tumors were excised and RNAs were extracted from both FVB and FVBN202 carrier mice based on the presumption that the biology of the former would be representative of active tumor rejection and that of the latter would be representative of tumor tolerance. Thus, the timing of tumor harvest was chosen to capture transcriptional signatures associated with the active phase of the tumor rejection process in wild-type FVB mice in comparison with the corresponding tolerance of spontaneous mammary tumors in the FVBN202 mice. We speculated that this comparison would allow distinguishing whether tolerance was due to inhibition of T-cell function within the tumor microenvironment of spontaneous mammary tumors or to a complete absence of such responses. To enhance the robustness of the comparison, a similar analysis was performed extracting total RNA from spontaneous tumor in FVBN202 mice. In addition, RNA was extracted from MMC tumors in wild-type FVB mice that experienced tumor recurrence following the initial rejection of MMC. This second analysis allowed the comparison of mechanisms of tumor evasion in the absence of known tolerogenic effects. Microarray analyses were then performed on the amplified RNA (aRNA) extracted from these tumors using 36K oligo mouse arrays. Hence, genes considered as differentially expressed in the study groups could represent either MMC tumor cells or host cells infiltrating the tumor site. Probes with missing values >80% or a change <3-fold were excluded from further analysis. Unsupervised clustering showed outstanding differences among the three experimental groups (Fig. 2*A*). Genes of spontaneous mammary tumors (samples 13, 15, 16, and 17) clustered closely to those of transplanted MMC (samples 14 and 18) excised from FVBN202 mice, suggesting that the biology of MMC tumors remains comparable between these two experimental models of tolerance. Global transcriptional patterns associated with tumor relapse (samples 1–8) were instead clearly different from those of spontaneous mammary tumors or MMC transplanted in tolerant FVBN202 mice, suggesting that a completely different biological process was at the basis of tumor evasion through loss of target antigen expression. Finally, MMC tumors undergoing rejection

(samples 9–12) were clearly separated from either kind of nonregressing tumors.

**Biomarkers of rejection.** Our first class comparison searched for differences between the four tumor samples undergoing rejection and the rest of the MMC tumors whether belonging to the tolerogenic or the evasion process. This approach followed the exclusion principle whereby factors determining the occurrence of a phenomenon should be discernible from unrelated ones independent of the causes preventing its occurrence. An unpaired Student's *t* test with a cutoff set at $P < 0.001$ identified 2,449 genes differentially expressed between regressing and nonregressing tumors (permutation $P = 0$), of which 1,003 genes were up-regulated in regressing tumors clearly distinguishing the two categories (Fig. 2*B*). Of those, a large number were associated with immune regulatory functions. Gene Ontology databank was queried to assign genes to functional categories and up-regulated pathways were ranked according to the number of genes identified by the study belonging to each category (Fig. 2*C*). The top categories of genes that were up-regulated in primary rejected MMC tumors were cytokine-cytokine interaction, mitogen-activated protein kinase signaling, cell adhesion–related transcripts and axon guidance, T-cell receptor, Janus-activated kinase-signal transducer and activator of transcription (STAT), and Toll-like receptor (TLR) signaling pathways. Natural killer cell–mediated cytotoxicity and calcium signaling pathways were also enriched in up-regulated genes. In contrast, very little evidence of immune activation could be observed in either category of nonregressing tumors, suggesting that lack of immune rejection is due to absent or severely hampered immune responses in the tumor microenvironment independent of the mechanisms leading to this resistance.

To better describe the immunologic pathways associated with tumor regression, we organized genes with immune function into three categories, including chemokines, IFN-α2, IFN-γ, and ISGs (Table 1), and cytokines and signaling molecules (Table 2). From this analysis, it became clear that T-cell infiltration into tumors was associated with activation of various pathways leading to the expression of IFN-α, IFN-γ, and several ISGs, including *IFN regulatory factor 4* (*IRF4*), *IRF6*, and *STAT2*. In addition, several cytotoxic molecules were overexpressed, including calgranulin A, calgranulin B, and granzyme B, all of them representing classic markers of effector T-cell activation in humans (10) and in mice (33). Thus, tumor rejection in this model clearly recapitulates patterns observed in various human studies in which expression of ISGs is associated with the activation of cytotoxic mechanisms among which granzyme B seems to play a central role.

**Is there a difference between signatures of immune evasion and immune tolerance?** As shown in Table 3, the high expression of IL-10 and the IL-10 receptor-β chain concordant with IRF1 in the tolerogenic model strongly suggests the presence of regulatory mechanism within the microenvironment of MMC-bearing FVBN202 mice. Preferential expression of SOCS-1 and SOCS-3 in the microenvironment of MMC tumors of FVBN202 mice also strongly suggests a marked activation of regulatory functions present in the tolerized host (Table 3).

To further investigate whether similar mechanisms were involved in failure of tumor rejection in tolerance and evasion models, we characterized potential differences between the two models of immune resistance; we compared statistical differences between the tolerogenic and the evasion model comparing the two nonregressing groups by unpaired Student's *t* test using as a significance threshold a *P* value of <0.001. This analysis was performed on preselected genes that had been filtered for an at least 80% presence of data in the whole data set and a minimal fold increase of 3 in at least one experiment (Fig. 3). This analysis identified 1,369 genes differentially expressed by the two groups (multivariate permutation test $P = 0$), of which 462 were up-regulated in the tolerogenic model and 907 were up-regulated in the tumor evasion model (Fig. 3*A*). Several of these genes were specifically expressed by either group, although the expression of a few of them was shared by the regressing MMC tumors. Annotations and functional analysis based on Gene Ontology database showed that the predominant functional classes of genes transcriptionally active in one of the other type of nonresponding MMC tumors were not associated with classic activation of T-cell effector functions but rather were associated with more general metabolic processes (Fig. 3*B* and *C*). However, detailed analysis of transcripts associated with immunologic function (Table 3) defined dramatic differences between the two mechanisms of immune resistance.

## Discussion

FVB mice reject primary MMC by T-cell–mediated neu-specific immune responses. However, a fraction of animals develop tumor relapse after a long latency. On the other hand, their transgenic counterparts, FVBN202, fail to mount effective neu-specific immune responses and develop tumors (19). Although FVBN202 mice seem to elicit weak immune responses against the neu protein within a certain window of time (22), the neu-expressing MMC tumors are still well tolerated and animals develop spontaneous mammary tumors. Despite the observation that T cells derived from FVB mice were capable of recognizing MMC and inducing apoptosis in these tumors *in vitro*, adoptive transfer of such effector T cells into FVBN202 mice failed to protect these animals against challenge with MMC.

It has been suggested that T cells play a significant role in determining the natural history of colon (14–16) and ovarian (17) cancer in humans. Transcriptional signatures have been identified that suggest not only T-cell localization but also activation through the expression of IFN-γ, ISGs, and cytotoxic effector molecules such as granzyme B (10). We have recently shown that rejection of basal cell cancer induced by the activation of TLR agonists also is mediated, at least in part, by localization and activation of CD8-expressing T cells with increased expression of cytotoxic molecules (34). Yet, a comprehensive experimental overview of the biological process associated with tumor rejection in its active phase has not been reported. Thus, our first class comparison searched for differences between the four tumor samples undergoing rejection and the rest of the MMC tumors whether belonging to the tolerogenic or the evasion process. Unlike nonregressing tumors (tolerance and evasion models), regressing tumors (rejection model) showed up-regulation of immune activation genes, suggesting that failure in tumor rejection is due to immune evasion or severely hampered immune responses in the tumor microenvironment. A particularly interesting observation was the relative low expression of ISGs, with the exception of Irf2bp1. Although the transcriptional patterns differentiating regressing from nonregressing tumors were striking and in many ways representative of previous observations in humans by our and other groups (35), differences among MMC tumors nonregressing in FVBN202 mice and those relapsing after regression in FVB mice were subtle. We have previously proposed that lack of regression of human tumors is primarily associated with indolent immune responses rather

**Table 3.** Manually selected genes with immunologic function based on supervised comparison of evasion and tolerogenic tumor models and tumor rejection model

| Symbol | Description | Evasion | Tolerogenic | Rejection |
|---|---|---|---|---|
| Chemokines | | | | |
| *Ccl2* | Chemokine (C-C motif) ligand 2 | 2.304 | 0.614 | 0.392 |
| *Ccl4* | Chemokine (C-C motif) ligand 4 | 1.899 | 0.478 | 0.838 |
| *Ccl6* | Chemokine (C-C motif) ligand 6 | 2.525 | 0.536 | 0.400 |
| *Ccr7* | Chemokine (C-C motif) receptor 7 | 1.441 | 0.696 | 0.829 |
| *Cxcl10* | Chemokine (C-X-C motif) ligand 10 | 2.341 | 0.781 | 0.264 |
| *Cxcl9* | Chemokine (C-X-C motif) ligand 9 | 1.440 | 0.354 | 2.288 |
| *Xcl1* | Chemokine (C motif) ligand 1 | 4.913 | 0.279 | 0.282 |
| *Cx3cl1* | Chemokine (C-X3-C motif) ligand 1 | 5.120 | 0.393 | 0.155 |
| ILs and signaling | | | | |
| *Il12b* | IL-12b | 1.768 | 0.866 | 0.397 |
| *Il13* | IL-13 | 1.475 | 0.779 | 0.669 |
| *Il17d* | IL-17D | 1.690 | 0.674 | 0.633 |
| *Il23r* | IL-23 receptor | 2.048 | 0.457 | 0.772 |
| *Il2rg* | IL-2 receptor, γ chain | 1.798 | 0.742 | 0.484 |
| *Il4* | IL-4 | 2.342 | 0.497 | 0.521 |
| *Il4i1* | IL-4 induced 1 | 2.290 | 0.701 | 0.325 |
| *Il6* | IL-6 | 1.105 | 0.504 | 2.291 |
| *Il7r* | IL-7 receptor | 1.218 | 0.439 | 3.063 |
| *Il9* | IL-9 | 1.711 | 0.801 | 0.477 |
| *Tlr11* | TLR11 | 2.656 | 0.435 | 0.540 |
| *Blnk* | B-cell linker | 1.457 | 0.749 | 0.726 |
| *Bok* | Bcl-2–related ovarian killer protein | 2.071 | 0.432 | 0.822 |
| *Vpreb3* | Pre-B lymphocyte gene 3 | 2.712 | 0.148 | 2.398 |
| *Lcp2* | Lymphocyte cytosolic protein 2 | 1.640 | 0.588 | 0.824 |
| *Ly6d* | Lymphocyte antigen 6 complex, locus D | 4.093 | 0.223 | 0.567 |
| *Nfkb1* | NF-κB light chain gene enhancer 1, p105 | 1.737 | 0.785 | 0.477 |
| *Pias2* | Protein inhibitor of activated STAT2 | 1.336 | 0.502 | 1.458 |
| *Pias3* | Protein inhibitor of activated STAT3 | 1.763 | 0.828 | 0.427 |
| *Stat4* | STAT4 | 1.579 | 0.685 | 0.630 |
| *Il10* | IL-10 | 0.788 | 2.528 | 0.400 |
| *Il1r2* | IL-1 receptor, type II | 0.804 | 2.504 | 0.391 |
| *Il10rb* | IL-10 receptor, β | 0.422 | 2.839 | 1.174 |
| *Socs1* | SOCS-1 | 0.566 | 4.683 | 0.308 |
| *Socs3* | SOCS-3 | 0.621 | 1.751 | 1.120 |
| *Bak1* | BCL2-antagonist/killer 1 | 0.663 | 2.698 | 0.514 |
| *Lsp1* | Lymphocyte specific 1 | 0.609 | 1.468 | 1.514 |
| *Tank* | TRAF family member-associated NF-κB activator | 0.718 | 2.204 | 0.351 |
| *Tlr6* | TLR6 | 0.480 | 1.141 | 3.559 |
| ISGs | | | | |
| *Ifnb1* | IFN-β1, fibroblast | 1.388 | 0.644 | 1.003 |
| *Irf7* | IRF7 | 1.500 | 0.677 | 0.406 |
| *Ifrd1* | IFN-related developmental regulator 1 | 2.248 | 0.337 | 1.011 |
| *Ifnar1* | IFN (α and β) receptor 1 | 1.884 | 0.855 | 0.356 |
| *Igtp* | IFN-γ–induced GTPase | 1.863 | 0.671 | 0.524 |
| *Irf1* | IRF1 | 0.549 | 2.715 | 0.671 |
| *Irf3* | IRF3 | 0.845 | 1.759 | 0.479 |
| *Irf6* | IRF6 | 0.601 | 1.816 | 1.282 |
| *Ifngr1* | IFN-γ receptor 1 | 0.582 | 4.515 | 0.308 |
| *Ifrg15* | IFN-α–responsive gene | 0.591 | 1.913 | 1.168 |

than dramatic changes in the tumor microenvironment enacted to counterbalance a powerful effector immune response (13, 31, 35). The MMC tolerance model allowed investigating this hypothesis at least in this restricted case. Spontaneous mammary tumors or transplanted MMC tumors in FVBN202 mice displayed immune-suppressive properties that were identified by transcriptional profiling through the activation of genes associated with regulatory function. This would occur only in case an indolent adaptive immune response occurred in these transgenic mice and was hampered at the tumor site by a mechanism of peripheral suppression. If, however, central tolerance was the reason for the lack of rejection, minimal changes should be observed in tolerogenic model similar to those detectable in the tumor evasion model where MMC tumors lost expression of HER-2/*neu* and become

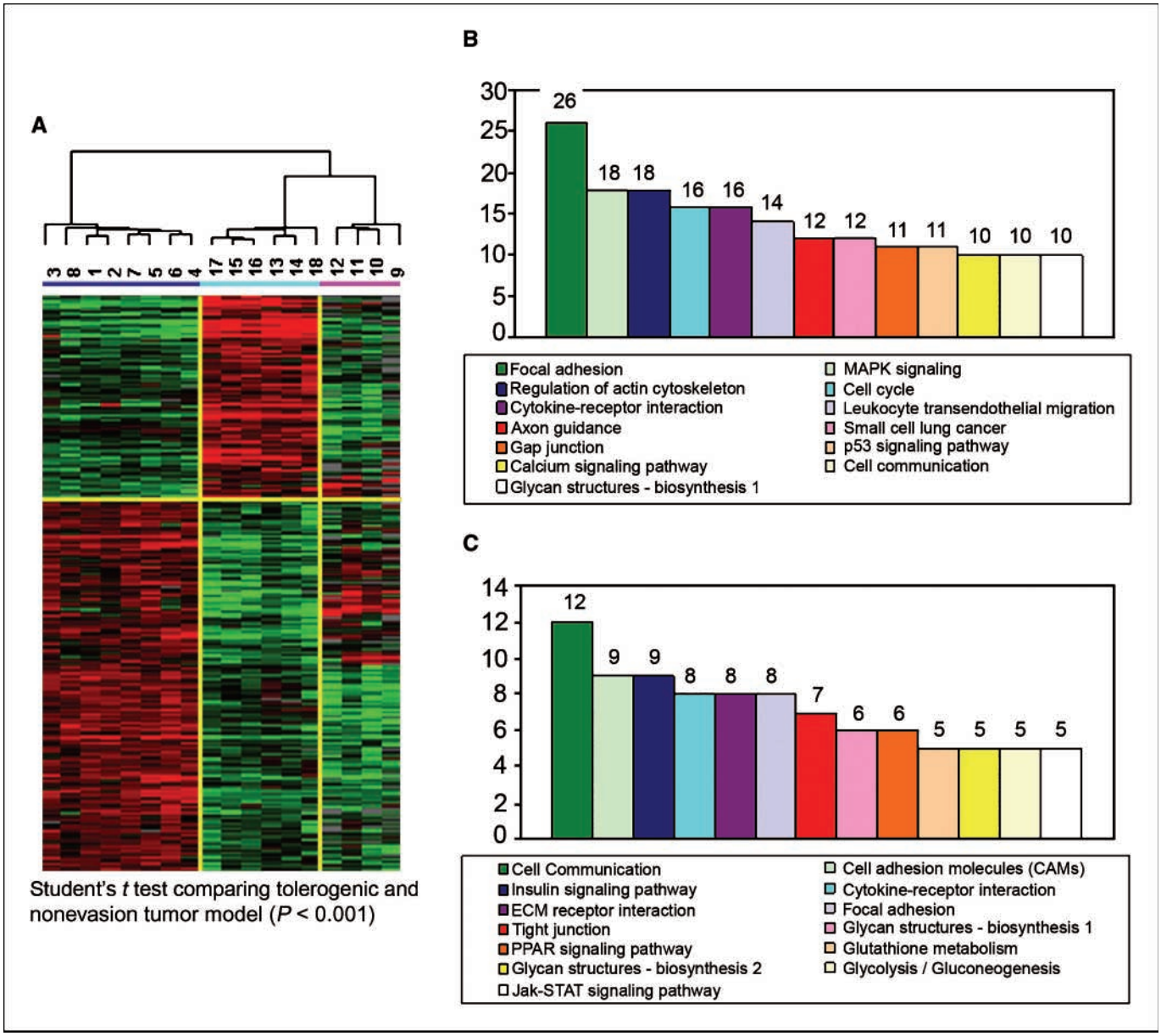


**Figure 3.** Gene expression profiling and gene oncology pathway analyses in tolerance and evasion models. *A,* supervised cluster analysis ($P < 0.001$, Student's *t* test; fold change, >3) comparing evasion (*blue bar*) and tolerogenic group (*turquoise bar*). Differentially expressed genes (1,326) have been visualized also including tumor regression samples (*pink bar*). Gene ontology pathway analysis projecting either up-regulated pathways in evasion group (*B*, 854 genes) or tolerogenic tumor models (*C*, 475 genes).

irrelevant targets for HER-2/*neu*–specific T-cell responses. The presence of regulatory mechanism within the microenvironment of MMC-bearing FVBN202 mice was associated with increased IL-10 as well as increased expression of SOCS-1 and SOCS-3. It has recently been shown that myeloid-derived suppressor cells (MDCS) induce macrophages to secrete IL-10 and suppress antitumor immune responses (36). Importantly, it was shown that high levels of MDCS in neu transgenic mice would suppress antitumor immune responses against tumors (37). IL-10 is increasingly recognized to be strongly associated with regulatory T-cell (38) and M2-type tumor-associated macrophage function (39), and its expression is mediated in the context of chronic inflammatory stimuli by the overexpression of IRF1. SOCS-1 inhibits type I IFN response, CD40 expression in macrophages, and TLR signaling (40–42). Expression of SOCS-3 in dendritic cells converts them into tolerogenic dendritic cells and supports Th2 differentiation (43). Importantly, tumors that express SOCS-3 show IFN-γ resistance (44).

Unlike tolerance model, recurrence model revealed expression of Igtp, suggesting the involvement of IFN-γ in this model (Table 3). This observation is consistent with our previous findings on the role of IFN-γ in neu loss and tumor recurrence (19). MMC tumors evading immune recognition had undergone a process of complex immune editing that resulted not only in the loss of the HER-2/neu target antigen but also in the up-regulation of various Th2-type cytokines, such as IL-4 and IL-13 (45), and the corresponding transcription factor IRF7 overexpression predominantly associated to a deviation from cellular Th1 to Th2 and humoral type immune

responses (46). In addition, the microenvironment of recurrent tumors was characterized by the coordinate expression of STAT4, IL-12b, IL-23 receptor, and IL-17; this cascade has been associated with the development of Th17-type immune responses that play a dominant role in autoimmune inflammation (47, 48) and T-cell–dependent cancer rejection (49, 50). Because both humoral and cellular immune responses are potentially involved in the rejection of HER-2/neu–expressing tumors (51), these data suggest that a cognitive and active immune response is still attempting to eradicate MMC tumors that may still express subliminal levels of the target antigen. However, the overall balance between host and cancer cells favors, in the end, tumor cell growth because the expression of HER-2/neu, the primary target of both cellular and humoral responses, is critically reduced.

Altogether, these observations suggest that neu antigen loss and subsequent immunologic evasion from cellular Th1 to Th2 and humoral type immune response is a major mechanism in evasion model, whereas peripheral suppression, such as sustained IL-10, SOCS-1, and SOCS-3 expression, is a major player in tolerance model. This conclusion provides a satisfactory explanation for the lack of rejection of MMC tumors in FVBN202 mice receiving adoptively transferred HER-2/neu–specific T cells. In this case, effective T-cell responses exclude central tolerance or peripheral ignorance as the only mechanism potentially hampering their effector function at the tumor site, suggesting that other regulatory mechanisms such as peripheral suppression could be responsible for inactivation of donor effector T cells. High levels of MDSC in neu transgenic mice support this possibility, and the role and mechanisms of MDSC in suppression of adoptively transferred neu-specific T cells remain to be determined in FVBN202 mice.

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## 5.2 Systemic treatment of xenografts with Vaccinia Virus GLV-1h68 reveals the immunologic facet of oncolytic therapy – Research article 2

# Systemic treatment of xenografts with vaccinia virus GLV-1h68 reveals the immunologic facet of oncolytic therapy

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## Systemic treatment of xenografts with vaccinia virus GLV-1h68 reveals the immunologic facet of oncolytic therapy

RNA extraction, amplification and microarray hybridizations of infected and non infected tumor tissue and human cancer cell lines, statistical data analyses, cDNA sequence analyses and partial interpretation and formulation of the results were performed by the author of this thesis with help and supervision of the following co-authors who contributed to the manuscript:

Dr. N. Chen, Dr. Y. A. Yu and Dr. Q. Zhang generated the viral construct.

Dr. N. Chen provided viral DNA and RNA from both, virus infected and control tumor cells.

Dr. Y.A. Yu provided all infected and uninfected tumor xenografts.

Dr. Q. Zhang assisted with computerized VACV genome regulation analyses.

Dr. Z. Pos provided help for statistical interpretation of the results.

Ms. V. Raab and Dr. S. Weibel carried out all tumor immunohistochemistry .

Dr. M. Sabatino, Dr. V. Monsurró, Dr. R. M. Buller and Dr. D. F. Stroncek contributed to the data analysis of findings and helped in writing the paper.

Mr. A. Monaco and Ms. H.Liu helped in cDNA sequence analyses.

T. Trevino provided all cell cultures.

Dr. E. Wang and Dr. F. M. Marincola supervised microarray experiments and gene expression profiling, participated in designing the study and helped to write the paper.

Prof. Dr. A. A. Szalay supervised and designed the study and helped to write the paper.

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# Systemic treatment of xenografts with vaccinia virus GLV-1h68 reveals the immunologic facet of oncolytic therapy

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## Abstract

**Background:** GLV-1h68 is an attenuated recombinant vaccinia virus (VACV) that selectively colonizes established human xenografts inducing their complete regression.

**Results:** Here, we explored xenograft/VACV/host interactions *in vivo* adopting organism-specific expression arrays and tumor cell/VACV *in vitro* comparing VACV replication patterns. There were no clear-cut differences *in vitro* among responding and non-responding tumors, however, tumor rejection was associated *in vivo* with activation of interferon-stimulated genes (ISGs) and innate immune host's effector functions (IEFs) correlating with VACV colonization of the xenografts. These signatures precisely reproduce those observed in humans during immune-mediated tissue-specific destruction (TSD) that causes tumor or allograft rejection, autoimmunity or clearance of pathogens. We recently defined these common pathways in the "immunologic constant of rejection" hypothesis (ICR).

**Conclusion:** This study provides the first prospective validation of a universal mechanism associated with TSD. Thus, xenograft infection by oncolytic VACV, beyond offering a promising therapy of established cancers, may represent a reliable pre-clinical model to test therapeutic strategies aimed at modulating the central pathways leading to TSD; this information may lead to the identification of principles that could refine the treatment of cancer and chronic infection by immune stimulation or autoimmunity and allograft rejection through immune tolerance.

## Background

In the past, we applied inductive reasoning [1] to identify immunologic signatures associated with tumor rejection, clearance of pathogen, acute allograft rejection or autoimmunity. This exercise leads to the formulation of the "immunologic constant of rejection" (ICR) hypothesis: "*immune-mediated tissue specific destruction (TSD) follows a common final pathway independent of the originating cause and the disease context*" [2]. 4 axioms were proposed at the basis of the ICR: *i*) TSD does not necessarily occur because of non-self recognition but also occurs against self or quasi-self; *ii*) the requirements for the induction of a cognate immune response differ from those necessary for the activation of an effector one; *iii*) although the prompts leading to TSD vary in distinct pathologic states, the effector immune response converges into a single mechanism; and *iv*) adaptive immunity participates as a tissue-specific trigger, but it is not always sufficient or necessary. Here, we applied deductive reasoning to test whether immunologic markers of ICR could be predictably observed in a controllable experimental model. We selected a promising pre-clinical endeavor where the systemic administration of oncolytic VACV induces xenograft regression in immune deficient mice through an, at least in part, immunologically-mediated mechanism. In addition, we tested the validity of the fourth axiom, which postulates, in concordance with others' observations, that tumor rejection does not require adaptive immunity [3].

Systemic delivery of oncolytic viruses leads to their specific localization to established tumors and to viral replication followed by oncolysis [4]. VACV, in particular, possesses strong antitumor properties [5] while its history as vaccine against smallpox proves it safe in humans. Further attenuation by disruption of non essential viral genes such as J2R (coding for thymidine kinase) [6,7] and A56R (coding for haemagglutinin) [8] increased the therapeutic potential of VACV as an oncolytic agent. In addition, the same construct could be used for tumor-specific delivery of light-emitting proteins for real-time imaging [9] or therapeutic proteins such as tumor suppressors [10], anti-angiogenesis factors [11] or immune modulators [12]. The design of a VACV construct, GLV-1h68, derived from LIVP wild-type strain by insertion of 3 expression cassettes encoding *Renilla* luciferase-*Aequorea* green fluorescent fusion protein (RUC-GFP), β-galactosidase and β-glucoronidase [9,13] lead to a highly attenuated oncolytic virus capable of targeting established human xenografts. The ability to replicate specifically within tumors and to leave non malignant tissues virus-free makes GLV-1h68 systemic administration a promising pre-clinical tool capable of safely eradicating pancreatic cancer [14], malignant pleural mesothelioma [15], breast carcinoma GI-101A xenografts [13] and anaplastic thyroid cancer [16].

Eradication of established human breast cancer GI-101A xenografts can be reproducibly induced in nude mice injected intravenously with $1 \times 10^7$ plaque forming units (PFU) of GLV-1h68. Tumor eradication occurs in 95% of treated animals within 130 days from injection and it is associated with pristine tropism of GLV-1h68 for the xenograft and lack of systemic toxicity or mortality. Because GLV-1h68 encodes a luciferase reporter, it is possible to estimate kinetically virus titers in tumor xenografts and correlate this parameter with treatment outcome [13].

Experimental observations demonstrated a tight relationship between virus replication within the tumor xenograft and response to oncolytic treatment. However, the mechanisms leading to tumor regression by oncolytic virus remain unknown [17,18]. While it is possible that a direct oncolytic activity may be responsible for tumor regression, it is also possible that tumor eradication is the result of a complex interplay among virus, cancer cells and the host [19]. Expression profiling of xenografts responding to treatment with GLV-1h68 based on a mouse-specific platform and hence representative of the host's response to the GLV-1h68-infected human xenograft suggested that their eradication is associated with the over-expression of signatures consistent with innate immune defense activation. These signatures are inclusive of interferon-stimulated genes (ISGs) such as *STAT-1* and *IRF-7*, chemokines (*Ccl2*, *Ccl9*, *Ccl27*, *Cxcl9*, *Cxcl10*, *Cxcl12*), chemokine receptors (*Ccr2*), interleukins (*IL-18*) and innate immune effector functions (IEF) [13]. The participation of immune cells was supported by immunohistochemistry, which demonstrated active peri-tumoral and intra-tumoral infiltration of monocytes in treated samples [13]; however, the specificity of the association between xenograft eradication and immune activation could not be determined since non-responding xenografts were not included in the previous analysis.

We hypothesized that in this model the eradication of responding xenografts is, at least in part, mediated through innate immune mechanisms and, as a consequence, this model could provide important insights about the role played by innate immunity in mediating tissue rejection in the immune incompetent host [2]. Recent animal studies suggest that immune-mediated eradication of syngeneic tumors is independent of adaptive immune responses [3], and the involvement of cytotoxic T cells may provide primarily help for the *in situ* targeting and/or activation of innate immune effector cells [20]. Therefore, progression of events leading to xenograft rejection in a mouse model deprived of adoptive immune function may simplify the identification of the requirements for tumor rejection and, more broadly, those necessary for TSD [2].

To determine which innate responses and virus replication characteristics specifically correlated with oncolytic GLV-1h68-mediated tumor rejection, we tested human cancer cell lines of different tissue derivation for their *in vitro* permissivity to GLV-1h68 replication, their *in vivo* colonization and their susceptibility to VACV-mediated eradication. In addition, we used 3 array platforms to characterize VACV, human (tumor cells) and mouse (inflammatory cells) gene expression in GLV-1h68-infected xenografts *in vivo*. The results demonstrated that tumor rejection is associated with activation of innate immune mechanisms in the host that recapitulate faithfully the biological pathways observed in association with immune-mediated TSD in humans [2]. Thus, this model suggests that immune rejection does not depend upon adaptive immunity as long as the initiating mechanism (in this case selective viral localization at the tumor) is specific to a particular tissue. The demonstration that immune deficient mice can reject human xenograft following a pathway common to other human immune pathologies suggests that the ICR is a universal phenomenon across species and may represent a target for immune modulation in the context of various diseases.

## Results

### *Variability of xenograft responses to the systemic administration of GLV-1h68*

A panel of human cancer cell lines of different histological derivation was tested for their sensitivity to the oncolytic activity of intra-venously injected GLV-1h68 [21-25]. 2 characteristic patterns were identified: some cell lines progressively continued their growth independent of therapy (i.e. HT-29), while others followed 3 growth phases: first, a slightly faster growth of infected compared to control tumors, then a period of no or minimal growth, and finally, complete regression (i.e. Gl-101A) [13] (Figure 1A). Tumor growth or regression patterns were cell line-specific, highly reproducible, and independent of number of cancer cells administered or dose of GLV-1h68 injected [13]. A therapeutic index (T.I.) was calculated to provide a single parameter descriptive of each cell line's responsiveness to VACV therapy by integrating the areas between the median growth of control and treated xenografts (eight animals in each group in all experiments described here and thereafter) (Table 1). The same cell lines were subjected to an *in vitro* assay in which their permissivity to GLV-1h68 replication was tested (**data not shown**, see Additional file 1). We observed that 3 of 3 cell lines that resisted replication during the first 24 hours following infection (MDA-MB-231, SiHa and NCI-H1299) uniformly produced xenografts partially or non-responding to VACV therapy *in vivo*. However, 8 of 10 cell lines that allowed viral replication in the first 24 hours yielded xenografts responsive *in vivo* to VACV treatment while 2 (HT-29 and 1936-MEL) yielded xenografts that did not respond. The relationship between the permissivity of a given cell line to *in vitro* replication of GLV-1h68 and the *in vivo* responsiveness of the corresponding xenograft was significant (Fisher exact test $p_2$-value = 0.005) but not absolute.

Of particular interest was a pair of cell lines: the colorectal carcinoma HT-29 (non responding) and the breast adenocarcinoma GI-101A (responding) cell lines [13] that displayed in vitro a similar degree of permissivity to VACV replication. Since the distinct behavior of the 2 cell lines could have been due to their diverse ontogeny [26], we tested a pair of autologous melanoma cell lines, 888-MEL and 1936-MEL derived from the same progenitor cell clone though established from 2 metachronous metastases [24,27]; 888-MEL was generated in 1989 during the earlier stage of disease at a time when the patient underwent a complete remission following adoptive transfer of tumor infiltrating lymphocytes; 1936-MEL was expanded 12 years later from a metastasis excised at a time when the patient was rapidly progressing and did not respond to further therapy [24]. The cell lines displayed the same degree of permissivity in vitro to GLV-1h68 replication, but yielded xenografts with disparate sensitivity to VACV treatment in vivo (Table 1). These data suggest that even in autologous systems responsiveness is related to biological characteristics of the tumors that are independent of their ontogeny, and are more likely related to evolving phenotypes during the natural history of the disease.

We then analyzed VACV replication *in vivo* in a responding (GI-101A), a non-responding (HT-29) and another presently less characterized (PC-3) line. Twenty-one days post GLV-1h68 administration; viral titers were lower in non-responding xenografts (Figure 1B). The difference was less pronounced after 42 days, suggesting that the lack of responsiveness to oncolytic therapy may be associated with delayed but not completely absent VACV replication.

### *Transcriptional profiling of VACV/tumor/host interactions*

To gain better insights on the mechanisms governing xenograft rejection, we compared simultaneously the transcriptional patterns of VACV, human cancer cells and mouse host cells in responding and non-responding xenografts excised at time points associated with tumor and viral growth (day 21 after injection) or at the plateau phases preceding tumor rejection (day 42). This was achieved by the adoption of organism-specific platforms.

### *Transcriptional differences between xenografts responding or non-responding to systemic GLV-1h68 administration: the VACV signatures*

VACV-gene expression was assessed by a custom-made VACV array platform to compare the expression of VACV transcripts *in vivo* in the responding GI-101A and the non-

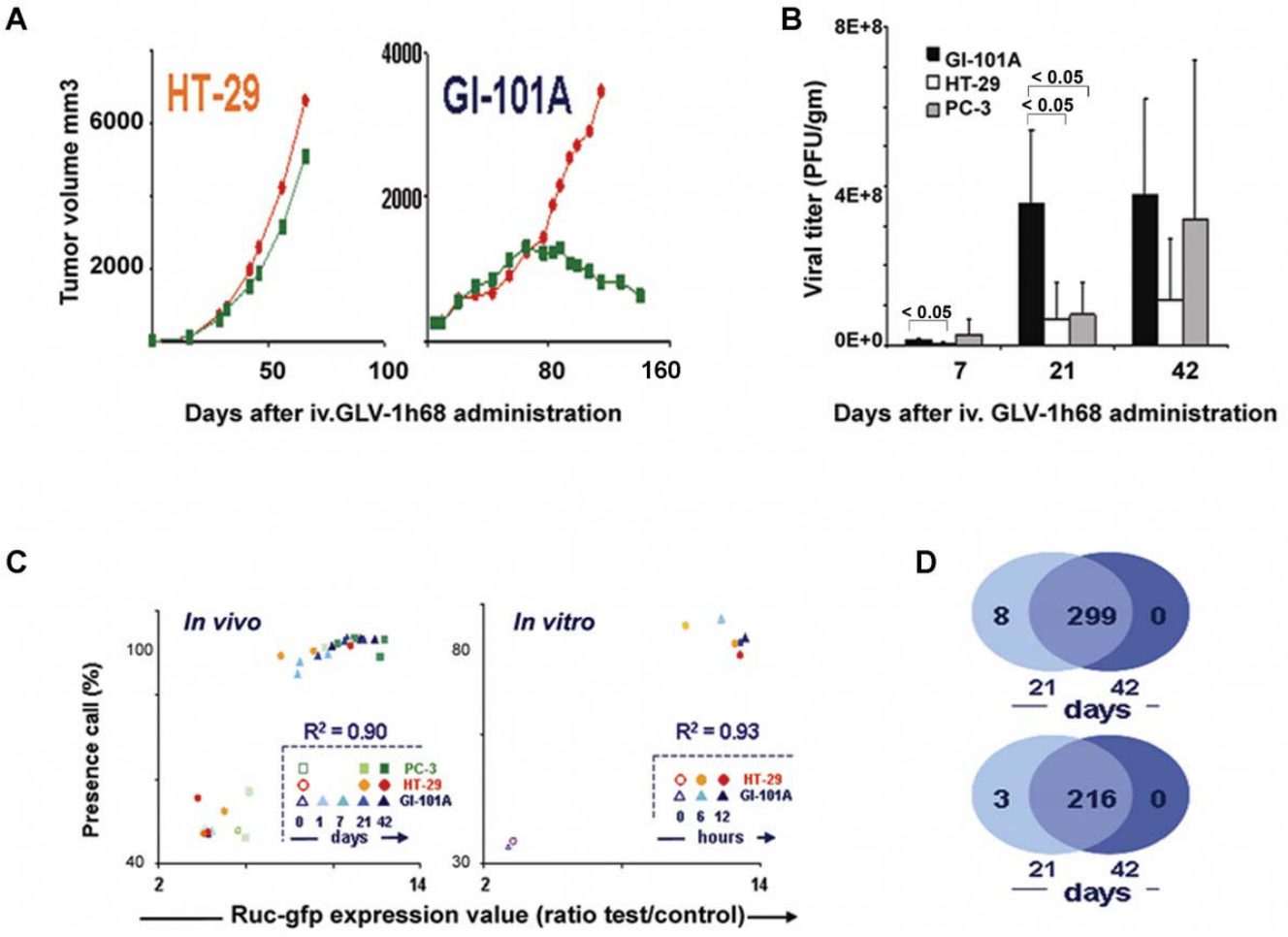


**Figure 1**

**Characterization of human xenografts and Vaccinia Virus signatures**. (**A**) Representative growth curves (n = 8 animals) for 2 xenografts from HT-29 and GI-101A cell lines; red dots represent control, green boxes the post treatment groups; for further details about the xenograft model refer to references [9,13]. (**B**) Viral titer (PFU/gram of xenograft; n = 4) comparing the permissivity of 3 xenografts derived from GI-101A, HT-29 and PC-3 cell line whose responsiveness is under characterization 7, 21 and 42 days after GLV-1h68 administration. (**C, left**) Scatter plot correlating the level of *Renilla* luciferase-*Aequorea* green fluorescent protein messenger RNA expression with the presence call of probes above the set threshold level for the VACV expression array platform. VACV gene expression in non-infected PC-3, HT-29 and GI-101A xenografts was compared to infected xenografts 1, 7 (GI-101A only), 21 and 42 days before. High presence call (> 40%) in the non-infected xenografts could be expected due to the large number of mouse and human housekeeping genes present in the array platform); $R^2$ value refers to the correlation between RUC-GFP expression and number of VACV transcripts significantly up regulated in the same experiment. (**C, right**) Scatter plot as per panel **C, left**, comparing *in vitro* VACV gene expression of GLV-1h68-infected HT-29 and GI-101A cell lines at 6 and 12 hours with controls. (**D**) A Venn diagram displays the extent of overlap among VACV-specific probes (top) and VACV genes (bottom) differentially expressed by infected GI-101A xenografts at day 21 and 42 compared with day 1 after GLV-1h68 injection.

responding HT-29 (characterized by normal *in vitro* but delayed *in vivo* replication) xenografts. VACV transcriptional patterns correlated perfectly with viral titers. Moreover, in all cell lines there was a perfect correlation between RUC-GFP expression and overall expression of VACV genes ($R^2$ = 0.90) suggesting that this reporter gene accurately represents GLV-1h68 replication (Figure 1C). Variation in VACV gene expression was observed among cell lines or among xenografts derived from the same cell line. Furthermore, a clear dichotomy was observed in transcriptional patterns: VACV transcripts were either all up regulated or completely silent suggesting that the transition from early to late VACV gene expression occurred in rapid succession that could not be discriminated by the time points examined. Most GI-101A xenografts demonstrated early *in vivo* replication with 3 out of 4 expressing

**Table 1: Therapeutic Index (T.I) of responding compared to non-responding xenografts**

| Responders (R) | | T.I. | Poor/Non-Responders (NR) | | T.I. |
|---|---|---|---|---|---|
| 1858-MEL | Melanoma | 90.1 | MDA-MB-231 | Breast Adenocarcinoma | 21.6 |
| 888-MEL | Melanoma | 88.0 | SiHa | Cervical Squamous Cell Carcinoma | 15.6 |
| MIA PaCa-2 | Pancreatic Carcinoma | 80.1 | 1936-MEL | Melanoma | 13.7 |
| A549 | Lung Carcinoma | 62.8 | NCI-H1299 | Breast Adenocarcinoma | -2.3 |
| OVCAR-3 | Ovarian Adenocarcinoma | 56.2 | HT-29 | Colorectal Carcinoma | -19.0 |
| Panc-1 | Pancreatic Carcinoma | 50.9 | | | |
| DU-145 | Prostate Carcinoma | 48.4 | | | |
| GI-101A | Breast Carcinoma | 27.9 | | | |

VACV genes at day 7, and 4 out of 4 at day 21 and 42. In contrast, HT-29 suffered delayed replication *in vivo* with only a proportion displaying full VACV gene expression at day 21 (2 of 4 in either case). After 42 days the expression of VACV genes was turned on only in 1 of 4 HT-29 xenografts. VACV gene expression analysis confirmed lack of differences in VACV transcriptional patterns *in vitro* between HT-29 and GI-101A with 3 out of 3 cell cultures demonstrating active viral replication in either case in the first 12 hours after infection (Figure 1C).

Subsequently, comparisons were made between infected and non-infected GI-101A and HT-29 xenografts. Even though there might have been better responder/non-responder pairs to choose from we selected HT-29 tumors among non-responding cell lines because of its similar *in vitro* permissivity to GLV-1h68 that corresponded to a different behavior *in vivo* and previous characterizations in our laboratory. A high-stringency ($p_2$-value < 0.005) Student *t* test comparing the number of VACV genes differentially expressed at day 21 or 42 from infected animals with those from uninfected ones identified significant differences (multivariate permutation p-value < 0.001) only in GI-101A xenografts at day 21 and day 42 (Table 2). As previously discussed, the number of genes differentially expressed in xenografts with replicating VACV reflected completely the number of VACV-specific annotations present in the VACV-array platform demonstrating that GLV-1h68 replication is either absent or complete in xenografts at this time point. As to be expected, an almost complete overlap of VACV probes or genes expressed at day 21 and 42 was observed in the GI-101A xenografts (Figure 1D). Notably, a reverse behavior was observed in the pattern of expression of human house keeping genes represented in the VACV array platform. The expression of these genes was profoundly down-regulated in permissive cell lines suggesting a shut off of cellular metabolism in infected cells that correlated inversely with viral transcription as described by others [28]. It is noteworthy that, although HT-29 did not display significant up regulation of VACV genes using the high stringency parameters adopted here, it displayed a similar trend in gene expression with mild up-regulation of VACV genes and expression of GFP messenger RNA in some but not all xenografts (Figure 1C).

### *Transcriptional differences between xenografts responding or non-responding to systemic GLV-1h68 administration: the human cancer signatures*

A time course analysis evaluating the *in vivo* effects of viral replication on the permissive GI-101A human xenografts was performed using a custom-made 17.5 k human cDNA array platform [29]. 4 experimental groups of 4 mice each received systemic GLV-1h68 administration 1, 7, 21 and 42 days before xenograft excision (Figure 2A[30]). The transcriptional profile of infected GI-101A tumors was altered significantly by 21 days and increasingly so at 42 days after GLV-1h68 administration. Since the time course demonstrated that even in permissive xenografts significant changes occurred only at 21 and 42 days, we limited the subsequent analysis to these time points.

To better characterize the transcriptional program of VACV-infected cancer cell lines, we compared responding (GI-101A) and non-responding (HT-29) xenografts using a 36 K whole genome, human oligo array platform at day 21 and 42. Multiple dimensional scaling based on the complete data set demonstrated that infected GI-101A xenografts (2 darker blue color) segregated completely in Euclidian space from non-infected xenografts (2 lighter

**Table 2: Number of genes over-expressed in xenografts excised from GLV-1h68 infected animals**

| Experimental group | Days after VACV inj. | | | | |
|---|---|---|---|---|---|
| **VACGLa 520445F, Affymetrix platform** | | VACV only | Permutation | House keeping (human/mouse) | Permutation |
| (cut off $p^2$-value < 0.005 (unpaired Student *t* test) | | (∑219) | test p-value | (∑337) | test p-value |
| GI-101A | 7 | 0 | N.S. | 3 | N.S. |
| GI-101A | 21 | 219 | < 0.001 | (232) | < 0.001 |
| GI-101A | 42 | 216 | < 0.001 | (237) | < 0.001 |
| HT-29 | 21 | 0 | N.S. | (3) | N.S. |
| HT-29 | 42 | 0 | N.S. | (10) | N.S. |
| | | | | | |
| **37 K whole genome HUMAN array** | | All genes | Permutation | | |
| (cut off $p^2$-value < 0.001 (unpaired Student *t* test) | | (∑37 K) | test p-value | | |
| GI-101A | 21 | 136 | < 0.05 | | |
| GI-101A | 42 | 91 | < 0.05 | | |
| HT-29 | 21 | 4 | N.S. | | |
| HT-29 | 42 | 10 | N.S. | | |
| | | | | | |
| **37 K whole genome Mouse array** | | All genes | Permutation | | |
| (cut off $p^2$-value < 0.001 (unpaired Student *t* test) | | (∑37 K) | test p-value | | |
| GI-101A | 21 | 105 | < 0.05 | | |
| GI-101A | 42 | 1026 | < 0.001 | | |
| HT-29 | 21 | 7 | N.S. | | |
| HT-29 | 42 | 14 | N.S. | | |

* In parenthesis, genes down-regulated in GLV-1h68-infected animals referring to human/mouse house-keeping genes in the VACV chip

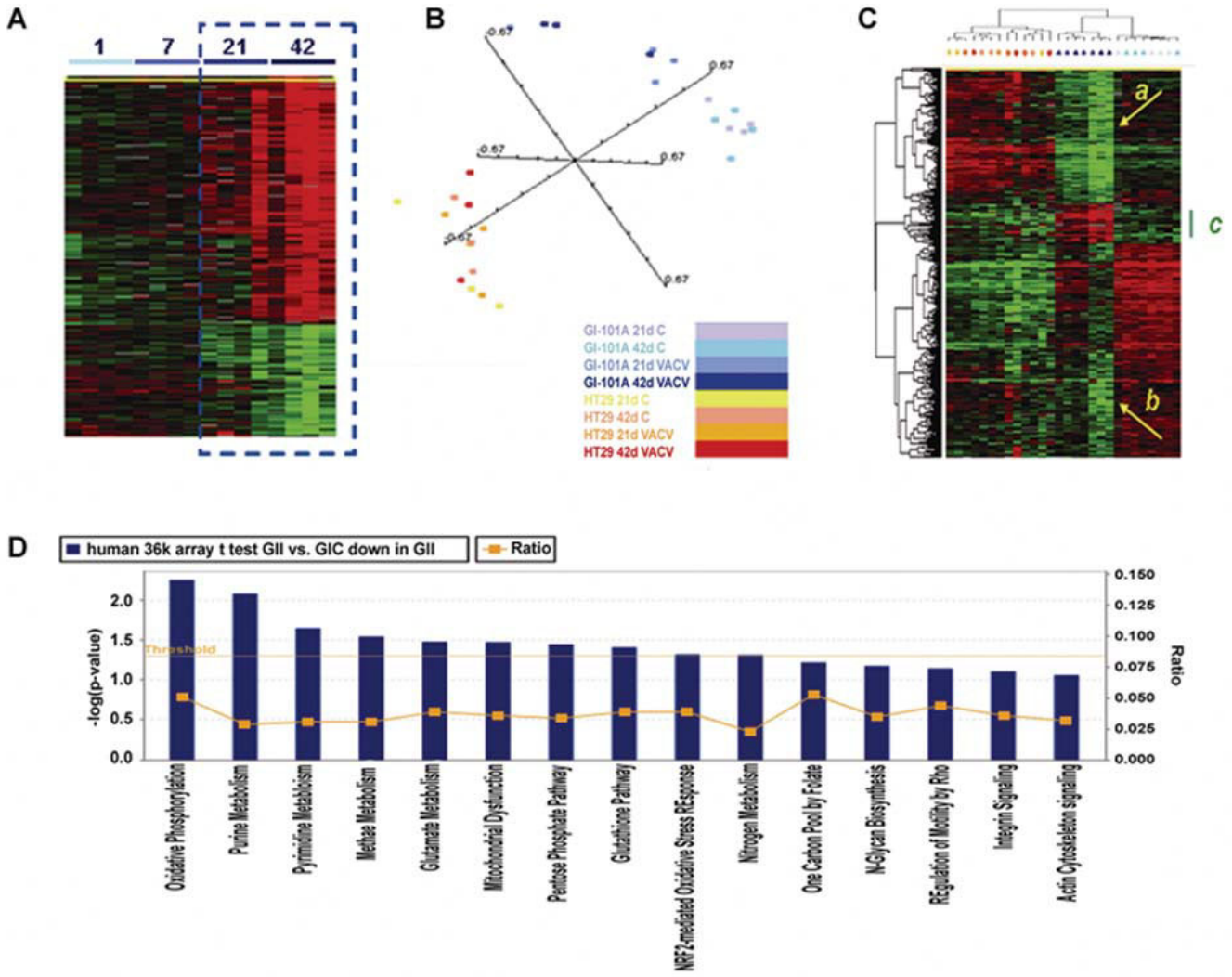


**Figure 2**

**Human Cancer signatures**. (**A**) Time course analysis of infected GI-101A xenografts (parameters for gene selection; F test p-value < 0.005, 80% presence call, ratio of > 2 and false discovery rate < 0.1). Gene distribution is shown based on 893 genes of 17,500 present in the human cDNA platform that passed the statistical criteria and presented according to Spearman rank correlation. The dashed box outlines the 2 time points most affected by VACV infection. The heat map information is presented according to the central method for normalization [30]. (**B**) Multiple dimensional scaling based on the 36 k oligo array human platform comparing HT-29 and GI-101 xenografts. (**C**) Self organizing heat map based on 841 out of 1,073 genes differentially expressed between GI-101A xenografts from infected compared to non-infected mice that passed the standard filter conditions (presence call in at least 80% and at least 3 fold ratio change). HT-29 samples are also represented as a reference, color coding of samples is as per panel (B). The green bar underlines the genes specifically expressed by GI-101A xenografts from infected animals; the 2 yellow arrows (*a*) and (*b*) point at genes who expression was profoundly depressed in xenografts from infected animals. (**D**) Ingenuity pathway analysis showing canonical pathways significantly down-regulated in GI-101A xenografts at day 41 following GLV-1h68 injection; IPA analysis based on an unpaired, two-tailed Student *t* test comparing infected to non-infected GI-101A xenografts at day 42 (threshold $p_2$-value < 0.001).

blue colors) while HT-29 xenografts intermingled whether they were from infected or non-infected animals (Figure 2B).

To test overall differences between xenografts from infected and non-infected animals, we applied a Student *t* test (cut-off $p_2$-value < 0.001) comparing infected to non-infected GI-101A and HT-29 xenografts. Comparison of GI-101A identified 1,073 genes differentially expressed between infected and non-infected xenografts (permutation test p value = 0). On the contrary, only 9 genes were found to be differentially expressed by HT-29 xenografts from infected compared to non-infected animals (permutation test non significant). Among genes differentially expressed in the

GI-101A xenografts from GLV-1h68 infected animals, the large majority were down-regulated, particularly, in xenografts excised at day 42 suggesting that viral replication depresses cellular metabolism (Figure 2C, yellow arrows annotated with *a* and *b*) consistent with the down-regulation of house keeping genes observed in the VACV-chip. Ingenuity pathway analysis (IPA) demonstrated that, among the canonical pathways, the top 10 categories of down-regulated genes represented depressed cellular function including alterations in oxidative pathways, mitochondrial dysfunction, and disruption of purine, pyrimidine and amino acid metabolism (Figure 2D). Interestingly, a smaller cluster of genes was over-expressed in GI-101A xenografts from infected animals (green bar, Figure 2C, Table 2). Among these genes, allograft inflammatory factor-1 (AIF-1), the tissue inhibitor of metalloproteinase 2 (TIMP-2) and the IL-2 receptor common γ chain were up regulated. Moreover, a multivariate analysis (F test, p-value cutoff < 0.001) based on the oligo arrays comparing the 4 groups at day 21 and 42 (HT-29 and GI-101A in infected and non-infected mice) identified respectively 2,241 and 1,984 clones differentially expressed among the 4 groups. Analysis with the 17 k cDNA arrays similarly identified 1,467 cDNA clones representative of the 4 categories at day 42. In both platforms, most of the differences in expression pattern involved tumor cell specific genes and both platforms segregated the HT-29 xenografts from GI-101A xenografts independent of GLV-1h68 administration according to their different ontogeny; a phenomenon we have previously described [26]; however, a subgroup of genes was observed to be specific for GI-101A infected xenografts. The GLV-1h68 infection-specific signatures were enriched for up regulated genes associated with immune function with a significantly higher than expected frequency (1.88) according to GeneOntology assignment. Among the genes up-regulated in the GI-101A xenografts excised from GLV-1h68 infected mice, several were associated with activation of innate immune mechanisms including the Toll-like receptor (TLR)-2, the interferon regulatory factor (IRF)-7, signal transducer and activator of T cell (STAT)-3 and tumor necrosis factor (TNF)-α. This enrichment was not observed in oligo-based arrays suggesting that these signatures could be potentially attributed to host infiltrating immune cells whose genes could cross-hybridize to the less stringent cDNA array probes; this could occur in spite of the intensity filter adjustment for sequences with high mouse to human similarity. Sequence verification, demonstrated that only STAT-3 and IRF-7 were indeed expressed by human cells while the other genes were mouse transcripts cross hybridizing to the cDNA probes but not to the more stringent oligo-probes (see Additional file 2).

In summary, analysis of human transcripts demonstrated that differences among xenografts from infected and non-infected mice are non-existent in non-responding tumors and limited to a small set of up-regulated genes in responding tumors several of them representing over-expression of host's genes cross hybridizing to the human platform. Most transcriptional differences in responding tumors were instead due to the shut down of cellular metabolisms induced by active viral infection while little evidence of apoptotic or necrotic induction by the oncolytic process could be identified at this early time point suggesting that at day 42 cells are starting to be strongly altered in their metabolism but are still alive; this finding correlates with the presence of viable cancer cells observed by histopathological examination [13].

### *Transcriptional differences between xenografts responding or non-responding to systemic GLV-1h68 administration: the mouse host's signatures*

To define the host's involvement in the early phases of the oncolytic process when tumor cells are still present and alive [13], we analyzed HT-29 and GI-101A xenografts using a custom-made, whole genome 36 K mouse array platform. In this case, all 4 GI-101A xenografts excised at day 42 from infected mice could be utilized while only 3 of 4 could be utilized for the human arrays because of degradation of human mRNA in one of the regressing xenografts. Gene expression was affected significantly only in GI-101A xenografts (Table 2). A statistical overview of gene expression modulation of GI-101A xenografts from GLV-1h68 infected grafts gave a completely opposite picture compared to the human arrays in which a predominant down-regulation of cellular metabolism was observed. In particular, most mouse genes were up regulated in xenografts excised from infected animals suggesting that, while the metabolism of cancer cell was declining (Figure 2C), the host response was enhanced (Figure 3A). An F test was performed for a global comparison of all experimental groups; at day 21, 1,066 genes demarcated the differences among the 4 experimental groups. This number increased to 1,471 by day 42 (permutation test p-value < 0.001 in either case).

At day 21, IPA reveled that the 2 canonical pathways predominantly affected in GI-101A xenografts from VACV-infected mice reflected chemokine and IFN signaling (Figure 3B). At day 42, additional canonical pathways became affected including those associated with cellular stress (Figure 3C). There was significant overlap among the 2 time points when only genes associated with immune function were compared (Table 3 and 4), while most differences between the 2 time points were observed among genes associated with cellular stress and altered metabolism. Since this manuscript focuses on the immune aspects of oncolytic therapy, we will restrict the discussion to immunologic signatures from now on.

In general, immunologic differences between the early (day 21 from VACV injection) and the later (day 42) time points were quantitative rather than qualitative, There-

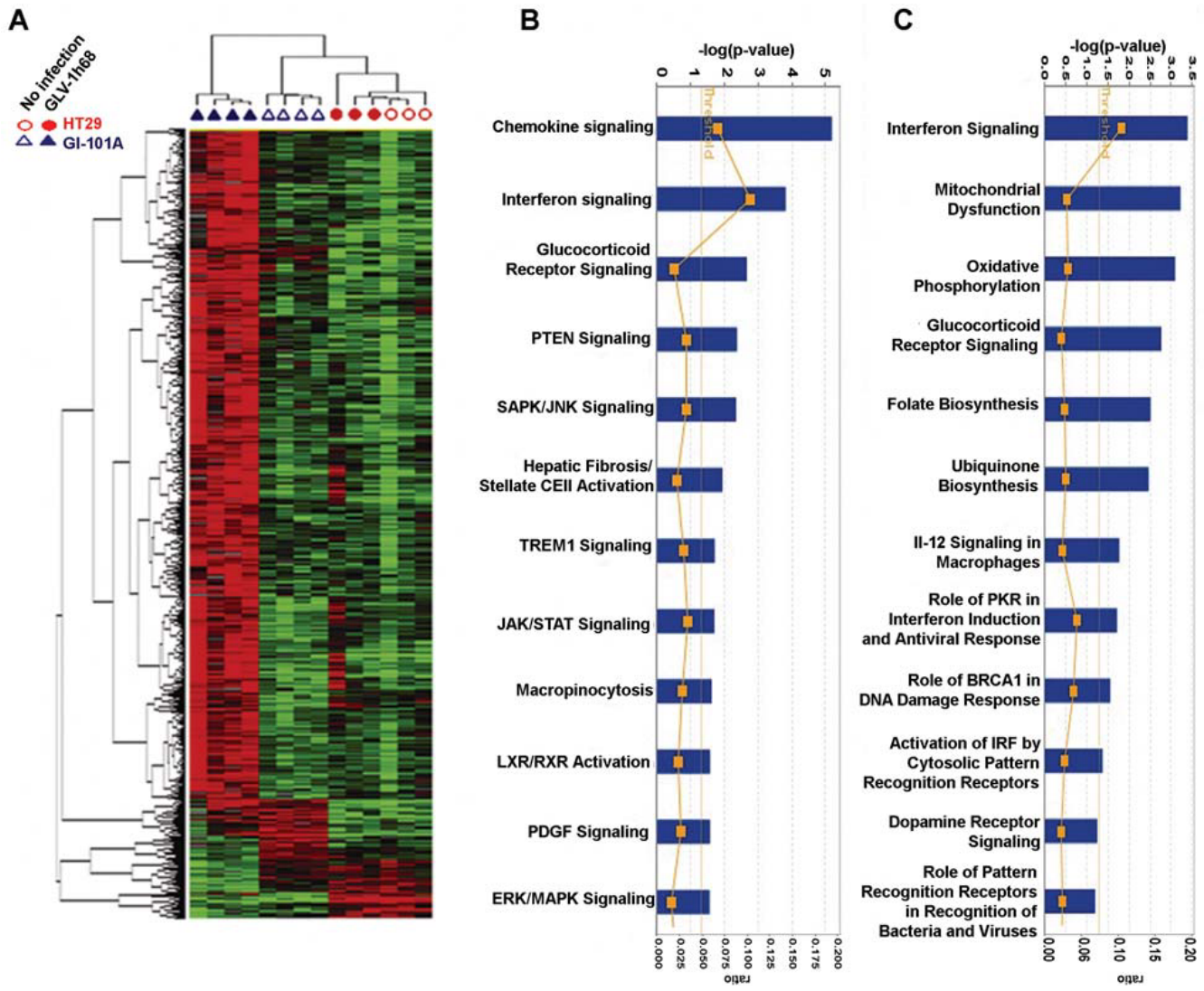


**Figure 3**

**Mouse host's signatures**. (**A**) Self-organizing heat map of mouse genes differentially expressed among the 4 experimental groups (HT-29 and GI-101A xenografts from GLV-1h68-infected or non-infected mice) according to an F test at day 42 after infection. Standard filter was applied (80% presence call, 3 fold ratio cut off) that allowed 819 out of 1,066 genes at day 21 and 1,159 out of 1,471 genes at day 42. IPA of canonical pathways over-induced in infected compared to non-infected GI-101A xenografts at day (**B**) 21 and (**C**) 42 based on an unpaired, two-tailed Student *t* test comparing infected to non-infected GI-101A xenografts (threshold $p_2$-value < (A) 0.001 and (B) 0.0001).

fore, although some transcripts from VACV infections were significantly up regulated at day 21 a similar trend could be observed at day 42 though it did not reach the same level of statistical significance (F-test p-value < 0.001). Additionally, in the majority of cases, the host's transcription was enhanced at the later time point when tumor growth reached a plateau and the rejection process was presumed to start.

Among interleukins, IL-18 and the IL-18 binding protein played a prominent role early in the course of infection, while later IL-15 became increasingly up regulated (Table 3 and 4). CCR2, CCR3 and CCR5 ligand chemokines played a predominant role at day 21 while CXCR3 and CXCR4 ligand chemokines up-regulation became more prominent later. Among the CXCL chemokines, CXCL-12/SDF-1 was previously associated with the rejection of metastatic melanoma during IL-2 therapy [31] and together with CXCL-9 through -11 chemokines in association with the rejection of basal cell carcinomas (BCCs) treated with TLR7 agonists [32]. ISGs and other genes associated with the IFN signaling were among the most

**Table 3: Mouse immune genes up-regulated in regressing GI-101A tumors at day 21 (F test p2-value < 0.001)**

| Gene LLID # | Symbol | Name | HT-29 Control | HT-29 GLV-1h68 | GI-101A Control | GI-101A GLV-1h68 |
|---|---|---|---|---|---|---|
| **Interleukins** | | | | | | |
| 16068 | *Il18bp* | *interleukin 18 binding protein* | 0.58 | 0.98 | 1.00 | 5.70 |
| 16173 | *Il18* | *Il18 – interleukin 18* | 1.24 | 1.25 | 1.00 | 4.10 |
| **Chemokines** | | | | | | |
| 20296 | **Ccl2** | **Ccl2/MCP-1 (human nomenclature)** | 1.65 | 2.12 | 1.00 | 10.84 |
| 20292 | **Ccl11** | **Ccl11/Eotaxin** | 0.64 | 1.10 | 1.00 | 9.47 |
| 20307 | **Ccl8** | **Ccl8/MCP-2** | 1.51 | 3.30 | 1.00 | 7.16 |
| | *Cxcl11* | *CXCL11/I-TAC* | 0.82 | 1.04 | 1.00 | 5.69 |
| 17329 | **Ccl12** | **Ccl12/MCP-5** | 1.22 | 1.44 | 1.00 | 4.97 |
| 20306 | *Ccl7* | *Ccl7/MCP-3.MARC* | 0.72 | 0.90 | 1.00 | 4.71 |
| | **Cxcl10** | **Cxcl10/IP-10** | 0.83 | 0.84 | 1.00 | 4.08 |
| 17329 | *Cxcl9* | *Cxcl9/Mig* | 0.72 | 0.72 | 1.00 | 3.80 |
| 20308 | *Ccl9* | *Ccl9/MRP-2/CCF18/MIP-1g* | 1.06 | 1.08 | 1.00 | 3.12 |
| 20315 | *Cxcl12* | *Cxcl12/SDF-1/PBSF* | 0.75 | 0.84 | 1.00 | 3.07 |
| 20304 | *Ccl5* | *Ccl5/RANTES* | 0.66 | 0.97 | 1.00 | 2.56 |
| **ISGs** | | | | | | |
| 16145 | *Igtp* | *interferon gamma induced GTPase* | 0.50 | 1.13 | 1.00 | 10.11 |
| 76933 | *Ifi27* | *interferon, alpha-inducible protein 27* | 0.87 | 1.14 | 1.00 | 10.04 |
| 231655 | **Oasl1** | **2'-5' oligoadenylate synthetase-like 1** | 0.66 | 0.73 | 1.00 | 6.93 |
| 17857 | *Mx1* | *myxovirus (influenza virus) resistance 1* | 0.77 | 0.89 | 1.00 | 6.41 |
| 16145 | *Igtp* | *interferon gamma induced GTPase* | 0.89 | 0.80 | 1.00 | 5.62 |
| | *Ifi204* | *interferon activated gene 204* | 0.71 | 1.06 | 1.00 | 5.44 |
| 246730 | **Oas1a** | **2'-5' oligoadenylate synthetase 1A** | 0.64 | 1.16 | 1.00 | 5.38 |
| | *Iigp2* | *interferon inducible GTPase 2* | 0.63 | 0.83 | 1.00 | 5.25 |
| 26388 | **Ifi202b** | **interferon activated gene 202B** | 0.54 | 1.17 | 1.00 | 4.61 |
| | *Ifi47* | *interferon gamma inducible protein 47* | 0.54 | 0.67 | 1.00 | 4.56 |
| 15957 | *Ifit1* | *interferon-induced protein with tetratricopeptide repeats 1* | 1.06 | 0.94 | 1.00 | 4.15 |

**Table 3: Mouse immune genes up-regulated in regressing GI-101A tumors at day 21 (F test p2-value < 0.001)** *(Continued)*

| | | | | | | |
|---|---|---|---|---|---|---|
| 20847 | **Stat2** | **signal transducer and activator of transcription 2** | 0.81 | 0.85 | 1.00 | 4.06 |
| 20846 | *Stat1* | *signal transducer and activator of transcription 1* | 0.74 | 0.68 | 1.00 | 4.02 |
| 16362 | *Irf1* | *interferon regulatory factor 1* | 0.91 | 1.21 | 1.00 | 3.78 |
| 65972 | **Ifi30** | **interferon gamma inducible protein 30** | 0.64 | 0.70 | 1.00 | 3.67 |
| 246728 | **Oas2** | **2'-5' oligoadenylate synthetase 2** | 1.08 | 1.10 | 1.00 | 3.29 |
| | | | | | | |
| **Other** | | | | | | |
| 17067 | *Ly6c* | *lymphocyte antigen 6 complex, locus C* | 0.90 | 0.99 | 1.00 | 5.77 |
| 17067 | *Ly6c* | *Lymphocyte antigen 6 complex, locus C* | 0.95 | 1.12 | 1.00 | 5.27 |
| 17071 | *Ly6f* | *lymphocyte antigen 6 complex, locus F* | 1.01 | 1.08 | 1.00 | 5.01 |
| 20715 | **Serpina3g** | **serine (or cysteine) peptidase inhibitor, clade A, member 3G** | 0.88 | 0.68 | 1.00 | 4.77 |
| 18636 | **Cfp** | **complement factor properdin** | 0.75 | 0.86 | 1.00 | 4.62 |
| 15331 | **Hmgn2** | **high mobility group nucleosomal binding domain 2** | 1.05 | 1.05 | 1.00 | 4.30 |
| 13032 | **Ctsc** | **cathepsin C** | 0.60 | 0.60 | 1.00 | 4.24 |
| 64685 | **Nmi** | **N-myc (and STAT) interactor** | 1.04 | 1.11 | 1.00 | 4.12 |
| 20343 | *Sell* | *selectin, lymphocyte* | 0.95 | 0.75 | 1.00 | 3.91 |
| 12370 | **Casp8** | **caspase 8** | 0.82 | 0.51 | 1.00 | 3.74 |
| 16423 | ***Cd47*** | ***CD47 antigen (Rh-related antigen, integrin-associated signal transducer)*** | 0.89 | 0.83 | 1.00 | 3.72 |
| 14962 | **Cfb** | **complement factor B** | 0.77 | 0.89 | 1.00 | 3.58 |
| | **Pla2g7** | **phospholipase A2, group VII (platelet-activating factor acetylhydrolase, plasma)** | 0.66 | 0.53 | 1.00 | 3.55 |
| 13025 | *Ctla2b* | *Cytotoxic T lymphocyte-associated protein 2 beta* | 0.64 | 1.03 | 1.00 | 3.40 |
| 18595 | **Pdgfra** | **platelet derived growth factor receptor, alpha polypeptide** | 0.98 | 1.99 | 1.00 | 3.34 |
| 12267 | **C3ar1** | **complement component 3a receptor 1** | 0.72 | 0.84 | 1.00 | 3.34 |
| 16653 | **Kras** | **v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog** | 2.15 | 1.36 | 1.00 | 3.22 |
| 64138 | **Ctsz** | **cathepsin Z** | 0.50 | 0.43 | 1.00 | 3.11 |

* In italic transcripts common to day 21 and 42; in bold those unique to each category

http://www.biomedcentral.com/1471-2164/10/301

**Table 4: Mouse immune genes up-regulated in regressing GI-101A tumors at day 42 (F test p2-value < 0.001)**

| Gene LLID # | Symbol | Name | HT-29 Control | HT-29 GLV-1h68 | GI-101A Control | GI-101A GLV-1h68 | GI-101A GLV-1h68 (day 21) |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| **Interleukins** | | | | | | | |
| 16068 | *Il18bp* | *interleukin 18 binding protein* | 1.31 | 2.18 | 1.00 | 13.28 | 5.70 |
| 16173 | *Il18* | *interleukin 18* | 1.12 | 1.40 | 1.00 | 10.89 | 4.10 |
| 16168 | **Il15** | **interleukin 15** | 1.02 | 1.51 | 1.00 | 5.20 | 2.86 |
| 16154 | **Il10ra** | **interleukin 10 receptor alpha** | 0.74 | 0.90 | 1.00 | 3.51 | 2.08 |
| | | | | | | | |
| **Chemokines** | | | | | | | |
| | *Cxcl11* | *Cxcl11/I-TAC* | 0.92 | 1.75 | 1.00 | 13.57 | 5.69 |
| 17329 | *Cxcl9* | *Cxcl9/Mig* | 1.01 | 1.07 | 1.00 | 11.74 | 3.80 |
| 20304 | *Ccl5* | *Ccl5/RANTES* | 1.00 | 2.69 | 1.00 | 13.33 | 3.23 |
| 20308 | *Ccl9* | *Ccl9/MRP-2/CCF18/MIP-1g* | 1.56 | 3.14 | 1.00 | 12.03 | 6.60 |
| 20304 | *Ccl5* | *Ccl5/RANTES* | 1.11 | 2.57 | 1.00 | 9.81 | 2.56 |
| 20306 | *Ccl7* | *Ccl7/MARC* | 0.84 | 1.18 | 1.00 | 5.86 | 4.71 |
| 20315 | *Cxcl12* | *Cxcl12/SDF-1/PBSF* | 0.41 | 0.58 | 1.00 | 5.23 | 3.07 |
| 20301 | **Ccl27** | **Ccl27/ALP/CTACK/ILC/Eskine** | 1.86 | 1.91 | 1.00 | 5.17 | 1.66 |
| 20308 | *Ccl9* | *Ccl9/MRP-2/CCF18/MIP-1g* | 1.26 | 1.42 | 1.00 | 4.04 | 3.12 |
| | | | | | | | |
| **ISGs** | | | | | | | |
| 16145 | *Igtp* | *interferon gamma induced GTPase* | 1.15 | 3.31 | 1.00 | 48.21 | 10.11 |
| 76933 | *Ifi27* | *interferon, alpha-inducible protein 27* | 0.76 | 0.91 | 1.00 | 12.84 | 10.04 |
| | *Ifi47* | *interferon gamma inducible protein 47* | 0.66 | 0.94 | 1.00 | 11.09 | 4.56 |
| | *Iigp2* | *interferon inducible GTPase 2* | 0.64 | 1.41 | 1.00 | 10.05 | 5.25 |
| 16145 | *Igtp* | *interferon gamma induced GTPase* | 0.70 | 1.28 | 1.00 | 9.70 | 5.62 |

**Table 4: Mouse immune genes up-regulated in regressing GI-101A tumors at day 42 (F test p2-value < 0.001)** *(Continued)*

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 17857 | *Mx1* | *myxovirus (influenza virus) resistance 1* | 0.62 | 1.46 | 1.00 | 9.46 | 6.41 |
| | *Ifi204* | *interferon activated gene 204* | 0.86 | 1.77 | 1.00 | 8.94 | 5.44 |
| 16362 | *Irf1* | *interferon regulatory factor 1* | 0.59 | 0.98 | 1.00 | 7.28 | 3.78 |
| 20846 | *Stat1* | *signal transducer and activator of transcription 1* | 0.57 | 0.81 | 1.00 | 6.84 | 4.02 |
| 20846 | *Stat1* | *signal transducer and activator of transcription 1* | 0.66 | 0.97 | 1.00 | 6.17 | 2.79 |
| 15957 | *Ifit1* | *interferon-induced protein with tetratricopeptide repeats 1* | 0.79 | 1.03 | 1.00 | 5.77 | 4.15 |
| 60440 | **Iigp1** | **interferon inducible GTPase 1** | 0.77 | 1.00 | 1.00 | 4.19 | 3.00 |
| 15976 | **Ifnar2** | **Interferon (alpha and beta) receptor 2** | 1.10 | 1.60 | 1.00 | 3.73 | 2.19 |
| | **Irf5** | **interferon regulatory factor 5** | 0.53 | 0.70 | 1.00 | 3.47 | 2.02 |
| | | | | | | | |
| **Others** | | | | | | | |
| 17071 | *Ly6f* | *Lymphocyte antigen 6 complex, locus F* | 0.56 | 0.73 | 1.00 | 8.56 | 2.68 |
| 11629 | **Aif1** | **allograft inflammatory factor 1** | 0.90 | 1.33 | 1.00 | 8.46 | 2.62 |
| 17067 | *Ly6c* | *Lymphocyte antigen 6 complex, locus C* | 1.24 | 1.22 | 1.00 | 7.35 | 5.27 |
| 17067 | *Ly6c* | *lymphocyte antigen 6 complex, locus C* | 0.99 | 1.12 | 1.00 | 6.03 | 5.77 |
| 17071 | *Ly6f* | *lymphocyte antigen 6 complex, locus F* | 0.80 | 0.89 | 1.00 | 5.66 | 5.01 |
| 20343 | *Sell* | *selectin, lymphocyte* | 0.61 | 0.79 | 1.00 | 5.03 | 3.91 |
| 76281 | **Tax1bp1** | **Tax1 (human T-cell leukemia virus type I) binding protein 1** | 1.01 | 1.51 | 1.00 | 5.02 | 1.98 |
| 230233 | **Ikbkap** | **inhibitor of kappa light polypeptide enhancer in B-cells** | 0.81 | 0.93 | 1.00 | 4.55 | 1.43 |
| 110454 | **Ly6a** | **lymphocyte antigen 6 complex, locus A** | 0.96 | 0.98 | 1.00 | 4.13 | 2.42 |
| 13025 | *Ctla2b* | *Cytotoxic T lymphocyte-associated protein 2 beta* | 0.68 | 1.20 | 1.00 | 4.12 | 3.40 |
| 71966 | **Nkiras2** | **NFKB inhibitor interacting Ras-like protein 2** | 1.07 | 1.27 | 1.00 | 3.80 | 1.93 |
| 17087 | **Ly96** | **lymphocyte antigen 96** | 1.13 | 1.43 | 1.00 | 3.77 | 1.44 |
| 17069 | **Ly6e** | **Lymphocyte antigen 6 complex, locus E** | 0.77 | 1.15 | 1.00 | 3.28 | 1.87 |

* In italic transcripts common to day 21 and 42; in bold those unique to each category

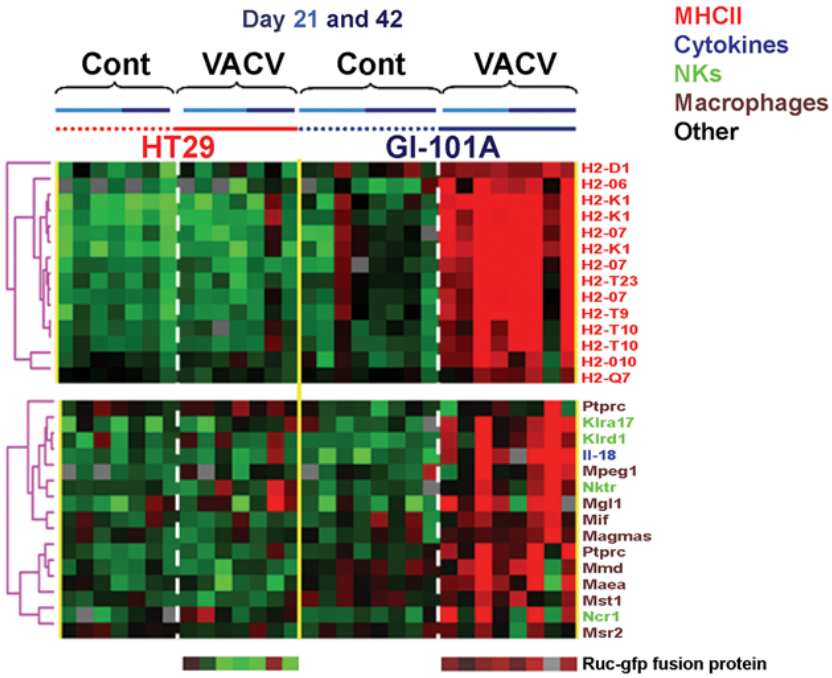


**Figure 4**

**Mouse immune gene signatures**. Self-organizing heat map based on genes selected according to macrophage (brown), natural killer cell (light green), cytokine (blue) or major histocompatiblity class II (red) annotations among those up-regulated in GI-101A xenografts excised from VACV-infected animals (Student *t* test $p_2$-value < 0.001). Genes presented in Figure 6 as representative of the ICR were omitted here to avoid redundancy.

up-regulated at either time point studied; these included IFN-γ induced GTPase, whose expression was increased 48-fold at day 42 in GI-101A tumors from GLV-1h68-infected animals compared to controls. IPA suggested that the majority of up-regulated genes reflected predominantly IFN-γ stimulation, a phenomenon we have observed in BCCs regression upon treatment with TLR-7 agonists [32] and, more generally, in association with TSD [2]; among them, STAT-1 and IRF-1 were previously described in association with TSD [2,31,32] and play a central role in the signaling of IFN-γ and other pro-inflammatory cytokines such as IL-2 and IL-15 [33].

Macrophage presence/function also played an important role (Figure 4) and was associated with over-expression of major histocompatiblity class II genes supporting the presence of activated macrophages in infected GI-101A xenografts. Furthermore, this prominent and specific infiltration could be substantiated by immunohistochemical analyses that demonstrated a strong peri- and intra-tumoral infiltration of MHC class II-expressing host's cells surrounding virally-infected cancer cells (Figure 5).

Although there was only partial overlap between genes up-regulated at day 21 and 42, most overlap was due to genes related to immune function. Applying a stringent Student *t* test ($p_2$-value < 0.001) comparing infected to non-infected GI-101A xenografts at the 2 time points, similar results were observed; although less genes were significantly up-regulated at day 21 (compared to the F test) a good proportion overlapped with day 42 and those overlapping genes were exclusively related to immune function (Table 2). We then re-directed genes with immune function significantly up-regulated in infected GI-101A xenografts into self-organizing biological pathways using IPA; this analysis identified those genes most tightly associated with the immunological network leading to TSD during rejection of GI-101A xenografts; while at day 21 (Figure 6A) CCL chemokines and STAT-2 played a central role, at day 42, IL-15, STAT-1 and IRF-1 played a central role (Figure 6B). Interestingly, IL-18, which was identified as playing a central role in this immune-deficient mouse model; was not previously observed as a component of the ICR in immune competent human tissues affected by immune-pathology. Finally, as expected no genes associated with B or T cell signaling or function in the grafts were significantly up-regulated at this phase of the immune-response against infected GI-101A xenografts, in accordance with the biology of the host's model system. This data suggest that at least in this model, adaptive immunity is not necessary for TSD.

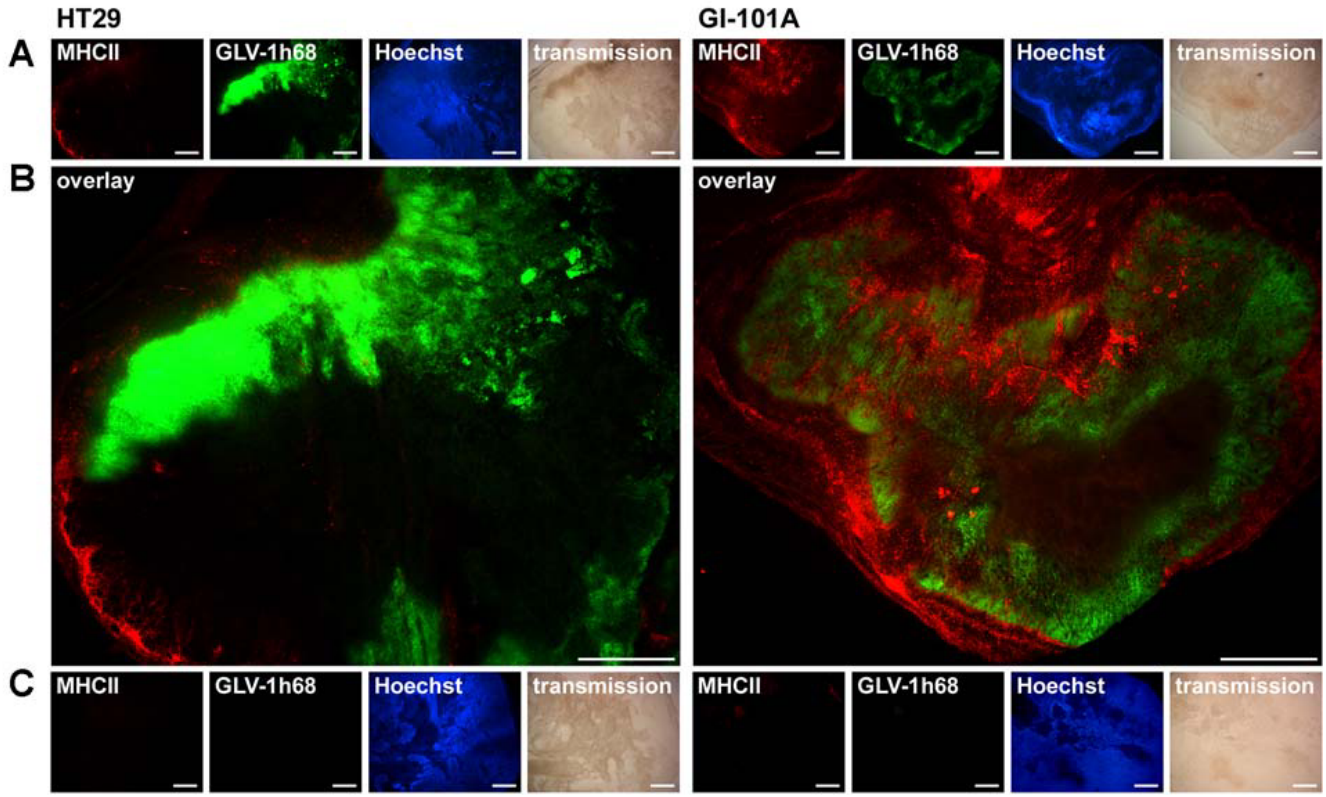


**Figure 5**
**Immunohistochemistry staining of MHC class II positive cells**. Scale bars are equal to 1 mm and 10× magnification was applied. (**A**) 42 days after GLV-1h68 administration HT-29 (left) and GI-101A (right) xenografts were excised, sectioned and labeled for MHCII and vital DNA (Hoechst). In addition, GFP signals from VACV infected cells and transmission images are shown. (**B**) Overlay of MHCII and GFP signals in HT-29 tumors (left) and GI-101A tumors (right). (**C**) Uninfected HT-29 (left) and GI-101A (right) xenografts were excised at day 42 and treated identical to their infected counterparts. Tissue sections were stained for MHCII and vital DNA (Hoechst). As expected, no Virus-derived GFP signal could be detected.

Based on GeneOntology annotations, we then compiled a database of genes associated to TSD in various immune pathologies in accordance to the ICR hypothesis [2,34]. These genes have been described as highly associated with TSD in the context of acute allograft rejection, pathogen clearance during acute infection and tumor rejection during immunotherapy [2]. We displayed these *a priori* and arbitrarily selected genes as a self organizing heat map based on the data from the present study (Figure 6C). All were specifically expressed in infected responding tumors compared with non-infected GI-101A and the infected or non-infected HT-29 xenografts. This display represents a prospective validation of a universal mechanism leading to TSD in mice as well as in humans.

## Discussion

Immune-mediated TSD is the ultimate manifestation of the effector phase of the immune response and, as we recently argued, may follow a common final pathway independent of the pathological circumstances leading to its occurrence [2,34]. Thus, although the mechanisms originating acute allograft rejection, clearance of pathogens during acute infection, flares of autoimmunity or cancer regression may be different, in the end, they all converge into a cascade of immunologic steps capable of turning a chronic and lingering inflammatory process into an acute and destructive one. We argued that, among the 4 axioms upon which the ICR is founded, adaptive immune responses are neither necessary nor sufficient to induce tissue-specific rejection but rather start a tissue-specific reaction in cases in which such specificity is not determined by other factors. Indeed, others have shown that tumor rejection can be determined by innate immune mechanisms [3] and adaptive T cell responses play a role as helpers to stimulate more powerful innate immune effector mechanisms [20]. Thus, in conditions in which a switch from a chronic to an acute inflammatory process can be induced by other factors like the immune-stimulation induced by the presence of a virus in the target tissue, adaptive immune responses may not be necessary and

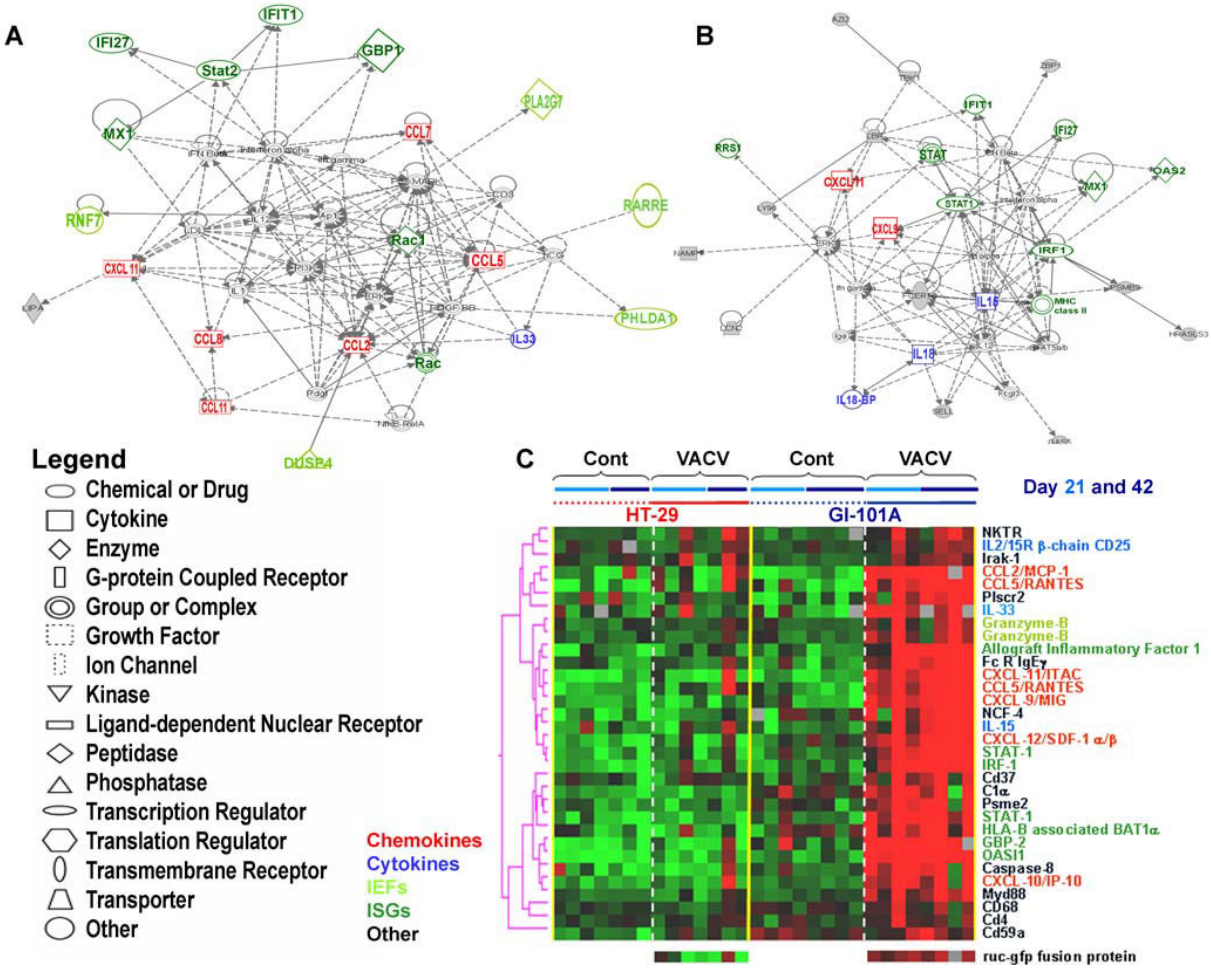


**Figure 6**

**Mouse immune genes associated with the Immunologic Constant of Rejection Hypothesis**. IPA self-organizing network based on genes with immune annotations whose expression was significantly up-regulated in VACV-infected GI-101A xenografts at day (**A**) 21 and (**B**) 42 from VACV-infection. (**C**) Self-organizing heat map based on genes associated with the ICR hypothesis. The genes were arbitrarily selected a priori based on previous studies as summarized in [2] and are displayed based on their expression in the current study without further selection.

immune-mediate rejection can occur without the assistance of T or B cells.

This hypothesis is suggested by some human observations. The rejection of skin cancers by the local application of TLR-7 agonists occurs without direct evidence of adaptive immune responses [32,35-37]. Also renal cell carcinomas are as sensitive to systemic administration of IL-2 as metastatic melanoma yet, while in the latter adaptive immune responses are easily demonstrable, in the former, they have been quite elusive, and most likely of secondary significance [38,39].

Xenografts growing in primarily T cell-depleted and secondarily B cell-deficient animals provide the best evidence that in the absence of non-self discrimination, allograft rejection does not occur. In this model, although xenografts by themselves do not provide sufficient pro-inflammatory signals to induce acute inflammation, the presence of viral replication provides the "tissue-specific trigger" that activates the immune response. According to our hypothesis, the ICR is activated when chronic inflammation is switched into an acute one. A critical step in this process is the expression of IFN-γ dependent pathways probably by activated mononuclear cells; this is clearly demonstrable in most cases in which TSD has been studied in humans by the requirement for the expression of IRF-1 [2,31,40,41]; a transcription factor closely related to IFN-γ signaling. IFN-α and IFN-γ regulate directly or indirectly the production of CXCR3, CXCR4 and CCR5 ligands among which the CXCL-9 through -11 chemokines (Mig, IP-10/Crg-2 and ITAC), CCL5 (RANTES) and CXCL12 (SDF-1) appear to play a prominent role [42]. Indeed, this expression pattern has been consistently

observed in most cases in which TSD was studied in the involved tissue by transcriptional profiling [2,34] including animal rejection models [20]. This study provides experimental evidence that such signatures are associated with TSD and, potentially, immune-mediate rejection independent of the presence of adaptive immunity. Moreover, this model provides evidence that non-self discrimination plays at best a partial role in a host that cannot eliminate xenografts unless appropriate danger signals are provided by a pathogen [43-46]. Contrary to acute allograft rejection occurring in humans [47], no B lymphocyte signatures (CD20, immunoglobulin genes) could be observed clearly demonstrating that reconstitution of a potential B cell response could not have been responsible for the inflammatory switch and the production of CXCL and CCL chemokines [48]. Furthermore, contrary to a similar rejection model that we presently analyzed in a syngeneic mouse system [49], no involvement of T or B cell signatures participated in the rejection of GI-101A xenografts. Furthermore, contrary to the syngeneic model of HER-2/*neu*-expressing mammary tumor rejection [49] where clear up-regulation of type I and type II IFNs could be documented, in this model ISG expression was not directly accompanied with the over expression of IFN-α, IFN-β or IFN-γ suggesting that, as recently demonstrated in a cytomegalovirus model [50], stimulation of interferon response genes could occur independently of de novo synthesis of IFNs through a direct interaction of viral proteins with cellular transcription factors.

Although most differences in the transcriptional pattern of human cancer cells were associated with arrested or dampened metabolism (Figure 3D) a handful of genes were up-regulated specifically in infected GI-101A xenografts. Among those, only IRF-7 and STAT-3 could be proven to be specifically expressed by human cells (Figure 4B). The expression of IRF-7 is not surprising considering the presence of replicating VACV in those cells compared with control xenografts from non-infected animals [18], while the expression of STAT-3 in the absence of over-expression of STAT-1 contrasts with the analysis of host's transcripts in which STAT-1 over-expression dominated (Table 3). As IRF-7 and STAT-3 were also expressed by host cells, it could be hypothesized that transfer of VACV from cancer cells to host cells infiltrating the xenografts reproduced patterns observed in human cancer cells, while host's immune cells followed the classical up-regulation of pro-inflammatory pathways through STAT-1, IRF-1 signaling [18,42,51].

The over-expression of IL-18 and the IL-18-binding protein in this model is of particular interest. IL-18, originally called IFN-γ inducing factor, has not been previously observed by us or others as consistently associated with TSD [2]. It is possible, that IL-18 over expression is a specific causative mechanism in this model as VACV particles have been described as inducers of this cytokine by direct monocyte activation through TLR signaling [52-54] a finding that needs to be corroborated by future studies. Contrary to IL-18, IL-15 is the most consistently observed cytokine in association with TSD [2]. Generally, we have observed this in association with the expression of IL-2 and, it is of interest that, in this T cell-depleted model only this monocyte produced cytokine is present. IL-15 is critical not only in expansion of memory CD8+ T cells in mice but also to maintain cytotoxic T cell effector functions. In fact, VACV clearance is delayed in IL-15 -/- mice due to a rapid loss of cytolytic function [55] most likely by natural killer cells [56]. Thus, the role that IL-15 may play in this immune-deficient model will need to be further investigated.

In practical terms, it would be important to understand why some tumors could be eliminated through viral oncolysis and/or a secondary immune rejection, while others are resistant. It appears that the degree of viral replication *in vivo* is a key determinant; however, it remains unclear the weight that direct viral oncolysis plays compared to immune-mediated rejection in this model. It appears that transcriptional changes associated with viral replication precede tumor destruction by a substantial amount of time and they are paralleled by the activation of immune signatures in the host, long before tissue destruction occurs. For instance, viral replication was quite active at day 21 in GI-101A xenografts; at the same time significant shut down of cancer cell metabolism (Figure 3C, D) and simultaneous activation of immune functions could be observed at that early time point (Table 2). Yet, tumors continued their growth at least till day 42 when their growth started to plateau. HT-29 allowed VACV replication *in vitro* similarly to GI-101A, but *in vivo* viral replication was substantially reduced in most though not all HT-29 xenografts (Figure 2C). This suggests that although the baseline biological phenotype of individual cell lines can influence viral replication, *in vivo* other factors may interfere with viral replication, and need to be further studied. This is suggested by the significant yet imperfect correlation between *in vitro* and *in vivo* replication data and, most importantly, by the individual variation among xenografts originated from the same cell line that can be permissive or non-permissive to viral replication *in vivo* (HT-29 example in Figure 2C). To clarify such subtleties, it will be necessary to investigate a larger panel of cell lines, and assess the growth patterns of individual xenografts. This could be achieved by the utilization of non-invasive strategies such as fine needle aspirations that allow direct linkage of the experimental results obtained by transcriptional profiling to the natural or therapy-induced history of each individual xenograft left in place [31,57,58].

## Conclusion

Although xenograft infection by oncolytic VACV offers a promising therapy of established cancer, it needs to be taken into account that the presence of adaptive immunity might change what is expected, perhaps inducing suppressive T cell responses that could abrogate the therapeutic effect of the virus in natural conditions.

The rejection of GI-101A tumors seems to be mediated by infiltrating leukocytes; thus, cancer cells not greatly affected by the viral infection *in vitro* may show resistance to *in vivo* oncolytic therapy. Future studies utilizing a broader panel of cell lines will be necessary to evaluate whether a correlation exists between *in vitro* replication pattern and *in vivo* regression following VACV infection. Alternatively, other factors related to the host response within the tumor microenvironment besides the in vitro permissiveness of cell lines may affect their in vivo permissiveness to VACV and/or their pattern of growth. Indeed, the nature of the tumor microenvironment might predict the success of the VACV therapy even though the treatment outcome seems to be mainly correlated with the ability of the infected tumor cells to provide the "tissue-specific signal" to activate the immune response and attract specific leukocytes.

In summary, this study provides the first prospective validation of a universal mechanism associated with TSD. This information may lead to the identification of principles that could refine the treatment of cancer and chronic infection by immune stimulation or autoimmunity and allograft rejection through immune regulation.

## Methods

### *Cell line culture*

All cell lines except when noted were purchased from American Type Culture Collection (Manassas). GI-101A cells were kindly provided by Dr. A. Aller, Rumbaugh-Goodwin Institute for Cancer Research, Inc., Plantation, Florida whereas the 3 melanoma cell lines from distinct cutaneous metastases were obtained from patient 888 as previously described [24]. MDA MB-231, PANC-1, CV-1 and PC-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (AA) (100 U/ml penicillin G, 250 ng/ml amphotericin B, 100 μg/ml streptomycin). MIA PaCa-2 cells have been cultured under similar conditions in DMEM media but supplemented with 12.5% FBS and 2 mM L-glutamine. SiHa and DU-145 cells were grown in Eagle's minimal essential medium (EMEM) which was enhanced with 10% FBS, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate and 1% AA.

All other cells were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with the following compounds: A-549 and HT-29 cells (10% FBS and 1% AA); GI-101A cells (20% FBS, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1% AA and 4 ng/ml β-estradiol/5 ng/ml progesterone); NCI-H1299 (10% FBS, 4.5 g/L glucose, 10 mM HEPES, 1 mM sodium pyruvate, 1% AA). OVCAR-3 culture media was prepared similarly to GI-101A media but supplemented with 2.3 g/L glucose instead and additional human Insulin; and 888-MEL, 1858-MEL and 1936-MEL cells (10% FBS, 1 mM HEPES, 1 mM Ciprofloxacin and L-glutamine/penicillin/streptomycin). All cell cultures were carried out at 37°C under 5% $CO_2$.

### *Viral construct*

The construction of the mutant GLV-1h68 virus was described previously [13]. Briefly, 3 expression cassettes (encoding for *Renilla* luciferase-*Aequorea* GFP fusion protein, β-galactosidase and β-glucoronidase) were recombined into the F14.5L, J2R and A56R loci, respectively, of the LIVP strain viral genome.

### In vitro *viral replication assay*

All cells were seeded in 6-well plates and infected with GLV-1h68 at the multiplicity of infection of 0.01 as we have previously described [13]. The infected cell cultures were harvested in triplicate up to 72 hours post infection (hpi). Viral titers were determined by plaque assays on CV-1 cell monolayers and expressed as pfu/$10^6$ cells.

### *Virus titration of tumor tissue*

GLV-1h68 infected tumors were removed at day 7, 21 and 42, weighed and homogenized in DPBS containing proteinase inhibitor cocktail using MagNALyser (Roche Diagnostics) at a speed of 6500 for 30 s. After three freeze and thaw cycles to release the viral particles, the samples were sonicated and supernatants were collected by centrifugation at 1000 g for 5 min. Viral titers were determined in duplicates by standard plaque assays using CV-1 cells.

### *Animal models*

All mice were cared for and maintained in accordance with animal welfare regulations under an approved protocol by the Institutional Animal Care and Use Committee of LAB Research International Inc. (San Diego Science Center, San Diego, CA). Six to 8 weeks old nude mice (NCI:Hsd:Athymic Nude-*Foxn1*$^{nu}$, Harlan) were inoculated with $5 \times 10^6$ cells per mouse to obtain subcutaneous xenografts as previously described [13]. Tumor growth was measured once a week and tumor mass was reported in $mm^3$. Thirty days after implantation, $5 \times 10^6$ pfu of GLV-1h68 virus in 100 μl of PBS or 100 μl of PBS alone (control) was delivered by intravenous inoculation [13]. After inoculation with GLV-1h68 the expression of green fluorescent protein within the tumors could be monitored under UV-light. Twenty-one days and 42 days post inocu-

lation 3 or 4 animals from the treatment and the control groups were sacrificed and the tumors were excised.

#### *Immunohistochemistry*

GI-101A xenografts from GLV-1h68-infected and non-infected mice were removed at day 42 and snap-frozen in liquid $N_2$, followed by fixation in 4% paraformaldehyde/PBS pH 7.4 for 16 h at 4°C. Tissue sectioning was performed as previously described [59]. MHCII-positive cells were labeled using monoclonal rat anti-MHCII antibody (NatuTec, Frankfurt, Germany) and Cy3-conjugated donkey anti-rat secondary antibodies (Jackson ImmunoResearch, PA, USA). Hoechst 33342 (Sigma, Taufkirchen, Germany) was used to stain nuclei. The fluorescent-labeled sections were examined using the Leica MZ 16 FA Stereo-Fluorescence microscope equipped with a Leica DC500 Digital Camera (Leica, Solms, Germany). Digital images were processed with Photoshop 7.0 (Adobe Systems, CA, USA).

#### *Transcriptional profiling platforms*

VACV-gene expression was assessed by a custom-made VACV array platform (VACGLa520445F, Affymetrix, CA) including 308 probes representing 219 genes that covered the combined genome of several VACV strains (see additional file # 2), the *Renilla* luciferase-*Aequorea* green fluorescent fusion gene specific for GLV-1h68, and 337 human or mouse "house keeping" genes (393 probes). Time course analysis evaluating the *in vivo* effects of viral replication on the permissive GI-101A human xenografts was performed using a previously described custom-made 17.5 k human cDNA array platform [29]. Human or mouse arrays covered the complete genome of each species based on 36,000 oligos each. We have previously observed that the use of species-specific cDNA arrays as well as oligo probes can distinguish the expression patterns in mixed cell populations in which human tissues (cancer cells) are infiltrated with host cells. This is because of a lack or reduced cross-hybridization between non-related species compared to closely related ones such as primate to primate comparisons [13]. Although partial cross-hybridization may occur this can be detected and eliminated by applying an appropriate intensity signal cutoff. Since cDNA arrays contain probes of relatively large size (600 to 2,000 bases), to increase the specificity of the hybridization, we tested the same material on custom-made 36 kb oligo array platforms constituted of 70-base-length oligo-probes as well as cDNA probes using identical statistical parameters. With few exceptions (discussed in the results section) results were concordant between platforms and will be presented, thereof, in either format while comprehensive data are accessible through GEO.

#### *Total RNA isolation and amplification*

Total RNA (tRNA) from excised tumors was isolated after homogenization using Trizol reagent according to the manufacturer's instructions. tRNA from tissue cultures was isolated with the Qiagen RNeasy Mini kit and the quality of obtained tRNA was tested with the Agilent Bioanalyzer 2000 (Agilent Technologies). For expression studies based on cDNA and oligo array techniques, tRNA was amplified into antisense RNA (aRNA) as previously described [60,61].

Mouse reference RNA was prepared by homogenization and pooling of selected mouse tissues (lung, heart, muscle, kidneys, liver and spleen) from 3 female C57Bl/6 mice. Reference for human arrays was obtained by pooling PBMCs from 4 normal donors. Both, human and mouse reference tRNA was amplified into antisense RNA in large amounts [60,61]. Five μg tRNA of selected tumor and cell samples were amplified according to the Affymetrix manual using the GeneChip® One-Cycle Target Labeling and Control kit.

#### *Microarray performance and statistical analysis*

Array quality was documented as previously described [29]. For 36 k whole genome mouse and human array performances both reference and test aRNA were directly labeled using ULS aRNA Fluorescent Labeling kit (Kreatech) with Cy3 for reference and Cy5 for test samples and co-hybridized to the slides [49]. 17 k human cDNA arrays were carried out as described according to our standard method for labeling and array hybridization [62]. A customized VACV-GLV-1h68 Affymetrix expression array was specifically prepared for this study. Amplified RNA from tumor or cell samples was handled according to the manufacture's instructions for eukaryotic sample processing and hybridized to the arrays. After a 16 h incubation in the hybridization oven at 45°C, the arrays were washed and stained in the Fluidics station using the GeneChip® Hybridization, Wash, and Stain Kit.

The data was uploaded to the mAdb databank http://nciarray.nci.nih.gov and further analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute http://linus.nci.nih.gov/BRB-ArrayTools.html[63] and Cluster and TreeView software [64]. Multiple dimensional scaling was performed on the BRB-array tool.

Data retrieved from the Affymetrix platform was normalized using median over entire array as reference because of single color labeling technology. For all array type's unsupervised analysis was used for class confirmation using the Stanford Cluster program (80% gene presence across all experiments and at least 3-fold ratio change) and Treeview program for visualization. Gene ratios were average corrected across experimental samples and displayed according to uncentered correlation algorithm. Class comparison was performed using parametric unpaired Student's t test or 3-way ANOVA to identify differentially expressed genes among GLV-1h68 infected and unin-

fected tumors or cells at various time points using different significance cutoff levels as demanded by the statistical power of each test. Subsequent filtering (80% gene presence across all experiments and at least 3-fold ratio change) narrowed down the number of genes that were expressed differentially between experimental groups.

Statistical significance and adjustments for multiple test comparisons were based on univariate and multivariate permutation test as previously described.

No quantitative polymerase chain reaction-based (q-PCR) validation of the gene sets identified in this study was performed since we have previously extensively shown that the present method for RNA amplification is robust and yields results comparable to those obtained by qPCR [29,65,66], and the primary purpose of the analysis was to evaluate general patterns of expression rather than identifying and characterize single gene expression levels.

Gene function interpretation was based on GeneOntology software while pathway analysis was based on Ingenuity Pathways Analysis software.

#### *Sequence analysis*

To determine species origin of selected genes we designed primers flanking array probe positions within coding regions (Additional file 2). Gene transcript sequences were obtained from Ensembl database. To rule out any cross hybridizations we did extensive BLAST search of the designed primer sequences.

Specific primers were used to reverse transcribe 500 ng tRNA from excised tumors and amplify the messages subsequently. The resulting PCR products were analyzed with the Agilent Bioanalyzer to proof their length and presence. All amplicons have been cleaned up with ExoSAP-IT® (United States Biomedical/Affymetrix, Cleveland, OH, USA) and transferred to the sequencing reactions performed with BigDye® . Before loading into the 48-capillary 3730 DNA Analyzer (Applied Biosystem, Foster City, CA, USA) all reactions were purified with DyeEx 2.0 Spin Kit (Qiagen, Valencia, CA, USA).

## Competing interests

this work was supported by Genelux Co.; Andrea Worschech, Nanhai Chen, Yong A Yu, Qian Zhang, Stephanie Weibel, Viktoria Raab and Aladar A Szalay have received payment or are employees of Genelux Co.

## Authors' contributions

AW performed experiments, data analysis, conceived and designed the study and wrote the paper under supervision of EW, AAS and FMM who designed the study and drafted the paper. NC, YAY, QZ helped to design the study and performed some experiments. VM, RMB, MS and DFS contributed to the interpretation of the results and reviewed the paper. ZP, SW, VR, AM and HL participated or helped to carry out some experiments. All authors read and approved the final manuscript.

## Additional material

### Additional file 1

***Replication ability of GLV-1h68 in multiple human cancer cell lines.*** *Viral titers were examined 24, 48 and 72 hpi in 13 human cancer cell lines representing highly susceptible and delayed* in vitro *replication models.*

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### Additional file 2

***Sequence verification of genes which have potentially cross hybridized between human and mouse arrays****. The data provided represent the sequence analysis of selected genes which have been described as up regulated in infected GI-101A xenografts based on human 17 K cDNA arrays but not based on human 36 K oligo arrays. Some of the genes were in fact expressed by the host and cross-hybridized to less specific cDNA probes on the human platform.*

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[http://www.biomedcentral.com/content/supplementary/1471-2164-10-301-S2.pdf]

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## 5.3 *In vitro* permissivity of 75 human cancer cell lines to Adenovirus 5 and oncolytic Vaccinia Virus – Manuscript for Research article 3

# *In vitro* permissivity of 75 human cancer cell lines to Adenovirus 5 and oncolytic Vaccinia Virus and the correlation with intrinsic immune activation levels

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## *In vitro* permissivity of 75 human cancer cell lines to Adenovirus 5 and oncolytic Vaccinia Virus and the correlation with intrinsic immune activation levels

Amplification and microarray hybridizations of infected and non infected human cancer cell lines, statistical data analyses, VACV and Ad5 infections and FACS measurements, qPCR performance, partial interpretation of the results and writing of the manuscript were performed by the author of this thesis with help and supervision of the following co-authors who contributed to the manuscript:

Dr. N. Chen, Dr. Y. A. Yu and Dr. Q. Zhang generated the viral construct.

Dr. N. Chen provided virus infected and control tumor cells for VACV gene expression study.

M.L. Ascierto helped to isolate RNA, designed qPCR primer and helped to perform qPCR experiments.

G. DiPasquale provided assistance with Ad5 infections and data interpretation.

Z. Pos helped to perform FACS measurements and analyses.

T. Trevino expanded all uninfected cell cultures.

Dr. E. Wang and Dr. F. M. Marincola supervised microarray experiments and gene expression profiling, participated in designing the study and helped to write the paper

Prof. Dr. A. A. Szalay supervised and designed the study and helped to write the paper.

Ms. A. Worschech, was a visitor at NIH, and is a graduate student in Dr. Szalay's laboratory in the Department of Biochemistry, University of Würzburg, Germany, and is supported by a graduate stipend and foreign travel grant from Genelux Corporation which provided free housing and daily allowance fellowship.

I hereby confirm the a ove statements:

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### 5.3.1 Background

Despite recent improvements in conventional cancer treatment such as radiation and chemotherapy the number of cancer-related deaths is still increasing and novel therapies are needed to introduce vital advantages in the patients' outcome. Promising results are noteworthy in the context of oncolytic therapy and it is worthwhile to be discussed as an alternative treatment option for cancers.

Oncolytic viruses were first noticed in the early 20$^{th}$ century when some cancer patients were noted to undergo tumor regression after systemic viral infections (1). A leukemia patient, for instance, was reported to undergo remission after acute infection with influenza virus (2).

The mutant adenovirus ONYX-015, which carries a deletion for E1B-55K, was the first replication-competent modified virus that displayed anti-cancer effects in humans (3), and was originally believed to target p53-deficient cancer cells. The use of this genetically engineered virus as a therapeutic agent has progressed to phase III clinical trials only 4 years after its first application in patients (4).

It is important to consider that safe administration of oncolytic viruses in humans depends on an exclusive tropism for cancer cells. This has been achieved, among other methods, by the disruption of non-essential viral genes in viruses such as in HSV (5), adenovirus (6) and vaccinia (7) that, for unclear reasons, alters the replicative capacity of viruses in a tissue-specific fashion.

VACV is a promising candidate poxvirus for oncolytic therapy due to its extensive use in humans for vaccination worldwide against smallpox. This experience has clearly been demonstrated it to be safe. We recently introduced a novel VACV strain (GLV-1h68) derived from the LIVP progenitor strain that was modified by insertion of three expression cassettes (Renilla luciferase-Aequorea green fluorescent fusion protein, β-galactosidase and β-glucoronidase) into the F14.5L, J2R and A56R loci of the viral genome respectively. Because of its light emitting properties GLV-1h68 can be used simultaneously as an imaging tool to detect malignant cells in the body while exerting its oncolytic effects (8).

The NCI-60 cell lines were assembled by the National Cancer Institute as an *in vitro* anticancer drug screen (9,10), which went into operation in 1990. The panel comprises 60 human cancer cell lines representing nine tissues of origin types:

breast, colon, central nervous system, renal, lung, melanoma, ovarian, prostate, and hematogenous. More than 100,000 compounds have been screened for anticancer activity against the NCI-60 which has been profiled more extensively at the molecular level than any other set of cells in existence (11). The resulting data have proved rich in information about the mechanisms of action and resistance of those compounds (12-14). The cells have also been profiled more extensively at the DNA, RNA, protein, chromosomal, and functional levels than any other set of cells (15).

We previously studied an oncolytic xenograft model in which the eradication of responding tumors was at least partially mediated through innate immune mechanisms. This study provided important insights about the role of innate immunity in the context of tissue rejection in an immune incompetent host (16) which was clearly cell line specific and not a general phenomenon. In the same study, we also subjected a smaller panel of cancer cell lines to an *in vitro* assay to test their susceptibility to the replication-competent VACV GLV-1h68 and found cell line specific divergent patterns of high and low viral replication. We also identified a significant but not absolute correlation (Fisher exact test p2 value = 0.005) between the *in vitro* replication and *in vivo* responsiveness to oncolytiv therapy in the given xenograft (16).

Interestingly, in a related study we observed two clearly distinguishable phenotypes of primary pancreatic adenocarcinomas by applying global transcriptional profiling. The tumors could be separated according to the expression of ISGs and the two phenotypes could be readily recognized by immunohistochemical detection of the MxA protein, whose expression reflects the activation of interferon dependent pathways. The two molecular phenotypes discovered in primary carcinomas *in vivo* were also observed among established pancreatic adenocarcinoma cell lines, suggesting that these phenotypes are an intrinsic characteristic of cancer cells independent of their interaction with the host's microenvironment. The two pancreatic cancer phenotypes are characterized by different permissivity to viral vectors used for gene therapy, as cell lines expressing ISGs resisted to Ad5 mediated lysis *in vitro*. Similar results were observed when cells were transduced with AAV 5 and 6 (17).

Based on this background information we decided to screen the NCI-60 cancer cell line panel (plus few more cell lines which were available in our laboratory) regarding their susceptibility to Ad 5 and the oncolytic VACV GLV-1h68. We also

applied whole genome human 36k oligo arrays to all samples to test their endogenous activation level of immune related genes and possibly relate this activation status to the respective replication efficiency.

### 5.3.2 Materials and Methods

Cancer cell lines

60 cell lines from the NCI-60 cell line panel were purchased from NCI-Frederick Cancer Center DCTD Tumor/Cell Repository. GI-101A cells were kindly provided by Dr. A. Aller, Rumbaugh-Goodwin Institute for Cancer Research, Inc., Plantation, Florida and Huh7.5.1 by Dr. Richard Wang, Department of Transfusion Medicine, NIH, Bethesda, MD. The three melanoma cell lines (888-MEL, 1858-MEL and 1936-MEL) from distinct cutaneous metastases were obtained from patient 888 as previously described (18). MIAPaCa2, HT29, A549, OVCAR3, Panc-1, Siha, MDA-MB-231, NCI-H1299 and PC-3 were purchased from American Type Culture Collection (Manassas) in the past and were included in the study as a control.

All cells were cultured in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% FBS, 10mM HEPES, 1% antiobiotic/antimycotic solution and 1mM and cell cultures were carried out at 37°C under 5% $CO_2$.

During prolonged cell culture and immediately before RNA isolation all cells have been tested for mycoplasma contamination with the Venor®GeM Mycoplasma Detection kit (Sigma) and only negative results have been obtained.

Viral constructs

The construction of the mutant Vaccinia virus GLV-1h68 virus was described previously (8). Briefly, 3 expression cassettes (encoding for *Renilla* luciferase-*Aequorea* GFP fusion protein, β-galactosidase and β-glucoronidase) were recombined into the F14.5L, J2R and A56R loci, respectively, of the LIVP strain viral genome.

The Ad5-CMV-GFP recombinant Ad5 was purchased from Applied Viromics (Freemont, CA).

Viral infections

All cells were seeded in 6-well plates and infected with GLV-1h68 at the multiplicity of infection (MOI) of 0.3 and 0.6 and with Ad5 at the MOI of 100 and 300. The cells were incubated at 37°C for 1h with brief agitation every 20min to allow infection to occur. The VACV infection medium (RPMI with 2% FBS) was removed and cells were incubated in fresh cell culture medium until cell harvest (18h for VACV; 48h for Ad5).

FACS-analysis of GFP Protein expression

18 hours post infection (hpi) with VACV and 48hpi with Ad5 respectively, infected cells were trypsinized (where applicable) and fixed with paraformaldehyde. After two wash steps with AUTOMacs Running buffer, cells were resuspended in 300µl AUTOMacs running Buffer and GFP expression was analyzed with a FACSCalibur flow cytometer. Data were evaluated by the FlowJo software. To ensure between-batch reproducibility, all FACS experiments were done including BD's Rainbow Calibration Particles (six peaks) as standards, and measured signal intensities were immediately converted in molecules of equivalent fluorescein, phycoerythrin, or allophycocyanin (MEFL, MEPE, MEAPC) in each experiment.

Additionally, A549 was repeatedly included in all individual batches as a positive control for viral infection.

Quantitative real-time PCR validation of VACV gene expression

For validation of FACS data, differentially expression of three virus genes was detected by using TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). RNA was extracted from mock and infected cells using the RNeasy Protect Mini Kit (Qiagen) according to the manufacturer's recommendation and reverse transcribed with random oligo primers. Primer Express 2 (PE2) (Applied Biosystems, Foster City, CA) was used to generate primers and a TaqMan probe specific for the virus sequence (see Table). Real-time PCR was performed on Real-time thermal cycler 7900HT (Applied Biosystems, Foster City, CA). The differences of expression were determined by relative quantification method; the Ct values of the virus genes were normalized to the Ct values of endogenous control (18s rRNA).

| Gene name | Sequence 5'-3' |
|---|---|
| *IMV surface protein* | *Fw: TTTCCAAATTAGTTAGCCGTTGTTT* |
| | *RvAGCAATTGTTAAAGCCGATGAAG* |
| | *Probe: (6FAM)AGAGTTTCCTCATTGTCG* |
| *Interferon resistance protein* | *Fw:GATAGCTTCAGAGTGAGGATAGTCAAAA* |
| | *Rv:CAATGCGGGTGATGTAATAAAGG* |
| | *Probe:(6FAM)AGAGCATAATCATTCTCGTATACT* |
| *Green fluorescence protein (GFP)* | *Fw:TGGAACTGGATGGCGATGT* |
| | *Rv:TTCACCCTCTCCGCTGACA* |
| | *Probe: (6FAM)TGGGCACAAATTT* |

tRNA isolation and amplification

tRNA from tissue cultures was isolated with the Qiagen miRNeasy Mini kit and the quality was tested with the Agilent Bioanalyzer 2000 (Agilent Technologies). Reference was obtained by pooling PBMCs from 4 normal donors. For expression studies based on oligo array techniques, both reference and test tRNA was amplified into antisense RNA (aRNA) as previously described (19, 20).

Microarray performance and statistical analysis

Array quality was documented as previously described (21). For 36k human array performances both reference and test aRNA were directly labeled using ULS aRNA Fluorescent Labeling kit (Kreatech) with Cy3 for reference and Cy5 for test samples and co-hybridized to the slides. After 20h incubation at 42°C the arrays were washed, dried and scanning using the Agilent scanner.

Obtained data files were uploaded to the mAdb databank (http://nciarray.nci.nih.gov) and further analyzed using BRBArrayTools developed by the Biometric Research Branch, National Cancer Institute (http://linus.nci.nih.gov/BRB-ArrayTools.html) (22) and Cluster and TreeView software (23). Multiple dimensional scaling was performed on the BRB-array tool as well.

Unsupervised analysis was used for class confirmation using the Stanford Cluster program and Treeview program for visualization. Gene ratios were average corrected across experimental samples and displayed according to uncentered correlation algorithm. Class comparison was performed using parametric unpaired Student's t test to identify differentially expressed genes among different cell line categories using different significance cutoff levels as demanded by the statistical

power of each test. Subsequent filtering (80% gene presence across all experiments and at least 2-fold ratio change) narrowed down the number of genes that were expressed differentially between experimental groups. Statistical significance and adjustments for multiple test comparisons were based on univariate and multivariate permutation test as previously described. No quantitative polymerase chain reaction-based (q-PCR) validation of the gene sets identified in this study was performed since we have previously extensively shown that the present method for RNA amplification is robust and yields results comparable to those obtained by qPCR (21, 24, 25), and the primary purpose of the analysis was to evaluate general patterns of expression rather than identifying and characterize single gene expression levels. Gene function interpretation was based on Ingenuity Pathway analysis.

DNA isolation

Automated DNA isolation was performed from 75 non-infected human cancer cell lines using Fujifilm's Quickgen DNA Whole Blood kit and Nucleic Acid Isolation System-810, as per manufacturer's instructions.

### 5.3.3 Results

#### *5.3.3.1 Microarray analysis of 75 cancer cell lines and validation of the results*

To gain better insights in the characteristics and transcriptional status of the examined cancer cell lines we applied 36k whole genome human arrays to validate our dataset and performed unsupervised cluster analysis with all cancer cells. The two repeated hybridizations (Siha and SNB19) as well as five cell line pairs (MDA-MB231, HT29, A549, Ovcar3 and PC-3) consistent of an older and younger passage were found to consistently cluster together suggesting that the technical array quality was highly reproducible (**Figure 1A**).

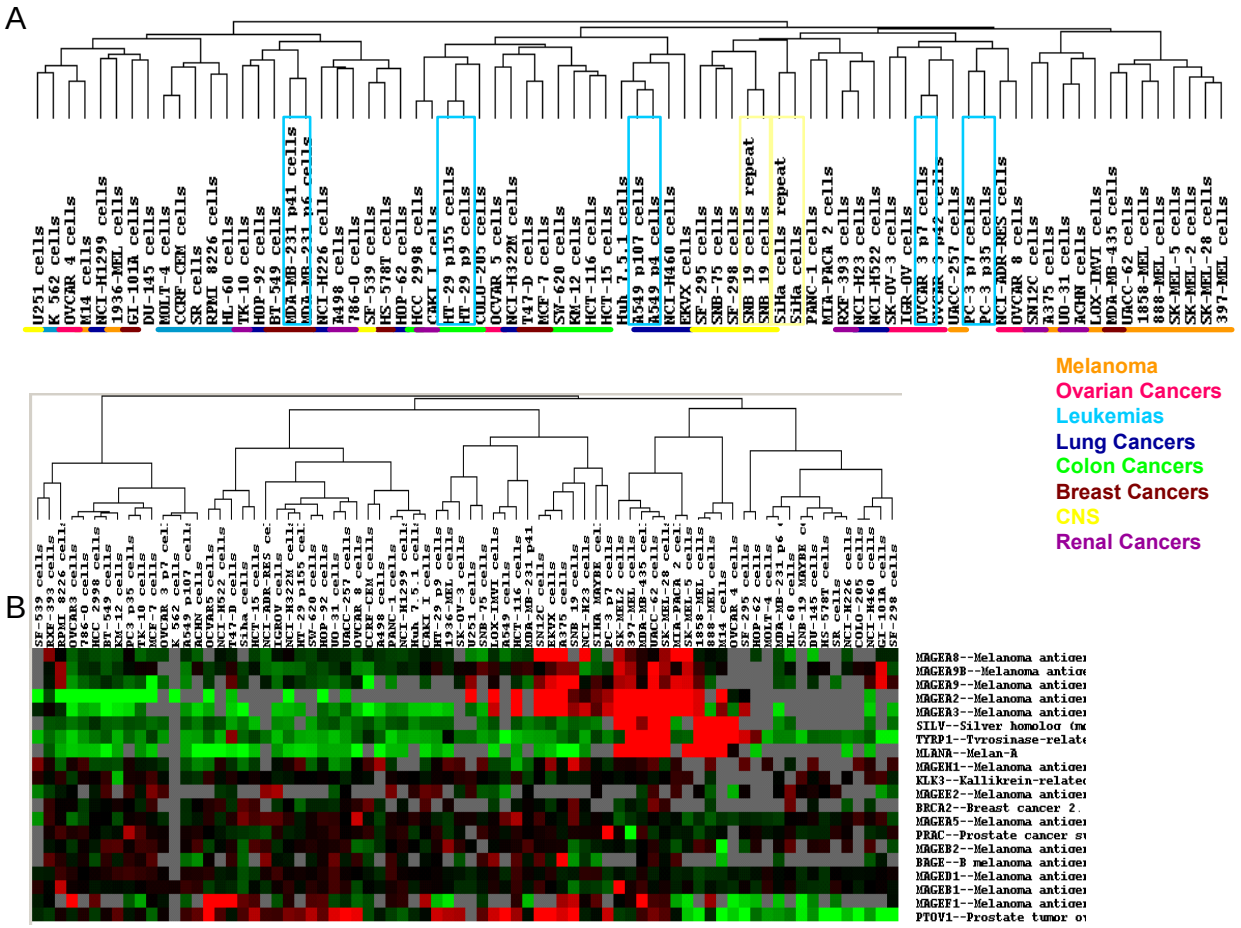



***Figure 1*** – ***(A)*** Unsupervised cluster analysis of 75 human cancer cell lines. Histological similar tumors and repeated hybridizations, as indicated by color-coding, are phenotypical similar and cluster closely together. ***(B)*** Expression analysis of common TAAs.

In a next step we checked for phenotypical markers of the respective cancers. We searched for melanoma-antigens, cancer-testis antigens and other markers specific for different malignancies based on literature findings. We identified the classic melanoma antigens (gp100, MelanA and Tyrosinase) to be expressed in most of the melanoma cell lines which were included in this study. Other melanomas, e.g. 1936-MEL and A375 were already proven to have lost the melanoma specific markers and we didn't expect them to show expression of the respective genes. Furthermore, we were able to detect PRAC (prostate cancer susceptibility candidate) exclusively in both, the early and late PC-3 cell line clones (**Figure 1B**).

### 5.3.3.2 Infection with GLV-1h68

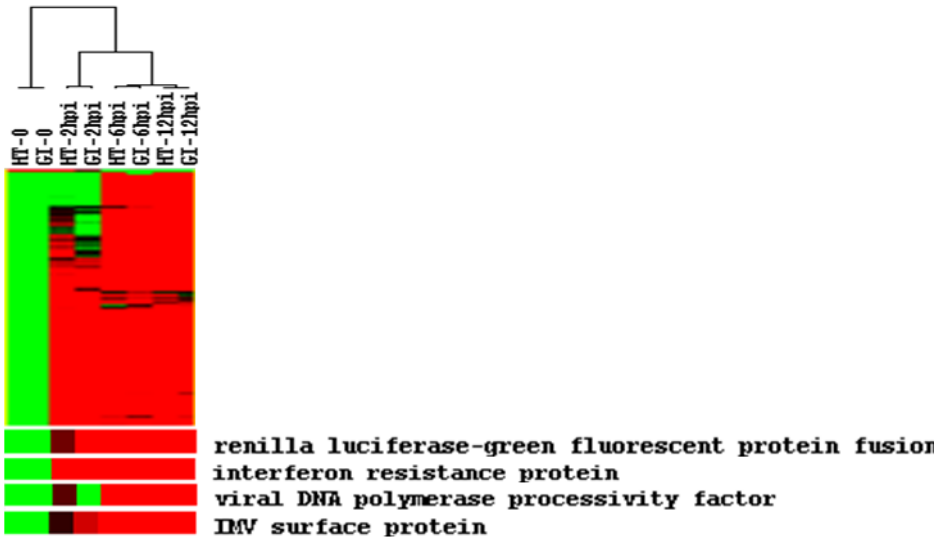
GFP gene expression correlates well with the remaining viral transcriptom

We hybridized two infected tumor cell lines, HT-29 and GI-101A and xenografts grown from the respective cell lines to customized VACV arrays and monitored the expression of the ruc-gfp-fusion transcript together with 219 viral genes on the chip. We observed that gfp expression correlates well with the majority of the viral transcripts and can be detected as early as 2hpi in HT-29 and GI-101A cells (**Figure 2A**). In infected GI-101A xenografts the gfp signal was visible as soon as 7dpi and fully expressed at day 21 (**Figure 2B**). The gfp expression was much more variable in HT-29 tumors and even after 42 days two out of three infected xenografts displayed down-regulation of the gfp message RNA. Interestingly, gfp seems to be among the first transcripts to be expressed in the xenografts as seen in one GI-101A tumor after 7 days and one HT-29 day 21 tumor in which most of the other viral genes are not expressed yet.

We also compared the ruc-gfp expression in GLV-1h68 infected HT-29 and GI-101A cells with the expression of the IMV-surface protein, Interferon resistance protein and DNA-polymerase processivity factor (**Figure 3A**). Scatterplot analysis revealed that there was a strong correlation for all three comparisons (R2 = 0.86/0.87).

Furthermore, the gfp signal detected with the customized microarrays and the overall presence call of all viral transcripts on the chip correlation was nearly perfect (R2 = 0.90 for the tumors, 0.93 for the cell lines) suggesting that the reporter gene gfp accurately represents the GLV-1h68 transcriptom and the replication of the virus (**Figure 3B**).

A




B

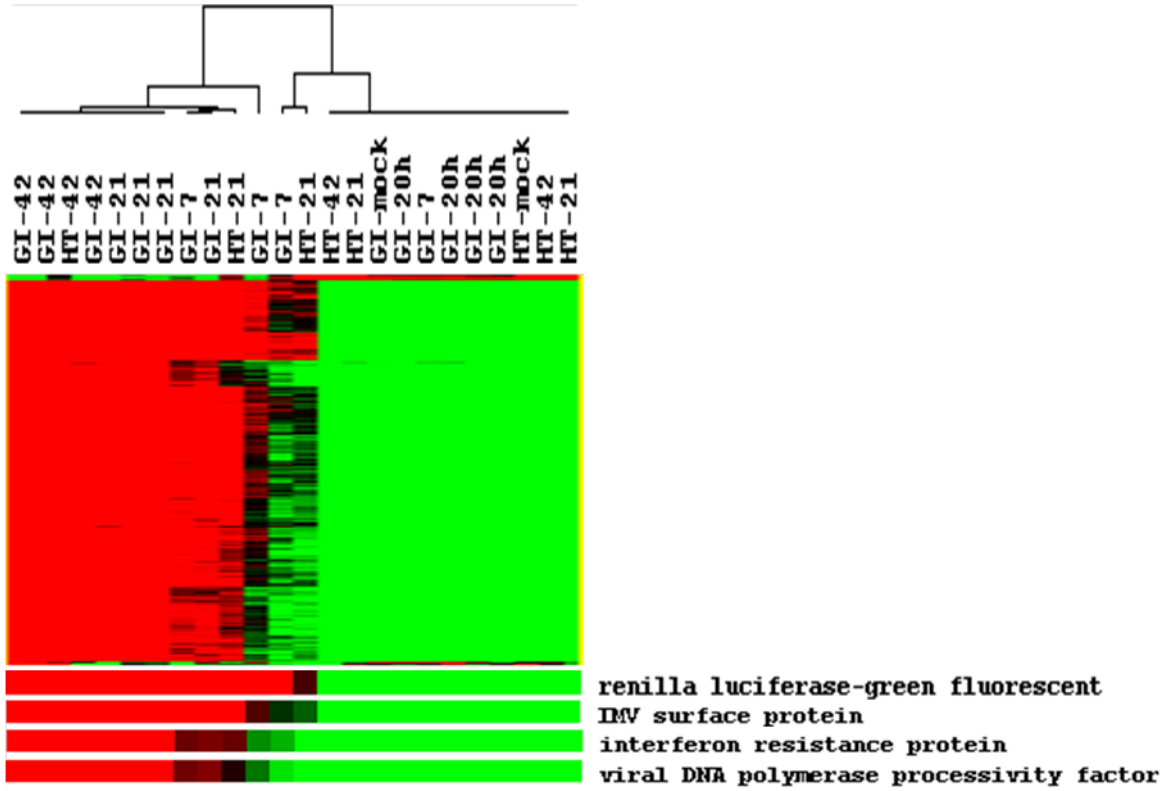



***Figure 2*** – ***(A)*** Time course analysis of GLV-1h68 infected HT-29 and GI-101A cells after two, six and 12 hpi. ***(B)*** Time course analysis of GLV-1h68 infected HT-29 and GI-101A Xenografts after tone, seven 21 and 42 days post infection.

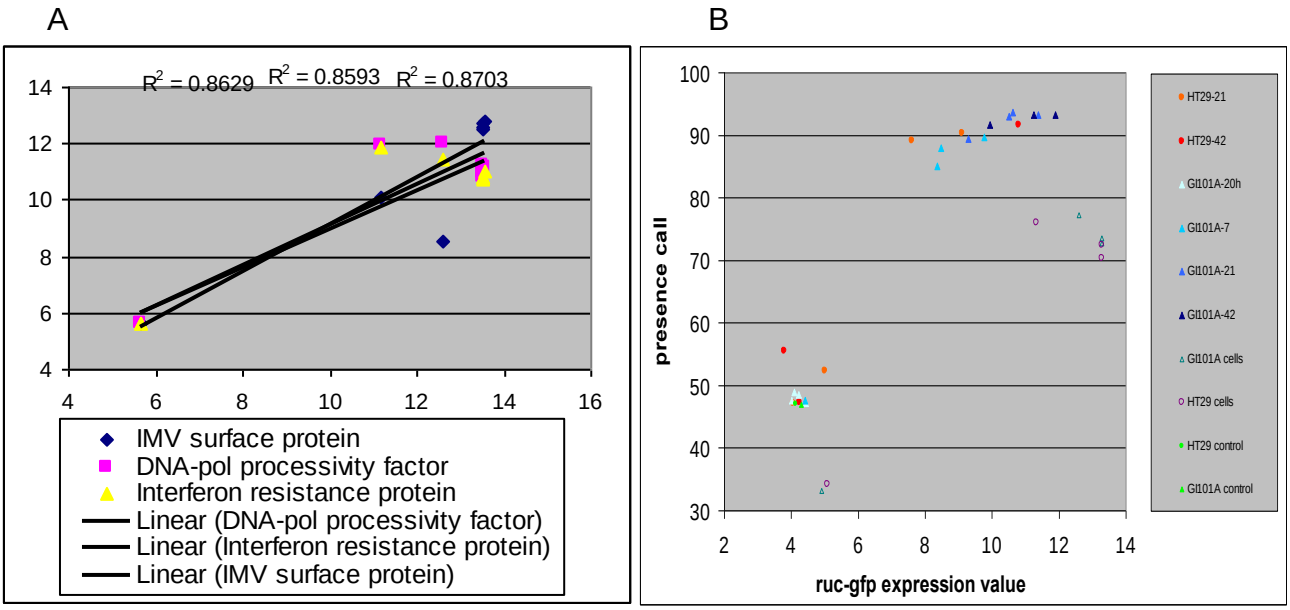


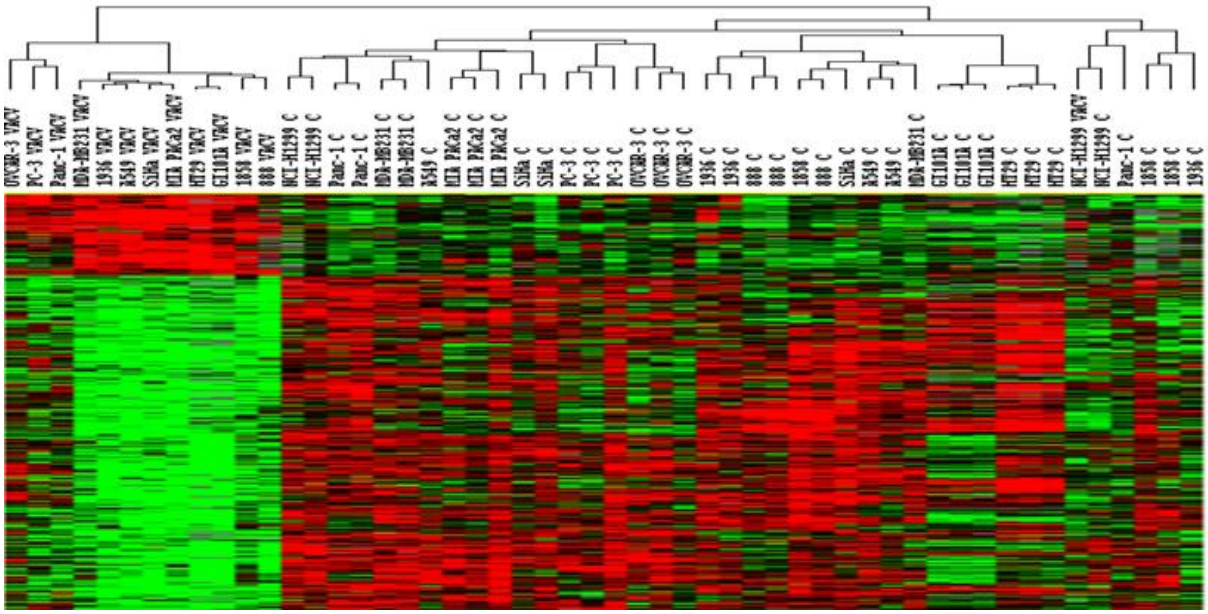

***Figure 3*** – ***(A)*** Correlation analysis of GfFP expression vs. IMV-surface protein, DNA-polymerase processivity factor and Interferon resistance protein. ***(B)*** Scatterplot analysis showing the correlation between GFP expression and overall presence call of the VACV microarray platforms for GLV-1h68 infected HT-29 and GI-101A cells and xenografts

VACV infection causes apoptosis through an oxidative phosphorylation mediated stress response

13 human cancer cell lines were infected with GLV-1h68 at an MOI of 0.01 and cells were harvested at three and 12hpi. Microarray analysis revealed a distinct signature in infected cells 12 hpi when compared to non infected controls (**Figure 4A**). This pattern mainly consisted of down-regulation of the host's metabolism such as protein ubiquitination, purine metabolism and nucleotide excision repair pathway (**Figure 4B**). Interestingly, the three top down-regulated pathways were oxidative phosphorylation, mitochondrial dysfunction and NRF2-mediated oxidative stress response. Cells that are relying on ATP production through oxidative phosphorylation undergo apoptosis if this main energy source is cut off. It is believed that most cancer cell lines rely on glycolysis as main energy source even in presence of high $O_2$ concentration and Warburg stated in 1956 that cancers originate from normal body cells in two phases; the first one being the irreversible injury of respiration (26). But even though all tumor cells show an enhanced glycolytiv flux not all of them have a diminished mitochondrial metabolic capacity. In fact, Moreno-Sanchez et al

summarized that in some cancer types oxidative phosphorylation contributes with an percentage as high as 97% to the ATP synthesis source (27).

A



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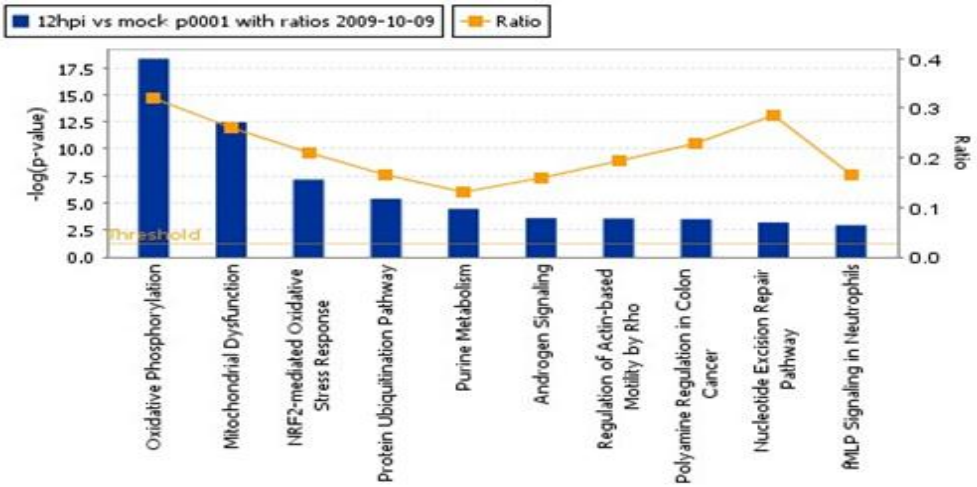



For Figure 4C see following page

C

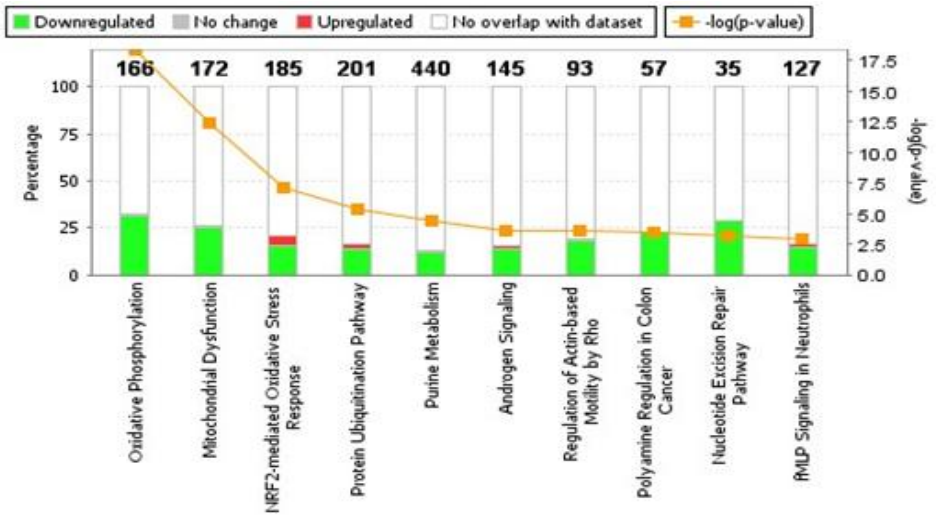



***Figure 4*** – ***(A)*** Microarray analysis of uninfected and 12hpi with GLV-1h68 human cancer cells. Student's t test analysis revealed 2564 differentially expressed genes at a p-value cut off >0.001 between the two groups. For headmap display, 80% presence call and 3fold change filters were applied. ***(B)*** Canonical pathway analysis of 2564 differentially expressed genes between mock infected and GLV-1h68 infected human cancer cells 12hpi and ***(C)*** proportion of up- and down-regulation in the respective pathway.

GFP protein expression using FACS analysis

After the validation of our infection model we decided to focus on gfp expression as a parameter to assess the extent of VACV (and later Ad5) replication in the cells. We tested different MOIs and timepoints of cell harvesting in a pilot study and concluded that infections with MOI 0.3 and 0.6 together with cell harvest 18 hpi were suitable to detect slight differences between the cell lines (data not shown).

75 cancer cell lines were infected with GLV-1h68 in groups of five cell lines and A549 was consistently used as a positive control in each batch. We identified two distinct patterns of GFP expression, i.e.; viral replication: some cell lines were highly susceptible to the infection (e.g. A549) in a dose dependent manner whereas others (e.g. SNB19) didn't display a strong replication pattern (**Figure 5**).

All measurements were normalized using BD's Rainbow Calibration Particles (6 peaks) as standards to ensure between-batch reproducibility and A549 infection was highly reproducible throughout the whole experiment (data not shown).

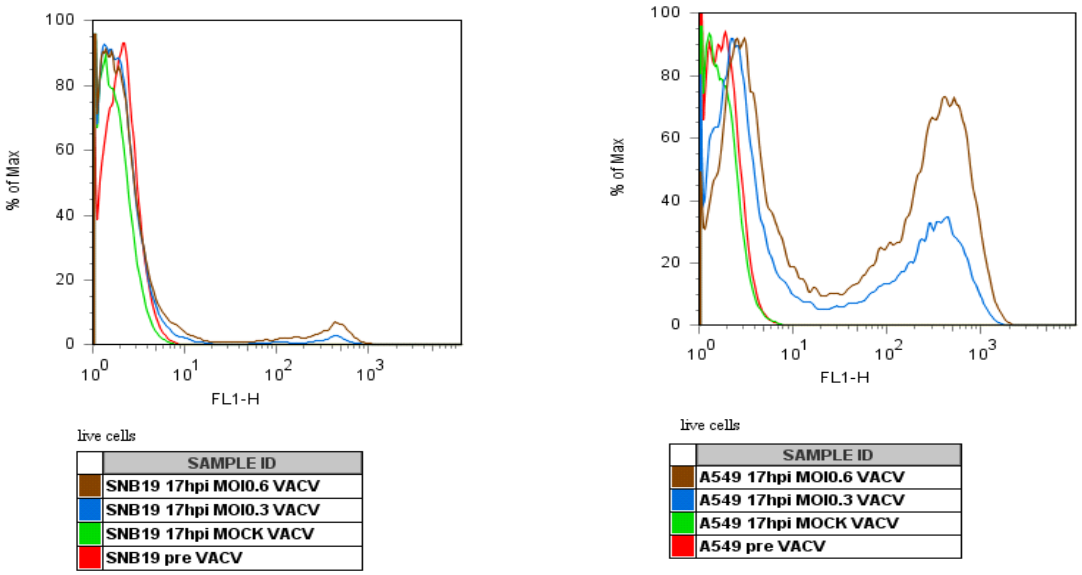



***Figure 5*** – GFP protein level of a poor (SNB-29, left) and a good (A549, right) VACV replicator cell line.

We measured the frequency of $GFP^+$ cells and the geometric mean in the respective population and defined the infectivity index (II) as the product of the two parameters. We plotted the VACV II after infection with the two different MOIs against each other (**Figure 6**) and found a perfect correlation between MOI 0.3 and 0.6 (R2= 0.96) with the ratio between the two parameters being 2.03. This suggests that our infection model is in the dynamic range and we decided to focus on MOI 0.6 data thereafter.

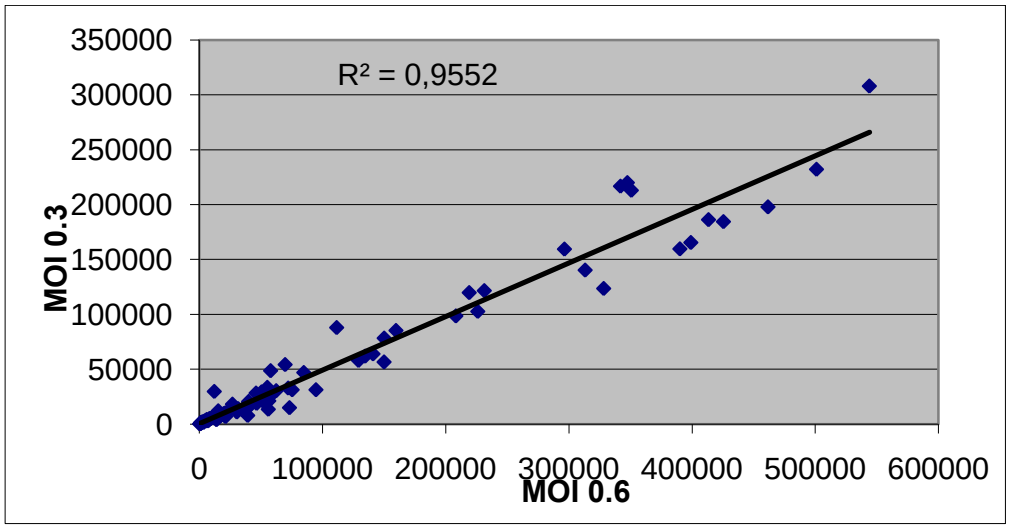



***Figure 6*** – Scatterplot analysis comparing Infectivity Indices after infection with GLV-1h68 at MOI 0.3 and 0.6.

We then ranked all 75 cell lines according to their infectivity index and found 10 cell lines (13.3 %; A549 p4, Ovcar 3 p7, A549 p120, HCT 116, HT29 p6, IGR-OVI, 397-Mel, 1858-Mel, Ovcar 4, Colo 205) to be very susceptible to VACV infection, 42 intermediate cell lines (56%) and 23 cell lines (30.7) were less permissive to the virus. Interestingly, 6 out of 6 tested haematopoetic cancer cell lines were among the lowest replicator models (Table 1).

Furthermore, 4 out of 5 cell line pairs with one new passage and an older one which has been cultured for a longer time showed that the younger passage was more susceptible to the virus infection suggesting that cell aging might play a role in the infection process.

***Table 1*** **-** Infectivity indices of 75 human cancer cell lines after infection with GLV-1h68

| MOI 0.6 | | | |
|---|---|---|---|
| Samples | Freq. | corrected GFP | index |
| A549 p4 | 0.70 | 604384.3 | 425962.9 |
| OVCAR-3 p7 | 0.62 | 682424.4 | 425559.9 |
| A549 p120 | 0.67 | 584812.6 | 409912.1 |
| HCT 116 | 0.72 | 561504.6 | 406258.9 |
| HT29 p6 | 0.42 | 378101.1 | 159709.9 |
| IGR-OVI I | 0.37 | 386358.5 | 141059.5 |
| 397-MEL | 0.36 | 370231.0 | 134616.0 |
| 1858-MEL | 0.29 | 445888.1 | 129263.0 |
| OVCAR-4 | 0.38 | 294742.0 | 111530.4 |
| Colo 205 | 0.34 | 285047.8 | 101576.1 |
| M14 | 0.40 | 235048.7 | 94724.6 |
| HT29 p155 | 0.25 | 343594.8 | 84833.6 |
| SK-MEL2 | 0.25 | 307659.0 | 75407.2 |
| SK-OV-3 | 0.41 | 178673.2 | 73220.3 |
| Siha | 0.42 | 173515.0 | 72182.2 |
| NCI-H322M | 0.39 | 178138.8 | 69687.9 |
| MALME 3M | 0.23 | 268488.3 | 62450.4 |
| PC3 p7 | 0.27 | 218151.0 | 57919.1 |
| KM12 | 0.17 | 334384.2 | 57112.8 |
| DU-145 | 0.28 | 204003.4 | 56733.3 |
| 888-MEL | 0.23 | 244953.5 | 56437.3 |
| SF 295 | 0.24 | 230609.0 | 56038.0 |
| MCF-7 | 0.21 | 260769.3 | 55230.9 |
| SN12C | 0.32 | 156316.2 | 50505.8 |
| TK-10 | 0.37 | 132233.0 | 49256.8 |
| NCI-ADR_RES | 0.43 | 107875.4 | 46569.8 |
| SF539 | 0.16 | 283998.5 | 46093.0 |
| PC3 p35 | 0.15 | 269836.7 | 40124.7 |
| NCI-H23 | 0.12 | 321226.5 | 40056.9 |
| Huh 7.5.1 | 0.17 | 232535.0 | 39321.7 |

| MOI 0.3 | | | |
|---|---|---|---|
| Samples | Freq. | corrected GFP | Index |
| OVCAR-3 | 0.39 | 476448.1 | 184433.1 |
| A549 p120 | 0.43 | 393581.4 | 182782.1 |
| A4549 p4 | 0.44 | 410321.0 | 178741.5 |
| HCT 116 | 0.46 | 382216.5 | 175816.9 |
| OVCAR-4 | 0.30 | 288485.9 | 87959.3 |
| HT29 p6 | 0.31 | 273708.1 | 85314.8 |
| IGR-OVI I | 0.21 | 300434.1 | 63992.5 |
| 397-MEL | 0.24 | 259295.4 | 61867.9 |
| 1858-MEL | 0.17 | 345082.3 | 58008.3 |
| NCI-H322M | 0.33 | 163152.5 | 54280.8 |
| PC3 p7 | 0.26 | 188753.0 | 48698.3 |
| HT29 p155 | 0.17 | 280559.8 | 47021.8 |
| Colo 205 | 0.20 | 177643.3 | 38005.5 |
| MCF-7 | 0.14 | 241434.7 | 33559.4 |
| Siha | 0.29 | 112648.1 | 32870.7 |
| M14 | 0.22 | 144656.2 | 31347.0 |
| SK-MEL2 | 0.14 | 216913.7 | 31322.3 |
| MALME 3M | 0.17 | 179240.3 | 30488.8 |
| NCI-H226 | 0.11 | 269728.8 | 29778.1 |
| SN12C | 0.25 | 116902.3 | 29342.5 |
| SF539 | 0.12 | 235164.9 | 28313.9 |
| DU-145 | 0.16 | 163835.8 | 26328.4 |
| TK-10 | 0.27 | 95157.6 | 25321.4 |
| KM12 | 0.10 | 257565.7 | 25009.6 |
| 888-MEL | 0.13 | 159746.1 | 21198.3 |
| NCI-H23 | 0.10 | 205786.3 | 20660.9 |
| NCI-ADR_RES | 0.26 | 71633.2 | 18911.2 |
| OVCAR-3 p42 | 0.10 | 182136.4 | 18231.9 |
| RXF-393 | 0.10 | 161757.3 | 16934.9 |
| PC3 p35 | 0.09 | 169741.7 | 15853.9 |

Table 1 continues on following page

| MOI 0.6 | | | |
|---|---|---|---|
| Samples | Freq. | corrected GFP | index |
| A498 | 0.17 | 218743.6 | 36486.4 |
| EKVX | 0.16 | 228304.9 | 36117.8 |
| RXF-393 | 0.14 | 240258.7 | 35969.7 |
| ACHN | 0.32 | 99935.4 | 31639.6 |
| OVCAR-8 | 0.36 | 85914.0 | 31066.5 |
| MDA-MB231 p41 | 0.17 | 177467.2 | 30471.1 |
| OVCAR-3 p42 | 0.12 | 224702.3 | 26919.3 |
| SNB75 | 0.09 | 249449.6 | 22849.6 |
| BT549 | 0.11 | 210463.3 | 22182.8 |
| SNB19 | 0.18 | 119269.9 | 21349.3 |
| 1936-MEL | 0.16 | 132661.7 | 21079.9 |
| MDA-MB435 | 0.17 | 121543.9 | 20698.9 |
| HOP62 | 0.12 | 162569.6 | 19768.5 |
| MIA PaCa2 | 0.17 | 103369.8 | 17376.5 |
| SK-Mel 28 | 0.15 | 110045.0 | 16737.8 |
| Caki I | 0.09 | 174012.0 | 15869.9 |
| UACC 257 | 0.13 | 114623.8 | 15405.4 |
| GI-101A | 0.14 | 107339.6 | 14791.4 |
| Hs578T | 0.08 | 167415.7 | 13912.2 |
| NCI-H226 | 0.07 | 177078.2 | 12183.0 |
| U-251 | 0.11 | 106490.4 | 11245.4 |
| UACC 62 | 0.08 | 136255.0 | 10437.1 |
| NCI-H460 | 0.09 | 98167.8 | 9247.4 |
| SF268 | 0.10 | 90469.5 | 8703.2 |
| SK-Mel 5 | 0.07 | 118884.4 | 7917.7 |
| Panc1 | 0.08 | 96173.4 | 7876.6 |
| MDA-MB231 | 0.11 | 70431.3 | 7500.9 |
| 786-0 | 0.15 | 47672.2 | 7141.3 |
| HCC2998 | 0.05 | 124047.0 | 6537.3 |
| A375 | 0.09 | 68745.3 | 6049.6 |
| UO-31 | 0.09 | 66299.9 | 5960.4 |
| HOP92 | 0.10 | 46249.7 | 4430.7 |
| RPMI-8226 | 0.04 | 92443.9 | 3891.9 |
| NCI-H1299 | 0.05 | 63868.4 | 3500.0 |
| T47D | 0.07 | 43097.5 | 2831.5 |
| LOX-IMVI | 0.04 | 59170.4 | 2591.7 |
| HCT15 | 0.07 | 31525.7 | 2121.7 |
| OVCAR5 | 0.04 | 49914.0 | 1961.6 |
| NCI-H522 | 0.03 | 59233.8 | 1723.7 |
| SR | 0.04 | 36772.3 | 1390.0 |
| SW-620 | 0.06 | 21137.4 | 1223.9 |
| K562 | 0.03 | 35710.5 | 1185.6 |
| CCRF_CEM | 0.08 | 11360.8 | 890.7 |
| HL-60 | 0.07 | 11962.5 | 890.0 |
| MOLT4 | 0.02 | 9768.2 | 242.3 |

| MOI 0.3 | | | |
|---|---|---|---|
| Samples | Freq. | corrected GFP | Index |
| A498 | 0.10 | 156625.4 | 15224.0 |
| SK-OV3 | 0.19 | 79394.6 | 15005.6 |
| EKVX | 0.10 | 145845.8 | 14059.5 |
| ACHN | 0.17 | 81170.6 | 14010.0 |
| OVCAR-8 | 0.20 | 66982.6 | 13691.2 |
| SF 295 | 0.11 | 122850.3 | 13550.4 |
| UACC 257 | 0.12 | 104444.5 | 12136.4 |
| MDA-MB231 p31 | 0.09 | 119430.8 | 11023.5 |
| SNB75 | 0.06 | 169541.2 | 10715.0 |
| BT549 | 0.06 | 170059.8 | 10407.7 |
| 1936-MEL | 0.11 | 96281.5 | 10215.5 |
| HOP62 | 0.08 | 117864.7 | 9594.2 |
| MIA PaCa2 | 0.11 | 74149.1 | 8460.4 |
| Caki I | 0.07 | 117698.4 | 8215.3 |
| Huh 7.5.1 | 0.07 | 121778.7 | 7952.1 |
| MDA-MB435 | 0.09 | 85855.0 | 7761.3 |
| SK-Mel 28 | 0.08 | 84743.6 | 7152.4 |
| SNB19 | 0.11 | 63484.9 | 6932.5 |
| U-251 | 0.08 | 84774.6 | 6434.4 |
| UACC 62 | 0.05 | 110735.9 | 5880.1 |
| GI-101A | 0.08 | 69806.8 | 5500.8 |
| SF268 | 0.07 | 77908.3 | 5157.5 |
| NCI-H460 | 0.07 | 70416.3 | 5147.4 |
| Panc1 | 0.07 | 71715.8 | 4790.6 |
| UO-31 | 0.07 | 59925.6 | 4212.8 |
| SK-Mel 5 | 0.04 | 97376.4 | 4080.1 |
| Hs578T | 0.05 | 86516.3 | 4057.6 |
| 786-0 | 0.10 | 37145.3 | 3640.2 |
| A375 | 0.06 | 55348.8 | 3426.1 |
| MDA-MB231 | 0.07 | 48072.7 | 3418.0 |
| HOP92 | 0.08 | 59925.6 | 4212.8 |
| HCC2998 | 0.04 | 69076.5 | 2811.4 |
| RPMI-8226 | 0.03 | 74995.6 | 2467.4 |
| T47D | 0.05 | 37639.4 | 2058.9 |
| OVCAR5 | 0.04 | 46024.6 | 1827.2 |
| LOX-IMVI | 0.04 | 46085.3 | 1774.3 |
| NCI-H1299 | 0.04 | 44676.1 | 1590.5 |
| NCI-H522 | 0.03 | 44717.1 | 1524.9 |
| HCT15 | 0.06 | 24326.9 | 1501.0 |
| SW-620 | 0.06 | 24840.1 | 1485.4 |
| K562 | 0.04 | 35073.5 | 1238.1 |
| SR | 0.03 | 27322.6 | 765.0 |
| HL-60 | 0.05 | 12811.9 | 590.6 |
| CCRF_CEM | 0.05 | 9591.2 | 515.0 |
| MOLT4 | 0.02 | 9980.5 | 246.5 |

We also analyzed different parameters such as age and gender of the patients, histological classification and p53 mutation status based on available data in the literature but we couldn't identify any correlation to the VACV infectivity index. Furthermore, the sensitivity to the oncolytic VACV was highly cell line specicific and even three autologous melanoma cell lines derived from the same progenitor cell clone though established from 3 metachronous metastases were phenotypical different based on our assay. While the 888-MEL cell line (II=56437, intermediate) was generated in 1989 during the earlier stage of the disease and the patient underwent complete remission after adoptive therapy, the 1858-MEL cell line (II=129263, high) was established 10 years later in 2000 after a recurrence of the disease in the patient and unsuccessful treatment with a β-catenin reactive clone. Lastly, 1936-MEL (II=21080, intermediate) was expanded in 2001 after the patient was rapidly progressing and did not respond to further treatment (**Table 1**).

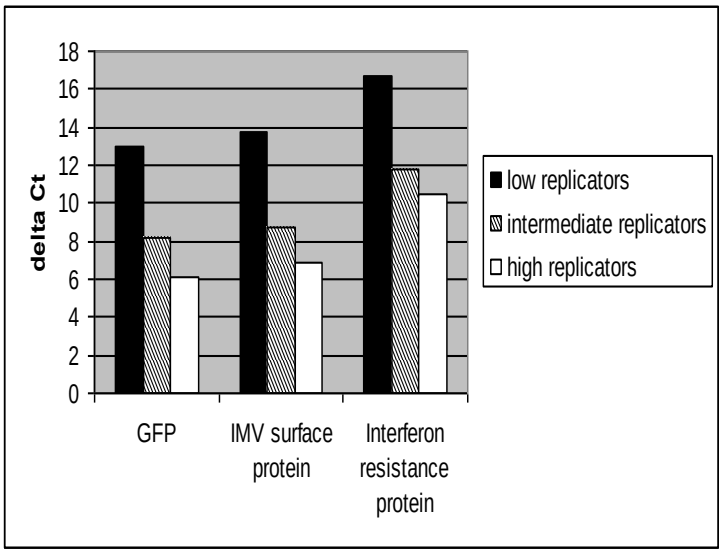
qPCR quantification of viral gene expression

In order to test our FACS based GFP protein assay we looked at three viral genes applying quantitative real-time PCR. Besides the RUC-GFP fusion protein we choose to monitor also the Interferon-resistance protein (Interf; K3L) and the IMV surface protein (IMV; A27L).

We grouped the cells according to the VACV II and normalized the Ct values with the endogenous control 18S rRNA for each sample. Due to the assay design and analysis of viral genes uninfected control samples couldn't be used as negative control and only the Δ Ct (instead of ΔΔCt) was calculated. For all three markers (GFP, IMV and Interf) we found statistical significant differences ($p<0.003$ for all comparisons) based on a two-tailed Student t test with two sample unequal variance settings (**Figure 7A**).

Overall correlation analysis revealed a moderate correlation ($R2=0.66$) between the GFP protein level (measured by FACS analysis) and the mRNA transcript level (qPCR results; **Figure 7B**). The two outliers were two leukemia cell lines, HL-60 and MOLT4, which almost persisted infection with VACV and behaved similar to non-infected controls. When taken out from the scatterplot analysis the overall correlation value immediately increased to $R2=0.75$.

A




B

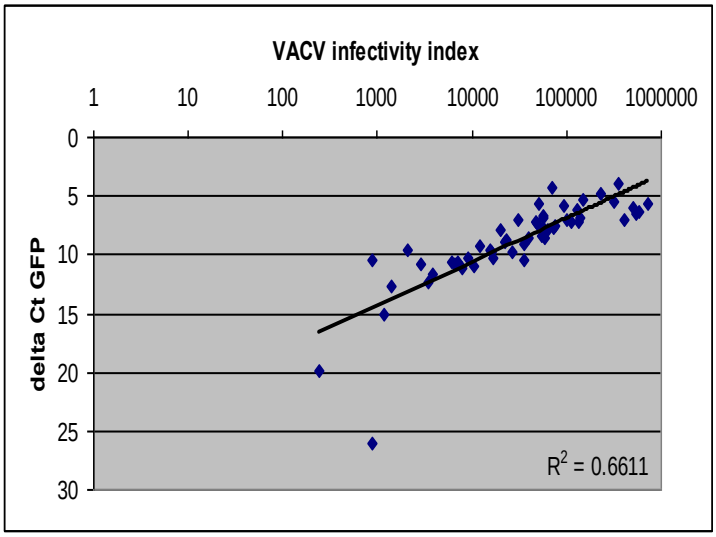



***Figure 7*** – ***(A)*** qPCR results of infected human cancer cell lines and displayed as ΔCt. Cells were grouped in high, intermediate and low replicators based on FACS analysis. ***(B)*** Scatterplot analysis comparing ΔCt value and infectivity indices derived from GFP expression based on FACS analysis.

<u>Microarray analysis of 75 cancer cell lines (VACV classification)</u>

Secondarily amplified RNA from 75 untreated cell lines was hybridized to whole genome human arrays in order to assess the endogenous transcript level. Based on the classification derived from the FACS analysis we applied a Student t test to test overall differences (cut-off p2 value <0.01) comparing high vs. low replicator cell lines. The analysis revealed 335 differentially expressed genes between the two groups (permutation p value 0.08) with 168 down-regulated and 167 up-regulated transcripts in the low replicator cell lines (**Figure 8A**).

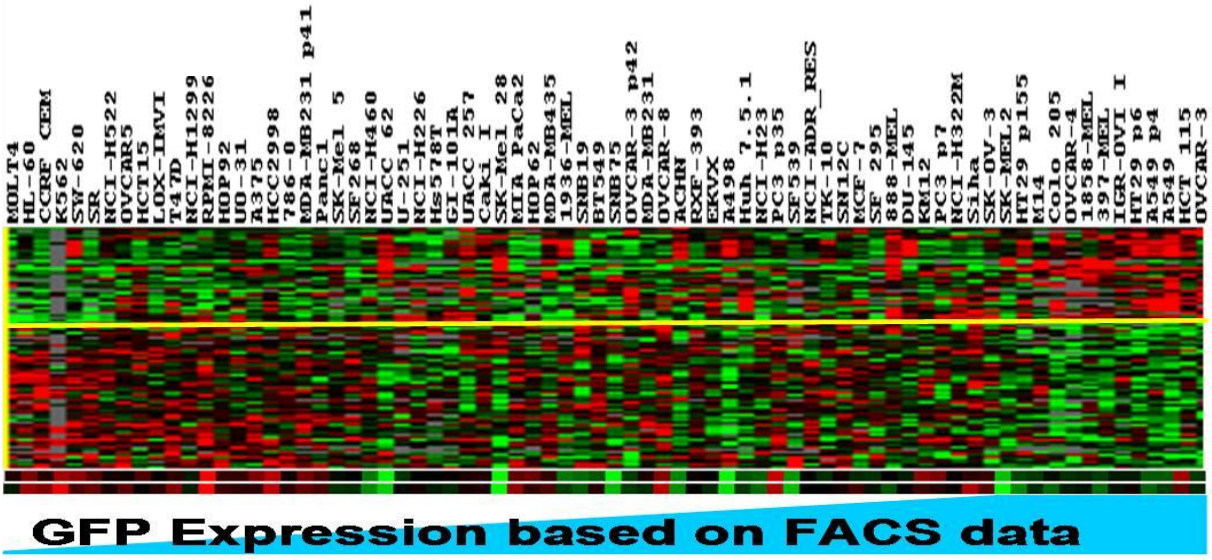



***Figure 8*** – ***(A)*** Microarray analysis of 75 human cancer cells ordered according to the VACV infectivity index. Student's t test analysis between high and low replicators revealed 335 differentially expressed genes. For visualization, intermediate replicators are also shown.

Interestingly, one of the most involved networks is centered around the up-regulated NfκB complex (**Figure 8B**). Among the down-regulated genes involved in the same network is IFN-α and –β, Il-12 complex and Il-12B, CCL1 and GDF-15, a new member of the TGF-β family. GDF-15, also called Macrophage inhibitory cytokine 1 (MIC-1) is shown to be associated with progression of cervical cancer and high grade prostate tumors. High MIC-1 serum levels in clinical studies were linked with the progression of prostate cancer to metastasis, implicating a role of MIC-1 in prostate tumorigenesis and metastasis (28).

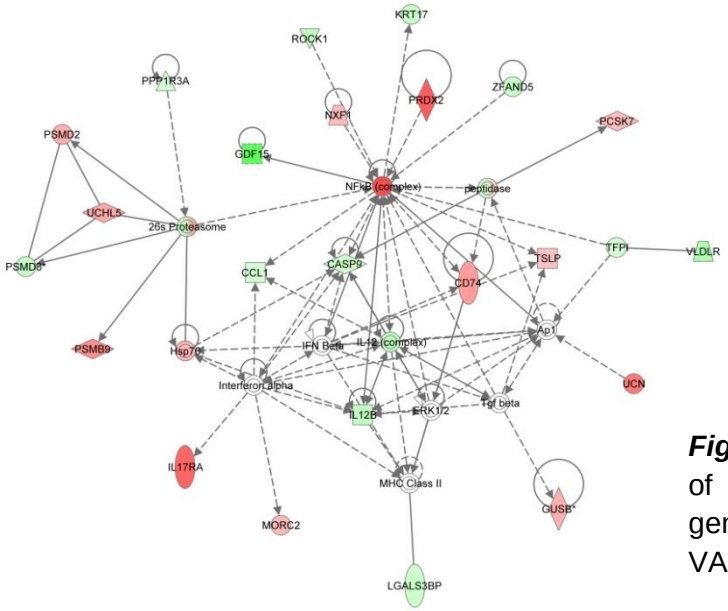
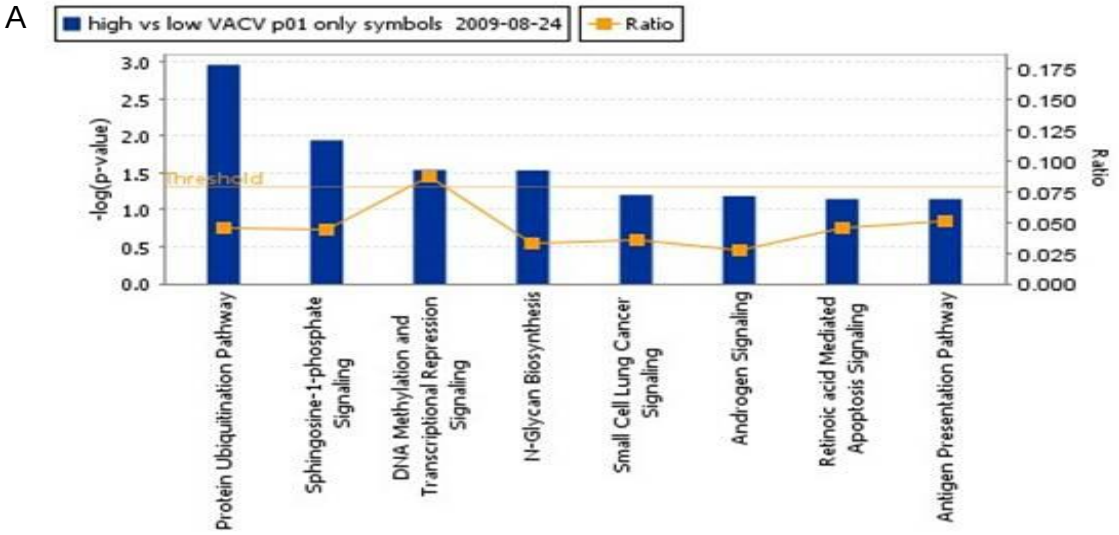



***Figure 8 – (B)*** Network analysis of 335 differentially expressed genes between high and low VACV replicators.

Ingenuity Pathway Analysis revealed up-regulated pathways in low replicators (i.e. down-regulated in high replicators) such as DNA-Methylation and Transcriptional Regression signaling (**Figure 8C and D**).

A




B

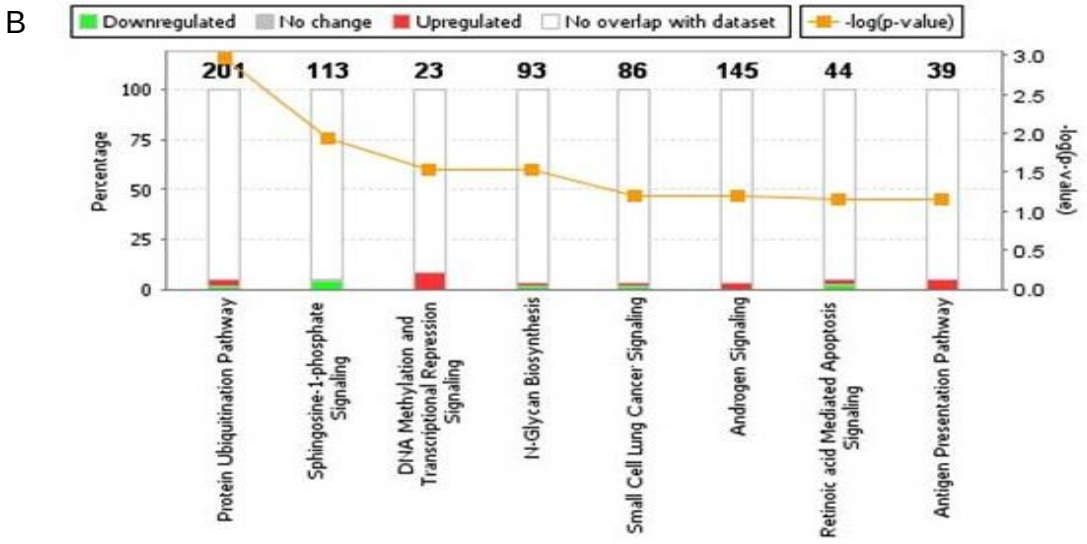



***Figure 8*** – ***(C)*** and ***(D)*** Canonical Pathway analysis was performed using IPA software

Furthermore, two probes of the Finkel-Reilly-Biskis murine sarcoma virus-associated ubiquitously expressed gene (Fau) were consistently up-regulated in the low replicator cell lines (**Figure 8A**). This gene has been identified in previous studies in our lab when we compared microarrays from a smaller subset of uninfected human cancer cell lines and fau was associated with implicated VACS permissivity also at that time (data not shown). Pickard et al. identified fau as a novel apoptosis-regulator and candidate tumor suppressor with oncogenic properties in different contexts (29).

Fau also carries an ubiquitin-like FUBI domain which is known to target the Bcl2 ligand 14, a pro-apoptotic member of the Bcl-2 family of apoptosis-controlling proteins.

#### *5.3.3.3 Infection with Ad5*

All cell lines were simultaneously infected with Ad5, a non-oncolytic member of the Adenovirus C species. Similar to VACV, Ad5 uses the host's replication machinery but in contrast to VACV Ad5 replicates intra-nuclear.

FACS analysis of GFP expression

In a pre-study we tested different MOIs and timepoints for cell harvesting and also monitored GFP expression under a fluorescence microscope (data not shown). Based on these preliminary findings we decided to harvest cells at 2 days post infections with MOI 100 and 300, respectively.

Table 2 Infectivity indices of 75 human cancer cell lines after infection with Ad5.

| MOI 300 | | | |
|---|---|---|---|
| Samples | Freq | corrected GFP | index |
| SNB 19 | 0.87 | 1206458.6 | 1043948.6 |
| U-251 | 0.82 | 1009315.2 | 831272.0 |
| MIA PaCa2 | 0.77 | 903603.6 | 700021.7 |
| SiHa | 0.80 | 683036.9 | 544790.2 |
| LOX-IMVI | 0.31 | 1056991.7 | 330204.2 |
| OVCAR8 | 0.60 | 528694.1 | 317163.6 |
| 397-MEL | 0.63 | 463757.6 | 290034.0 |
| A375 | 0.41 | 629801.5 | 260548.9 |
| HCT 116 | 0.58 | 364305.8 | 227020.8 |
| NCI-ADR_RES | 0.51 | 406655.3 | 208329.5 |
| OVCAR3 p42 | 0.42 | 336167.9 | 140854.3 |
| NCI-H522 | 0.53 | 251068.5 | 132965.9 |
| 1858-MEL | 0.34 | 357082.6 | 121836.6 |
| SF 268 | 0.32 | 376279.3 | 120672.8 |
| SK-Mel 28 | 0.55 | 183295.4 | 100720.8 |
| SK-Mel5 | 0.29 | 330664.5 | 96421.8 |
| BT549 | 0.41 | 218617.4 | 89720.6 |
| 888-MEL | 0.40 | 225987.7 | 89491.1 |
| SF 295 | 0.37 | 238359.8 | 87597.2 |
| MDA-MB231 p41 | 0.33 | 214176.8 | 70571.3 |
| OVCAR3 p7 | 0.46 | 142274.8 | 65887.5 |
| 786-0 | 0.35 | 180776.1 | 63705.5 |
| SK-MEL2 | 0.38 | 141676.8 | 53143.0 |
| MDA-MB231 p6 | 0.35 | 153300.0 | 53011.1 |
| Huh7.5.1 | 0.25 | 204617.8 | 50131.4 |
| PC3 p35 | 0.21 | 235940.5 | 48698.1 |

| MOI 100 | | | |
|---|---|---|---|
| Samples | Freq | corrected GFP | index |
| SNB 19 | 0.60 | 541326.6 | 325012.5 |
| U-251 | 0.45 | 490278.7 | 222390.4 |
| MIA PaCa2 | 0.47 | 428562.7 | 199453.1 |
| SiHa | 0.45 | 267343.4 | 119368.8 |
| LOX-IMVI | 0.14 | 662586.5 | 92033.3 |
| A375 | 0.23 | 382947.5 | 89954.4 |
| 397-MEL | 0.33 | 257358.2 | 86163.5 |
| HCT 116 | 0.34 | 202565.9 | 72362.0 |
| OVCAR8 | 0.24 | 252630.8 | 61086.1 |
| SK-Mel5 | 0.22 | 277900.0 | 59998.6 |
| OVCAR3 p42 | 0.25 | 169570.8 | 42494.4 |
| NCI-ADR_RES | 0.21 | 183860.1 | 39254.1 |
| 1858-MEL | 0.17 | 216026.2 | 35795.5 |
| NCI-H522 | 0.22 | 148337.3 | 32767.7 |
| 888-MEL | 0.19 | 148955.3 | 27869.5 |
| BT549 | 0.19 | 140537.2 | 26280.5 |
| SK-Mel 28 | 0.23 | 109433.8 | 25596.6 |
| SF 295 | 0.17 | 149991.3 | 24913.6 |
| MDA-MB231 p41 | 0.15 | 147215.5 | 22332.6 |
| SF 268 | 0.12 | 176824.4 | 20706.1 |
| 786-0 | 0.18 | 115865.8 | 20404.0 |
| MDA-MB231 p6 | 0.16 | 112837.8 | 17941.2 |
| MALME 3M | 0.10 | 161042.2 | 16684.0 |
| PC3 | 0.11 | 138370.6 | 15829.6 |
| Huh7.5.1 | 0.12 | 122558.3 | 15197.2 |
| SK-MEL2 | 0.19 | 79244.8 | 15016.9 |

Table 2 continues on following page

| MOI 300 | | | |
|---|---|---|---|
| Samples | Freq | corrected GFP | index |
| MALME 3M | 0.21 | 216085.5 | 45875.0 |
| TK10 | 0.27 | 152084.8 | 41154.1 |
| SF-539 | 0.15 | 277008.3 | 40415.5 |
| NCI-H1299 | 0.29 | 125060.5 | 36780.3 |
| RXF-393 | 0.26 | 127575.8 | 36163.4 |
| HOP92 | 0.22 | 151037.4 | 32639.2 |
| NCI-H226 | 0.14 | 231414.5 | 32560.0 |
| HOP62 | 0.24 | 132321.6 | 31466.1 |
| OVCAR4 | 0.20 | 154163.4 | 31063.9 |
| NCI-H23 | 0.21 | 132290.0 | 27847.1 |
| EKVX | 0.20 | 132592.4 | 26942.8 |
| SN12C | 0.22 | 115862.1 | 25825.7 |
| A498 | 0.19 | 133291.5 | 25565.3 |
| ACHN | 0.19 | 129811.0 | 24806.9 |
| HT29 p9 | 0.30 | 83782.2 | 24724.1 |
| SNB75 | 0.15 | 159882.6 | 23998.4 |
| A549c | 0.25 | 79328.4 | 22090.4 |
| SR | 0.24 | 89279.9 | 21382.5 |
| DU-145 | 0.26 | 79504.5 | 20599.6 |
| PC3 p7 | 0.22 | 89183.5 | 19780.9 |
| NCI-H460 | 0.17 | 116599.2 | 19763.6 |
| IGR-OVI | 0.35 | 50069.5 | 17354.1 |
| Hs 578T | 0.13 | 121459.1 | 15206.7 |
| SK-OV3 | 0.24 | 59835.8 | 14270.8 |
| HCT15 | 0.21 | 67586.8 | 14071.6 |
| UACC257 | 0.08 | 162103.7 | 12368.5 |
| RPMI 8226 | 0.45 | 26343.1 | 11767.5 |
| KM12 | 0.12 | 94730.3 | 11168.7 |
| A549 p4 | 0.21 | 50675.3 | 10722.9 |
| Panc1 | 0.18 | 54628.3 | 9887.7 |
| UO-31 | 0.09 | 99635.2 | 9395.6 |
| UACC62 | 0.09 | 84933.4 | 7610.0 |
| GI-101A | 0.13 | 50669.7 | 6379.3 |
| T47D | 0.12 | 40109.3 | 5005.6 |
| HCC2998 | 0.07 | 67084.9 | 4924.0 |
| M14 | 0.23 | 15933.4 | 3664.7 |
| CCRF_CEM | 0.36 | 9820.4 | 3551.1 |
| HT29 p155 | 0.08 | 41241.1 | 3216.8 |
| MDA-MB435 | 0.08 | 38131.0 | 2978.0 |
| K562 | 0.06 | 43618.5 | 2403.4 |
| OVCAR5 | 0.04 | 52569.7 | 2034.4 |
| Caki I | 0.05 | 37481.2 | 1919.0 |
| NCI-H322M | 0.04 | 34065.5 | 1481.9 |
| MCF-7 | 0.05 | 25781.8 | 1402.5 |
| SW620 | 0.11 | 12075.4 | 1307.8 |
| 1936-MEL | 0.04 | 20512.6 | 863.6 |
| Colo 205 | 0.04 | 14277.5 | 539.2 |
| HL60 | 0.02 | 13798.8 | 321.5 |
| MOLT4 | 0.04 | 8601.8 | 317.4 |

| MOI 300 | | | |
|---|---|---|---|
| Samples | Freq | corrected GFP | index |
| RXF-393 | 0.13 | 92486.3 | 12929.8 |
| OVCAR3 p7 | 0.17 | 70979.1 | 11810.9 |
| TK10 | 0.12 | 90521.5 | 10853.5 |
| NCI-H226 | 0.07 | 151422.0 | 10841.8 |
| A498 | 0.10 | 110561.3 | 10691.3 |
| RPMI 8226 | 0.31 | 33475.5 | 10514.7 |
| NCI-H23 | 0.10 | 102477.7 | 10319.5 |
| SR | 0.14 | 69746.6 | 9883.1 |
| HOP62 | 0.11 | 88499.4 | 9726.1 |
| OVCAR4 | 0.09 | 111873.9 | 9699.5 |
| SF-539 | 0.06 | 145942.9 | 9136.0 |
| HOP92 | 0.10 | 95617.5 | 9083.7 |
| EKVX | 0.10 | 91954.4 | 9066.7 |
| HT29 | 0.15 | 61016.5 | 8993.8 |
| SNB75 | 0.08 | 117242.5 | 8898.7 |
| NCI-H1299 | 0.11 | 74405.4 | 8407.8 |
| ACHN | 0.08 | 97846.6 | 8199.5 |
| A549 p120 | 0.12 | 59989.4 | 8190.7 |
| A549 p4 | 0.16 | 46247.0 | 7316.3 |
| NCI-H460 | 0.09 | 70881.3 | 6542.3 |
| SN12C | 0.09 | 70297.5 | 6439.2 |
| DU-145 | 0.11 | 56199.5 | 6361.8 |
| IGR-OVI | 0.15 | 38261.3 | 5888.4 |
| PC3 p7 | 0.10 | 59842.0 | 5882.5 |
| HCT15 | 0.12 | 45778.9 | 5269.1 |
| UACC257 | 0.04 | 118290.0 | 5051.0 |
| Hs 578T | 0.07 | 71071.5 | 4619.6 |
| UO-31 | 0.06 | 73789.1 | 4508.5 |
| KM12 | 0.06 | 73198.4 | 4055.2 |
| Panc1 | 0.09 | 43184.4 | 3942.7 |
| M14 | 0.24 | 14772.7 | 3536.6 |
| UACC62 | 0.05 | 67989.3 | 3358.7 |
| SK-OV3 | 0.08 | 38481.0 | 3143.9 |
| CCRF_CEM | 0.27 | 9641.2 | 2557.8 |
| GI-101A | 0.07 | 33144.0 | 2389.7 |
| HCC2998 | 0.05 | 48251.6 | 2205.1 |
| OVCAR5 | 0.04 | 44393.1 | 1904.5 |
| T47D | 0.05 | 34409.8 | 1875.3 |
| HT29 | 0.05 | 35627.1 | 1742.2 |
| K562 | 0.04 | 32149.4 | 1408.1 |
| MDA-MB435 | 0.04 | 28797.7 | 1195.1 |
| NCI-H322M | 0.03 | 30875.2 | 1059.0 |
| Caki I | 0.03 | 30034.6 | 1036.2 |
| MCF-7 | 0.05 | 19802.4 | 1000.0 |
| SW620 | 0.05 | 10406.2 | 504.7 |
| Colo 205 | 0.03 | 12808.7 | 419.1 |
| 1936-MEL | 0.02 | 18209.4 | 387.9 |
| MOLT4 | 0.04 | 8781.0 | 344.2 |
| HL60 | 0.02 | 14157.2 | 256.2 |

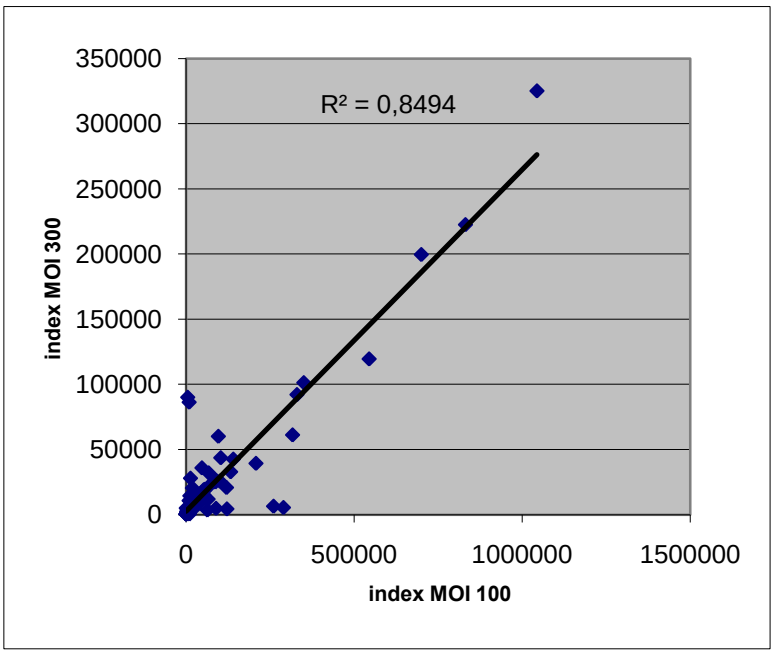
Analysis revealed a perfect correlation between MOI100 and 300 data points (R2=0.97) with a ratio of 3.4 (**Figure 9A**). Calculation of the Ad5 infectivity index was as described for the VACV infections (briefly: frequency of GFP+ cells multiplied with the geometric mean of GFP+ cells) and all cell lines were ranked according to the MOI 300 Index value (**Table 2**). This analysis revealed 15 cell lines (20%) (SNB 19, U-251, MIA PaCa2, SiHa, LOX-IMVI, Ovcar 8, 397-Mel, A375, HCT 116, NCI-ADR-RES, Ovcar 3 p42, NCI-H522, 1858-Mel, SF 268, SK-Mel 28) as highly susceptible to Ad5 infection, 40 intermediate replicators (53.3%) and 20 cell lines with low permissivity (26.7%) (Panc-1, UO-31, UACC62, GI-101A, T47D, HCC2998, M14, CCRF- CEM, HT29 p155, MDA-MB435, K562, Ovcar 5, Caki I, NCI-H322M, MCF-7, SW620, 1936-Mel, Colo 205, HL-60 and MOLT4).
In contrast to infection with VACV, in 4 out of 5 pairs (Ovcar 3, MDA-MB-231, A549 and PC-3) the younger passages were less susceptible to infection with Ad5.

Microarray analysis of 75 cancer cell lines (Ad5 classification)

To better characterize the transcriptional program of cancer cell lines with various susceptibility to Ad5 we compared high and low replicators applying a student t test with the cut off p value of 0.01 (**Figure 9B**). 722 genes separated the two phenotypical classes (permutation p value 0).

Canonical pathways analysis using IPA software revealed basic metabolic pathways such as protein ubiquitination and o-glycan Biosynthesis to be mainly down-regulated in the low replicator group (**Figure 9C and D**). Interestingly, the most significant down-regulated pathway among cancer cell lines with low susceptibility to Ad5 is tight junction signaling. Ad5, in contrast to VACV, uses a specific receptor for virus entry into the host cells. The coxsackievirus and adenovirus receptor (CAR) mediates attachment and entry of a number of adenoviruses but interacts also in homotypic intercellular interactions. Coyne and Bergelson describe that CAR is closely associated with the tight junction with contribution to the barrier to paracellular flow of solutes and macromolecules and it also seems to play a role during embryonic development and cell proliferation (30).

A




B

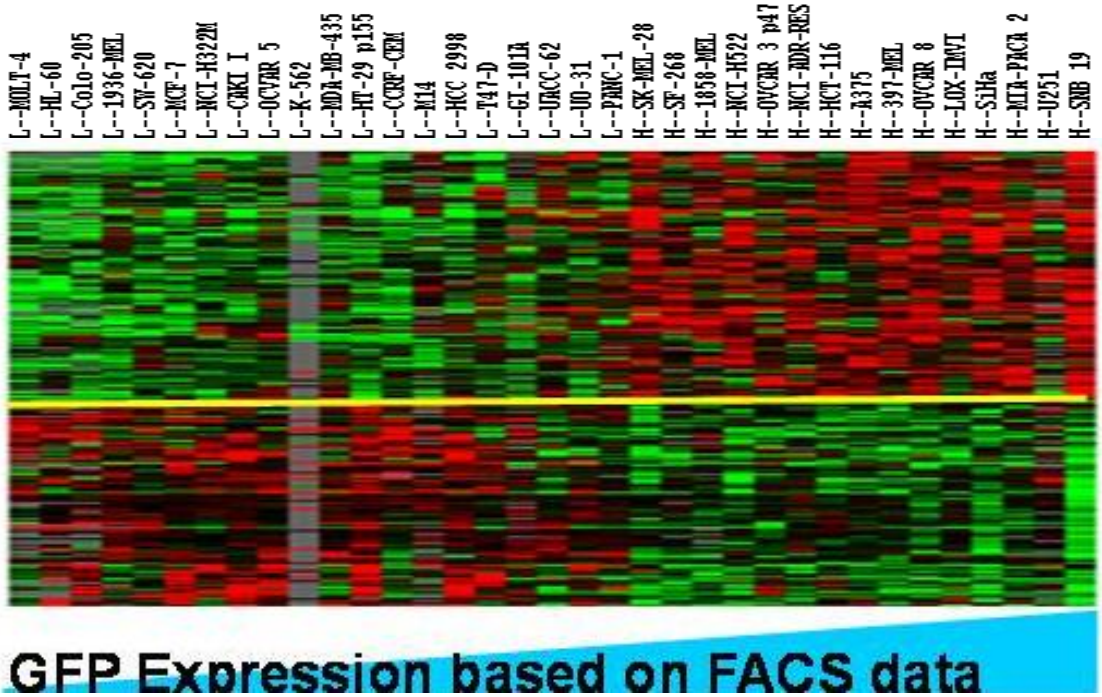
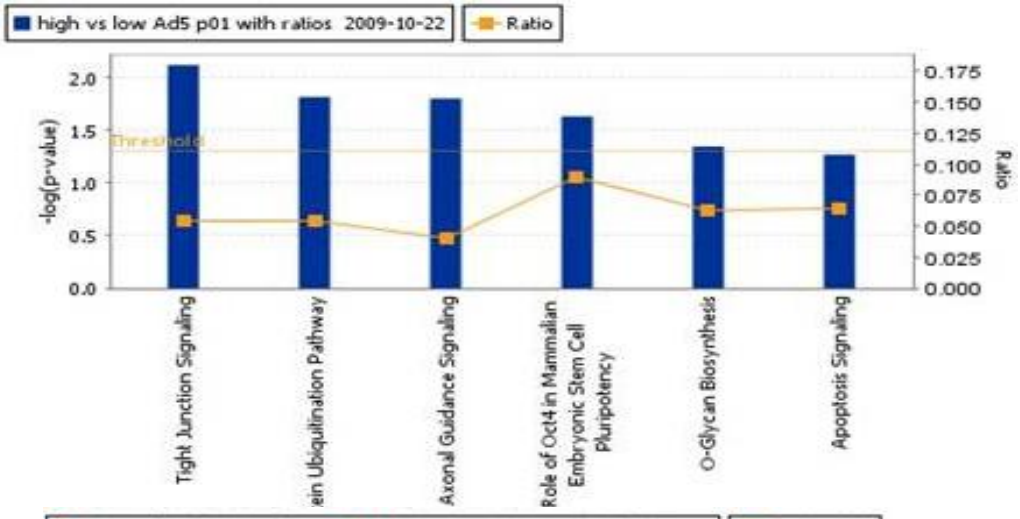



***Figure 9*** – ***(A)*** Scatterplot analysis comparing Ad5 infectivity indices after infection with MOI 100 and 300. ***(B)*** Microarray analysis of 75 human cancer cells ordered according to the Ad5 II. Student's t test analysis between high and low replicators revealed 722 differentially expressed genes.

C




D

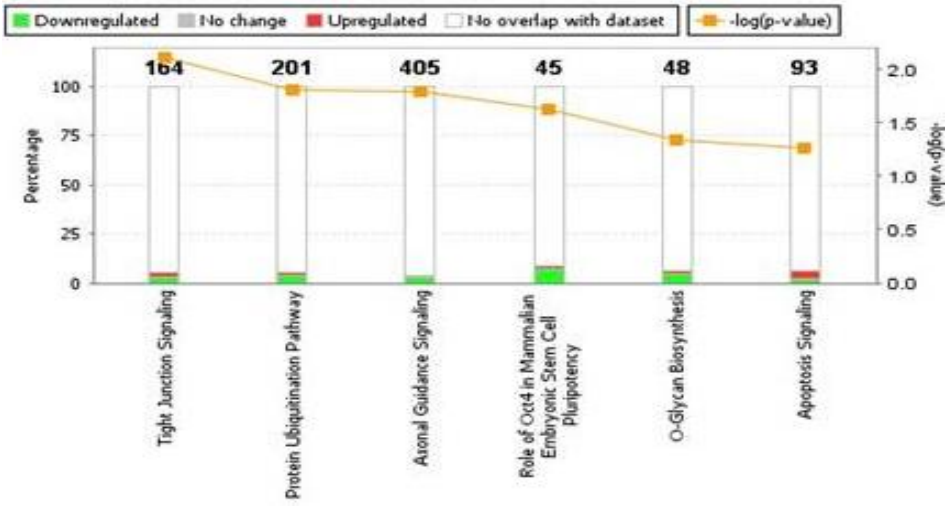



***Figure 9*** – Canonical Pathway analysis ***(C)*** and ***(D)*** was performed using IPA software and displayed the most affected networks and the proporation of up- and down-regulated, respectively.

We then analyzed molecule networks which were created based on the input of 722 differentially expressed genes between high and low Ad5 replicator cell lines and Mx1 was among up-regulated genes in low replicators (**Figure 10**). We previously identified Mx1, whose expression is indicative of the activation of Interferon dependent pathways, to be associated with impaired Adenovirus replication in both, pancreatic cancer cell lines and primary tumors in vivo (17). Furthermore, the highest up-regulated gene in low replicators (15fold compared to high replicators) was immune-responsive gene 1 (IRG1) which has never been associated with restricted Adenovirus replication before.

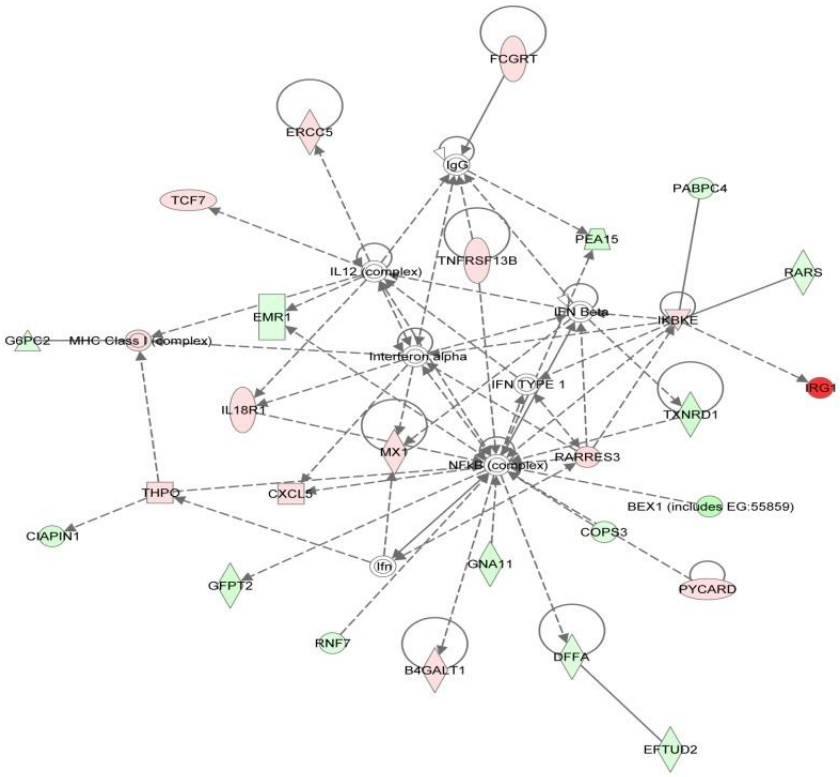



***Figure 10*** – Network analysis of 722 differentially expressed genes between high and low Ad5 replicators reveals the up-regulation of IRG-1 and Mx-1.

### *5.3.3.4 Comparison of Ad5 and VACV susceptibility in human cancer cell lines*

A scatterplot analysis comparing the Infectivity indices from both VACV and Ad5 infection showed that there is no correlation between the quantitative susceptibility of the two viruses that we examined (**Figure 11**). However, 10 cell lines were less susceptible to both viruses, 25 showed intermediate permissivity and 3 cell lines were highly susceptible to both agents accounting for half (38 out of 75) of the cancers cells.

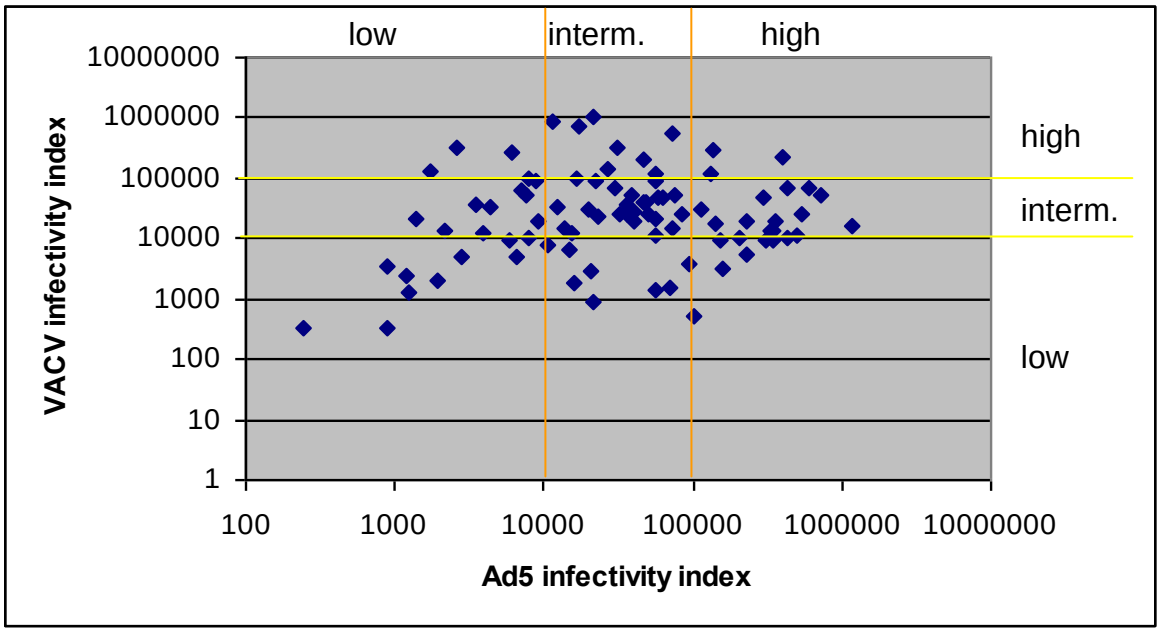



***Figure 11*** – Correlation analysis between VACV and Ad5 infectivity indices. While we couldn't detect any direct, quantitative correlation half of the cancer cell lines could be grouped in corresponding replication classes.

### 5.3.4 Discussion

The innate host's immune response is believed to limit the effects of oncolytic therapy by limiting viral replication. Recent data, however, suggest that the immune response also promotes tumor rejection during the oncolytic process (16) following pathways similar to those observed in other immune pathologies leading to immune-mediated tissue destruction (31). Paradoxically, endogenous activation in cancer cells of innate immunity, particularly, interferon-stimulated genes (ISGs) hamper the secondary activation of the host's immune response *in vivo* by limiting viral replication (17).

To characterize the relationship between baseline immune activation of cancer cells and viral replication, we screened the NCI-60 cancer cell lines plus other cell lines developed in house for their permissivity to infection with VACV and Adenovirus Ad5, two leading candidates for oncolytic therapy. Although overall permissivity to both viruses was similar, there was poor correlation among individual cell lines suggesting that factors in addition to constitutive immune activation modulate viral replication and intrinsic factors more than histological background is important (**Tables 3 and 4**).

***Table 3 –*** Classification of 75 human cancer cell lines according to their susceptibility to Ad5 infection.

| | | Ad5 replication | | |
|---|---|---|---|---|
| **Tumor Origin** | **∑ Cell Lines** | **high** | **interm.** | **low** |
| Lung Cancer* | **10** | **1** | **9** | **1** |
| Renal Cancer | **8** | **-** | **6** | **2** |
| Melanoma | **13** | **5** | **5** | **3** |
| CNS | **6** | **3** | **3** | **-** |
| Prostate Cancer* | **2** | **-** | **3** | **-** |
| Pancreatic Cancer | **2** | **1** | **-** | **1** |
| Liver Cancer | **1** | **-** | **1** | **-** |
| Colon Cancer* | **7** | **1** | **3** | **4** |
| Cervical Cancer | **1** | **1** | **-** | **-** |
| Leukemia | **6** | **-** | **2** | **4** |
| Breast Cancer* | **7** | **-** | **4** | **4** |
| Ovarian Cancer* | **7** | **3** | **4** | **1** |

*** Duplicates which originated from different labs; different passages**

***Table 4 -*** Classification of 75 human cancer cell lines according to their susceptibility to VACV infection

| | | VACV replication | | |
|---|---|---|---|---|
| **Tumor Origin** | **∑ Cell Lines** | **high** | **interm.** | **low** |
| Lung Cancer* | **10** | **1** | **5** | **4** |
| Renal Cancer | **8** | **-** | **6** | **2** |
| Melanoma | **12** | **2** | **8** | **3** |
| CNS | **6** | **-** | **5** | **1** |
| Prostate Cancer* | **2** | **-** | **3** | **-** |
| Pancreatic Cancer | **2** | **-** | **1** | **1** |
| Liver Cancer | **1** | **-** | **1** | **-** |
| Colon Cancer* | **7** | **3** | **1** | **3** |
| Cervical Cancer | **1** | **-** | **1** | **-** |
| Leukemia | **6** | **-** | **-** | **6** |
| Breast Cancer* | **7** | **-** | **6** | **2** |
| Ovarian Cancer* | **7** | **3** | **4** | **1** |

*** Duplicates which originated from different labs; different passages**

In this context it also needs to be taken into consideration that Ad5 -in contrast to VACV- utilizes CAR for specific cell entry but a growing number of studies have demonstrated that expression of CAR on primary human tumor cells is highly variable. This has been reported for ovarian, cervical, prostate, head and neck, and bladder cancer, melanoma, glioma and others (32-40)

Recently, it has been suggested that the low expression of CAR is the rate-limiting factor for infectivity with serotype 5 Ad5 (41) but to data there is no known cellular receptor selective for VACV infection and its tropism is more promiscuous.

Microarray analysis of uninfected cancer cell lines revealed in case of Ad5 and VACV, respectively the involvement of the Nfκb pathway which is intrinsicly upregulated in low replicator cell lines. The most common form of NF-kB in mammals is a dimer composed of subunits termed p50 and p65 (RelA). Many different stimuli including bacteria, viruses and cytokines promote degradation of Ik-Bs allowing NF-kB translocation from the cytoplasm to the nucleus, where NF-kB binds to a set of related DNA target sequences, called kB sites (42). VACV is known to closely interact with the host's innate immune system (43) and encodes protein such as M2L which can interfere with Nfκb signaling (44).

Recombinant adenoviral vectors have been shown to activate NF-kB in murine hepatocytes, murine dendritic cells and human vascular smooth muscle cells (45-47)

but no reports describe the implication of preexisting activation of such markers. However, in a recent study we demonstrate the role of endogenously up-regulated Interferon-related-pathways, mainly characterized by MX-1 activation and the correlation with impaired Ad replication in primary pancreatic adenocarcinomas and pancreatic tumor cell lines (17).

Interestingly, we identified Fau as a marker for decreased permissivity to oncolytivc VACV infection. Fau has been characterized by others as a novel apoptosis-regulator and candidate tumor suppressor with oncogenic properties in different contexts (29). Fau also carries an ubiquitin-like FUBI domain which is known to target the Bcl2 ligand 14, a pro-apoptotic member of the Bcl-2 family of apoptosis-controlling proteins.

Furthermore, GDF-15 (also called Macrophage inhibitory cytokine 1), a new member of the TGF-β family, was up-regulated in high VACV replicators, suggesting a beneficial role for the infection/replication cycle. Altough, GDF-15 is shown to be associated with progression of cervical cancer (48) and high grade prostate tumors (28) no study showed the relationship with Adenovirus 5 susceptibility so far.

The mechanistic explanation for these phenomena remains to be elucidated in the future. Infections with other non-receptor dependent Viruses such as Vesicular Stomatitis Virus or other VACV constructs will provide evidence whether the tropism for any given cell is truly viral construct specific or part of a more general phenomenon. Blockage of NFκB signaling with NBD peptides or siRNA approaches will show whether the replication pattern can be turned around and the permissivity of low replicator cell lines can be improved.

Lastly, in the context of oncolytic therapy appropriate infection and targeting of the malignant lesion is an important prerequisite for the treatment outcome and patients' survival. Previous studies in our laboratory suggested that there might be a relationship between *in vitro* replication ability and *in vivo* outcome in the respective tumors (16). To address this important question in depth, a large scale experiment using fine needle aspiration techniques will be launched soon and prospective follow up studies will be done. The outcome might have implications in the design and prescreening of future clinical studies and help to improve the treatment success.

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# 6 Discussion

Different attempts to study the microenvironment of tumors all lead eventually to the same outcome: The study of the interaction of the host's immune system and the malignancy. Virchow suggested already in 1863 that the presence of leucoreticular infiltrates is the origin of cancers and chronic inflammation fosters at such sites (1). Over the last years evidence has accumulated in the literature that the relationship between cancers and the immune system is more dynamic. Under certain circumstances inflammation can be beneficial to cancer growth and promote carcinogenesis whereas in other cases the inflammation is destructice and facilitates tumore regression. However, in most situations the inflammation is chronically manifested and characterized by production of angiogenic and growth factors. In a study from Shankaran et al (2) (it is shown that lymphocytes and IFN-γ collaborate to protect against development of carcinogen-induced sarcomas and spontaneous epithelial carcinomas and knockout mice are more prone to develop tumors at higher frequencies. Additionally, congenital and acquired immunodeficiencies are also associated with increased prevalence of malignancies (3).

Thus, these findings suggest that the immune response functions as an effective extrinsic tumour-suppressor system and that immunosurveillance has an active protective role against cancer. On the other hand, the ability of the immune system to select for tumors of lower immunogenicity has the capacity to promote tumor growth. These dual effects of the immune system on developing tumors prompted Dunn et al. (4) to refine the cancer immunosurveillance hypothesis and they introduced the concept of immunoediting. Once tumors surpass this control mechanism they usually develop a phenotype capable of manipulating immune cells and established their autonom autocrine and paracrine cytokine and chemokine microenvironment. This in turn leads to progressive growth of the respective lesion and can only be altered by immunotherapy which triggers a more acute inflammation that favors tumor regression (**Figure 1**).

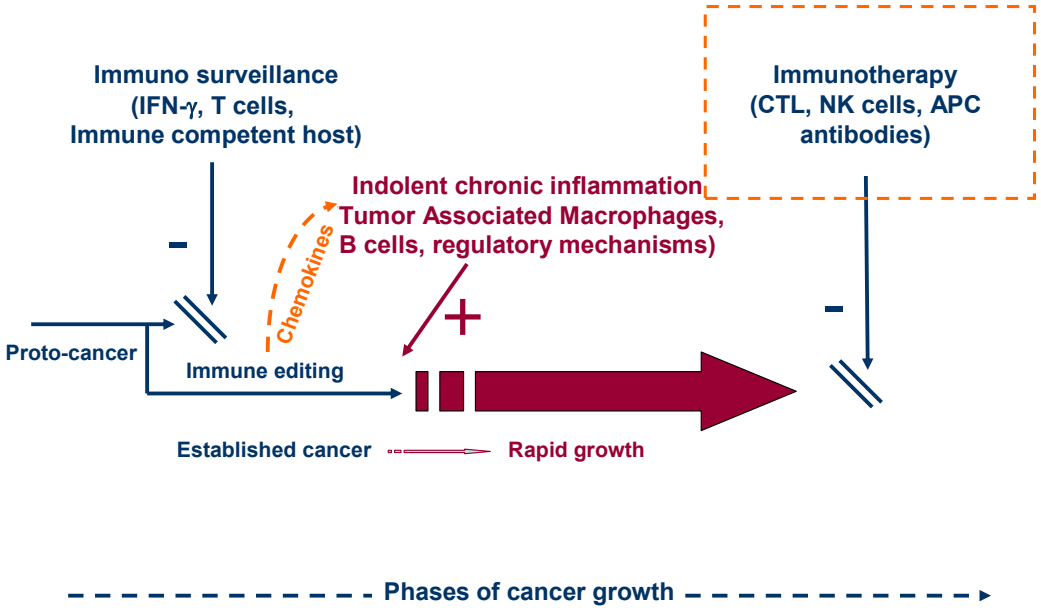


Figure 1 – Postulated interactions between immune and cancer cells at various stages of carcinogenesis and progression (5).

In fact, the fostering inflammation not only makes the tumor a unique neoplastic “organ” in the body but also offers the opportunity for possible treatment options. When stimulated properly the chronic inflammation (similar to autoimmunity diseases and chronic allograft rejection) when the disease persists or tissues are tolerated can be switched to an acute inflammation (such as acute allograft rejection and cancer regression) when the disease is cleared or tissues are rejected (6).

Even though this phenomenon can happen spontaneously it is very rare and underlying machanisms are not fully understood yet. Cancers themselves generally, do not launch danger signals and therefore, cannot initiate, sustain and complete tissue rejection (5). Synthetic molecules that are used as cancer vaccines have been developed that mimic pathogen invasion and stimultate the danger signaling pathways. Naturally, danger signaling in response to foreign pathogens and microbes, for instance, is triggered by certain pattern recognition receptors, such as TLRs and the innate immune system becomes activated. Updated definitions of the traditional understanding of self and non-self discrimination however, lead to the more recent concept suggesting that danger signaling occurs in response to molecular patterns that can be associated with either pathogens (nonself) or also normal cell components (self) (7). One such endogenous danger signal, e.g. is High mobility group box 1 (HMGB1) which is not only passively released during cellular necrosis (8) but also actively by immune cells such as monocytes, macrophages and

DCs. HMGB1 therefore belongs to the group of alarmins which are characterized by the ability to signal tissue and cell damage.

We studied in our nude mice model (Research article 2) immune effector mechanisms that lead to tumor rejection in GI-101A xenografts solely through innate immunity since these mice lack T-cells and secondarily B-cell function. We hypothesize that the presence of the oncolytiv VACV in the target tissue is a sufficient stimulus to bypass the requirements for adaptive immunity and is able to trigger an acute inflammation and immune-mediated rejection occurs without the assistance of T or B cells.

This hypothesis is suggested by some human observations. The rejection of skin cancers by the local application of TLR-7 agonists occurs without direct evidence of adaptive immune responses (9, 10). Also renal cell carcinomas are as sensitive to systemic administration of IL-2 as metastatic melanoma yet, while in the latter adaptive immune responses are easily demonstrable, in the former, they have been quite elusive, and most likely of secondary significance (11, 12). In this model, although xenografts by themselves lack the ability to signal danger and do not provide sufficient proinflammatory signals to induce acute inflammation, the presence of viral replication provides the "tissue-specific trigger" that activates the immune response. According to our hypothesis, the ICR is activated when chronic inflammation is switched into an acute one. A critical step in this process is the expression of IFN-γ dependent pathways probably by activated mononuclear cells; this is clearly demonstrable in most cases in which TSD has been studied in humans by the requirement for the expression of IRF-1 (6); a transcription factor closely related to IFN-γ signaling. IFN-α and IFN-γ regulate directly or indirectly the production of CXCR3, CXCR4 and CCR5 ligands among which the CXCL-9 through -11 chemokines (Mig, IP-10/Crg-2 and ITAC), CCL5 (RANTES) and CXCL12 (SDF-1) appear to play a prominent role (13). Indeed, this expression pattern has been consistently observed in most cases in which TSD was studied in the involved tissue by transcriptional profiling (6) including animal rejection models (14, 15). Moreover, this oncolytic xenografts model provides evidence that non-self discrimination plays at best a partial role in a host that cannot eliminate xenografts unless appropriate danger signals are provided by a pathogen (15). Contrary to acute allograft rejection

occurring in humans (16), no B lymphocyte signatures (CD20, immunoglobulin genes) could be observed clearly demonstrating that reconstitution of a potential B cell response could not have been responsible for the inflammatory switch and the production of CXCL and CCL chemokines (17).

Furthermore, contrary to the syngeneic neu-overexpressing mammary carcinoma mouse model (Research article 2) where clear up-regulation of type I and type II IFNs could be documented, no involvement of T or B cell signatures participated in the rejection of the GI-101A xenografts and ISG expression was not directly accompanied with the over expression of IFN-α, IFN-β or IFN-γ suggesting that, as recently demonstrated in a cytomegalovirus model (18), stimulation of interferon response genes could occur independently of de novo synthesis of IFNs through a direct interaction of viral proteins with cellular transcription factors (partially modified from Research article 1).

Tumor rejection in this nude mice model occurred exclusively in GI-101A xenografts whereas HT-29 tumors persisted the treatment and continued to grow. The genetic background of the mice as well as the viral construct is identical and we therefore hypothesized that the different behavior is instrinsicly encoded in the cancer cells. This in turn, might lead to impaired danger signaling in the non-responding tumors (e.g. HT-29). To broaden the anaylsis and increase the statistical power we screened 75 human cancers mainly consistent of the NCI-60 cell line panel (Manuscript for Research article 3). We previously reported a slight correlation between *in vitro* permissivity to VACV infection and *in vivo* outcome of the respective xenograft (Research article 2). Thus, virus encoded GFP protein analysis was reflective of VACV replication and allowed us to separate the tumors in different replicator groups and possible have an impact on *in vivo* behavior later on. Microarray analysis of untreated tumor cells revealed endogenous activation of NfκB signaling in cells with low permissivity to VACV and Ad5 replication but we couldn't detect a strong pattern of ISG up-regulation as suggested in case of primary pancreatic carcinomas and pancreatic cell lines. In this study, Monsurro et al (19) describe two phenotypes ("inflammatory" vs "quiescent") of tumors characterized by endogenous expression of ISGs which have also been reported for melanoma before (20) and could possibly be a widespread phenomenon among cancers. Futhermore,

the activation of ISGs in this model was due to two independent taxonomies of cancer cells and not to the host's reaction to the cancer as it is was observed in primary xenografts growing in immune deficient animals and in *in vitro* cultured cell lines. Infection with Ad and Ad-associated viruses was impaired in activated cancers indicating the existence of a true "anti-viral" state.

It remains to be elucidated why these two phenotypes exist. One possibility is that the cancer cells bearing the "anti-viral" state are chronically infected with a latent virus that could induce endogenous activation of innate cellular immune responses. Alternatively, it might represent an endogenous activation of anti-viral pathways associated with the mutagenic process. This phenomenon has been clearly described for Epstein-Barr virus (21) or papilloma virus related cancers (22) and could apply to other viruses as well.

However, in a smaller study, we compared a number of cancer cell lines bearing either phenotype by hybridizing their mRNA to a commercially available pathogen chip containing probes for all known viruses (Agilent Technology) and we could not identify any viral sequence in the cell lines (Worschech A et al., unpublished observation). Thus, the "anti-viral state" is a characteristic molecular phenotype of a subset of pancreatic cancers that may be the result of a specific mutational profile of cancer cells which is difficult to be understood at this time (23).

Whereas the relationship between cancerogenesis and chronic inflammation was noted already more than 100 years ago and has been extensively studied in the last decades the mechanisms leading to TSD in various contexts and underlying acute inflammatory processes remains to be fully elucidated in the future. However, as described in the Immunologic Constant of Rejection (Review 1) although the mechanisms prompting TSD differ among immune pathologies the effect phase converges and central molecules (such as CXCR3 ligands, CCL5, STAT1 etc.) can be detected over and over every time TSD occurs. This is evident not only in preclinical but also in clinical models studied by us and others as summarized in **Table 1.**

***Table 1 –*** The Immunologic Constant of Rejection

| | ***STAT-1/IRF-1 T-bet+/IFN-γ/IL-15*** | ***GNLY/GZM TIA*** | ***CXCL-9 to -11 CXCR3*** | ***CCL5 CCR5*** | *Ref* |
|---|---|---|---|---|---|
| ***Cancer*** | | | | | |
| ***Prognosis*** | | | | | |
| Colon *hu* CA | ↑ | ↑ | | | (Camus et al. 2009;Pages et al. 2005) |
| Lung *hu* CA | ↑ | ↑ | | | (Dieu-Nosjean et al. 2008) |
| Melanoma *hu xeno* | n.t. | n.t. | ↑ | ↑ | (Harlin et al. 2009) |
| Ovarian *hu* CA *xeno* | ↑ | ↑ | ↑ | ↑ | (Benencia et al. 2005) |
| ***Rejection*** | | | | | |
| Mastocytoma *mus* | ↑ | ↑ | | | (Shanker et al. 2007) |
| Breast *hu* CA *xeno* | ↑ | ↑ | ↑ | ↑ | (Worschech et al. 2009) |
| BCC *hu* CA | ↑ | ↑ | ↑ | ↑ | (Panelli et al. 2006) |
| ***Allo Tx*** | | | | | |
| ***Rejection*** | | | | | |
| Kidney *hu* | ↑ | ↑ | ↑ | ↑ | (Reeve et al. 2009;Saint-Mezard et al. 2009;Sarwal et al. 2003;Sarwal et al. 2001) |
| Heart *hu* | n.t. | n.t. | ↑ | n.t. | (Karason et al. 2006) |
| Islet *pig* | n.t. | ↑ | ↑ | ↑ | |
| ***GVHD*** | ↑ | ↑ | ↑ | n.t. | (Imanguli et al. 2009) |
| ***HCV*** | | | | | |
| ***Viral clearence*** | | | | | |
| Chimp | ↑ | ↑ | ↑ | | (Bigger, Brasky, & Lanford 2001;Nanda et al. 2008) |
| Human | ↑ | | ↑ | | (Asselah et al. 2008;Feld et al. 2007;He et al. 2006) |
| ***Acute Cardivascular events (human)*** | ↑ | | ↑ | | (Okamoto et al. 2008;Zhao et al. 2002) |
| ***COPD (human)*** | | | ↑ | | (Costa et al. 2008) |

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# 7 List of Abbreviations

| | |
|---|---|
| Ad | Adenovirus |
| Ag | Antigen |
| AIF-1 | Allograft inflammatory factor 1 |
| BCC | Basal cell carcinoma |
| BCR | B-cell receptor |
| CAR | Coxsackie- and Adenovirus receptor |
| CTL | Cytotoxic T-lymphocyte |
| CTLA-4 | Cytotoxic T-lymphocyte antigen 4 |
| DC | Dendritic cell |
| EEV | Extracellular enveloped virus |
| fau | Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed |
| HCV | Hepatitis C virus |
| hpi | hours post infection |
| ICR | Immunologic constant of rejection |
| IEFs | Immune effector functions |
| IFN | Interferon |
| II | Infectivity Index |
| IL | Interleukin |
| IPA | Ingenuity Pathway Analysis |
| IRDS | Interferon-related DNA damage resistance signature |
| IRF | Interferon regulatory factor |
| ISGs | Interferon stimulated genes |
| MHC | Major histocompatibility complex |
| MMC | Mammary carcinoma |
| MxA/1 | Myxovirus-resistance A/1 |
| NK | Natural killer |
| PAP | Pathogen associated pattern |
| s.c. | subcutaneous |
| SOCS | Suppressor of cytokine signaling |
| STAT1 | Signal transducer and activator of transcription 1 |
| T.I. | Therapeutical Index |
| TAA | Tumor-associated antigens |
| TCR | T-cell receptor |
| TGF | Transform growth factor |
| TIMP-1 | Tissue inhibitor of Metalloproteinases 1 |
| TLR | Toll-like receptor |
| TNF | Tumor necrosis factor |
| Tregs | T regulatory cells |
| TSD | Tissue-specific destruction |
| VACV | Vaccinia Virus |
| VEGF | Vascular endothelial growth factor |

# 8 Publications

## Publications directly related to the PhD thesis:

***Original Articles***

2009
**A. Worschech**, ML. Ascierto, G. DiPasquale, N. Chen, Q. Zhang, YA. Yu et al.
**In vitro permissivity of 75 human cancer cell lines to Adenovirus 5 and oncolytic Vaccinia Virus and the correlation with intrinsic immune activation levels**
In preparation

2009
**A. Worschech**, N. Chen, YA. Yu, Q. Zhang, Z. Pos, S. Weibel et al.
**Systemic treatment of xenografts with vaccinia virus GLV-1h68 reveals the immunologic facet of oncolytic therapy**
BMC Genomics. 2009 Jul 7;10:301

2008
**A. Worschech***, M. Kmieciak*, K. L. Knutson, H. D. Bear, A. A. Szalay, E. Wang et al.
**Signatures associated with rejection or recurrence in HER-2/neu-positive mammary tumors** (*, both authors contributed equally)
Cancer Res, 68: 2436-2446

***Reviews***

2009
**A. Worschech**, D. Haddad, E. Wang, D.F. Stroncek, F.M. Marincola, A. A. Szalay
**The immunologic aspects of poxviruses oncolytic therapy**
Cancer Immunol Immunother. 2009 Sep;58(9):1355-62. Epub 2009 Mar 6

2008
E. Wang, **A. Worschech**, F. M. Marincola
**The immunologic constant of rejection**
Trends Immunol, 29(6): 256-62

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