Quantitative Analysis of Adenoviral Vector Modification of a Cytokine-Mediated Cell Death Decision

by

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ABSTRACT

Intracellular networks arise from complex interactions between proteins that relay signals and control cellular responses. Viruses, with limited genetic material, can modify network signals and change cell behavior. Replication-deficient viruses are used extensively as delivery vectors in clinical gene therapy and in molecular biology, but little is known about how the viral carrier itself contributes to cellular responses.

In this thesis, we explored the link between viral vector modifications of signaling networks to changes in cellular phenotype. We approached this problem by studying a therapeutically relevant model in which an adenoviral vector (Adv) sensitizes human tumor epithelial cells to tumor necrosis factor (TNF)-induced apoptosis. We first measured TNF-stimulated signaling profiles over a range of Adv infection levels for a distribution of kinases centrally involved in the TNF signaling network. We then applied quantitative analytical techniques to determine the most important signals contributing to Adv-induced changes in TNF-mediated apoptosis.

We experimentally derived a mathematical equation describing the saturation of anti-apoptotic Akt effector signaling in the presence of high levels of Adv infection, which could predict TNF-induced apoptosis in HT-29 cells. However, the same equation did not apply in HeLa cells, suggesting that one-signal models are insufficient to account for complex network interactions. Therefore, we applied a systems-modeling approach to our Adv–TNF system and mathematically identified a multivariate signal-processing function sufficient to predict Adv–TNF induced apoptosis in both HT-29 cells and HeLa cells. The common-processing model identified critical Adv-induced cell-specific signaling modifications, and accurately predicted apoptosis following perturbation with pharmacological inhibitors of Akt and IKK. Thus, by combining experimental and computational approaches, this thesis has identified an important biological principle, common signal processing, for studying cell-specific responses to viral infections and rational drug therapies.

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Kathryn Miller received a bachelor of arts in Engineering Sciences in 1997 from Dartmouth College in Hanover, NH. While at Dartmouth, Kathryn worked in the laboratory of Dr. Lee Lynd studying recombinant microorganisms for converting cellulosic biomass into ethanol. Upon graduation, she was awarded a fellowship to complete a one-year bachelor's degree in Chemical Engineering at the Thayer School of Engineering at Dartmouth College, which she was awarded in 1998. Following receipt of her degrees, she worked for two years at the Monitor Consulting Group in Cambridge, MA.

Kathryn started her graduate work in the Chemical Engineering Department at MIT in the fall of 2000. Under the supervision of Dr. Douglas Lauffenburger, she completed her thesis entitled “Quantitative Analysis of Adenoviral Vector Modification of a Cytokine-Mediated Cell Death Decision.” For three years, she was supported by an NIH Biotechnology Training Grant. As part of this fellowship, she spent six months in 2004 at Merck & Co. under the supervision of Dr. Jean-Luc Bodmer, working on an adenoviral HIV vaccine project. In January through March of 2006, Kathryn had the opportunity to pursue her long-time interest in science and technology policy as a Christine Mirzayan Science and Technology Fellow at the National Academy of Science.

Upon leaving MIT, Kathryn will take a postdoctoral position in the laboratory of Dr. David V. Schaffer at the University of California at Berkeley.
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CHAPTER 1

1. Introduction

1.1. Adenoviruses as tools for biological discovery and gene therapy

1.1.1. Introduction

Recombinant adenoviruses are commonly used as delivery vectors in gene therapy clinical trials and in basic bioscience studies for the delivery of transgenes. In both of these areas, it is important to understand how the adenoviral vector (Adv) alters cells during infection. Adv-induced cellular changes might synergize or antagonize the effects of certain transgenes, confounding interpretation of their normal role in cells. Deconstructing the contributions of Adv and the transgene is also relevant in vivo, because gene products intrinsic to Adv may cause host cells to react differently to proinflammatory cytokines that circulate during infection [1]. The virology of wild type adenovirus has been studied extensively [2], but there is not the same molecular-level understanding of the engineered Advs used in gene therapy applications. In this Introduction, we describe the complexity of the interaction network between wild type Ad and recombinant Adv and inflammatory cytokines of the immune system. We then discuss quantitative systems biology approaches that could be useful in deconstructing how these interactions contribute to changes in cellular response.

1.1.2. Adenovirus biology and vector design

Adenoviridae have been isolated from multiple species and tissue types, and have been extensively studied and developed for gene therapy applications (for a full review of adenovirus biology, see [2]). The human adenovirus (Ad) family consists of more than
50 serotypes that can infect and replicate in a wide range of tissues including the respiratory tract, the gastrointestinal tract, the eye, and the liver. Adenoviruses have nonenveloped capsids with a 30- to 40-kb linear double-stranded DNA genome that encodes for more than 50 proteins through extensive splicing.

The adenovirus has an icosahedral capsid composed of hexon proteins coming together at a point to join with the penton base (Fig. 1-1). The fiber shaft extends from the penton base, ending in the fiber knob. The fiber knob binds to the coxsackie and adenovirus receptor (CAR) found on most cell types [3], but entry of the Ad particle proceeds via endocytosis mediated by the $\alpha_v\beta_3$ integrins [4]. Subsequently, Ad capsids are transported to the host nucleus where they insert their DNA and initiate the replication cycle.

Ad binding and entry initiate a number of signaling pathways (Fig. 1-3, right side). Internalization via $\alpha_v$ integrins requires association with the Crk-associated substrate p130CAS and activation of phosphotidylinositol-3-OH kinase (PI3K) [5, 6]. In addition, the Ad capsid stimulates activation of the MKK6–p38–MK2 pathway which enhances microtubule-mediated viral nuclear targeting [7]. Inhibition of either of these pathways significantly inhibits infection.

The Ad lifecycle occurs in an early and late phase, divided by the onset of viral replication. This division also describes how Ad regulates cell viability, inhibiting apoptosis in the early stages of infection such that viral replication is maximized, and promoting apoptosis (and ultimately cell lysis) late in infection to release progeny virions [8]. To execute this lifecycle, Ad genes are transcribed in a complex temporal manner and are divided on this basis into three major groups: early (E1A, E1B, E2, E3, and E4), intermediate or delayed (IX and Iva2) and the major late transcription unit (processed in 5 mRNAs L1–L5) [2, 8] (Fig. 1-2). The E1A proteins function to trans-activate the other Ad early transcription units (E1B, E2, E3, and E4) to induce the cell to enter S phase in order to create an environment optimal for virus replication [9]. The E2 region encodes proteins necessary for replication of the viral genome, including the DNA polymerase, the preterminal protein, and a DNA-binding protein (for a more detailed review, see [10]). Proteins of the E3 region function primarily to subvert the host immune response and E4 proteins regulate the cell cycle, both contributing to a productive infection [11].
Proteins of the late genome region are primarily involved in vector packaging.

Adenoviruses have been extensively studied, designed and redesigned for use as gene therapy delivery vectors, due to several attractive features including their wide tropism (range of cell types and tissues in which a virus can sustain a productive infection), ease of production, and large gene carrying capacity [12]. Adenoviral vectors comprise 25% of all past and current gene therapy clinical trials, second only to retroviral vectors. The most commonly used Adv is the so-called first-generation Adv (FG-Adv). This virus has been deleted for the adenoviral early regions E1 and E3, eliminating or greatly impairing viral replication. Despite this genetic modification, E1/E3-deleted Advs are not biologically inert. Cells infected with E1/E3-deleted Adv still express low levels of other wild-type gene products, which are known to cause potent immunogenic responses [13]. In an attempt to mitigate this problem, E2 and/or E4 coding sequences were removed from second-generation Advs [13]. These vectors have shown reduced toxicity in vivo [14, 15]. Construction and production of these multiply-deleted viruses

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is difficult, however, due to the need for isolation of cell lines expressing the missing functions \textit{in trans} [11]. The development of helper-dependent Adv (HD-Adv), vectors devoid of all viral genes, has resulted in the most significant improvements in Adv safety and efficacy [16]. A direct in vivo comparison between an FG-Adv and HD-Adv carrying the same expression cassette showed that TNF and IL-6 levels were upregulated in response to the FG-Adv but not for the HD-Adv [17]. However, because these vectors have no coding sequences, a helper-virus is required for their propagation, and the resulting vector product is invariably contaminated with helper-virus [16]. In addition, acute toxicity is still present after high-dose systemic injection of HD-Advs into nonhuman primates [18], due to the intact innate immune response that is induced by the Ad capsid (discussed below). While the development of helper-dependent Advs continues to progress, to date this type of vector has been used only once in a clinical trial\textsuperscript{1}.

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\textsuperscript{1} Adapted from [11]. The central, solid line represents the viral genome. Positions of the left and right ITRs, the packaging sequence (P), early transcription units (E1A, E1B, E2, E3, and E4), and the major late transcription unit (major late promoter [MLP], L1–L5) are shown. Arrows indicate the direction of transcription. Below are genome structures of first-generation, second-generation, and helper-dependent vectors. Open boxes indicate regions that have been deleted.
1.1.3. Gene therapy challenge: Adenovirus and the immune system

A major limitation in the application of Adv gene therapy is the activation of the host immune response. Much work has been done to decouple the early innate immune response induced by Ad binding and entry from the later adaptive immune response. The adaptive immune response is largely directed to the newly synthesized viral proteins produced at low levels in cells transduced by FG- (E1/E3-deleted) Adv, consequently resulting in transient transgene expression and long-term toxicity [19]. In contrast, acute inflammation occurs within 24 hours of transduction, is dose-dependent, and is independent of viral transcription [20]. Following vector administration, resident macrophages efficiently take up adenoviral vectors and release inflammatory cytokines within the tissue to eliminate infected cells [21, 22]. In vivo evidence exists for induction of tumor necrosis factor (TNF), interleukin 6 (IL-6), IL-1β, interferon-γ (IFN-γ) and numerous chemokines [23-25].

Ad proinflammatory gene expression is mediated through a number of signaling events which usually peak within 30 minutes of the initial infection [26]. In epithelial cells, ERK and p38 are activated within minutes of Adv entry, leading to induction of the chemokine IP-10 [27]. Capsid activation of ERK, initiated by αv integrin interaction, also leads to the production of IL-8 [28]. Adv vectors induce translocation of NF-κB to the nucleus, presumably through activation of IKK [29], resulting in the transcriptional activation of IP-10 and RANTES [30, 31]. Finally, all three major MAPK pathways (ERK, p38 and JNK1) appear to cooperate with NF-κB in the induction of ICAM-1 [32]. Little work has been done to characterize altered cell responses in this complicated multi-stimulant environment.

1.2. Signaling network interactions between virus and host
1.2.1. Introduction

The inflammatory cytokine, tumor necrosis factor-α (TNF), plays a key role in the early innate immune response and subsequent elimination of E1/E3-deleted Adv from the host [21, 33, 34]. Consequently, Ads have evolved complex counterstrategies to evade TNF inflammatory responses and complete the viral replication cycle [35]. A key role of TNF is to regulate programmed cell death, or apoptosis. TNF-induced apoptosis is controlled by a complex intracellular signaling network of kinases, proteases, and transcription factors that is well characterized (for a complete review, see [36]).

In this section we describe the TNF-activated signaling network controlling apoptosis and the mechanisms evolved by wild type Ad to block the cell death response. We then consider signaling network interactions between Adv and TNF and describe how we can take advantage of the TNF signaling network to understand underlying Adv perturbations.

1.2.2. TNF-activated signaling and apoptosis

The TNF-activated signaling network is generally considered common across cell types. Briefly, binding of the trimeric TNF ligand to the TNF receptor (TNFR1) causes recruitment of the TNFR1-associated death domain (TRADD). TRADD subsequently recruits downstream adaptors resulting in activation of IκB-α kinase (IKK), p38-mitogen-activated protein kinase (p38), and c-Jun N-terminal kinase (JNK1) [37]. These pathways initiate a variety of biological responses, both prodeath and prosurvival.

IKK mediates the classical NF-κB activation pathway. IκB sequesters the NF-κB p50-p65 heterodimer in the nucleus by masking the nuclear localization sequence (NLS) of p65. Activated IKK, a complex containing two kinase subunits (IKKα and IKKβ) and a regulatory subunit (NEMO), phosphorylates IκB on two serine residues [29]. Phosphorylated IκBs are then polyubiquitinated and subsequently degraded by the proteasome [38]. Because the NLS of the NF-κB heterodimer is then exposed, the dimer
freely translocates to the nucleus where it can bind to \( \kappa B \) binding sites of promoter regions and stimulate transcription. Phosphorylated JNK1 translocates directly to the nucleus where it phosphorylates c-Jun and ATF2, which both bind to AP-1 sites in the \( c-jun \) promoter, thus enhancing its transcriptional activity [39]. ERK and p38 have also been shown to activate AP-1 genes [39].

TNF also stimulates caspase activation. Caspases (or cysteine proteases that cleave after an aspartate residue in the substrate) are a conserved family of enzymes that execute the apoptotic (cell death) process [40]. TRADD and the subsequent recruitment of the Fas-associated death domain (FADD) and the initiator caspase 8 forms the death-inducing signaling complex (DISC) [40]. DISC activation leads to cleavage and activation of the executor caspase 3 and induction of apoptosis [41]. This is known as the extrinsic apoptotic pathway.

The intrinsic apoptotic pathway, also known as the mitochondrial pathway, can be indirectly activated by TNF [42]. The Bcl-2 family of proteins, consisting of both anti-apoptotic and pro-apoptotic members, regulate apoptosis by controlling the release of cytochrome c from the mitochondria [43]. TNF is linked to the intrinsic pathway via caspase 8-mediated activation of Bid, a pro-apoptotic Bcl-2 family protein [42]. Activated Bid is subsequently translocated to the mitochondria and induces cytochrome c release, initiating formation of the apoptosome and activation of caspase 9 [44]. These interactions are summarized in Fig. 1-3 (left side).

### 1.2.3. Transcription-dependent mechanisms of wild-type Ad to counteract TNF

In addition to stimulating inflammatory cytokines, gene products of wild type Ad have both pro- and anti-apoptotic functions, creating complex interactions with the TNF network (Fig. 1-3). The Ad E1A protein promotes or induces cell death via multiple mechanisms. E1A induces apoptosis directly through the p53 pathway [8]. In addition, E1A stimulates the production of TNF [45] and then exacerbates the prodeath stimulus by down-regulating expression of anti-apoptotic c-FLIP [46]. Rather than serving a
**Fig. 1-3.** Schematic representation of the signaling pathways induced by TNF and Adv.
Protein products of Ad transcription are highlighted in blue outside the main network. The kinases measured in the latter part of this thesis (MK2, IKK, JNK1, Akt, and ERK) are highlighted in yellow. Blue Ad capsids show kinases activated by the capsid without an established pathway. Figure is modified from [47].
functional purpose, E1A promotion of cell death is most likely an unwanted consequence of deregulating the cell cycle for transcription and subsequent replication [8]. Therefore, Ad has evolved several mechanisms to inhibit the apoptotic response in order to complete the replication cycle, including another E1 protein product, E1B-19K [48]. The E1B-19K protein is a member of the Bcl-2 family and is functionally interchangeable with the anti-apoptotic protein Bcl-2 [49, 50]. E1B-19K blocks apoptosis by binding and inhibiting Bax, after Bax has undergone a conformational change induced by TNF [51], and thus preventing release of cytochrome c from the mitochondria. E1B-55K inactivates and degrades p53, allowing host cell replication to proceed without being inhibited by the p53-induced cell cycle check point [52]. E3 products primarily function to inhibit death signals initiated by the host immune system [8] and are not necessary for efficient viral replication. The E3-10.4/14.5K complex inhibits activation of the IKK complex, thus preventing the release of NF-κB to the nucleus and blocking TNF-induced NF-κB activation [53]. E3 proteins are also involved in the down-regulation of TNF-family receptors from the cell surface, including Fas [54, 55] and TRAIL [56], although there is no specific evidence for down-regulation of TNFR1 itself. Retention of the E3 locus is a strategy for mitigating the Adv-mediated immune response [57], however generally it is removed to take advantage of additional space for packaging a transgene.

In contrast to Adv E3-deleted mutants, Adv deleted for the E4 locus, specifically ORF 6 and ORF 3, are severely impaired for growth [58]. In the absence of the proteins encoded by E4orf3 and E4orf6, the DNA of the Ad genome is joined into concatemers too large to be packaged into the capsid [59]. The E4 proteins inactivate the DNA repair complex and thus prevent end-joining of Ad genomes [60]. E4orf4 has been associated with a pro-apoptotic role in infection, correlated with its binding to protein phosphatase 2A (PP2A) [61], however this mechanism has not yet been elucidated. More recently, Ad E4orf4 and Ad E4orf1 have been shown to induce proliferative signals in infected cells, presumably to induce cell (and thus viral) replication [62]. Ad E4orf4 substitutes for glucose-mediated signaling to the mammalian target of rapamycin (mTOR) and Ad E4orf1 mimics growth factor signaling via activation of PI3K. Ad E4orf1 selectively stimulates PI3K via interaction
with its PDZ domain, resulting in downstream activation of both Akt and p70S6-kinase [63]. This explains recent evidence that the Ad E4 gene promotes endothelial cell survival through activation of Akt [64]. While wild type Ad proteins operate together to optimize infection, deletion of one or more of these gene loci, such as E1 and E3 deletion in FG-Adv, could result in unforeseen effects.

1.2.4. The intersection of Adv–TNF signaling

Wild type Ad uses transcription-dependent mechanisms to promote cell survival — and counteract TNF-induced apoptosis — long enough to complete the viral replication cycle. However, gene therapy vectors are transcription-limited and replication-deficient due to the absence of the E1 trans-factor and are deleted for all E3-mediated mechanisms. Based on the overlap in the signaling networks activated by TNF and Adv infection (summarized in Table 1-1), we hypothesized that Adv would still alter signaling and cell-fate responses to TNF. If true, this finding would have important implications for interpreting both gene therapy and basic bioscience studies in which Adv and TNF are involved.

We also hypothesized that the study of Adv cellular responses would benefit from a quantitative systems biology methodology. The intracellular signal transduction network stimulated by TNF and altered by Adv infection acts as a processor that converts multiple input cues into an output cell fate decision. Ultimately the cell fate decision involves a balance between prodeath and prosurvival signals [65]. Although it is possible to assign functions to individual viral-host protein-protein interactions, we do not yet understand how all interactions in concert contribute to the binary decision of life and death. However, by taking a more systems-oriented approach to the problem, we may be able to understand signaling mechanisms without a detailed knowledge of specific interactions. We therefore considered current methods in computational systems biology that could be applied to our system.
### Table 1-1. Evidence for TNF and Adv activation of the protein kinase network controlling apoptosis

<table>
<thead>
<tr>
<th>Kinase pathway</th>
<th>Involvement in TNF-induced apoptosis</th>
<th>Involvement in Ad or Adv infection</th>
</tr>
</thead>
</table>
| PI3K–Akt       | Akt activated by TNF in certain circumstances [66]  
                     Akt inactivates multiple pro-apoptotic proteins [67] | PI3K required for Ad endocytosis via α₅ integrins [5]  
                     Association of p130CAS with PI3K mediates Ad entry [6]  
                     Akt activated by Ad E4 gene [64] |
| Raf–MEK–ERK    | ERK activated indirectly by TNF-induced autocrine signaling [68, 69]  
                     ERK inhibits caspase-9 processing [70], inhibits Bad translocation via RSK [71], and downregulates Bim [72] | Ad infection stimulates ERK and induces IL-8 [28]  
                     ERK signaling during Adv cell entry leads to IP-10 expression [27] |
| IKK–NF-κB      | IKK activated directly by TNF [73, 74]  
                     NF-κB inhibits apoptosis [75-77] | NF-κB mediates Adv-induced IP-10 expression [30]  
                     NF-κB required for Adv-capsid induction of RANTES [31]  
                     NF-κB contributes to ICAM-1 gene expression in early phase of Ad infection [32] |
| p38–MK2        | p38 activated directly by TNF [78]  
                     p38 promotes prosurvival NF-κB signaling in certain circumstances [79]  
                     MK2 required for stabilization of proapoptotic transcripts [80] | MK2 accelerates Ad nuclear targeting [7]  
                     p38 signaling during Adv cell entry leads to IP-10 expression [27] |
| JNK1           | JNK1 activated directly by TNF [81]  
                     JNK1 promotes c-FLIP, degradation [82] and reactive oxygen species [83, 84] | JNK1 rapidly activated after Ad exposure [32] |
1.3. A systems biology approach to understand how viruses perturb cell response functions

1.3.1. Introduction

The fundamental components of many intracellular signaling pathways are common to all cells [85]. Intracellular networks arise from complex modular interactions between the receptors, adaptors, and enzymes that together relay signals [86]. Computational models are useful tools for organizing and analyzing individual component interaction data obtained through extensive experimental measurements.

It is increasingly recognized that useful models of biological networks – models that provide insight into unanswered biological questions – will require a spectrum of computational approaches, varying in their level of abstraction and specificity [87, 88]. This spectrum ranges from detailed mechanistic models (consisting of transport- and reaction-based equations) to probabilistic models (e.g., Bayesian networks and Markov chains) to statistical models (e.g., partial least squares, decision tree analysis, and clustering) [88, 89]. A range of these approaches have already been applied to questions of viral–host interactions at the population and gene expression level. For example, compartmentalized models of HIV replication dynamics in a T-cell population have provided significant insight into HIV propagation [90]. Cluster analysis of gene expression changes from multiple datasets, heterogeneous for both cell type and pathogen species profiled, revealed a common host-transcriptional-program shared among host-pathogen pairs [91]. Both of these approaches contribute to our understanding and treatment of infectious diseases.

This spectrum of computational approaches is increasingly being applied to the analysis protein signaling networks. Models have been used to analyze data quality and information content ([92], [47]) and to deduce signaling network structure ([93],[94]). Dinh et al recently developed a spatial–temporal model of the adenviral trafficking
network for the purpose of understanding which network parameters influence viral gene delivery [95]. This model related molecular-level trafficking events to whole-cell distribution of viruses and is one of the few examples of quantitative modeling of a viral signaling network.

Models have also been applied to understanding how signaling networks control functional responses. However, in the face of limited knowledge of the intracellular network, most of these models are constructed by correlating experimental measurements to measured cellular responses ([96],[97],[88]). Network-function models have not yet been used as tools to deconstruct pathogen–host signaling networks, but there is growing interest in this application. In a recent review on DNA viruses and cancer, O'Shea describes how DNA viral proteins and tumor cell mutations functionally converge in perturbing similar cellular pathways [98]. O'Shea suggests that a systems biology approach to the study of DNA viruses could further our understanding of and ability to treat network deregulation in tumor cells. Kitano and Oda hypothesize that the interactions between bacterial flora and the host immune system optimize robustness against pathogen attacks and nutrient perturbations in mammals [99]. Thus, applying quantitative modeling approaches to understand how pathogens modify cell responses may help us understand fundamental signaling network functions.

1.3.2. Model of a cytokine–growth factor signaling network

Partial least squares regression analysis (PLSR) has emerged as a powerful means of uncovering biological network functions by relating heterogeneous, multivariate measurements of cell signaling to measurements of cellular response for different stimuli. In the PLSR algorithm, the relative emphasis of a particular measurement is adjusted based upon its contribution to the outcome being modeled. In this way, the signals most closely related to cell response are highlighted while less important signals are deemphasized. In addition, PLS does not require detailed knowledge about the
interrelationship of network components, a significant advantage when studying complex, open-ended signaling networks.

Recently, a PLSR model was applied to understand how cells process the cytokine–growth factor-mediated cell death decision [97]. Specifically, the study examined the signaling networks that control the apoptosis–survival decision in HT-29 human colon adenocarcinoma cells pretreated with interferon-γ (IFN-γ), followed by treatment with combinations of the prodeath cytokine TNF and the prosurvival growth factors epidermal growth factor (EGF) and insulin [47]. Heterogeneous measurements of kinase activities, changes in protein phosphorylation, caspase cleavage, and changes in protein abundance were collected for 19 signaling proteins distributed across the TNF–growth factor networks, at 13 time points over a 24-hour period, under 10 different treatment combinations. Cellular response for each treatment was characterized by four different assays of apoptosis at three time points. The complete cytokine compendium included 8,340 individual biological measurements [47].

PLSR analysis of the cytokine compendium identified a molecular basis set of signals controlling cytokine-induced apoptosis [97]. This basis set was organized into axes described by linear combinations of the input signals, including a stress-apoptosis axis enriched for signals inducing apoptosis, and a survival signaling axis, enriched for pro-life signals [97]. These axes permit predictive analysis by calculating where a new set of signaling measurements induced by a different treatment combination would lie in the stress-survival signaling space, and using this information to predict the resulting level of apoptosis. The predictive capability of the cytokine compendium model was tested by perturbing two regulated autocrine stimuli known to contribute to TNF-induced molecular signals [69]. Specifically, TGF-α and IL-1α autocrine circuits were individually disrupted with antibodies or agonists, the set of resulting signals measured, and the PLSR model used to convert these signals into a prediction of apoptosis (or the apoptotic signature consisting of all four measurements at three time points.) There was a 90% correlation between the measured apoptotic outputs and the model predictions, confirming that the basis axes identified by PLSR capture the dynamic intracellular signal
processing of diverse stimuli [97]. Given that adenoviral infection perturbs the same underlying signals mediating the TNF response, we hypothesized that this approach may be useful in deconstructing underlying Adv signaling modifications.

1.3.3. Challenges in virus-host cell signaling analysis

Adenoviral gene therapy gives rise to complex patterns of signal transduction in the host cell, caused by binding and entry of the Adv capsid, background transcription of the viral backbone, and expression of the transgene. While there is an increased understanding of how individual signaling pathways are upregulated in response to Adv infection (Table 1-2), cells ultimately process cues through an interconnected network of these pathways. Therefore, we hypothesized that Adv-induced signals alter the response to TNF in a manner analogous to EGF, insulin, and the pretreatment cytokine INF-γ perturbation of the TNF response in HT-29 cells. In this case, quantitative measurement of signals distributed throughout the TNF network for different combinations of Adv and TNF and subsequent PLSR analysis of those datasets Adv would inform which signaling pathways modified by Adv contribute most to altering the cytokine-mediated cell response. The remaining chapters of this thesis summarize the results of this study.
CHAPTER 2

2. Experimental methods for quantitative Adv–TNF network analysis

2.1. Introduction

The objective of this thesis was to quantitatively deconstruct the contributions of Adv infection in the host cell response to the proinflammatory cytokine TNF that circulates during infection in vivo [21]. To do this, it was necessary to develop a quantitative experimental system with clear TNF-induced outcomes that were specific to the state of Adv infection. Ad and Adv have been shown to synergize with TNF in many cellular contexts [100-102]. We observed that infection with a first generation Adv synergized with TNF to induce apoptosis in human epithelial cells. We chose to study a first-generation Adv because these are the most common vectors used in both the laboratory and the clinic. We initially developed our experimental approach in the HT-29 human adenocarcinoma cell line consistent with the cytokine compendium work.

Our methodology was to explore the mechanism of Adv-mediated sensitization to TNF by measuring changes in the activity of signaling proteins centrally involved in the Adv–TNF network. We hypothesized that the Adv perturbations controlling changes in apoptotic response would be encoded in the upstream signals. We used statistical and computational analysis to identify the most important network signals controlling apoptosis. In Chapter 2, we describe our experimental methods for quantifying biological metrics and our computational methodology to analyze those measurements. In the final section of Chapter 2, we measure several biological responses specific to our Adv system that should be taken into account when interpreting our results presented in the following chapters.
2.2. Optimization of the Adv–TNF sensitization protocol

2.2.1. Apoptosis

Apoptosis, or programmed cell death, is central to metazoan homeostasis and development [103]. Apoptosis is characterized by highly conserved morphological and biochemical changes in the cell, including chromatin condensation, membrane fragmentation, and ultimately the formation of apoptotic bodies that are rapidly phagocytosed by neighboring cells [104]. These tightly regulated morphological and biochemical changes provide ideal hallmarks by which to quantify the amount of apoptosis occurring in a population.

We tested multiple combinations of Adv and TNF to determine a suitable experimental protocol to quantify the extent to which Adv infection alters the cell-death response to TNF stimulation. When designing our protocol, we sought to mimic the cytokine compendium approach discussed above, a protocol previously developed in our laboratory to understand the TNF-growth factor-mediated cell death decision [97]. Systematic collection of biological data under carefully matched experimental conditions makes it possible to compare, with high reliability, data that were not collected simultaneously in the same experiment [47]. Therefore, our objective here was to maximize comparability of the data for future biological investigations.

A general schematic of our protocol is illustrated in Fig. 2-1. Briefly, we infected HT-29 cells with either an E1/E3-deleted adenovirus carrying the CMV promoter and a β-gal reporter gene (Adv.β-gal) or an identical vector without a reporter gene (Adv.empty). Mock infections were carried out with media containing vector buffer only. The final Adv concentration in the infection media was $1.4 \times 10^{10}$ v.p./ml, resulting in approximately 100% of the cell population positive for Adv infection (see Fig. 2-3b). For control cells, buffer without Adv was added. TNF (100 ng/ml) was added 24 hours after the start of infection. Cells were collected 48 hours after TNF treatment and apoptosis was verified by three independent methods: quantitatively by flow cytometry with 1) an anti-cleaved caspase 3 antibody and the M30 antibody [105] against
Plate Cells 24h Infect with Adv for 6 hr 24h Add TNF 24-48h Measure signaling and apoptosis

Fig. 2-1. General schematic of the Adv-TNF sensitization protocol
Cells were plated at the density determined to be optimal for each individual cell line. 24 hours later the media was replaced with a reduced media volume containing the adenoviral vector. After 6 hours of exposure to the virus, the infection media was removed, cells were washed with PBS, and a full volume of media was replaced. 24 hours after the start of the infection, 100 ng/ml TNF was added to the cells. Signaling measurements were taken 0-24 hours after TNF addition. Apoptosis measurements were taken 24 or 48 hours after TNF addition. See Section 6.2 for additional detail.

caspase-cleaved cytokeratin (Fig. 2-2, a-d) or 2) annexin V and propidium iodide (PI) staining (Fig. 2-2, a-b); and verified visually by 3) DAPI-staining for chromatin condensation (Fig. 2-2, e-f). Adv infection alone caused only low levels of apoptosis compared to control cells; however, all three assays showed that the apoptotic population significantly increased upon TNF-treatment in Adv-infected cells as compared to uninfected cells. By annexin-PI and cleaved-caspase staining, the increase in apoptosis was two- to three-fold (Fig. 2-2, a-b; \( p < 0.01 \)). Adv constructs with and without a \( \beta \)-gal reporter enzyme resulted in similar rates of cell death, indicating that the presence of the \( \beta \)-gal transgene did not influence Adv sensitization. Furthermore, TNF-induced apoptosis required Adv preinfection in these cells, because TNF treatment by itself caused minimal cell death (Fig. 2-2, a, c, e). Two-factor analysis of variance (ANOVA) revealed a significant interaction effect between Adv infection and TNF treatment (\( p < 10^{-4} \)), indicating that Adv infection synergistically enhances TNF-induced apoptosis in HT-29 cells. Therefore, apoptosis provides a clear, quantitative response measurement for a system-level study. Measurements of cleaved-caspase products at 24 and/or 48 hours following TNF treatment were used for all subsequent studies.
Fig. 2-2. Adv infection sensitizes human epithelial cells to TNF-α-mediated apoptosis
(a-b) HT-29 cells were infected with either Adv.β-gal or Adv.empty (1.4 x 10^{10} vp/ml) or treated with buffer only and then stimulated with either 100 ng/ml TNF or carrier only. Cells were collected 48 hours after TNF addition, stained for either caspase-cleaved cytokeratin and cleaved (active) caspase 3 (white bars) or annexin V/PI (gray bars) and analyzed by flow cytometry. Data are presented as the mean of 3 biological replicates ± SE. Adv.β-gal was used for all subsequent experiments. (c-f) Visual comparisons of apoptosis in Adv-sensitized and control cells treated with TNF. (c-d) Sample flow cytometry plots for anti-caspase-cleaved cytokeratin and anti-cleaved (active) caspase 3 and (e-f) Fluorescent microscopy images of chromatin condensation (with DAPI-staining).
2.2.2. Infection efficiency

The β-gal reporter transgene allowed us to characterize the efficiency of Adv infection in the HT-29 cell line. HT-29 cells have been reported to have a low Adv infection efficiency [106] when considered in comparison with other cell lines [107]. Many variables affect net Adv dosage, including differences in cell plating density, infection time, and concentration-dependent rates of diffusion [108, 109]. To account for this, we altered total viral uptake by changing infection time and concentration (see Methods for details). Quantitative PCR of the Adv genome showed that infection with Adv for six hours at a concentration of $1.4 \times 10^{10}$ v.p./ml minimized exposure time with the virus while maximizing the number of transgene copies per cell (Fig. 2-3a). Therefore, we chose these infections conditions for our general protocol. The concentration of $1.4 \times 10^{10}$ v.p./ml, contains approximately 20 capsids per infectious particle by plaque assay, corresponding to a multiplicity of infection (MOI) of approximately 1000 infectious particles per cell. Although this MOI is high compared to

![Fig. 2-3.](image)

**Fig. 2-3.** High Adv concentrations are required for efficient infection of HT-29 cells (a) HT-29 cells were infected with two concentrations of Adv ($0.7 \times 10^{10}$ and $1.4 \times 10^{10}$ v.p./ml) for 0, 1, 3, 6, and 12 hours. The total number of Adv copies per cell was quantified by PCR. Values represent mean total β-gal DNA normalized to mean total β-actin DNA for duplicate samples. (b-c) HT-29 cells were infected for six hours at varying MOIs (with MOI 1000 = $1.4 \times 10^{10}$ v.p./ml), collected 48 hours later, and assayed for presence of β-gal. (b) Population positive for β-gal activity is determined by microscopy. 400 cells were counted and binned for two biological samples. Data is presented as the mean of the positive population (No. cells visibly expressing β-gal/400 cells). (c) Total β-gal enzymatic activity was measured and normalized to protein content of sample. Data are presented as the mean of 3 biological replicates ± SE.
other literature values, this was the lowest MOI tested that resulted in > 95% of the cell population positive for Adv infection (Fig 2-3b). Finally, we measured enzyme activity versus viral dose and found that β-gal activity increases with MOI, as expected (Fig. 2-3c). Therefore, we confirmed that Adv.βgal productively infects HT-29 cells, and that an MOI of approximately 1000 is necessary to infect almost 100% of the cell population.

It was possible that the high Adv titers used in the sensitization experiments had exceeded a threshold, above which apoptosis occurred non-physiologically. Therefore, we explored how Adv sensitization varied with Adv dosage. Quantitative PCR data of the Adv genome revealed that TNF-mediated apoptosis correlated linearly with dosage from 0 - 1400 total Adv copies per cell (Fig. 2-4; \( r = 0.95 \)), demonstrating that Adv sensitization to TNF is directly proportional to vector uptake in our in vitro experimental system. Moreover, synergistic apoptosis was evident with as few as 400 copies per cell. High infectivity levels are necessary to achieve efficient infection in vivo [20, 110], arguing that Adv sensitization to TNF-induced apoptosis can occur at clinically relevant infectivities.

Fig. 2-4. Sensitization dose response
HT-29 cells were infected with two concentrations of Adv (0.7 x 10^{10} and 1.4 x 10^{10} v.p./ml) for 0, 1, 3, 6, and 12 hours. The total number of Adv copies per cell was quantified by PCR. Values represent mean total β-gal DNA normalized to mean total β-actin DNA for duplicate samples. Variance in the Adv copy values ranged from 10-30% of the mean value. In parallel, HT-29 cells subject to the same infection conditions were treated with 100 ng/ml TNF and collected after 48 hours. Cleaved caspase 3-cytokeratin measurements are plotted as the mean apoptosis (above control level) of three biological replicates ± s.e.m. Pearson’s correlation coefficient for the mean values was \( r = 0.95 \).
Fig. 2-5. Adv sensitizes multiple human cell lines to TNF-induced apoptosis (a-b) HT-29 (black), A549 (green), HeLa (red), and C3A (blue) cells were infected with increasing doses of Adv (expressed as multiplicities of infection, p.f.u./cell) and treated with (a) carrier or (b) 100 ng/ml TNF. HT-29 and A549 cells were collected 48 hours post TNF treatment. HeLa and C3A cells were collected 24 hours post TNF treatment. Cleaved caspase 3-cytokeratin measurements are plotted as the mean of three biological replicates ± SE.

To determine whether Adv-TNF synergy existed in diverse tissue types, we compared the sensitization of HT-29 cells to that of other human cell lines, including HeLa cervical carcinoma cells, A549 lung carcinoma cells, and C3A human hepatocarcinoma cells over a range of infectivities (MOI: 30 -1000). Two-factor ANOVA revealed a significant interaction effect between Adv infection and TNF treatment in all cell types, even at the lowest MOIs tested ($p < 0.01$), indicating that Adv–TNF synergy was present to varying degrees in all cell types (Fig. 2-5, a-b). Surprisingly, of the cell types tested, Adv-infected HT-29 cells were the most insensitive to TNF-induced apoptosis. Apoptosis in Adv-infected A549 (Fig. 2-5, green) cells collected after 48 hours of TNF treatment was approximately two-fold greater than in HT-29 cells (Fig. 2-5, black) following the same treatment conditions. However, A549 cells were much more sensitive to Adv infection alone, in part accounting for this difference (Fig. 2-5a). Apoptosis in HeLa cells and C3A cells after 24 hours of TNF exposure was consistently two-fold greater than cell death in HT-29 cells after 48 hours of TNF exposure (Fig 2-5, a-b). HeLa cells were more sensitive than C3A cells to Adv infection alone at higher MOIs. These results demonstrate that Adv synergizes with TNF to induce apoptosis in many human cell types, and that these responses are cell-specific.
2.3. Large-scale signaling data collection

2.3.1. A high-throughput multiplex radioactivity-based kinase activity assay for quantitative signaling analysis

Once we had optimized an experimental system with which to quantitatively evaluate how Adv infection sensitizes cells to TNF-induced apoptosis, we sought to optimize a similarly quantitative procedure for measuring cell signaling. Evaluating how phenotypic information is encoded within cell-specific signaling requires dynamic and quantitative measurements of the intracellular network \[47, 97\]. Previously, it has been argued that measurements of kinase catalytic activity are more useful than measurements of simple protein abundance for monitoring information flow through a signaling network.

![Figure 2-6](image)

Fig. 2-6. Kinase activities are information-rich measures of signaling networks
Notched box plot of variable importance from a systems model of cytokine-induced apoptosis \[97\]. Variable importance is normalized so that variables with average information content in the model are assigned a value of one (see \[97\] for details). Measurements of kinase assays by kinase assay (left) were compared to measurements of protein state or protein level by Western blotting (center) or antibody array (right). Kinase measurements were significantly more informative on average than western blotting or antibody array (\(p < 0.05\) by signed rank test against a median value of 1). Figure courtesy of K. Janes.
network [111]. Recent analysis of a large-scale signaling compendium [47, 97] has further suggested that kinase activity measurements are more informative on average than measures of protein phosphorylation or cleavage state (Fig. 2-6).

A generalized assay in a 96-well format for the multiplex analysis of multiple protein kinase activities has been previously developed in our laboratory [111, 112]. This procedure, which utilizes kinase-specific immunopurification steps followed by rapid quantitative high-throughput activity measurements, has been applied to measure the activities of five kinases: ERK, Akt, IKK, JNK1, and MK2 [111] (for general assay schematic, see Fig. 2-7). The assay was previously optimized for HT-29 cells and HeLa cells to monitor information through the TNF, EGF, and insulin signaling networks in HT-29 cells sensitized with IFN-γ [47, 97, 111].

Using our general Adv–TNF sensitization protocol (Fig. 2-1) and the large-scale data collection procedure developed for cytokine compendium measurements (ERK, Akt, JNK1, IKK, and MK2) [47], we collected the analogous set of five kinase signals following TNF treatment in Adv-infected or uninfected HT-29 cells. Briefly, Adv-infected or uninfected HT-29 cells were treated with or without 100 ng/ml TNF and cell lysates were collected at 13 time points over 24 hours (see Chapter 6 for details).

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**Fig. 2-7. General schematic of the high-throughput kinase activity assay**

Reproduced from [111]. (1) Lysates are incubated with Protein A or G microtiter wells precoated with anti-kinase antibodies. (2) After several washes, an appropriate substrate and [γ-32P]-ATP are added to the plate to initiate (3) an in vitro phosphorylation reaction. Signal-to-noise ratio is optimized by varying the duration of the reaction for each kinase. (4) The reaction is terminated with H3PO4 (for ERK, Akt and JNK1 assays) or EDTA (for IKK and MK2 assays), and a fraction of the reaction mix is transferred to a phosphocellulose (PC) filter plate and washed to remove free 32P.
Our measurements show that kinase signaling time courses are quantitatively different between uninfected and Adv-infected cells treated with 100 ng/ml TNF and between Adv-infected cells with and without TNF treatment (Fig. 2-8). Measurements were sampled more densely in the first four hours because much of the signaling induced by TNF occurs shortly after cytokine addition [111]. Therefore, in addition to evaluating the significance of the difference between each pair of signaling curves for the full time course by two-factor analysis of variance (ANOVA), we also evaluated differences over the first four hours (7 time points) and the last twenty hours (6 time points) by the same method (Fig 2-8, table).

Akt, IKK, and MK2 time courses are significantly different between uninfected and Adv-infected cells treated with 100 ng/ml TNF (see Chapter 3 for more details). However, with the exception of Akt, signals during the first four hours of the time course are not significantly different between these two conditions. This may be due to the importance of instant, transcription-independent signals to TNF cell-fate decision, such as the acute activation of MK2 and JNK1 [113]. However, signals during the later hours of the time course are significantly different for all kinases except JNK1. Because we have not inhibited gene expression in our studies (unlike other studies with TNF [83] ), both TNF-induced and Adv-induced transcripts and proteins can affect the signaling network measurements, and therefore the later differences are likely transcription-dependent. In recent work growing out of the signaling compendium, the responses of HT-29 cells to the nine multi-input stimuli specified in the signaling and apoptosis datasets were transcriptionally profiled to interrogate the role of gene expression in the HT-29 system [114]. Preliminary analyses of these measurements revealed a strong clustering of TNF-treated samples. The data here suggest that the mechanism by which Adv alters the TNF-induced cell fate decision depends on late, possibly transcription-dependent, signals.

All signaling time courses are significantly different between Adv-infected cells with and without TNF treatment, as expected (Fig. 2-8, table), with the exception of early (0-4 h) Akt activity and late (4-24 h) IKK activity. Akt is significantly upregulated in Adv-infected cells prior to TNF treatment, consistent with literature linking Adv viral backbone proteins to Akt activation [62, 64]. The biological implications of Adv-induced Akt activity are explored in Chapter 3.
Fig. 2-8. Signaling time courses following TNF-activation in uninfected and Adv-infected cells. Uninfected and Adv-infected cells were treated with 100 ng/ml TNF at time t = 0 min. Cells infected with Adv in the absence of TNF treatment were included as a control. Lysates were collected at 0, 5, 15, 30, 60, and 90 minutes and 2, 4, 8, 12, 16, 20 and 24 hours and kinase activities for ERK (green), Akt (red), JNK1 (blue), IKK (purple) and MK2 (orange) were measured by a high throughput kinase activity assay and normalized to total protein content. Results are plotted as the mean relative activation of two biological replicates normalized to activity of the untreated control at 0 minutes (i.e., uninfected cells). The significance of the difference between each pair of signaling curves (e.g. ERK activity between Adv only and Adv + TNF conditions) was evaluated by two-factor ANOVA. Both differences in the full time course and partial time courses (0 to 4 hours and 4 to 24 hours) and comparisons between TNF only versus Adv + TNF and Adv only versus Adv + TNF were compared. P-values for each comparison are presented in tabular form and significant differences (p < 0.05) are shaded in gray.
Ad and Adv are also known to activate NF-κB in a variety of cell types [31, 32, 115] and recently an E1/E3-deleted Adv has been shown to attenuate cancer cell sensitivity to chemotherapeutic drugs via Adv-mediate NF-κB activation [116]. However, in all these studies, NF-κB was induced during the early phase of Adv infection leading to a host inflammatory response including secretion of IL-6 [20]. The cause of late IKK–NF-κB in Adv-infected cells with and without TNF is to our knowledge unexplored. One hypothesis is that this signal occurs indirectly in response to an autocrine signal from the IL-1α autocrine loop identified in the cytokine compendium study [69]. We discuss this hypothesis in more detail in Chapter 4.

2.3.2. Quantitative western blots measurements

Many critical signals for apoptosis cannot be measured via an enzymatic activity assay but instead require a protein state assay. For example, caspases are produced in cells as catalytically inactive protease precursors called zymogens. They are activated via proteolytic cleavage during apoptosis [40] and are thus effectively measured via size resolution and immunoblotting. Caspase 8 is an initiator caspase that is critical to mediating apoptosis of the extrinsic pathway, initiated by the binding of an extracellular death ligand such as TNF [117]. Cytokine-signal mapping of the TNF–growth factor signaling compendium revealed that caspase 8, in addition to JNK1 and MK2, co-varied most closely with TNF-treatment [47]. Therefore, we sought to augment our data set with the addition of caspase 8 measurements via Western blot.

Cleaved caspase 8 is membrane-associated and therefore requires a whole-cell homogenate (observations by S. Gaudet). To add caspase 8 measurements to our time course, we repeated the three HT-29 conditions (see Fig. 2-8) on a single day. Caspase 8 cleavage does not occur until approximately 4-8 hours post TNF addition [47]. Therefore, we collected an abbreviated time course with samples taken at 0, 4, 8, 12, 16, 20, and 24 hours. In our experimental system, cleaved caspase 8 was undetectable in Adv–TNF treated cells until 16 hours and it rose steadily as measured at 20 and 24 hours (Fig. 2-9). There was no cleaved caspase 8 detectable in uninfected cells treated with
Fig. 2-9. Caspase 8 cleavage in Adv–TNF HT-29 cells
Caspase 8 time abbreviated time course following TNF-activation in uninfected and Adv-infected cells. Uninfected and Adv-infected cells were treated with 100 ng/ml TNF at time t = 0 min. Cells infected with Adv were included as a control. Lysates were collected at 12, 16, 20, and 24 hours and caspase 8 (pro- and cleaved-) were measured by quantitative western blot. (a) Pro- and cleaved-caspase 8 at 12 h and 24 h. (b) Fraction of cleaved caspase 8 (cleaved/pro) as a function of time. Approximately equal amounts of protein content were loaded in each lane. Results are plotted as the mean ratio of three biological replicates ± SE.

TNF or in Adv-infected cells without TNF treatment, consistent with apoptosis measurements.

Because the caspases are direct effectors of apoptosis, adding these measurements to a network model of apoptosis would likely increase the predictive power. However, our final network model did not require the pro- or cleaved-caspase 8 signals to predict apoptosis in Adv-infected epithelial cells (see Chapter 4). This is consistent with the findings of the cytokine compendium that the caspase effector signals are encoded by the upstream signaling network [97].
2.4. Partial least squares regression analysis

2.4.1. Measurement processing and metric extraction

One aim of this work was to combine our measurements with the cytokine compendium for the purpose of predicting apoptotic response. If possible, this would augment the findings of Gaudet, et al. [47] to show that data sets collected at different times and by different pairs of hands can be compared with confidence. To do this, we processed our measurements using the same method of signal-specific normalization: 1) correcting each measurement for the total amount of cellular protein in the sample and 2) adjusting kinase activity measurements for changes in the specific activity of [γ-32P]ATP [47]. In the cytokine compendium, each signal was referenced to its own background (t = 0 min average) activity in the sample. However, while observations in the cytokine compendium were all pretreated with IFN-γ, the pretreatment in our measurements, i.e., ± Adv infection, was the variable of interest. Differences between baselines contained biologically relevant information, creating a key difference in our data set. To explore this, we processed our data in two ways: 1) signals for each pretreatment (i.e., Adv @ 1000 MOI) were normalized to the baseline signals for the same time course pretreatment (internal normalization) or 2) signals for each pretreatment were normalized to the baseline signal for the uninfected control (control normalization). Measurements processed via both methods were evaluated in subsequent PLS analysis.

For PLS analysis all measurements are combined into a single matrix; therefore the temporal organization of the data is lost. It was unknown whether time-dependent features such as the steady-state signal or the maximum activity contained important information metrics were empirically derived from [97]. Therefore, we used the method developed by Janes and Kelly [89] in which time-dependent signaling metrics are derived empirically from a dynamic data set (Table 2-1). Each metric is then entered as a separate variable into the data matrix. A total of 35 metrics were calculated for each signal for a combined total of 175 measurements per observation.
Table 2-1. Signaling metrics extracted from dynamic network measurements
Adapted from [97].

<table>
<thead>
<tr>
<th>Metric class</th>
<th>Metrics extracted</th>
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<tbody>
<tr>
<td>Local metrics</td>
<td>Time points</td>
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<td></td>
<td>Point-to-point derivatives</td>
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<td>Summary metrics</td>
<td>Area under the curve</td>
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<td>Mean signal</td>
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<td>Maximum signal</td>
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<td></td>
<td>Steady-state signal</td>
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<td>Peak metrics</td>
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<td>Activation slope</td>
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2.4.2. Predictions with TNF–GF compendium

We recalculated the cytokine–growth factor PLSR model using only the five dynamic kinase-activity profiles from the nine cytokine–growth factor treatments fitted to two apoptosis measurements (cleaved caspase products at 24 and 48 hours). We then used this model to predict apoptosis for our combinations of TNF only, Adv only, and Adv+TNF in HT-29 cells based on the analogous set of signaling measurements. While the model accurately predicted apoptosis in cells infected with Adv and treated with TNF, the model could not differentiate those cells that were not sensitized to apoptosis and instead predicted significant levels of apoptosis for all treatments (Fig. 2-10a).

Although this result was disappointing, the conditions used to train the model were designed to study the combination of growth factors and TNF on the cell fate decision. We reasoned that this would not provide enough information to differentiate between infected and uninfected conditions where TNF is the major stimulus; instead, these conditions would correlate most closely with TNF conditions in the compendium, resulting in a positive prediction of apoptosis.

The PLSR model is derived as a series of latent variables called principal components, which contain linear combinations of the original kinase activity measurements [97, 118]. These principal components form a set of intracellular basis axes that define the dimensions of the signaling network that controls apoptosis. IFNγ +
Fig. 2-10. TNF-growth factor model predicts Adv-TNF-induced apoptosis but cannot differentiate uninfected cells
(a) Correlation between measured apoptosis and predictions of the TNF/GF model. Model training with cytokine compendium data (black) is compared to model predictions of Adv+TNF (blue) and Adv only or TNF only apoptosis (green) at 24 hr (filled) and 48 hr (hollow). Data are presented as the mean of three biological replicates ± S.E., and model uncertainties were estimated by jack-knifing[89]. (b) TNF and insulin axes are recapitulated using only kinase activity data. Adv+TNF (blue), TNF only (green) and Adv only conditions are shown.

TNF treatments projected strongly along principal component 1, while insulin measurements projected along principal component 2, as was found in the full compendium model [97]. (Interestingly, EGF treatment did not project as strongly as found in the original compendium model). When the TNF treatments ± Adv-infection were plotted on this treatment mapping, they projected strongly onto the TNF axis (Fig. 2-10b). The Adv only condition did not fall within the region outlined by the TNF and growth factor conditions. This supported our hypothesis that information on how Adv changes apoptotic response to TNF was needed to construct a useful model. We pursue this in Chapter 4.

2.5. Other biological considerations
2.5.1. Adenoviral replication in carcinoma cell lines

As described above, E1/E3-deleted Advs are not biologically inert. Therefore, by using a first generation Adv (FG-Adv), our results are specific to cytokine-induced cell signaling networks in the context of Adv background transcription. This system is appropriate because this is the most common vector used; however, to understand its limitations, we quantified aspects of Adv biology that may be contributing to the sensitization response.

Ad E1A products function as transcription factors to promote viral replication by trans-activating other Ad transcription units, namely the E2 and E4 regions, which encode proteins for necessary for Ad DNA replication [2]. Thus, Adv that have been deleted for the E1A region are highly attenuated [119]. However, human tumor cell lines derived from colon, liver, cervical, and lung cancer support replication of Adv deleted for the E1A/E1B genes [107]. It has been hypothesized that cellular proteins can substitute functionally for E1A in the trans-activation of viral proteins and cause this phenomenon [107, 120].

Given the high MOI used in our experimental system, we suspected that Adv genome replication was occurring. Using quantitative PCR, we confirmed nearly 100-fold replication of the Adv genome in HT-29 cells over 72 hours following the start of infection (Fig. 2-1a). Exposure to TNF exacerbated this effect: the amount of Adv genome replication in the 24 hours after TNF addition was almost 4-fold greater in the presence of TNF (Fig. 2-1b). To our knowledge, no literature evidence exists documenting this effect, although it is likely that TNF treatment results in an influx of proteins to the nucleus that can act as trans-factors for Adv genome replication.

For most tumor cell lines, the cellular trans-complementing factors substituting for E1A and causing Adv DNA replication remain unclear. However, in human papilloma virus (HPV)-associated cervical HeLa carcinoma cells, HPV 18 DNA is integrated into the genome and is amplified [121]. One of the HPV 18 DNA products, the E7 protein, structurally and functionally similar to Ad E1A [122]. In HeLa cells, the HPV E6 and E7 proteins can substitute for adenoviral E1A and E1B, allowing for replication of Adv [123]. Based on this information and the data for HT-29 cells, we
Fig. 2-11. HT-29 cells support Adv genome replication
(a) HT-29 cells were treated with $1.4 \times 10^{10}$ v.p./ml Adv and 100 ng/ml TNF (●) or mock (O) as previously described and collected at 6, 24, and 48, and 72 hours post infection. The total number of Adv copies per cell was quantified by PCR. Data are presented as the mean total fiber knob DNA normalized to mean total β-actin DNA for three biological replicates ± S.E. (b) TNF increases Adv genome replication. Data are presented as [mean copies/cell @ 48 h ± TNF] / [mean copies/cell @ 24 h].

conclude that Adv genome replication is occurring in HeLa cell lines in our experimental system, although we did not measure it directly.

2.5.2. Transcriptional shedding of adenoviral backbone proteins

Gene products of the Adv early transcription region 4 (E4) operate through a complex network of protein interactions with cellular regulatory components of important host cell functions, including transcription, apoptosis, and DNA repair [124], and signaling. Commonly, vectors contain the E4 gene (Adv.E4+) and these vectors selectively elicit phosphorylation of Akt in endothelial cells [64]. E4orf1 has also been shown to mimic growth factor signaling via activation of PI3K [63]. In addition, E4orf4 activates mTOR [62], and likely Akt as well through mTOR regulation [125]. Because our vector is positive for E4, we reasoned that E4 proteins are responsible for Akt activation in Adv-infected HT-29 cells prior to TNF exposure (Fig. 2-8). Construction of an Adv vector without a full deletion of the E4 region is difficult because E4orf6 is critical for adenoviral replication [124]. Therefore, we tested our hypothesis
indirectly by measuring the shedding of E4 transcripts using RT-PCR.

We found evidence of significant E4 transcription 24 hours following Adv infection (Fig. 2-12a). Adding TNF 24 hours after the initial infection enhanced E4 transcription more than 50% over 24 hours as compared to infected cells that were not treated with TNF (Fig. 2-12b). Our system is consistent with the demonstrated mechanisms linking Ad E4 and Akt activation and therefore we conclude Adv E4 has a role in Adv–TNF synergy. We explore the implications of this high level of Akt activation in Chapter 3.

2.5.3. Helper-dependent adenoviral vector infection to test effect of viral transcription

Because of the known activity of Adv E4 and the presence of Adv genome replication, it is likely that wild type adenoviral genes remaining in the vector backbone account at least in part for Adv–TNF synergy. As described above (Section 1.1.2), helper-dependent Adv (HD-Adv), deleted for all adenoviral genes, show the most
promise for eliminating vector-induced toxicity. Therefore, we sought to determine if this new generation of vector would mitigate Adv–TNF synergy.

We compared our standard E1/E3-deleted Adv (Adv.dE1E3 with a CMV promoter and β-gal transgene) to an HD-Adv carrying the β-gal transgene under control of the Rous sarcoma virus (RSV) promoter (Adv.HD). We matched MOI based on blue-forming units (b.f.u.), a measure of infectivity in Adv.HD analogous to p.f.u. for FG-Adv. TNF-induced apoptosis in cells infected with Adv.HD was reduced as compared to Adv.dE1E3-infected cells but was still significantly above apoptosis in control (uninfected) cells (p < 0.05; Fig. 2-13a). To confirm a productive infection, we measured β-gal activity. Cells infected with Adv.dE1E3 exhibited strong β-gal activity while enzyme activity in Adv.HD-infected cells was undetectable (Fig. 2-13b). This difference could reflect difference in infectivity, thus raising questions about the comparability of our apoptosis data. However, the presence of Adv genome replication in HT-29 cells infected Adv.dE1E3 may result in amplification of the β-gal gene that is unrelated to infectivity. In addition, the different promoters in the vectors likely lead to different amounts of activity. A more direct and sensitive measure of viral infection by Q-PCR
would be necessary to compare infection efficiency. We also measured apoptosis in the presence of PI3K inhibition, the upstream activator of Akt, using 20 μM LY294002. Surprisingly, Adv.HD were very sensitive to PI3K–Akt pathway inhibition, with apoptosis increasing more than 5-fold to 40% and the size of the apoptotic population rose to almost 30% (Fig. 2-13c). This data indicates that Adv-infected cells’ heightened dependence on Akt activity to confer protection against TNF-mediated apoptosis may not be entirely caused by the presence of wild-type viral genes. However, another convoluting factor is that propagation of Adv.HD vectors requires a helper virus, which invariably contaminates the final vector product [16]. The most effective way to test the effects of Adv wild-type gene products would be to inactivate transcription using UV-psoralen exposure [126]. This is an important control for future work.
3. Insight into the mechanism of Adv–TNF synergy through quantitative signaling measurements

3.1. Introduction

In this study we apply our quantitative methods of signaling and apoptosis analysis to further the molecular-level understanding of the engineered Advs used in gene therapy applications. Specifically, we deconstructed the contributions of Adv and the transgene to understand how Adv-induced cellular changes synergize or antagonize the effects of certain transgenes, confounding interpretation of their normal role in cells.

3.2. Adv modification of the TNF-induced signaling network

Because Adv infection sensitizes cells to TNF-induced cell death, we reasoned that Adv might modulate one or more kinase signaling pathways centrally involved in the TNF-activated network (Fig. 3-1A). Cell death responses to TNF are highly regulated by a number of kinase signaling pathways [127-129]. In addition, Adv E4 is known to mimic growth factor signaling, leading to activation of PI3K and Akt [62, 64]. Therefore, we measured three kinases (MK2, IKK, and JNK1) that are distributed within the TNF signaling network (Fig. 3-1A, blue shading) as well as two growth-factor activated signals (Akt and ERK; Fig. 3-1A, lavender shading). To measure Adv-induced activity changes leading to sensitization, we treated Adv-infected and uninfected HT-29 cells with TNF (100 ng/ml) and collected cell lysates at 13 time points over 24 hours. From these cell extracts, we measured MK2, IKK, JNK1, Akt, and ERK activities by using a high-throughput quantitative multiplex kinase assay [111].
Fig. 3-1. (Previous page) Adv infection upregulates TNF-induced pro- and anti-apoptotic signaling. 

A, Schematic representation of the signaling pathways induced by TNF (shaded in blue) and insulin (shaded in lavender). The kinases measured (MK2, IKK, JNK1, Akt, and ERK) are highlighted in yellow. Figure is modified from [47] with permission. B-C, Dynamic activation status and inhibitor response data for (B) TNF-activated pathways and (C) growth factor-activated pathways in uninfected cells treated with 100 ng/ml TNF (TNF; blue) and Adv-infected cells treated with 100 ng/ml TNF (Adv + TNF; red). Lysates were collected at 0, 5, 15, 30, 60, and 90 minutes and 2, 4, 8, 12, 16, 20 and 24 hours and kinase activity was measured by a high throughput kinase activity assay and normalized to total protein content. Results are plotted as the mean relative activation of two biological replicates normalized to activity of the untreated control at 0 minutes (i.e., uninfected cells). The significance of the difference between each pair of curves was calculated by two-factor ANOVA (MK2: p < 0.01; IKK: p < 0.001; JNK1: p = 0.71; Akt: p < 10^-4; ERK: p = 0.43). Apoptosis in the presence of kinase inhibition was measured by flow cytometry for cleaved caspase-cytokeratin. The following inhibitor concentrations were used: 10 μM SB202190 (SB); 20 μM SC-514 (SC); 10 μM SP600125 (SP); 20 μM LY294002 (LY); 10 μM U0126 (U0). Cells were collected at 24 hours, rather than 48 hours, to minimize baseline apoptosis resulting from inhibition. Measurements are plotted as the mean % change in apoptosis resulting from inhibition (i.e., mean % apoptosis in the presence of inhibitor – mean % apoptosis without inhibitor) of three biological replicates (SC, SP, U0) or six biological replicates (SB, LY) ± s.e.m. Note that LY plot ranges from -40 to +40 (as compared to -20 to +20 for other inhibitor plots). Changes are labeled as significant (*) if p < 0.05.

D, Adv-TNF synergy via pro-apoptotic p38-MK2 signaling and anti-apoptotic PI3K-Akt signaling. Synergy is illustrated schematically as an operational amplifier, with the strength of the Adv or TNF input into either the MK2 or Akt signaling time course indicated by line weight. Amplification of the inputs results in activation (MK2) or inhibition (Akt) of apoptosis.

the contribution of Adv infection alone, kinase activities were also measured in Adv-infected cells after mock stimulation. Finally, to determine the phenotypic consequences of each kinase activity, we measured the change in TNF-stimulated apoptosis in the presence of pathway-specific, small-molecule inhibitors.

We observed potent activation of the TNF signaling network when either uninfected or Adv-infected cells were treated with TNF (Fig 3-1B). Among TNF pathways, Adv infection significantly increased TNF-induced MK2 and IKK signaling (p < 0.01, two-factor ANOVA) but not JNK1 (p = 0.71). These altered intracellular patterns were a product of Adv-TNF synergy, because Adv infection alone contributed minimally to JNK1, IKK, and MK2 activation (Fig 3-2). Small-molecule inhibition of the measured pathways confirmed the importance of MK2 and IKK for Adv sensitization. Inhibition of the upstream activator of MK2 with SB202190 significantly decreased apoptosis (p < 0.01), suggesting a pro-apoptotic role. Conversely, IKK inhibition with SC-514 resulted in a small but significant increase in apoptosis (p < 0.05), consistent with the recognized anti-apoptotic functions of IKK [38]. JNK1 was transiently activated by TNF but was unchanged by Adv infection; thus, as expected, JNK1 inhibition with SP600125 did not
Fig. 3-2. Adv infection alone upregulates Akt but not other TNF-growth factor-activated pathways. Dynamic activation status for TNF-activated pathways and growth factor-activated pathways in Adv-infected cells following mock stimulation (Adv; green). Activation status of Adv-infected cells treated with 100 ng/ml TNF (Adv + TNF; red) is plotted for reference (see also Fig. 3-1B-C). Lysates were collected at 0, 5, 15, 30, and 60 minutes and 2, 4, 8, 12, 16, 20 and 24 hours and kinase activity was measured by a high throughput kinase activity assay. Results are plotted as the mean relative activation of two biological replicates normalized to activity of the untreated control at 0 minutes (i.e., uninfected cells). The significance of the difference between each pair of curves was evaluated by two-factor ANOVA (MK2: \( p < 10^{-12} \); IKK: \( p < 0.001 \); JNK1: \( p < 10^{-6} \); Akt: \( p < 0.01 \); ERK: \( p < 10^{-6} \)). Note that Akt activity is plotted on a linear scale (as compared to a semi-log scale for the other kinases).

significantly affect Adv-sensitized cell death. We conclude that, of the TNF-dependent kinases measured, the p38-MK2 pathway is the most important pro-apoptotic signal contributing to Adv-TNF synergy.

Multiple growth factor pathways were also strongly activated by Adv and TNF (Fig. 3-1C). Similarly to TNF-induced JNK1, ERK was transiently activated by TNF but was not differentially affected by prior Adv infection. Thus, inhibition of the upstream ERK kinase with U0126 did not significantly affect Adv-TNF synergy. In contrast, Adv infection alone induced a three- to five-fold increase in Akt activity for up to 24 hours (\( p < 0.01 \); 3-2), consistent with the PI3K-activating role of E4 [62, 64]. Akt activation was further increased by TNF treatment, resulting in eight- to ten-fold greater activity than in uninfected TNF-treated cells (\( p < 10^{-10} \)). Akt transmits many key pro-survival signals [130], and we found that upstream PI3K inhibition with LY294002 dramatically
increased synergistic apoptosis mediated by Adv and TNF ($p < 10^{-4}$). Together, these experiments indicate that Adv infection synergizes with TNF by augmenting an existing p38-MK2 pro-death signal and adding a new PI3K-Akt pro-survival signal to the network (Fig. 3-1D).

3.3. Role of Akt in TNF sensitization

3.3.1. Upregulation of endogenous Akt activity in Adv-infected cells by insulin

Despite potent Akt signaling in Adv-infected cells (Fig. 3-1C), synergistic apoptosis still occurred after TNF-stimulation. The LY294002 inhibitor experiment confirmed a pro-survival role of Akt by downregulation (Fig. 3-1C). However, the contribution of some pro-survival molecules has been shown to vary depending upon whether protein function is increased or decreased [94]. To activate endogenous Akt, we therefore used the growth factor insulin, which has previously been shown to be reasonably specific in its activation of Akt in HT-29 cells [47]. Additionally, insulin is known to inhibit TNF-induced cell death [131] through upregulation of Akt activity in HT-29 cells sensitized with interferon-$\gamma$ (IFN) [132]. To test if insulin similarly provides protection from TNF-induced apoptosis in HT-29 cells sensitized by Adv, we added 100 nM insulin together with TNF. In contrast to IFN-sensitized cells, insulin did not decrease TNF-induced apoptosis in Adv-infected cells ($p = 0.15$; Fig. 3-3A). Surprisingly, this was not due to a lack of Akt signaling, since insulin increased Akt activity to a similar extent in both Adv-sensitized cells and IFN-treated cells ($p < 0.05$; Fig. 3-3B). There was not a significant decrease in TNF-induced MK2 activity in Adv-infected cells as a result of insulin stimulation ($p = 0.49$; Fig. 3-3C), confirming the selectivity of insulin as an Akt-selective agonist. To verify that IFN- and Adv-sensitized cells behaved similarly when Akt was inhibited, we measured TNF-induced apoptosis and Akt activity in the presence of LY294002. For both sensitizing agents, LY294002
Fig. 3-3. Insulin increases Akt activity but does not reverse TNF-induced apoptosis or reduce MK2 activity in Adv-infected cells

Adv- and IFN-sensitized HT-29 cells were treated with TNF (100 ng/ml and 50 ng/ml, respectively) in the presence and absence of (A-C) 100 nM insulin or (D-F) 20 μM LY294002. Cells were lysed at 12 hours and assayed for kinase activity or collected after 24 hours and assayed for apoptosis by flow cytometry for cleaved caspase-cytokeratin. A,D, Change in TNF-induced cell death. Measurements are plotted as the mean of six biological replicates (2 independent experiments) ± s.e.m. B,E, change in TNF-induced Akt activity. Measurements are plotted as the mean of six biological replicates (2 independent experiments) ± s.e.m. C,F, Change in TNF-induced MK2 activity in Adv-sensitized cells. Measurements are plotted as the mean of three biological replicates ± s.e.m. Changes are labeled as significant (*) if p < 0.05.

inhibition significantly increased TNF-induced apoptosis (p < 10^-9; Fig. 3-3D) and reduced Akt activity (p < 0.001; Fig. 3-3E), as expected. Again, there was no change in MK2 activity in Adv-sensitized cells as a result of Akt inhibition (p = 0.45; Fig. 3-3F), indicating that MK2 pro-apoptotic signaling is separable from Akt activity changes in Adv-infected cells. The lack of apoptotic rescue shows that the insulin-induced Akt activation in Adv-infected cells does not result in functional anti-apoptotic signaling.
3.3.2. Saturation of downstream Akt effectors by Adv

The lack of pro-survival signaling by insulin-stimulated Akt could be explained by an inability to phosphorylate downstream Akt effectors in Adv-infected cells. To test this, we measured phosphorylation levels of multiple Akt substrates in Adv- and IFN-sensitized HT-29 cells after TNF stimulation alone and in the presence of LY294002 or insulin. We used an Akt-phosphosubstrate (Akt-pSub) antibody that binds specifically to phosphorylated substrates of Ser/Thr kinases that recognize the RxRxxS/T motif [133]. In addition, we measured levels of phosphorylated glycogen synthase kinase-3 α and β (pGSK-3α/β), an established substrate of Akt [134]. Western blotting of HT-29 lysates showed that pGSK-3α/β and multiple Akt-pSubs were significantly upregulated in Adv-sensitized cells as compared to IFN-sensitized and untreated cells, even before the addition of TNF (Fig. 3-4A). This is consistent with the high baseline Akt activity induced by Adv (Fig. 3-3C). Two Akt-inducible proteins, pGSK-3α and Akt-pSub42kDa (Fig. 3-4A), were quantified by densitometry and normalized to the Adv- or IFN-sensitized zero-minute band. For both IFN and Adv pretreatments, TNF-inducible phosphorylation of GSK-3α and Akt-pSub42kDa was reduced with LY294002 treatment (Fig. 3-4,B-C), confirming that both proteins are reliable indicators of functional Akt activity. In IFN–TNF-treated cells, insulin significantly increased pGSK-3α and Akt-pSub42kDa, but there was no significant insulin-induced phosphorylation of these proteins in Adv-TNF-treated cells, despite clear insulin-induced activation of Akt in Adv-infected cells (Fig. 3-3B). Thus, Akt activation in Adv-infected cells sustains phosphorylation of downstream effectors, but further Akt activation induced by insulin fails to increase Akt effector phosphorylation. We conclude that Adv infection in combination with TNF saturates the ability of Akt to phosphorylate its substrates, contributing to the observed loss of anti-apoptotic signaling.
A

B pGSK-3α

C Akt-pSub

E Akt-phospho-(Ser/Thr) Substrates

IFN + TNF

Adv + TNF

Controls IFN Adv

+ LY alone + Ins

+ LY alone + Ins

+ LY alone + Ins

+ LY alone + Ins

+ LY alone + Ins

+ LY alone + Ins

+ LY alone + Ins

+ LY alone + Ins

75 kDa

50 kDa

42 kDa

37 kDa

25 kDa

52
3.4. Construction of a global Akt-survival dose-response curve combining Adv- and IFN-mediated sensitization

Insulin caused a similar absolute increase in Akt signaling in uninfected and Adv-infected cells, both adding roughly three Akt-activity units after stimulation (Fig. 3-3B). However, relative to the baseline Akt signal for each treatment (Fig. 3-5A), the “fold activation” of Akt induced by insulin was larger for IFN-sensitized cells (3.7-fold) than for cells infected with Adv (2-fold). Recently, it has been suggested that cells become quickly desensitized to absolute levels of signaling and are often more responsive to gradients of signals [135]. To examine the importance of fold changes, we measured Akt activity and TNF-induced apoptosis under a set of Adv, IFN, LY, and insulin conditions that gave a range of TNF-induced apoptotic responses (see Fig. 3-5 legend and Methods). Then, we preprocessed the data by normalizing IFN- and Adv-sensitized cells to their respective zero-minute values (Fig. 3-5A). When cell viability was plotted against this fold activation of Akt, we found that both IFN-TNF and Adv-TNF treatments collapsed onto a common sigmoidal dose-response curve (Fig. 3-5B). Preprocessing measurements of Akt-effector substrates in the same manner resulted in a dose-response identical to that of Akt, with the exception of a two-fold faster transition steepness between minimum- and maximum-observed viability (Fig. 3-5C). The faster viability transition for Akt effector substrates is consistent with the known ultrasensitivity that arises from sequential signaling cascades [136]. The existence of a single function—relating fold activation of the PI3K-Akt pathway to viability for a wide range of treatment conditions—suggests that the relative change in Akt signaling is more important than its absolute level for determining TNF-induced cell fate.
Fig. 3-5. Adv-infected are trapped near the plateau of a global Akt-survival dose-response curve. A, Cells were infected with Adv or treated with IFN, lysed after 24 hours and measured for Akt activity. B-C, Following Adv or IFN sensitization, cells were treated with TNF (100 ng/ml and 50 ng/ml, respectively), TNF + LY294002 (20 μM), TNF + insulin (100 nM), or TNF + LY294002 + insulin (B only). Conditions were chosen to generate a range of Akt activities. Measurements were taken at 12 hours for Akt activity and effector phosphorylation and at 24 hours for apoptosis as described in Fig. 3. B, Cell survival versus fold activation Akt activity. Akt activities were normalized to sensitized 0-min values from A. Akt measurements are the mean of six biological replicates ± s.e.m. Survival measurements are 100% - the mean of six biological replicates ± s.e.m. The arrow indicates Adv-infected cells without LY inhibition. C, Plot of survival (from B) versus effector phosphorylation levels quantified in Fig. 4. Solid red markers indicate IFN-sensitized conditions and open red markers indicate Adv-sensitized conditions (same convention as in B). B-C, Sigmoid function and parameters were calculated as described in Methods. Transition steepness (Ts) and the upper and lower bounds of the 90% confidence interval (parentheses) are labeled on the graphs.

The calculated Akt-survival dose-response provides a quantitative explanation for how insulin is unable to attenuate synergistic TNF-induced apoptosis in Adv-infected cells (Fig. 3-3A). First, by dramatically increasing the baseline Akt signal (Fig. 3-5A), Adv infection reduces insulin’s ability to induce a strong fold activation of the PI3K-Akt pathway (Fig. 3-3B). This prevents Adv-infected cells from moving far rightward on the dose-response curve in Fig. 3-5B. Second, our panel of experimental treatments indicates that the role of Akt signaling in these cells is most apparent when pathway activity is reduced to levels that are below baseline signaling. Adv-infected cells are thus positioned very near the upper viability-plateau of the dose-response (Fig. 3-5B, arrow), suggesting a limited capacity to reduce TNF-induced apoptosis further. Together, this indicates that Adv infection traps cell in a network state that is unable to transmit additional anti-apoptotic information via the Akt pathway.
3.5. Further considerations

Our aim in this study was to explore how Adv alters human epithelial cell signaling and apoptotic responses to the inflammatory cytokine TNF. Compared with wild-type adenovirus, there are relatively few studies on Adv infection and its interaction with host-cell signaling pathways. Understanding how Adv-infected cells respond in the context of a cytokine-rich environment, such as an inflamed tissue, is critical to improving gene therapy applications. MOIs at the injection site can be very high in vivo [20, 110], similar to the ratios used in this study. High concentrations of Adv in vivo activate innate immune responses independent of viral gene transcription [24, 25, 137]. Neutrophils, natural killer cells, and macrophages are recruited to the site of infection where they secrete cytokines such as TNF. TNF and TNF-family cytokines are directly responsible for apoptosis in the target cells [24, 138]. Adv infection, immune cell activation, cytokine release, and host cell death are complex, overlapping events in vivo. By studying Adv infection and apoptosis in the context of one defined cytokine in vitro, we were able to discover that Adv and TNF cooperate to promote cell death in infected cells. Adv-TNF synergy could be an important contributor to gene therapy side effects, which have been reported in Adv clinical trials [139-141].

E1/E3-deleted Ads are commonly used in laboratory experiments as delivery vectors to overexpress or inhibit pathways and elucidate function. Such studies assume that changes caused by Adv infection and changes caused by the transgene are linearly cumulative. If true, then infection with an Adv that lacks the transgene is an adequate control. However, the results here show that Adv itself can significantly alter signaling pathways in the cell, both at the basal level (e.g., Akt) and in response to TNF (e.g., MK2). For Adv and TNF, cell response to Adv and TNF is highly non-linear, with the underlying impact of infection becoming apparent only after cytokine stimulation. This reveals an important caveat for interpreting laboratory studies involving Adv. Controls with “empty” Adv might well be complemented by delivering the transgene (and associated control) with a different vector, such as liposomes for transient expression or lentiviruses or adeno-associated viruses for stable expression.
A surprising result of our work is that regimes exist where increased Akt activity produces limited pro-survival benefit. We showed that this is partly due to saturation of Akt effector phosphorylation, but it remains unclear why saturation occurs. Saturation of the PI3K-Akt pathway has also been reported in other contexts—for instance, in response to high concentrations of certain growth factors [142]—suggesting that saturation is not Adv-specific. The simplest molecular explanation for saturation in Adv-infected cells is that Akt and its substrates are negatively regulated by different protein phosphatases. Akt forms complexes with protein phosphatase 2A (PP2A) [143] and a protein phosphatase 2C (PP2C) family member [144], which dephosphorylate the T308 and S473 sites on Akt. By contrast, the Akt substrate GSK-3β coassociates with protein phosphatase 1 (PP1) [145], as well as a PP1 regulatory subunit [146], which is a recognized substrate of GSK-3β [147]. Thus, PP2A and PP2C could allow Adv- and insulin-mediated activation of Akt, but PP1 may block additional phosphorylation of GSK-3β. Such Akt-independent control of GSK-3β and the closely related isoform GSK-3α could be particularly important for Adv-TNF synergy and apoptosis, because GSK-3β signaling is important for resistance to TNF-induced cell death [148].

Our results suggest several mechanisms by which Adv and TNF may synergize to induce apoptosis. One pathway we identified to be directly involved was via the stress kinase MK2. Among its substrates, MK2 directly phosphorylates small heat shock protein 27 (Hsp27), which reduces its ability to protect against oxidative stress [149]. Reactive oxygen has recently been implicated in TNF signaling [38, 83], which together is consistent with the pro-apoptotic role we observed for MK2. At the post-transcriptional level, MK2 contributes to the regulation of several cytokines, including TNF [80]. Adv-TNF activation of MK2 in vivo may start a positive feedback loop in cells of the innate immune system that augments cytokine production. Adv infection may also induce secretion of interferon-alpha (IFN-α) in host epithelial cells. Upregulation of IFN-α could partially inhibit protein synthesis within the cell [150], limiting NF-κB-mediated transcription of molecules, such as c-FLIP [151], that are activated in response to TNF and prevent apoptosis. Any attenuation of NF-κB signaling would need to occur downstream of IKK, because TNF-induced IKK activation was not decreased but increased after Adv infection.
One possible systems-level explanation for Adv sensitization is that IKK–NF-κB and PI3K-Akt collaborate via a common dose-response for survival. In this case, saturation of survival signaling by Adv-mediated upregulation of Akt may proportionally reduce the pro-survival contribution of IKK. NF-κB is a recognized determinant for survival in the presence of TNF [75-77], but we found that small molecule inhibition of IKK did not cause a pronounced increase in TNF-induced apoptosis in Adv-sensitized cells (Fig. 3-1B). These data support the hypothesis that Akt activation by E4 of Adv [62] could saturate total pro-survival signaling from both Akt and IKK-NF-κB. Akt and IKK have been implicated in a common signaling pathway under certain circumstances [152, 153] and apoptosis has been shown to be determined by combinations of multiple intracellular signaling pathways [89, 97]. Cooperation between Akt and IKK may therefore uncouple the contribution of IKK to TNF anti-apoptotic signaling.
CHAPTER 4

4. Testing a fundamental biological principle through data-driven modeling of Adv–TNF synergy

4.1. Introduction: cell-specific versus common processing

The fundamental components of many intracellular signaling pathways are common to all cells [85]. Intracellular networks arise from complex modular interactions between the receptors, adaptors, and enzymes that together relay signals [86] (Fig. 4-1a). Experimental [154] and literature-based [155] reconstructions of signaling networks often assume that there exists a common network that is qualitatively the same for all cells [88]. However, stimulating or perturbing the molecular network as a whole with mitogenic, pathogenic, or inflammatory stimuli often causes distinct phenotypes that are specific to cell type [36, 156]. For example, apoptosis [157] and chemokine release [158] are two cell-specific responses induced by the cytokine, tumor necrosis factor (TNF). Likewise, the cellular outcomes of virus infection depend upon the particular host tissue [159]. How are cell-specific responses achieved via a common signaling network?

Essential to answering this question are the downstream effector proteins (including cytoplasmic substrates, transcription factors, and terminal enzymes; Fig. 4-1a), which ultimately convert (or “process”) signals into cell-specific phenotypes. Cell specificity is thus an important obstacle to understanding how signaling networks control cellular behavior and to developing drugs that treat cellular dysfunction [160, 161].

In this Chapter, we use our Adv-TNF experimental system to explore the fundamental biological question of signaling cell specificity by demonstrating that a single PLSR model can predict Adv-induced apoptosis across cell types. In addition, we use principal component analysis to develop hypotheses about Adv modification of TNF-induced signaling and propose areas for further analysis.
4.2. Experimental and computational approach to test common effector-processing mechanism

4.2.1. Signaling inputs from multiple cell lines

We began by addressing the problem of cell-specificity in a defined experimental setup with clear outcomes that are cell-specific. Adenovirus (Ad) and adenoviral gene-therapy vectors (Adv) have been shown to synergize with TNF in many cellular contexts [100-102]. Adv uptake by cells of the immune system results in TNF release [20], and Adv binding and entry activates many signaling pathways shared with the TNF network. In epithelial cells, we have found that synergistic apoptosis caused by Adv–TNF is a result of Adv-induced changes in multiple key signaling pathways of the TNF network [162]. Interestingly, the extent of Adv–TNF synergy is specific to cell type: HeLa cervical carcinoma cells are much more sensitive to apoptosis than HT-29 colon adenocarcinoma cells when pre-infected with the same virus-to-cell ratio and stimulated with the same concentration of TNF [162] (Fig 4-2a).
One can envision two extreme strategies for mediating cell-specific outcomes to a single stimulus. The first possibility is that both signal activation and effector processing are cell specific (Fig. 4-1b). In this scenario, the same signaling event (for instance, the fold activation of a protein kinase) would contribute differently to a phenotype, depending on the particular cell type in which the signal is activated. A simpler alternative is that differences in signal activation are sufficient to confer cell-specific variations in phenotype (Fig. 4-1c). In this case, different cell types could use a common effector-processing mechanism to convert cell-specific signals into cell-specific responses. Typically, the details linking signals to effectors and responses are incomplete. Therefore, we set out to test these two hypotheses by measuring the intracellular network and then mathematically calculating the bulk molecular contribution of multiple critical signals to effector processing (Fig. 4-1b,c). We show that the resulting model strongly supports a role for common effector processing in cell specificity (Fig. 4-1c).

Our experiments focused on an Adv–TNF treatment combination in which the extent of HeLa apoptosis was 2.5-fold higher than that observed for HT-29 cells (Fig. 4-2a; see Methods for details). HeLa cell-specific sensitivity to apoptosis is a result of Adv–TNF synergy, because TNF-induced cell death was reduced five-fold in the absence of Adv pre-infection (Fig. 4-2b). We have previously shown that measurements of kinase catalytic activity are particularly useful signals for monitoring information flow.

![Fig. 4-2. Cell-specific apoptotic responses to Adv–TNF stimulation of HT-29 and HeLa cells](image-url)

(a) Apoptosis in HT-29 and HeLa cells following Adv infection at 100 m.o.i. and stimulation with 100 ng/ml TNF for 48 hours. Data are presented as the mean of three biological replicates ± S.E. (b) Apoptosis in HeLa cells uninfected or Adv-infected at 100 m.o.i. and stimulated with 100 ng/ml TNF for 24 hours.
Fig. 4-3. Cell-specific signaling responses to Adv infection and TNF stimulation of HT-29 and HeLa cells

TNF-activated kinase activity time courses in (a) HeLa cells uninfected (blue) or infected with 100 m.o.i. Adv (red) and (b) HT-29 (black) and HeLa (red) cells following Adv infection at 100 m.o.i. (HeLa data same as in (a)). Lysates were collected at 0, 5, 15, 30, and 60 minutes and 2, 4, 8, 12, 16, 20 and 24 hr and kinase activities were measured by a high throughput kinase activity assay[111]. Results are plotted as the mean relative activation of two biological replicates normalized to activity of the untreated, uninfected control at zero minutes.

through a network [47, 97, 111]. Consequently, for Adv–TNF treatment combinations, the dynamics of five protein kinase activities (ERK, Akt, JNK1, IKK, and MK2) were measured in both HT-29 and HeLa cells at 13 time points from 0–24 hr after TNF addition. Kinase activities were also measured in uninfected HeLa cells stimulated with TNF to confirm that Adv had perturbed these pathways en route to synergizing apoptosis. Two-way analysis of variance (ANOVA) revealed significant alterations in ERK, JNK1, IKK, and MK2 activity time courses between uninfected and Adv-infected HeLa cells (p < 0.05; Fig. 4-3a), consistent with the known crosstalk between Adv and TNF signaling pathways. Importantly, when the network activation of HT-29 cells and HeLa cells was compared, we observed complex differences in the dynamics of all pathways–ERK, Akt, IKK, JNK1, and MK2–between cell types (p < 0.05; Fig. 4-3b). Thus, Adv infection
perturbs the TNF-activated kinase network in epithelial cells [162] and the extent of this perturbation is cell specific.

We compared the new kinase-activity data with time courses previously measured [69, 162] in HT-29 cells stimulated with various combinations of Adv, interferon-γ (IFNγ, another TNF-sensitizing factor [163]), and TNF (Fig. 4-4a). The total dataset included seven treatment combinations of Adv, IFNγ, and TNF in HT-29 cells and two combinations in HeLa cells. All measurements were processed via internal normalization, rather than control normalization (see Section 2.4.1), consistent with the findings in Chapter that fold activation is more important than relative activation. One-way hierarchical clustering of these network time courses indicated that the two treatment combinations in HeLa cells paired more closely with HT-29 treatments than with each other. This suggested that the HeLa activity profiles, although different from HT-29 cells for the same stimulus, were still within the realm of network states achievable by HT-29 cells.

Because signaling dynamics between HeLa and HT-29 cells were comparable overall (Fig. 4-4a) but the apoptotic responses were clearly different, the two effector hypotheses could be directly tested. If the common-processing hypothesis were true, then a processing function that is deduced from HT-29 signaling and apoptosis data (Fig. 4-1c; f(x)) should accurately predict the conversion of HeLa signals into HeLa-cell-specific

![Colorgram of time-dependent kinase activities in HT-29 and HeLa](image)

**Fig. 4-4.** Colorgram of time-dependent kinase activities in HT-29 and HeLa HT-29 cells (black) and HeLa cells (red) treated with different combinations of Adv, IFNγ, and TNF [69, 162]. Data are presented as the mean of duplicate (Adv conditions) or triplicate (IFNγ conditions) biological samples relative to the zero-minute activity for each condition.
apoptosis. Failure of such a model would suggest that cell-specific processing was required (Fig. 4-1b).

4.2.2. PLS model construction and testing through prediction

To calculate the effector-processing function, we used partial least squares regression (PLSR), which has been shown to connect cytokine-induced apoptosis to measurements of the underlying intracellular signaling network (see Section 1.3.2) [97]. The PLSR model was constructed from the five dynamic kinase-activity profiles for each of the seven HT-29 treatment conditions shown in Fig. 4-4, including signaling metrics calculated from each time course (see Section 2.4.1). Using flow cytometry, we analyzed caspase substrate cleavage in HT-29 cells at 24 and 48 hours after TNF stimulation to provide two quantitative measures of the apoptotic phenotype that the kinase-network dynamics must fit. The apoptotic effector-processing function of the PLSR model was derived as a series of latent variables called principal components, which contain linear combinations of the original kinase activity measurements [97, 118]. Principal components are calculated iteratively according to the following general steps: 1) a loadings vector and a scores vector are calculated that describe the line of best fit through the data (primary variation in the data); 2) this product (the principal component representation of the data) is subtracted from full data matrix leaving a residual (or unexplained variation in the data); 3) the next principal component is regressed against the residual variation in the data [118]. Therefore each successive PLS dimension contains information not captured by the preceding component.

The root mean square error (RMSE) of apoptotic predictions, a measure of the difference between the predicted value and the observed experimental value, decreases monotonically for the calibrated model because all of the input treatments are included. After several iterations, however, the residuals become very small, and new dimensions capture only uninformative variations in the data, such as experimental noise and measurement error. Such information is undesirable for modeling true biological variation. It is therefore necessary to optimize the number of principal components. This
Fig. 4-5. Test of a common effector-processing hypothesis in epithelial cells treated with Adv and TNF. 
(a) RMSE of calibration and prediction of apoptotic outputs by the PLS model as a function of increasing 
number of principal components. An optimum model with three components was selected. (b) Correlation 
between measured apoptosis and predictions of the common-processing model. Model training with HT-29 
apoptosis (black) is compared to model predictions of HeLa apoptosis (red) at 24 hr (filled) and 48 hr 
(hollow). Data are presented as the mean of three biological replicates ± S.E., and model uncertainties were 
estimated by jack-knifing[89].

is done using a method known as cross-validation. In cross-validation, one observation is 
left out and the principal components are used to predict the excluded sample. The 
RMSE of prediction is then recalculated; however in this case it is optimized after a 
smaller number of components and increases when the “noisy” dimensions are included. 
When we applied this optimization method to our model, the RMSE was minimized with 
three principal components (Fig. 4-5a).

When the resulting model was then used to predict TNF-induced apoptosis in 
HeLa cells with and without Adv pre-infection, we found that the model predictions 
captured apoptosis measured directly in HeLa cells to within 99% accuracy (Fig. 4-5b). 
These predictions of HeLa apoptosis were as close to experimental observation as the 
calibrated fitting of the HT-29 data used to train the model. The remarkable accuracy of 
the HT-29 model for predicting HeLa-specific responses suggests that these two cell 
types share a common effector-processing mechanism that converts signal activation to 
apoptosis (Fig. 4-1c).
4.2.3. Biological insights through principal component analysis

The apoptotic effector-processing function of the PLSR model was derived as a series of latent variables called principal components, which contain linear combinations of the original kinase activity measurements [97, 118]. These principal components form a set of intracellular basis axes that define the dimensions of the signaling network that controls apoptosis. IFNγ + TNF treatments projected strongly along one of the model basis axes of the common-processing model predicting HT-29 and HeLa apoptosis (Fig. 4-4c). This axis corresponded to maximum TNF-sensitivity and included early JNK1 activity and late IKK activity (Table 4-1). The JNK1–IKK axis recapitulated the IFNγ + TNF basis axis of an earlier model of a TNF-growth factor network in HT-29 cells [97], indicating that this combination of cytokines is a potent network activator and a strong pro-death stimulus [163]. In addition, the common-processing model relied upon a second axis consisting of TNF treatments without any sensitizing agents. Biochemical

Table 4-1. Top 20 kinase metrics contributing to the JNK1–IKK axis of the common-processing model. See [97] for a complete description of the loading coefficients and signaling metrics.

<table>
<thead>
<tr>
<th>Loading coefficient</th>
<th>Kinase</th>
<th>Signaling metric</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.177</td>
<td>IKK</td>
<td>8 hr derivative</td>
</tr>
<tr>
<td>0.164</td>
<td>JNK1</td>
<td>4 hr derivative</td>
</tr>
<tr>
<td>0.160</td>
<td>JNK1</td>
<td>30 min time point</td>
</tr>
<tr>
<td>0.153</td>
<td>IKK</td>
<td>Area under the curve, peak #1</td>
</tr>
<tr>
<td>0.150</td>
<td>IKK</td>
<td>16 hr derivative</td>
</tr>
<tr>
<td>0.146</td>
<td>IKK</td>
<td>20 hr time point</td>
</tr>
<tr>
<td>0.141</td>
<td>JNK1</td>
<td>5 min derivative</td>
</tr>
<tr>
<td>0.139</td>
<td>IKK</td>
<td>24 hr time point</td>
</tr>
<tr>
<td>0.139</td>
<td>Akt</td>
<td>5 min derivative</td>
</tr>
<tr>
<td>0.137</td>
<td>JNK1</td>
<td>Activation slope, peak #1</td>
</tr>
<tr>
<td>0.136</td>
<td>JNK1</td>
<td>4 hr time point</td>
</tr>
<tr>
<td>0.136</td>
<td>ERK</td>
<td>0 min derivative</td>
</tr>
<tr>
<td>0.135</td>
<td>JNK</td>
<td>15 min time point</td>
</tr>
<tr>
<td>0.135</td>
<td>JNK1</td>
<td>Maximum</td>
</tr>
<tr>
<td>0.135</td>
<td>MK2</td>
<td>60 min derivative</td>
</tr>
<tr>
<td>0.132</td>
<td>JNK1</td>
<td>Mean</td>
</tr>
<tr>
<td>0.130</td>
<td>JNK1</td>
<td>Area under the curve, peak #1</td>
</tr>
<tr>
<td>0.130</td>
<td>JNK1</td>
<td>60 min derivative</td>
</tr>
<tr>
<td>0.130</td>
<td>IKK</td>
<td>Steady state</td>
</tr>
<tr>
<td>0.122</td>
<td>JNK1</td>
<td>8 hr time point</td>
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</table>
activities overrepresented in this basis axis, which appeared to capture TNF-resistance, including late Akt activity and late MK2 activity (Table 4-2). HT-29 projections along the Akt–MK2 axis grouped closely with uninfected HeLa cells treated with TNF, again illustrating a greater commonality between these two cell types than was indicated by their raw network measurements (Fig. 4-4) or their apoptotic responses (Fig. 4-2a). Interestingly, treatments involving Adv + TNF synergy did not require a third principal component (data not shown), but rather fell in between the JNK1–IKK and the Akt–MK2 axes. From these reduced signaling dimensions, we conclude that cell-specificity is a result of a stronger HeLa projection along the TNF-sensitivity axis, characterized by increased early JNK1 activity and late IKK activity, at a lower level of infection compared to HT-29 cells (Fig. 4-6). Given the recognized antiviral properties of certain IFN-family members [164], it is likely that the increased projection along the JNK1–IKK network dimension is directly related to antiviral responses that are HeLa-cell specific.

Table 4-2. Top 20 kinase metrics contributing to the Akt–MK2 axis of the common-processing model. See [97] for a complete description of the loading coefficients and signaling metrics.

<table>
<thead>
<tr>
<th>Loading coefficient</th>
<th>Kinase</th>
<th>Signaling metric</th>
</tr>
</thead>
<tbody>
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<td>0.137</td>
<td>MK2</td>
<td>24 hr time point</td>
</tr>
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<td>0.126</td>
<td>MK2</td>
<td>12 hr derivative</td>
</tr>
<tr>
<td>0.126</td>
<td>IKK</td>
<td>Activation slope, peak #2</td>
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<tr>
<td>0.120</td>
<td>MK2</td>
<td>20 hr time point</td>
</tr>
<tr>
<td>0.117</td>
<td>Akt</td>
<td>20 hr time point</td>
</tr>
<tr>
<td>0.113</td>
<td>MK2</td>
<td>0 min derivative</td>
</tr>
<tr>
<td>0.112</td>
<td>MK2</td>
<td>5 min time point</td>
</tr>
<tr>
<td>0.112</td>
<td>MK2</td>
<td>Area under the curve, peak #2</td>
</tr>
<tr>
<td>0.112</td>
<td>Akt</td>
<td>24 hr time point</td>
</tr>
<tr>
<td>0.111</td>
<td>ERK</td>
<td>2 hr derivative</td>
</tr>
<tr>
<td>0.108</td>
<td>Akt</td>
<td>16 hr derivative</td>
</tr>
<tr>
<td>0.105</td>
<td>IKK</td>
<td>Activation slope, peak #1</td>
</tr>
<tr>
<td>0.104</td>
<td>MK2</td>
<td>4 hr derivative</td>
</tr>
<tr>
<td>0.104</td>
<td>MK2</td>
<td>20 hr derivative</td>
</tr>
<tr>
<td>0.104</td>
<td>MK2</td>
<td>Steady state</td>
</tr>
<tr>
<td>0.104</td>
<td>Akt</td>
<td>60 min time point</td>
</tr>
<tr>
<td>0.104</td>
<td>Akt</td>
<td>Steady state</td>
</tr>
<tr>
<td>0.101</td>
<td>Akt</td>
<td>4 hr time point</td>
</tr>
<tr>
<td>0.099</td>
<td>Akt</td>
<td>Maximum</td>
</tr>
<tr>
<td>0.099</td>
<td>Akt</td>
<td>Activation slope, peak #3</td>
</tr>
</tbody>
</table>
As demonstrated in the cytokine compendium work, interleukin-1α (IL-1α) cooperates with TNF to activate network signals in HT-29 cells [69]. In addition, IL-1α is released in primary hepatocytes following treatment with Adv and TNF (B. Cosgrove, unpublished data). While early IKK activity is generally considered to be anti-apoptotic [38], IL-1α upregulates a late, sustained IKK activity peak that is pro-apoptotic. This time-dependent nature of IKK activity in the apoptotic response was resolved in the cytokine compendium PLS model [97]. Therefore, we examined IKK activity in our model for evidence of the IL-1α autocrine loop.

We confirmed that the same dual role of IKK is revealed in the common processing model, as expected given that this is a TNF-dependent autocrine loop (Fig. 4-7a). We then compared IKK activity profiles in IFN-sensitized and Adv-sensitized HT-29 cells with Adv-sensitized HeLa cells (Fig. 4-7b). While the fold activation of IKK in HT-29 cells infected with Adv was significantly lower than in the same cells pretreated with IFN, the dynamics of IKK activation were nearly identical; however, temporal activation of IKK in HeLa cells was different. While the second IKK activation peak began to rise at approximately the same time as in HT-29 cells, it fell back to baseline levels by 20 hours, suggesting that an inhibitor of IKK or IL-1α may be active in HeLa cells. The IL-1 receptor antibody (IL-1ra) is another TNF-induced autocrine factor.
Fig. 4-7. Common-processing dimensions differentiate apoptotic role for early and late IKK activity (a) Principal-component loading values for early IKK (filled black), late IKK (open black), and apoptosis (blue) are plotted. (b) IKK activity in HT-29+IFN (filled black), HT-29+Adv (open black) and HeLa+Adv (filled red) cells following 100 ng/ml TNF. Description of measurements same as in Fig. 4-3. (c) Potential role of IL-1 autocrine loop in Adv-infected cells. HT-29 cells and HeLa cells were treated with 100 ng/ml TNF and pre-treated with either IFN or Adv (red) and HT-29 (black) in the presence of IL-1ra. Data are presented as the mean of 3 biological replicates ± S.E.

operating in HT-29 cells [69]. It is possible that this factor is also induced in HeLa cells, perhaps more strongly that in HT-29 cells.

Finally, we measured apoptosis Adv+TNF-treated HT-29 and HeLa cells following treatment with exogenous IL-1ra and compared the results to those in IFN+TNF-treated HT-29 cells (Fig. 4-7c). While IL-1ra significantly reduces apoptosis in IFN+TNF-treated HT-29 cells, it had no measurable effect in Adv+TNF treated cells. However, this result could be consistent with a strong IL-1α loop in HeLa cells. It has recently been shown that IL-1 (α and β) is a primary mediator of the host inflammatory response in the liver following intravenous injection of Adv and that anti-IL-1 antibodies are an effective strategy for attenuating this response [165]. Therefore, further investigation of this autocrine loop, including direct measurement of IL-1α and IL-1ra with an enzyme-linked immuno-specific assay (ELISA), is recommended.

The overrepresentation of Akt on the axis corresponding to TNF resistance is at first not surprising given its pro-survival role [130]. However, the only recognized growth factor stimulus in our experimental system is Adv shedding of E4ORF1 transcripts, which we hypothesize are subsequently translated into proteins that activate the PI3K–Akt pathway [63]. In this case, however, Akt would correlate with an infected
cell, and therefore apoptotic, phenotype. Therefore, we looked more closely at the loading weights for temporal measurements of Akt activity (Fig. 4-8a). As is evident in the top 20 metrics corresponding to the Akt–MK2 TNR-resistance axis, late Akt activity, specifically at 20 and 24 hours, maps far from the cleaved caspase-cytokeratin apoptotic markers. We plotted the signaling time courses for uninfected HeLa cells and HT-29 cells infected with Adv at 100 m.o.i. and observed a similar temporal pattern of Akt activity (p = 0.44 by two-factor ANOVA) including a substantial rise in activity at 20 hours (Fig. 4-8b, left). Although Ad E4ORF1 is known to activate the PI3K–Akt pathway [62, 63], it was surprising to observe a rise in Akt in uninfected HeLa cells.

As mentioned above, HeLa cells are transformed by the human papilloma virus (HPV) oncoproteins E6 and E7 [123]. Recently, HPV E7 was shown to increase Akt activity indirectly in response to serum stimulation by interacting with the catalytic domains of PP2A and inhibiting its ability to desphosphorylate Akt [166]. However, this does not fully explain what gives rise to Akt activation in the first place. It has been known for some time that both Ad E4ORF1 and HPV E6 inhibit tumor suppressor protein

Fig. 4-8. HPV E6 in HeLa cells may cooperate with Ad E4ORF1 in mediating TNF response. (a) Principal-component loading values for early Akt (filled black), late Akt (open black), and cleaved caspase-cytokeratin (blue) are plotted. (b) Akt activity plots. Left: HT-29 cells infected with Adv at 100 m.o.i. (black) and uninfected HeLa cells (red) following 100 ng/ml TNF. Right: HT-29 cells infected with Adv at 1000 m.o.i. (black) and HeLa cells infected with Adv at 100 m.o.i. (red) following 100 ng/ml TNF. Description of measurements same as in Fig. 4-3. (c) Schematic hypothesizing a pro-survival role for HPV E6 and Ad E4ORF1 at low levels and a cytotoxic role upon increasing levels of E4ORF1.
function via interaction with their PDZ domains, which gives rise to the oncogenic potential of these viral proteins [167]. Although to our knowledge there is no evidence that HPV E6 has been linked directly to Akt activation, it is possible that a HPV E6 PDZ interactions cause an increase in Akt activity in HeLa cells similar to Ad E4ORF1-mediated Akt activation in HT-29 cells. This would explain the similar pattern of Akt activation.

Interestingly, when we compared temporal Akt signaling in Adv-sensitized HeLa cells and HT-29 cells (100 and 1000 m.o.i., respectively), Akt activity also rose and fell in a similar pattern (p = 0.90; Fig. 4-8b, right). Given our results on the saturation of Akt anti-apoptotic signaling in HT-29 cells infected at high levels of Adv, we considered that the combination of Ad E4ORF1 and HPV E6 might have a similar effect in HeLa cells. In fact, a recent study in epithelial tumor cell lines demonstrated that low levels of HPV E6 were protective against TNF-induced apoptosis, while high levels of HPV E6 sensitized cells to TNF-induced apoptosis [168]. Therefore, we hypothesize that HPV E6 and Ad E4ORF1 are functionally interchangeable in this regard and, while low levels of either protein protect against TNF-mediated apoptosis, the presence of more Ad E4ORF1 at higher levels of infection reduces the anti-apoptotic signaling of Akt and potentially leads to a cytopathic effect (Fig. 4-8c). We recommend testing this hypothesis in follow-up experiments.

4.3. Common processing model as a tool for designing rational drug therapies

4.3.1. Predictions of Akt inhibition test robustness of the common effector-processing model

One immediate application of a common-processing model is for predicting cell-specific responses to rational drug therapies [169, 170]. In particular, drug safety in vivo is often compromised by unintended toxicity to off-target cell types [171]. We therefore
examined whether the common processing model could predict apoptotic outcomes across cell types when network signals were perturbed by inhibitors. Frequently, the drug response for a target cell type is already known, and this information can be used to inform predictions for another cell type. In Adv-infected HT-29 cells, for example, inhibition of the PI3K–Akt pathway (a clinically-relevant cancer target [172]) dramatically increases TNF-induced cell death [162]. By contrast, the role of PI3K–Akt in TNF-induced apoptosis of Adv-infected HeLa cells is unknown.

To predict the effects of PI3K inhibition on HeLa cell viability, we first included prior information about drug sensitivity in HT-29 cells. The model was updated with measured values [162] of Akt inhibition and apoptosis in HT-29 cells treated with LY294002, a small molecule inhibitor of PI3K. We assumed that the potency of LY294002 was constant throughout the 24 hours following TNF addition and that no other kinases were affected during this time period (see Methods for details). HeLa signaling and apoptosis data were withheld (as in Fig. 4-5b) to avoid biasing the common-processing model toward HeLa-specific outcomes. Next, we experimentally measured the extent to which LY294002 reduced Akt activity in HeLa cells (Fig. 4-9a). Using the LY294002-inhibited Akt activity in combination with the corresponding HeLa network measurements (Fig. 4-9a), we then used the updated model to predict the response to inhibition of the PI3K–Akt pathway when HeLa cells were preinfected with Adv and treated with TNF.

Surprisingly, the updated model predicted no significant increase in cell death in Adv+TNF-treated HeLa cells following PI3K inhibition (Fig. 4-9b; bars). This was despite clear inhibition of the Akt pathway by LY294002 in HeLa cells (Fig. 4-9a) and explicit model training on LY294002 sensitivity in HT-29 cells (see above). When apoptosis was measured directly in LY294002-treated HeLa cells, we found only a nominal increase in TNF-induced cell death (Fig. 4-9b; circles), in close agreement with the predictions of the common-processing model. HeLa apoptosis was also unchanged in the presence of the mechanistically distinct PI3K inhibitor wortmannin. Thus, both model and experiment indicate that the role of PI3K in Adv+TNF-induced apoptosis is cell specific: HT-29 apoptosis is PI3K-sensitive [173], whereas HeLa apoptosis is PI3K-resistant (Fig. 4-9b).
Fig. 4-9. Common effector processing uniquely predicts resistance of Adv-infected HeLa cells to pharmacological inhibition of PI3K. 
(a) Akt activity measured in Adv-infected HT-29 cells and HeLa cells 12 hr after TNF stimulation in the presence and absence of 20 μM LY294002 (LY). Data are presented as the mean of six (HT-29) or three (HeLa) biological replicates ± S.E. (b) Apoptosis predicted by the common-processing model (bars) compared with that measured experimentally (circles) in Adv-infected, TNF-treated HeLa cells in the presence and absence of 20 μM LY294002 (LY). (c) Apoptosis predicted by a HeLa cell-specific processing model (bars) compared with that measured experimentally (circles) in Adv-infected, TNF-treated HeLa cells in the presence and absence of 20 μM LY294002 (LY). (d) One-kinase relationship linking Akt activity to apoptosis of HT-29 cells (black) treated with combinations of IFNγ, Adv, and TNF[162]. 90% confidence interval (tan) is shown. HeLa cells (red) were treated with 100 moi Adv and 100 ng/ml TNF. (e) Apoptosis predicted by the Akt-apoptosis model (bars) and measured experimentally (circles) in Adv-infected, TNF-treated HeLa cells in the presence and absence of 20 μM LY294002 (LY). (f) Apoptosis predicted by the common-processing model for HT-29–HeLa hybrid-cell profiles containing mixtures of HT-29 signals (black) and HeLa signals (red). Data are presented as the mean of three biological replicates ± S.E., and model uncertainties were estimated by jack-knifing[89].

The accurate LY294002 predictions in HeLa cells now provided the opportunity to compare the common model directly against competing mechanisms of effector processing. To mimic cell-specific processing (Fig. 4-1b), we trained a separate PLSR
processing function based only on the HeLa signaling measurements and tested if the existing HeLa data itself was superior for relating signals to apoptosis correctly. The HeLa-specific model was far less accurate and highly uncertain as compared to the common-processing model (Fig. 4-9c). This emphasized that HT-29-specific information, and thus common-effector processing, was absolutely essential for prediction.

A reductionist alternative to common processing of the network is that there exists one signal, which is a master regulator of TNF-Adv synergy across cell types. Previously, we developed a mathematical relationship describing the dependence of TNF-induced apoptosis in sensitized HT-29 cells on the relative extent of Akt activation [162]. Using the activation of Akt at a single time point, we found that this relationship predicted apoptosis in Adv-infected HeLa cells within a 90% confidence interval (Fig. 4-9d), raising the possibility that Akt signaling alone was sufficient to predict cell-specific apoptosis. However, when the Akt model was used to test the result of LY294002 inhibition, it incorrectly predicted a four-fold increase in Adv+TNF-induced apoptosis in HeLa cells (Fig. 4-9e), indicating the need for signaling information from multiple pathways. We demonstrated this requirement by constructing hybrid-cell profiles, in which HT-29 kinase measurements were individually substituted into the LY294002-inhibited HeLa dataset (see Methods for details). In the common-processing model, the HT-29-specific sensitivity to PI3K inhibition was captured by an ensemble of anti-apoptotic signals from ERK and IKK and pro-apoptotic signals from JNK1 and MK2 (Fig. 4-9f), in agreement with the recognized functions of these pathways (see Table 1-1). Among the competing mechanisms for achieving cell specificity, we therefore conclude that common effector processing is most consistent with experimental observations.

**4.3.2. Common effector-processing model predicts differential effects of IKK inhibition in HeLa cells and HT-29 cells**

The IKK–NF-κB pathway is another important therapeutic drug target for cancer as well as inflammatory diseases [174]. However, the value of pharmacological
inhibitors of IKK or NF-κB has been debated because the pathway operates very
differently across cell types [175]. We therefore used the common-processing model to
predict how small molecule inhibition of the IKK–NF-κB pathway would affect
apoptosis in HT-29 cells and HeLa cells. For the reversible IKK inhibitor SC-514 [176],
we simulated IKK inhibition as described for Akt (see Methods for details) and then had
the model predict apoptosis given the new SC-514-inhibited signaling inputs. No prior
information about IKK perturbation was used to update the model, because the response
of both HeLa and HT-29 cells to SC-514 was unknown.

The common-processing model predicted cell-specific differences in response to
Fig. 4-11. HeLa cells are more sensitive to IKK–NF-κB inhibition than HT-29 cells. Comparison of apoptosis in HT-29 cells (top) and HeLa cells (bottom) following IKK inhibition with 50 μM BAY 11-7082 (left) or NF-κB inhibition with 40 μM SN50 (right). Control cells were treated with 0.02% DMSO for BAY or the inactive SN50M peptide for SN50.

inhibition of the IKK–NF-κB pathway. In TNF-treated HT-29 cells, apoptosis was predicted to remain unchanged in both uninfected and Adv-infected cells (Fig. 4-10a, upper bars 1-2 and 3-4). In contrast, HeLa apoptosis was predicted to increase substantially in uninfected cells (Fig. 4-10a, lower bars 1-2) and to decrease slightly in Adv-infected cells (Fig. 4-10a, lower bars 3-4). When apoptosis was measured in the presence of 20 μM SC-514 experimentally, we found that IKK inhibition in HT-29 cells almost exactly mimicked the model predictions, with only a small absolute increase in apoptosis in HT-29 cells for both conditions (Fig. 4-10a, upper bars 5-6 and 7-8). By comparison, there was less agreement between model predictions and experiments in HeLa cells. Although the common-processing model captured the correct relative change in apoptosis for SC-514 inhibition of uninfected HeLa cells treated with TNF (3.0-fold increase predicted by model versus 4.3-fold increase measured experimentally; Fig. 4-10a, lower bars 5-6), the model predictions for Adv-infected HeLa cells were incorrect (Fig. 4-10a, lower bars 7-8). Apoptosis increased substantially after SC-514 inhibition, rather than decreasing as predicted. These experimental results were not unique to SC-
514, because we observed the exact same pattern of cell-specific apoptosis when using two other small-molecule inhibitors targeting different points of the IKK–NF-κB pathway (Fig. 4-11). Thus, the common-processing model had qualitatively predicted the existence of cell-specific responses to IKK–NF-κB pathway inhibition but did not quantitatively predict the extent of this specificity in Adv-infected HeLa cells treated with TNF.

The discrepancy between theory and experiment for the Adv-infected HeLa–SC-514 apoptosis prediction was not necessarily due to failure of common processing as a network mechanism for cell specificity. Rather, it was possible that the discrepancy reflected an inadequacy of the HT-29 data used to train the PLSR model. Adv-infected HeLa cells display a two-fold increase in IKK activity before four hours that falls outside the range of the HT-29 IKK activity data used for model training (Fig. 4-10b). Early IKK activity is generally considered to be anti-apoptotic [38], but because HT-29 cells do not activate IKK as strongly as HeLa cells, there may be a lack of experimental data relating early IKK activity to an anti-apoptotic phenotype. Without such data, the common-processing function would not interpret this HeLa-specific signaling variation properly, and thus would not accurately capture the change in HeLa apoptosis when IKK is inhibited with SC-514.

An important corollary to this explanation is that early IKK activity has an anti-apoptotic role in TNF-induced apoptosis in Adv-infected HeLa cells but not in Adv-infected HT-29 cells. To test for different roles of early IKK activity, we performed timed inhibition experiments with SC-514. Direct inhibition of early IKK signaling was not possible, because removal of SC-514 caused hyperactivation of IKK (Fig. 4-12), as reported previously [176]. To avoid possible misinterpretations stemming from IKK hyperactivation, we measured the role of early IKK activity indirectly by selectively inhibiting IKK from 4 to 24 hours and comparing the increase in apoptosis to that observed when IKK was inhibited from 0 to 24 hours (Fig. 4-6c). In HT-29 cells, IKK inhibition from 4 to 24 hours was the same as inhibiting IKK for the full 24 hours: both resulted in only a 15-20% relative increase in apoptosis, as seen earlier (Fig. 4-6a, upper bars 5-6). In contrast, nearly half of the SC-514-mediated increase in HeLa apoptosis could be attributed to early-phase inhibition of IKK (Fig. 4-6c). Therefore, a lack of
**Fig. 4-12.** SC-514 inhibition in vivo causes hyperactivation of IKK after removal. (a) Immunoprecipitates from HT-29 cells treated with 100 ng/ml TNF for 30 min were incubated with 100 μM SC-514 (SC) or 0.1% DMSO control for 15 min in vitro and then analyzed for IKK activity as described[111]. (b) HT-29 cells were preincubated with 100 μM SC-514 or 0.1% DMSO control for 1 hour in vivo and then stimulated with 100 ng/ml TNF for 15 minutes. Lysates were analyzed for IKK activity as described[111]. Washes during the IKK activity assay remove the SC-514 inhibitor and cause hyperactivation of IKK as reported previously[176]. Data are reported as the mean normalized activity ± range of 2-3 biological replicates.

input training data, rather than a failure of the effector-processing function, accounted for the inconsistency between model and experiment. However, this discrepancy allowed us to deduce the existence of an early IKK activity phase that was critical for anti-apoptotic signaling in Adv-infected HeLa cells and not present in HT-29 cells.

### 4.4. Further considerations

In metazoa, the need to achieve cell-specific responses with common network components can be considered analogous to the paradox of evolution, in that the need to change must be reconciled with the genetic conservation observed across species. A recent evolutionary theory proposes that certain “core processes” are conserved because they can easily be elaborated upon [179]. Although it is not yet clear whether common effector processing constitutes such a core process for multicellular organisms, our results do reveal an underlying similarity between disparate epithelial cell types. This similarity is uncovered only when signal processing is analyzed at the network level.
Reconstructions of cytokine-signaling networks have frequently observed a global hourglass topology of initiator signals (such as receptors and adaptors), transducer signals (such as enzymatic cascades), and effectors [180]. Many of the kinases measured here lie at the “waist” of these hourglasses, indicating a unique point of convergence in the network architecture. Our results suggest that initiator signals are highly cell-specific [86], whereas effector mechanisms are likely to be more widely conserved. Although there probably does not exist a common-processing function that captures the responses of all cell types, there may be common mechanisms for general cellular classes such as epithelial cells and hematopoietic cells.
CHAPTER 5

5. Conclusions and future directions

5.1. Contributions to signaling network analysis

Despite deletion of several immunogenic regions, Advs used in gene therapy still retain some of the host cell modulators found in the original adenoviral pathogen. The field is in need of new strategies for uncovering and addressing these problems at the molecular level. In epithelial cells, Adv infection is revealed at the phenotypic level by treatment with TNF. Intracellular Adv-TNF synergy was uncovered by dynamically measuring the TNF–Adv signaling network via five key protein kinases. By perturbing apoptotic response at high levels of adenoviral vector infection, we were able to demonstrate the saturation of Akt effector signaling underlying Adv–TNF synergy in HT-29 cells. Further systems analyses of the synergy between Adv and proinflammatory cytokines like TNF could aid the design of next-generation viral vectors for treating human disease [181].

In addition we have used our experimental system to uncover a fundamental biological principle, which we call common effector processing. By applying a data-driven modeling approach that converts important kinase signals into phenotype, we have identified a common effector-processing mechanism between tumor lines with cell-specific outcomes to the same stimulus. If the common-processing hypothesis had failed, it would have meant that each cell type would require its own in-depth experiments and model training. Our results raise the possibility that these types of models can be refined with new data to the point that they enable accurate predictions of phenotype that are broadly applicable. Already, the distinction between two carcinoma cell lines based on their apoptotic response to PI3K and IKK inhibition suggests immediate applications for combining pharmacological inhibitors [182] and cancer gene therapy [161] to treat certain tumors.
5.2. Application to the development of Adv-mediated cancer gene therapy

Adv-TNF synergy could be exploited to design Advs for cancer gene therapy, for instance by delivering a TNF-family cytokine as a transgene [183-186]. Many tumors are resistant to TNF-induced apoptosis [163], but presentation in the context of Adv infection can induce apoptosis in multiple carcinomas [183, 186]. The results presented here argue that the efficacy of these cancer gene therapy approaches is a direct product of Adv-TNF synergy. An important consideration for cancer therapies is the role of Akt in Adv-infected cells. Many tumors, especially brain, breast, and prostate cancer, have constitutive Akt activity due to a loss of function mutation in the PTEN phosphatase gene [187]. Our model suggests that, Adv-mediated gene transfer may serve as an even more potent pro-death stimulus in these tumors, because the anti-apoptotic function of the Akt pathway would already be saturated before infection. Subsequent cytokine stimulation would therefore activate only pro-apoptotic signals, leading to rapid apoptosis of the tumor cells. To our knowledge, there are as yet no examples of therapies that test this prediction.

In addition to delivering a death-inducing gene to a tumor via a viral vector, some cancer gene therapies are designed such that the virus itself is the oncolytic agent through selective replication in tumor cells [188]. This selective replication can be achieved with a tumor-specific promoter, as with CV706, an E3-deleted adenovirus with replication restricted to prostate tumors by inserting a prostate-specific antigen (PSA) promoter in front of the Ad E1A gene [189]. In other cases, selective replication is achieved by complementing a tumor mutation with a viral gene deletion, such that productive infection only results in tumor cells. This is the case for ONYX-015, a mutant adenovirus that lacks the E1B-55K protein and therefore cannot degrade p53 and carry-out a productive infection unless p53 is mutated, as is the case in many cancers [98, 190].

As discussed at length in this thesis, many Adv proteins have pro- and anti-apoptotic roles that could possibly be exploited to improve oncolytic therapies. However,
in some cases the wild type genes encoding these proteins must be removed to improve selective replication, which can have the unwanted result of an attenuated therapeutic response [191]. A network signaling study, comparing oncolytic adenoviruses systematically deleted for different genes, offers one approach to studying this problem. A cue–signal–response analysis could be pursued to identify signaling interactions that maximize apoptosis in cancer cell lines or interactions that minimize replication in primary cells. In addition to potentially revealing novel aspects of cancer biology, this approach may suggest effective combination Adv cancer gene therapy strategies.

5.3. Ongoing and future directions

5.3.1. Adenoviral gene therapy in the liver

Much effort has been put into developing adenoviral-mediated gene therapy for the liver, largely due to the natural hepatocyte-specific targeting of adenoviruses injected directly into the bloodstream [192]. However, the Adv-induced inflammatory response, mediated in part by TNF as discussed above, has been a major obstacle to the successful use of liver-directed Adv therapies [21, 22, 24]. Interestingly, a healthy liver has robust defense mechanisms with which it can exploit transient TNF-mediated stress signals to promote proliferation; however, a liver pre-exposed to certain toxins becomes susceptible to TNF-induced apoptosis [193]. Recently it has been demonstrated that infection with a first-generation Adv (the same vector used in these studies) renders primary hepatocytes sensitive to TNF-induced apoptosis (B. Cosgrove, unpublished data). With this experimental system and the approach demonstrated in this thesis, one can ask how Adv infection modulates cell signaling in the liver causing TNF to induce a stress response rather than a proliferative response. This work is being pursued currently in our laboratory.
5.3.2. NF-κB signaling in HIV latency

HIV latency is another viral–host interaction problem that would benefit from a systems biology analysis of the signaling network. The majority of HIV infection events in active T-cells result in rapid viral replication; however, a very small minority of infections can lead to a pool of latently infected memory T cell. Because the virus cannot directly infect memory T cells, it is unclear how this pool of cells develops. However, this reservoir is very long lived and represents the most significant barrier to complete elimination of virus from a patient. Infected patients must stay on costly anti-retroviral medications (which have significant side effects) for the remainder of their lives, because if they stop taking the medication, latent viral copies can reactivate and once again lead to full viremia.

Due to the extreme clinical importance of latency, there has been significant interest in understanding the molecular mechanisms that underlie its establishment [194-196]. It has recently been hypothesized that stochastic effects in viral gene expression can result in long delays before activation of viral gene expression [197], which could maintain the virus in a transcriptionally inactive state long enough for the host T-cell to make the slow conversion to a memory T-cell and thereby solidify the virus into a latent state. The IKK–NF-κB signal transduction pathway is known to be a crucial regulator of the HIV LTR promoter, and therefore it is important to understand how this signaling pathway affects stochastic gene expression of HIV genes, potentially contributing to latency.

One strategy for eliminating latent HIV is to purge the virus by chemically stimulating HIV LTR transcription, thus rendering such cells susceptible to antiretroviral agents [198]. In parallel, this approach may prevent the establishment of new latencies. The basal nuclear environment of NF-κB, which regulates transcriptional activation, is at least in part controlled by the upstream kinase IKK. We have demonstrated in this thesis and elsewhere that the information contained in kinase signaling dynamics can be used to predict cell function [97, 173]. It has also been demonstrated both computationally and experimentally that different inflammatory stimulants induce distinct IKK temporal profiles, which lead to different NF-κB dynamics and gene expression programs [199].
Thus, I have proposed a signaling network study of how chemical and pharmaceutical activators of IKK control NF-κB-mediated transcription dynamics in HIV latency as part of my post-doctoral work.
CHAPTER 6

6. Appendices

6.1. Materials

6.1.1. Adenoviral vectors

The recombinant adenovirus type 5 vectors with E1 and E3 regions deleted expressing either Escherichia coli β-galactosidase (β-gal) under control of the cytomegalovirus (CMV) enhancer/promoter (Adv.β-gal) or containing the CMV enhancer/promoter without a transgene (Adv.empty) were provided by the University of Michigan Vector Core. Virus was provided at a concentration of 4 x 10^{12} viral particles (v.p.) per milliliter and reported to have an infectious plaque-forming unit (p.f.u.) concentration of 1.7 x 10^{11} p.f.u./ml as determined by plaque assay. Working viral stocks were diluted in storage buffer (10 mM Tris-HCl pH 7.4, 137 mM NaCl, 5 mM KCl, 1 mM MgCl_2, 10% glycerol). Storage buffer was also used for controls.

6.2. Experimental Protocols

6.2.1. Cell culture and infection

HT-29 and HeLa cells (ATCC, Manassas, VA) were grown according to the manufacturer’s recommendations. Unless otherwise specified, HT-29 cells were seeded at 50,000 cells/cm^2, grown for 24 hours, and then infected for six hours in culture media (50% of normal media volume) containing 1.4 x 10^{10} v.p./ml Adv or an equivalent
volume of media containing storage buffer. At the end of the infection period, cells were washed once with PBS and a full volume of fresh culture media was replaced. Using these infection conditions, this was the lowest concentration tested that resulted in > 95% of the cell population positive for Adv infection (see Chapter 2 for details). A similar infection efficiency in HT-29 cells has been reported elsewhere [106]. Therefore, we used $1.4 \times 10^{10}$ v.p./ml for all experiments except dose response curves (for which concentrations are indicated in text). HeLa cells were seeded at 20,000 cells/cm² and infected as described for HT-29 cells at v.p. concentrations indicated in the text.

Helper-dependent adenoviral vector was provided by Anja Erdhart and Mark Kay at Stanford. Virus was supplied at a viral titer of $9 \times 10^9$ blue forming units (bfu) per ml in storage buffer. For experiments comparing Ad.dlE3.bgal and Ad.HD, Ad.dlE3.bgal working stock was diluted to $9 \times 10^9$ PFU/ml.

6.2.2. Beta-galactosidase Assays

Trypsinized cells were washed once in PBS and resuspended in 1x Reporter Lysis Buffer (Promega) for 15 minutes at room temperature. β-galactosidase activity was measured in a 96-well plate format according to manufacturers’ instructions. Total protein content of the sample was determined with a bicinchonic acid assay. Activity measurements were reported as milliunits β-galactosidase activity per μg protein.

6.2.3. Apoptosis assays

HT-29 or HeLa cells were seeded and infected as previously described. 24 hours after the start of infection, TNF (Peprotech; 100 ng/ml final concentration) or an equivalent volume of carrier (50:50 DMSO:water) was added to the culture media. For inhibitor studies, inhibitor or DMSO carrier was added to the medium one hour before TNF stimulation and collected 24 hours later. Inhibitors (Calbiochem) used were 20 μM LY294002, 10 μM U106, 10 μM SB202190, 10 μM SP600125, and 20 μM SC-514. For
growth factor studies, cells were treated with 100 nM insulin (Sigma), in parallel with
TNF treatment, and collected 24 hours after stimulation. At the indicated time, cells were
rinsed with PBS and trypsinized. The supernatant and rinse were saved and combined
with the trypsinized cells to ensure capture of both floating and adherent cells. The cells
were washed with PBS, and either all or a portion of the cells were fixed in 100%
methanol and stored at -20°C. The fixed cells were stained with a fluorescein-labeled
monoclonal antibody against caspase-cleaved cytokeratin (M30; Roche) and active
cleaved caspase-3 (BD Pharmingen) with Alexa 647-conjugated anti-rabbit secondary
(Molecular Probes) according to the manufacturer’s instructions. Cells were washed
once after staining and analyzed by flow cytometry (FACS-Calibur; Becton-Dickinson).
To perform parallel apoptosis assays, a portion of the original (unfixed) sample was
stained with an Alexa Fluor 488 annexin V conjugate (Molecular Probes), counterstained
with propidium iodide (PI; Molecular Probes) according to manufacturer’s instructions,
and analyzed by flow cytometry. In some cases, a portion of the original infected sample
was used for β-gal activity analysis to confirm a productive infection (data not shown).

6.2.4. Kinase activity assays

For kinase activity assays, HT-29 cells were grown and infected as previously
described. 24 hours after the start of infection, TNF was added to the culture media (100
ng/ml final concentration) for the indicated times. For Akt activity studies, 20 μM
LY294002 was added to the medium one hour before TNF stimulation and 100 nM
insulin was added in parallel with TNF stimulation, as indicated. Cells pretreated with
IFN were treated as previously described [111]. Cell lysates were prepared and analyzed
for ERK, Akt, IKK, JNK1 and MK2 activity as previously described [111]. Protein
concentrations of clarified extracts were determined with a bicinchonic acid assay
(Pierce) and all activity measurements were normalized to the total protein content of the
sample.
6.2.5. Quantitative polymerase chain reaction (PCR)

HT-29 cells were grown in 6-well plates and infected for the indicated times in either full media volume at a concentration of $0.7 \times 10^{10}$ v.p./ml or half volume at a concentration of $1.4 \times 10^{10}$ v.p./ml (all samples subject to the same overall MOI of 1000 p.f.u./cell). At the end of the infection time, cells were washed with PBS and trypsinized. DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genome quantification was performed using the ABIPrism 7700 (PE Biosystems) according to the method previously reported [200] with modifications. Taqman primers and probes were used to amplify the β-galactosidase reporter gene, and were designed using the Primer3 software package\(^2\) acquired from Applied Biosystems (PE Biosystems). The probe utilized a 3' TAMRA quencher molecule attached via a linker arm and a 5' covalently linked FAM fluorescent dye. The same Taqman primers and probe were used on known quantities of gWiz β-Gal plasmid standards (Aldevron) to calculate a quantitative viral copy number for each sample. Data was analyzed with ABI Sequence Detector v1.6.3 and total β-galactosidase DNA was normalized to total β-actin DNA for each sample using primers, probes and standards from the Human β-actin Gene kit (PE Biosystems). Results are presented as the average of total β-gal DNA normalized to total β-actin DNA.

For apoptosis measurements performed in parallel with quantitative PCR analysis infection media was aspirated at the indicated times, cells were washed once with PBS and fresh media was replaced. Cells were then treated with TNF, collected, and analyzed by flow cytometry as previously described.

6.2.6. RNA isolation and RT-PCR

HT-29 cells were grown in 12-well plates, infected and treated with TNF as described previously. At the time of collection, cells were trypsinized, transferred into an

\(^2\) http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi
Eppendorf tube and washed once with PBS. 1 ml of TRIzol® (Invitrogen) was added to the samples and vortexed until pellet was thoroughly lysed. The samples were then stored at -80°C until further processing. RNA was extracted using the RNeasy Tissue Kit (Qiagen) according to the manufacturer’s instructions, quantified by spectrophotometer, and stored at -80°C.

RNA samples were thawed on the day of cDNA preparation and approximately 200 ng of total RNA was used to prepare cDNA. Amplification grade DNAse I enzyme (1 unit activity/μg of RNA) and DNAse buffer (Invitrogen) were added to the samples. Samples were incubated at room temperature for 15 min, 1 μl of EDTA (25 mM; Invitrogen, MD) was added, and followed by denaturation of the enzyme at 65°C, for 10 min. Master mix solution was then added to the sample with final concentrations of 0.5 mM dNTPs (Qiagen, CA) 1mM random hexamers (Qiagen), 1x RT buffer (Qiagen), 2 units/μl Omniscript RT® (Qiagen), and 5 units/ml RNase (Ambion). The samples heated for one hour in a 37°C water bath for the conversion of the RNA to cDNA and then stored at -80°C.

Sequences for the RT-PCR E4orf3 primer were kindly provided by Matthew Weitzman’s laboratory (Salk institute). Primers were synthesized by Operon. 1 μl of cDNA was added to a mixture of 25 μl of SYBR Green Master-Mix (Qiagen), 21 μl of DEPC water (Qiagen), and 1.5 μl each of the forward and reverse primers. Amplification was carried out in an Opticon Monitor 2 system using standard SYBR Green RT-PCR annealing and melting protocols. Dilutions of disrupted Ad vector particles were used to generate a standard curve to convert cycle times to E4orf3 copy number.

### 6.2.7. Western blots

For determination of Akt substrate activity, 50 μg of protein lysate from 12 hour Akt activity experiment was resuspended in 40 μl sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM DTT, 0.01% bromophenol blue). Samples were boiled for 5 minutes, resolved on a 10% polyacrylamide gel and transferred to polyvinylidene difluoride (Biorad). Membranes were blocked with 5% nonfat skim milk
in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.1% Tween-20, and probed with anti-
phospho-GSK-3\alpha/\beta (1:1000; Cell Signaling) or anti-phospho-(Ser/Thr) Akt substrate
(1:1000; Cell Signaling). The membranes were then probed with horseradish peroxidase
conjugated anti-mouse or anti-rabbit secondary antibody (Amersham Pharmacia Biotech)
at 1:5000 dilution and visualized by enhanced chemiluminescence (Amersham Pharmacia
Biotech) on a Kodak Image Station (Perkin Elmer). Primary antibodies were removed
with stripping buffer (2% SDS, 62.5 mM Tris-HCl (pH 6.8), 100mM 2-mercaptoethanol).

6.2.8. PLSR model construction and refinement

Prior to all analyses, the signaling and apoptosis matrices were variance scaled to
non-dimensionalize the different measurements. The PLS model was constructed in the
SIMCA-P 10.0 (Umetrics) software suite according to the following iterative formulas:

\[ E_1 = X - t_1p_1^T; E_2 = E_1 - t_2p_2^T, t_2 = E_1w_1; E_i = E_{i-1} - t_ip_i^T, t_i = E_{i-1}w_i \]
\[ F_1 = Y - b_1t_1q_1^T; F_2 = F_1 - b_2t_2q_2^T; F_i = F_{i-1} - b_iq_i^T \]

where \( E_i \) represents the residual of the \( i \)th principal component, with score vector \( t_i \),
weight vector \( w_i \), and loading vector \( p_i \), and \( T \) represents transpose. \( F_i \) represents the
residuals of the \( i \)th dependent principal component, with score vector \( t_i \) and loading
vector \( q_i \), and \( b_i \) represents the coefficient characterizing the inner relation between the
independent and dependent principal components. Model predictions were made by
leave-one-out cross-validation for the IFN-Adv-TNF treatments and by unbiased
prediction for the HeLa predictions [47]. Model uncertainties were calculated by jack-
knifing [89]. Treatment projections (Fig. 4-4c) were plotted using \( t_1 \) and \( t_2 \) respectively
after a 50° subspace rotation [201]. Centered and scaled coefficients were used as the
regression weights.

To approximate the dynamic network behavior for a given treatment in the
presence of PI3K inhibition, the level of Akt was held constant at the LY294002-
inhibited activity level measured at 12 hours for HT-29 (0.17) and HeLa (0.33) compared
to a zero-minute baseline of one. Similarly, IKK activity levels were held constant at the SC-514-inhibited activity level measured in Supplementary Fig. S6 (0.52). With both inhibitor predictions, time-course values of the other kinases were kept identical to the network behavior measured in the absence of inhibitor, and metrics were extracted as described above. For HT-29–HeLa hybrid profiles, the measured HeLa kinase activities from Adv–TNF treatment were replaced with the corresponding kinase activities measured in HT-29 cells, and metrics were extracted as described above.

6.2.9. Statistical analysis

For comparing two individual means, a Student’s t test was used. For comparing differences in means on a Western blot across multiple blots, a paired Student’s t test was used. A Pearson’s correlation coefficient was calculated to assess linear correlation. For comparing two time courses, a two-factor analysis of variance (ANOVA) was used. The sigmoid function was defined as:

\[
y = \frac{base + max}{1 + \exp\left(\frac{x_{\text{half}} - x}{T_s}\right)}
\]

where \( y \) is percent viability, \( x \) is normalized Akt activity or level of phosphorylation, \( base \) is baseline percent viability, \( max \) is maximum increase in percent viability, \( x_{\text{half}} \) is the \( x \) value at which \( y \) is at \((base + max)/2\) and \( T_s \) is the transition steepness. (Note: Smaller \( T_s \) causes a faster rise). The sigmoid function was fit to data using Igor Pro Graphing software (WaveMetrics). \( Base \) and \( max \) values were held constant at 20% and 65%, respectively. The Levenberg-Marquardt non-linear, least-squares fitting algorithm was used to search for values of \( x_{\text{half}} \) and \( rate \) that minimize chi-square. 90% confidence interval for sigmoid curve fit was calculated using support plane analysis [202].
6.3. References


