Cell Shape and Treatment Duration: How They Influence a Cancer Cell's Response to TNF

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Cell shape and treatment duration:
how they influence a cancer cell's response to TNF

A dissertation presented
by
Xianfang Xia
to
The Division of Medical Sciences
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in the subject of
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Cell shape and treatment duration: how they influence a cancer cell's response to TNF

Abstract

The purpose of my research was to investigate the sources of heterogeneity in cellular decisions that are based on both external and internal cues. I used the signaling network induced by tumor necrosis factor (TNF) as a model system to examine how the duration of a stimulus and cell shape may affect signaling and cellular decisions. First, using a microfluidic device to achieve fine control of the ligand delivery to cells, my colleagues and I found that the duration of TNF stimulation is a factor that coordinates cell fate decisions in single cells. Specifically, we found that a few seconds of exposure to TNF is sufficient to activate the NF-κB pathway and induce apoptotic cell death and that, strikingly, a 1-min pulse of TNF can be more effective at killing cells than a 1-hour pulse. Second, to study the effects of cell shape, I used a two-pronged approach. Initially, I used live-cell imaging and single-molecule fluorescence in situ hybridization (smFISH) to examine whether descriptors of cell shape correlate with NF-κB nuclear translocation and NF-κB-dependent transcription in unperturbed populations of single cells. Next, I used surface micro-patterning to impose different geometry and degrees of spreading on cells and examine NF-κB-dependent transcription in these cells. I found that descriptors that quantify cell spreading, such as cell area and nuclear area, correlate with NF-κB nuclear translocation and NF-κB-dependent transcription. In addition, imposing bigger amount of spreading on cells increased the transcript abundance for two NF-κB-dependent genes, A20.
and IκBα. In contrast, the relationships between geometry-related cell shape descriptors and NF-κB-dependent transcription are more subtle and complex. Importantly, despite observing the correlations between cell spreading and NF-κB activity, I found that the effects of cell shape on NF-κB dynamics and on NF-κB-dependent transcription were small. Together, my investigations of TNF-induced signaling have shown that while the duration of treatment encodes information used in the TNF-induced cell death decision, NF-κB dynamics and NF-κB-dependent transcription are quite robust to changes in cell shape.
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(The following paragraph is the Chinese version of the last paragraph above.)

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Chapter 1 Introduction
Life is dynamic, even at its most basic level, that of single cells. Both the external and internal environments of individual cells are complex and constantly evolving. To survive in this constantly changing environment, cells must be able to detect multiple signals and compute a response and adapt. To integrate multiple signals in space and time, cells make use of cellular signal transduction networks, which are large, interconnected and highly dynamic networks, composed of interacting proteins and genes that coordinate cellular functions. Despite large amounts of data accumulated on signal transduction networks, our inability to predict individual cells’ exact responses to a given stimulus indicates that our understanding of the signal transduction networks is still incomplete.

Tumor Necrosis Factor (TNF) is a particularly interesting model system for studying signal transduction for two main reasons: first, because it is a ligand that activates multiple dynamic and interconnected downstream signaling pathways and second, because of the observed cell-to-cell variability in cellular response to TNF. Even clonal cancer cells treated with high TNF concentrations show variability: some cells die but others survive. In this thesis, using TNF as a model system, I will apply an array of single-cell approaches to exploit this cell-to-cell variability to gain a better understanding of TNF-induced cell decision processes.
1.1 The TNF signaling network - a tale of two complexes

TNF, a regulator of inflammation, is the prototypic member of a large family of cytokines, known as the TNF superfamily (reviewed in (Aggarwal, 2003)). At the organismal level, TNF has been implicated in the pathogenesis of a wide spectrum of diseases, including sepsis, diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, and cancer (reviewed in (Bradley, 2008)). Anti-TNF therapy has been approved for treatment of TNF-linked autoimmune diseases, such as rheumatoid arthritis (Bradley, 2008). At the cellular level, TNF induces a broad range of cellular responses, which is mirrored by the diverse set of signaling pathways activated by TNF (Wajant et al., 2003). Upon TNF binding to TNF Receptor 1 (TNFR1), two types of signaling complexes sequentially form (Micheau and Tschopp, 2003). Forming at the plasma membrane, the so-called Complex I is largely responsible for activation of the Nuclear Factor kappa B (NF-κB) pro-survival pathway (Figure 1.1). When TNFR1, with its associated Complex I, is eventually internalized by endocytosis, TNF-bound TNFR1 is degraded and the rest of Complex I is freed from the plasma membrane (Micheau and Tschopp, 2003; Watanabe et al., 1988). It is thought that the remaining members of Complex I serve as the platform for assembly of Complex II in the cytoplasm, with additional recruitment of the FADD adaptor protein and of initiator caspases, including caspase-8 (Micheau and Tschopp, 2003). Substantial accumulation of Complex II in the absence of inhibitory factors can lead to cell death through caspase-dependent apoptosis (Micheau and Tschopp, 2003). The wiring of the TNF signaling network suggests that the choice between TNF-induced cell fates, survival or apoptosis, may be partially dependent on the interaction between NF-κB-dependent pro-survival signaling and caspase-8 dependent pro-apoptotic signaling. There is growing evidence that these
two pathways are tightly connected and that both pathways are strongly connected to cell fate (reviewed in (Chen, 2002) and (Oberst and Green, 2011)). However, it is unknown what governs the strength of activation of these two opposing pathways when a cell is stimulated by TNF. Because Complex II only forms after endocytosis of Complex I, I hypothesize the duration of TNF treatment can determine the relative strength of activation of these two opposing pathways. In turn, this will affect the choice between TNF-induced cell fates, survival or apoptosis. The results from investigating this hypothesis are presented in Chapter 2.
Figure 1.1 Activation of TNF-Receptor 1 (TNFR1) via sequential formation of two signaling complexes.

Upon TNF binding to TNFR1, Complex I forms at the membrane and activates pro-survival signaling via NF-κB. When the receptor is internalized by endocytosis, Complex II forms and can initiate an apoptotic cascade via caspase-8. FLIP, induced by NF-κB, is a potent inhibitor of caspase-8.
1.2 TNF-induced NF-κB dynamics can determine NF-κB-dependent transcription

The NF-κB family of proteins is comprised of five structurally related transcription factors (reviewed in (Gilmore, 2006)). They all contain a highly conserved DNA binding and dimerization domain called the Rel Homology (RH) domain. NF-κB proteins must form dimers with one another, either homodimers or heterodimers, to bind to DNA. Some members of the NF-κB family contain a C-terminal transcription activation domain (RelA, RelB and c-Rel) while others do not (p50 and p52; Figure 1.2). Although p50 and p52 lack transcription activation domains (TADs), they can activate transcription through interaction with TAD-containing NF-κB subunits (Gilmore, 2006).
Figure 1.2 NF-κB subunit protein structures.

The Rel homology domain (RHD) is responsible for dimerization, nuclear translocation, DNA binding, and interaction with inhibitory IkB proteins. The transactivation domain (TAD) promotes transcription following NF-κB-DNA binding at target genes. The ankyrin domain (AD) repeat containing proteins p105 and p100 can be proteolytically processed to p50 and p52, respectively. Adapted from (Jost and Ruland, 2006).
TNF-induced Complex I signals through the so-called canonical NF-κB pathway, which is thought to be dependent on the dimer composed of RelA subunit and p50 subunit (Gilmore, 2006). The activity of RelA-p50 is primarily regulated by binding interaction with its inhibitory protein called IκBα. In unstimulated cells, RelA-p50 is primarily localized to the cytoplasm because it is actively shuttled out of the nucleus via the nuclear export sequence on IκBα. In response to Complex I signaling, IκBα is phosphorylated by the IKK complex (which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit IKKγ (NEMO)) and proteolytically degraded (Gilmore, 2006). This uncovers the nuclear localization signal sequence on the RelA-p50 dimer and allows it to accumulate in the nucleus and activate the transcription of target genes including, NFKBIA, the gene which encodes IκBα itself and TNFAIP3, the gene which encodes A20, another negative regulator of NF-κB activation (Tian et al., 2005). The re-synthesis of IκBα acts as negative feedback, promoting export of RelA-p50 to the cytoplasm and thus reducing RelA-dependent transcription (Sun et al., 1993).

Using fluorescent-protein-tagged RelA and time-lapse fluorescence imaging, several groups have now characterized the dynamics of the translocation of RelA between nucleus and cytoplasm upon stimulation of cells with TNF, or lipopolysaccharides (LPS), another stimulus that activates IKKα/β/γ and the canonical NF-κB pathway (Lee et al., 2009; Nelson et al., 2004). These single-cell measurements have shown that the sub-cellular distribution and activity of the RelA-p50 is dynamic – varying over time – and that these dynamics vary from cell to cell. For example, the temporal response of TNF-induced NF-κB nuclear activity was originally characterized to show markedly damped oscillations by observation at the population level using western blot and electrophoretic mobility shift assay (EMSA) (Hoffmann et al., 2002). Later, Nelson and colleagues, using single cells expressing fluorescent-protein-tagged RelA, showed
that the true dynamics in single cells are masked at the population level due to asynchronous oscillations following the first peak (Figure 1.3A) (Nelson et al., 2004). Single-cell data revealed that the subsequent oscillating peaks following the initial peak were actually of similar amplitude and the oscillations were sustained (Nelson et al., 2004).

Although the biological significance of this dynamic behavior is not fully understood, it raises the possibility that, RelA-p50 dynamics encode information to regulate transcription of some of its target genes (Ashall et al., 2009; Spiller et al., 2010). Indeed, our group recently observed that the maximum fold change of nuclear RelA (defined as the ratio of maximum to initial RelA fluorescence in the nucleus in TNF-stimulated cells) positively correlates with the transcript numbers of downstream early response target genes in single cells (Figure 1.3B) (Lee et al., 2014). In this study, the translocation of fluorescent RelA in single cells was recorded for 60 min post TNF stimulation, and single molecule fluorescent in situ hybridization (smFISH) was performed in the same cells to measure the amount of specific RelA-dependent transcripts. Interestingly, although there is a correlation of the maximum fold change of nuclear RelA with TNF concentration, there is much variability in the maximum fold change at each concentration. It is unclear what are the sources of this heterogeneity. Because cell shapes of cultured cancer cells vary from cell to cell, I hypothesize that variability in cell shape is one of the sources of heterogeneity in TNF-induced NF-κB dynamics. This hypothesis is explored in Chapter 3.
Figure 1.3 Single-cell data reveal NF-κB signaling regulatory mechanisms.

(A) Single-cell data showed that the strongly dampened NF-κB oscillations observed at the population level are due to loss of synchrony in oscillations following the first peak of translocation in individual cells. (B) By measuring NF-κB translocation and NF-κB-dependent transcription in the same single cells, it is clear that the Maximum Fold Change (MFC) in nuclear NF-κB – not its absolute abundance or concentration – more precisely determines transcriptional output in individual cells. MFC is defined as the ratio of maximum nuclear NF-κB intensity (F_{max}) to initial nuclear NF-κB intensity (F_i) in TNF-stimulated cells. Adapted from (Gaudet and Miller-Jensen, 2016).
1.3 TNF-induced apoptosis – extrinsic apoptosis initiated by caspase-8

In response to the activation of surface death receptor (such as TNFR1), Caspase-8, a cysteine protease, initiates a programmed cell death process known as extrinsic apoptosis (Muzio et al., 1996) and reviewed in (Elmore, 2007). The caspase-8 pro-enzyme (procaspase-8) remains monomeric and inactive until it is recruited by FADD to Complex II upon TNF stimulation (reviewed in (Wilson et al., 2009)). In Complex II, procaspase-8 is fully activated by induced dimerization followed by cleavage of its inter-domain linker (Wilson et al., 2009). There are two kinds of behavior in TNF-induced apoptosis (Aldridge et al., 2011; Barnhart et al., 2003). In Type I cells, the activation of effector caspases (procaspases-3/7) by caspase-8 is sufficient to trigger cell death. In Type II cells, activation of caspase-8 alone is insufficient to generate lethal levels of caspases-3/7. In this case, death requires a process called mitochondrial outer membrane permeabilization (MOMP; Figure 1.1), which serves both to activate the initiator caspase-9 and to antagonize the primary inhibitors of caspase-3/7 (Aldridge et al., 2011; Barnhart et al., 2003). MOMP is regulated by the Bcl-2 family of proteins and, in extrinsic apoptosis, is induced upon cleavage of Bcl-2 family member Bid to a truncated form (tBid) by caspase-8 (Tait and Green, 2010).

Quantitative analysis of pathways controlling extrinsic apoptosis in single cells has been performed in HeLa cells (type II cells) treated with TRAIL (TNF-related apoptosis-inducing ligand) (Albeck et al., 2008a; Hellwig et al., 2008), another member of the TNF superfamily (Aggarwal, 2003). TRAIL elicits a heterogeneous apoptotic response like the one seen with TNF (Albeck et al., 2008a; Hellwig et al., 2008). Albeck et al. used a FRET reporter of caspase-8 activity (IC-RP) and showed that, similar to TNF-induced RelA nuclear translocation, TRAIL-
induced caspase-8 activity time courses vary considerably from cell to cell. Yet, even though variable, caspase-8 activity was found to follow a stereotypical time course in these type II cells that commit to apoptosis (Figure 1.4) (Albeck et al., 2008a). For cells that underwent apoptosis, cleaved IC-RP accumulated sharply and the peaks of the time courses corresponded with apoptotic morphology before cell detachment, revealing the ‘time of death’. In cells that survived, fluctuations of the time courses were small.
Figure 1.4 Live-cell reporters for monitoring extrinsic cell death.

(A) Schematic diagram showing the design of a FRET-based live-cell reporter (IC-RP) for monitoring the activity of caspase-8. The reporter consists of CFP fused to YFP and linked by two IETD motifs, which are specifically recognized and cleaved by initiator caspase-8. (B) Time-lapse images of five IC-RP expressing HeLa cells treated continuously with 100 ng/mL TNF. (C) Single-cell time courses of caspase-8 activity are quantified by the CFP/YFP ratio. (D) A stereotypical single-cell time course of caspase-8 activity with the time of cell death marked.
1.4 How cells interpret temporal information when a stimulus is dynamic

Experiments designed to study cellular response to a stimulus often involve treating cells continuously for the duration of the experiments. However, the environment that surrounds cells *in vivo* can certainly fluctuate. Thus, a stimulus can be of short duration, or even pulsatile, as certain hormones are known to be (Goldbeter, 2007; Goldbeter et al., 2012). The frequency of periodic pulsatile stimuli and duration of each pulse can vary. For example, the gonadotropin-releasing hormone is released by the hypothalamus every hour in human whereas the growth hormone secreted by the hypothalamus every three to five hours (Goldbeter, 2007; Goldbeter et al., 2012). In some cases, the fluctuations can be part of a normal physiology, as for periodic hormone release. In other cases, the fluctuations can be caused by transient pathogens infections or sequential waves of cytokine release during inflammation (Han et al., 2012). In the latter scenarios, a question that remains unanswered is whether there is any information encoded in the duration of the inflammatory stimuli. If there is, how do cells interpret it and respond or adapt to the fluctuations in the environment?

Recently, several studies have looked at how time-varying stimulation with the same stimuli can lead to different cellular behaviors. For one, using fluorescent protein tagged RelA (FP-RelA), Ashall *et al.* recently observed various patterns of nuclear NF-κB oscillations when SK-N-AS neuroblastoma cells were exposed to several five-minute pulses of TNF at various intervals (Ashall *et al.*, 2009). Importantly, using RT-PCR, they showed the expressions of four NF-κB-dependent genes (IκBα, IκBε, MCP-1, RANTES) varied with different patterns of RelA oscillations. This suggests that the frequency of a pulsatile TNF stimulation can determine both the patterns of NF-κB dynamics and the NF-κB-dependent transcription profile in a population
of cells. More recently, Kellogg \textit{et al.} as well as Zambrano \textit{et al.} have shown that TNF pulses with certain periodic frequency could phase-lock and synchronize the NF-κB oscillations in single cells, thus reducing the cell-to-cell variability in NF-κB dynamics (Kellogg and Tay, 2015; Zambrano et al., 2016). Furthermore, Kellogg \textit{et al.} found that this synchronized NF-κB dynamics is also associated with enhanced NF-κB-dependent transcription (Kellogg and Tay, 2015). Zambrano \textit{et al.} found that while the expression of some NF-κB target genes also showed oscillatory patterns of expression, others, with long transcript half-life, did not track the nuclear NF-κB oscillations (Zambrano et al., 2016). Overall, these results suggest that the frequency of the TNF stimulation may reduce the noise in cellular response and enhance the output and also will affect the transcription profile of the responding cells.

In addition to the frequency of a time-varying stimulus, the duration of the stimulation may also contain information. Indeed, Kellogg, Tian \textit{et al.}, showed that a sustained and weak stimulation by LPS lead to delayed and variable NF-κB dynamics whereas, a transient, strong stimulus caused rapid and uniform NF-κB dynamics (Kellogg et al., 2015).

Despite these recent studies of the impact of oscillatory TNF stimulations, how the duration of a single TNF pulse treatment affects TNF-induced cellular responses has not been systematically explored. More specifically, how NF-κB dynamics, NF-κB-dependent transcription, and, especially, how apoptosis is affected by different durations of a TNF stimulation is unknown, and these are some of the questions that I will explore in Chapter 2.
1.5 How cells interpret information in cell shape

Since the earliest observations of cells under microscope, their shapes have been observed to be diverse. For example, adipocytes are round whereas hepatocytes are polygonal (Marieb, 2015). This observation has since inspired studies on the regulation and functional consequences of cell shape.

Cell shape, which herein I will define as the geometry of a cell and its degree of spreading, is regulated by both internal and external physical and chemical cues can affect the cell shape (reviewed in (Eyckmans et al., 2011; Paluch and Heisenberg, 2009)). Cell shape is the result of the mechanical balance of two countering forces exerted on the cell membrane; an outward force exerted by intra-cellular components and an inward force from the outside environment via interactions with neighboring cells or extracellular matrix (ECM) (Eyckmans et al., 2011; Paluch and Heisenberg, 2009).

While cellular process such as differentiation can change cell shape, changes in cell shape itself have been shown to play a role in cellular functions, such as survival and differentiation (Chen et al., 1997; McBeath et al., 2004). To examine the effects of cell shape on survival and apoptosis, Chen et al. used a micro-patterning technique to fabricate planar adhesive islands of defined size and geometry for attachment of a single cell per island (Chen et al., 1997). These adhesive islands were separated by non-adhesive regions. Therefore, in their system, the geometry and amount of spreading that the cells could adopt was finely controlled. In this study, Chen et al., observed a negative correlation between the amount of cell spreading and the apoptosis rates for human and bovine capillary endothelial cells in medium that contained saturating amounts of growth factors (Chen et al., 1997).
In addition to survival and apoptosis, cell shape has also been found to affect differentiation. When human mesenchymal stem cells (hMSCs) differentiate into adipocytes and osteoblasts, these cells adopt different cell shape, with adipocytes being round while osteocytes are elongated or cuboidal (Marieb, 2015; McBeath et al., 2004). In a study published in 2004, McBeath et al. set out to determine whether the reverse can be true: can changes in cell shape determine the differentiation? By seeding hMSCs on micro-patterns of different sizes, they found that differentiation into adipocytes occurred only on small islands (1024 μm²), differentiation into osteoblasts occurred only on larger island (10,000 μm²), and a mixture of both was found on intermediate-sized islands (2025 μm²), suggesting that the extent of cell spreading itself can affect differentiation (McBeath et al., 2004).

What are the molecular mechanisms responsible for the effect of cell shape on the survival and the differentiation? Dupont et al. elegantly identified the mechanosensitive transcriptional regulators YAP and TAZ to be the mediators of the effects of cell shape on the differentiation and survival (Dupont et al., 2011).

Although direct evidence is still lacking, it has been postulated that different cytoskeletal architectures (specified by the organization of filamentous actin, intermediate filaments and microtubules), which are associated with different cell shapes, can affect organelle distribution and the nuclear genomic structure and thus the accessibility of target promoters to their transcription factors (Eyckmans et al., 2011). Therefore, it is possible that cell shape can affect signaling pathway activity and its functional consequences, such as transcriptional output. In the context of the NF-κB pathway, a recent report by Sero et al. hinted that there were correlations between cell shape and TNF-induced NF-κB sub-cellular distribution in multiple breast cancer and primary cell lines (Sero et al., 2015). However, the strength of most of these effects was not
reported, and whether changes in cell shape were sufficient to affect NF-κB dynamic behavior and NF-κB-dependent transcription has not been examined. These are questions that I will investigate in Chapter 3.

Altogether, the problem of how cells integrate information from external and internal cues to respond to a given stimulus is complex and still an area of very active investigation in systems biology. This thesis discusses several external and internal cues that may affect the cellular responses to TNF. Particularly, in Chapter 2, I discuss our investigations on how cells respond to TNF stimulations of various durations. In Chapter 3, I examine the relationship between cell shape and NF-κB dynamics and NF-κB-dependent transcription. Together, this work represents examples of the study of how single cells decode information and of how they may adapt to changing external and internal environment.
Chapter 2 NF-κB signaling and cell fate decisions in response to a short pulse of tumor necrosis factor
Contributions:

This work was a collaborative project where I worked with Mohammad A. Qasaimeh, a visiting student from David Juncker’s laboratory at McGill University and Robin E.C. Lee, a postdoctoral fellow in the lab. My specific contribution to this work was designing, performing and analyzing the experiments measuring how duration of a TNF pulse influences the cell death response. Robin E.C. Lee and Suzanne Gaudet wrote the manuscript; Mohammad A. Qasaimeh, David Juncker and I edited the manuscript. This manuscript has been submitted for publication.

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The authors declare that there are no conflicts of interest.
2.1 Abstract

Tumor necrosis factor (TNF) is an inflammatory cytokine that can also induce apoptosis. In response to TNF, the canonical NF-κB pathway is activated, allowing the RelA transcription factor to accumulate in the nucleus and promote expression of pro-survival genes. In the same cell, caspases can also be activated to trigger apoptosis. While cells in the laboratory are often exposed to TNF for the duration of an experiment, in vivo exposure to TNF may be much shorter. To understand how opposing pro-survival and pro-apoptotic pathways respond to transient TNF exposure, we designed and built a microfluidic device, used it to deliver a short pulse of TNF to cells, and then monitored NF-κB and caspase activation by imaging. We find that a few seconds of exposure to TNF is sufficient to activate the NF-κB pathway and induce apoptotic cell death. Strikingly, a 1-min pulse of TNF can be more effective at killing than a 1-hour pulse, suggesting that in addition to TNF concentration, duration of exposure also coordinates cell fate decisions.
2.2 Introduction

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that modulates cellular decisions such as migration, proliferation, differentiation, and apoptosis (Aggarwal, 2003; Gaur and Aggarwal, 2003; Li and Schwartz, 2001; Waters et al., 2013). TNF was originally identified as an anti-tumor agent, although due to systemic toxicity, its therapeutic use is limited to local perfusion (Feinberg et al., 1988; Kramer et al., 2001; van Horssen et al., 2006). TNF-cancer connections are complex: despite some anti-tumor properties, TNF-associated inflammation is linked with progression of certain cancers (e.g. (Luo et al., 2004); reviewed in (Marx, 2004; Schottenfeld and Beebe-Dimmer, 2006)). Likewise, while TNF is important for normal immune cell functions (Aggarwal, 2003), chronically elevated TNF is associated with autoimmune diseases, many of which are treated with anti-TNF therapies (Croft et al., 2013).

The ability of TNF to regulate many cellular behaviors may stem from the fact that upon binding to a TNF receptor (TNFR1), TNF initiates a paradoxical network of pro-survival and pro-apoptotic intracellular signals. At the plasma membrane, TNF-bound receptors anchor the cytoplasmic components of “Complex I” to activate stress kinases and induce nuclear translocation of canonical NF-κB dimers (Micheau and Tschopp, 2003; Wajant et al., 2003). NF-κB then drives transcription of many inflammatory and anti-apoptotic genes (Tian et al., 2005). Cytoplasmic “Complex II” initiates the extrinsic apoptosis pathway by recruiting and activating initiator caspases. Although it is clearly established that internalization of TNFR1 is necessary for Complex II assembly and caspase activation (Micheau and Tschopp, 2003; Neumeyer et al., 2006; Schutze et al., 1999), little is known about the duration of TNF exposure that is required to induce apoptosis.
Laboratory experiments are commonly done with continuous TNF exposure yet *in vivo*, TNF exposure is likely transient. For example in rabbits, TNF released after lipopolysaccharide (LPS) treatment is rapidly cleared, and the half-life of TNF injected in the blood is ~6 min (Beutler et al., 1985). More recently, *in vitro* experiments have shown that NF-κB activation long outlasts treatment with a 1-, 2- or 5-min pulse of TNF and that, compared to continuous treatment, NF-κB activation is nearly indistinguishable in amplitude or timing when quantified by electromobility shift assays (Werner et al., 2008). Nevertheless, the NF-κB-driven transcription profile is sensitive to TNF treatment duration – a 5-min TNF pulse induces transcription of early, but not late, response genes (Ashall et al., 2009). The duration of TNF exposure required to activate NF-κB-driven transcription in single cells is not well-characterized experimentally, although a sub-minute LPS or TNF pulse induces NF-κB translocation in mouse fibroblasts (Kellogg et al., 2015), and it is unknown whether TNF exposure influences apoptosis.

Here, we set out to determine the TNF pulse duration required for NF-κB activation in single human cancer cells, and study how pulse duration affects TNF-induced apoptosis. We established a simple-to-use microfluidic system to expose cells to a short pulse of TNF. Using single-cell data, we quantified the threshold of NF-κB nuclear translocation required for inducing early gene transcription. We show that for high TNF concentrations, a 10-sec pulse was sufficient for NF-κB activation but a longer pulse is required at lower concentration. Extrinsic apoptosis was also strongly activated by a short TNF pulse. In HeLa cells, a 1-min TNF pulse induced apoptosis with a similar potency to 10-hr treatment whereas a 30- or 60-min pulse was less effective in cell killing. For Kym-1 cells, a 30-sec pulse of TNF induced as much death as continuous stimulation. Our study reveals that the duration of TNF exposure influences the TNF-induced cell fate decision.
2.3 Results

2.3.1 A brief TNF pulse induces nuclear localization of RelA

We designed and built a simple microfluidic system that uses laminar flow (Takayama et al., 2003) to provide spatiotemporal control over TNF delivery in cell cultures. Our device consists of two inlets, a three-chamber cell culture channel, and a single outlet (Figure 2.1A and Figure 2.2A). Reservoirs containing culture medium with or without TNF were attached to each inlet. Due to hydrostatic pressure differences between inlet reservoirs in the default operating mode, most of the channel was exposed to ‘Medium’ (Figure 2.1A, B, t = 0 sec). Cells exposed to the narrow laminar stream of ‘Medium+TNF’ that continuously flowed along one side of the channel were used as positive controls for each experiment. To expose the rest of the cells to a TNF pulse, the ‘Medium+TNF’ reservoir was temporarily raised to increase its hydrostatic pressure (Figure 2.1A, right). This reservoir also contained Alexa647-conjugated BSA allowing us to track the TNF-containing stream by imaging (Figure 2.1B). With this simple microfluidic system, we reproducibly achieved TNF pulses as short as 10 sec.
Figure 2.1 A microfluidic system to study cellular responses to short pulses of TNF.

(A) Schematic of the Y-junction microfluidic chip; the chambers are 2 × 2 mm² separated by a 1 mm wide connection. The microfluidic channels and chambers have a 40-μm depth. The flow is gravity-driven and in the default operating mode, before and after the pulse of TNF, the ‘Medium’ reservoir is positioned higher than ‘Medium + TNF’ (2 cm vs. 1 cm, left). The ‘Medium + TNF’ reservoir is manually raised to 8 cm for the duration of the pulse (right), and then lowered again. (B) Time-lapse epifluorescence images of the ‘Medium’ and ‘Medium + TNF’ laminar streams during a pulse of TNF; the ‘Medium + TNF’ stream was visualized using Alexa647-conjugated BSA (red). (C&D) Time-lapse images of EGFP-RelA HeLa cells treated with a 1-min pulse of Alexa647-BSA only, or a 30-sec pulse of Alexa647-BSA and 100 ng/mL TNF (left panels of C & D respectively). The time course of mean nuclear EGFP-RelA intensity (nFP-RelA intensity) for each cell was quantified from time-lapse images (right panels; n = 67 cells in C, n = 82 cells in D from one of four imaging positions from one representative experiment). The nEGFP-RelA time courses for two cells with different dynamics are highlighted in red and orange; a.u., arbitrary units.
To monitor TNF-induced NF-κB activation, we seeded the device with EGFP-RelA-expressing HeLa cells (Figure 2.2C) and quantified nuclear EGFP-RelA from time-lapse images as described previously (Lee et al., 2014). The calculated flow shear forces in the device were small, less than 0.2 Pa in any area that was imaged (Figure 2.2B), and therefore unlikely to stimulate the NF-κB pathway via mechanotransduction. Indeed, a mock 1-min pulse of medium without TNF had no observable effect on EGFP-RelA nuclear abundance (Figure 2.1C). In contrast, a 30-sec pulse of 100 ng/mL TNF resulted in transient and variable EGFP-RelA nuclear translocation in most cells (Figure 2.1D). Single-cell nuclear RelA time courses resembled those observed by us and others in response to continuous or 5-min TNF treatments (Ashall et al., 2009; Kellogg and Tay, 2015; Lee et al., 2014; Nelson et al., 2004; Sung et al., 2009; Tay et al., 2010a), experimentally demonstrating that a short TNF pulse can activate NF-κB and that its activation can be monitored in our device.
Figure 2.2 Microfluidic system to stimulate cells with a pulse of TNF under minimal shear stress.

(A) Image of the Y-junction microfluidic device, filled with a red dye. (B) Heat map of the simulated shear stress in a model of flow in the microfluidic system. Scale bar indicates the color correspondence for forces predicted to occur pre- and post-pulse (above) and during pulse (below). (C) Montage of tiled images showing the cell growth chambers and connecting channel. Images of the EGFP-RelA HeLa cells (green) and Alexa647-BSA (red) were overlaid.
2.3.2 Defining a transcription-inducing EGFP-RelA translocation

Cell-to-cell variability of nuclear EGFP-RelA following a 30-sec TNF pulse was substantial. Certain cells had large changes in nuclear EGFP-RelA abundance (Figure 2.1D, red trace) while others had high initial nuclear EGFP-RelA and only a small increase after TNF treatment (Figure 2.1D, orange trace). We set out to determine which cells were truly ‘responsive’ to the pulse of TNF, cells in which NF-κB activation should result in target gene transcription. We leveraged our published dataset of same-cell EGFP-RelA nuclear translocation time courses and target transcript numbers (Lee et al., 2014) to establish the EGFP-RelA nuclear translocation threshold under which cells are unlikely to induce NF-κB-dependent gene transcription in single cells. In that study, we determined that the maximal fold change of nuclear RelA (ratio of maximal to initial nuclear RelA in a cell) is predictive of transcript number for TNF-driven and RelA-dependent early response genes (Lee et al., 2014). Although those data are from cells treated continuously with TNF, we reasoned that if a given fold change is unlikely to induce transcription under continuous exposure, it is also unlikely to induce transcription after a short pulse. We therefore evaluated the error rate in determining whether or not a cell had a transcription-inducing EGFP-RelA translocation (‘responsive’ vs. ‘non-responsive’) while varying two parameters: fold-change threshold and cutoff value for transcript number.

We found a minimum of total error at ~1.22-fold-change for both IL8 and TNFAIP3, two NF-κB-inducible genes with no or few transcripts in unstimulated cells (Figure 2.3A and Figure 2.4; ~5% error). This fold-change threshold showed little dependence on the transcript number cutoff value, although values of 8 and 35 transcripts discriminated well between untreated and TNF-treated distributions for IL8 and TNFAIP3 respectively (Figure 2.3A, and Figure 2.4A,B).
Applying this threshold, the red-trace cell in Figure 2.1D (on page 25) was responsive to the 30-sec TNF pulse (max fold-change = 2.94), while the orange-trace cell was non-responsive (max fold-change = 1.06) despite having overall greater nuclear EGFP-RelA abundance. Altogether, our results suggest that the NF-κB system can sense and respond to an increase of nuclear RelA of as little as ~20%.
**Figure 2.3** The duration of TNF treatment required to elicit a transcriptionally significant NF-κB response is short but concentration-dependent.

(A) Total error in determining whether single cells underwent a transcription-inducing response to TNF based on same-cell EGFP-RelA translocation and transcripts number data for *TNFAIP3* (left) and *IL8* (right) (data from (Lee et al., 2014)). The error was evaluated for several mRNA number cutoffs used to distinguish TNF-responsive and non-responsive cells (shades of blue). For *TNFAIP3* and *IL8*, respective cutoff values of 35 and 8 mRNAs appropriately separated distributions of untreated cells from TNF-treated cells, (Figure 2.4) and therefore were used for final estimations of total error. The grey zone indicates fold-change levels that were deemed ‘non-responsive’. The data was collated from three independent experiments, with totals of n = 192 cells for *TNFAIP3* and n = 203 cells for *IL8*. (B&C) Single-cell nuclear EGFP-RelA (nEGFP-RelA) time courses after a 100 ng/mL (B) or 10 ng/mL (C) TNF pulse with indicated duration (n = 67 and n = 82 cells from one of four representative imaging frame in one experiment from at least two independent replicates). Gray zones represent the 1.22-fold cutoff above which cells were classified as having a transcription-inducing RelA translocation. Violin plots show the relative frequency distributions of nuclear EGFP-RelA fold-change in cells with transcription-inducing responses (blue, TNF pulse; red, continuous exposure) or sub-threshold translocations (gray). The median (thick black line) and upper and lower quartiles (thin black lines) are marked. P-values of a Kolmogorov-Smirnov test of the equality of each pair of distributions are reported above the graphs and percentages of cells classified as non-responders are shown in heatmaps below the plots. For each condition, data from at least two independent replicate experiments are combined, with between 69 and 389 total cells observed; violin plots were generated in MatLab from smoothened histograms (with normal kernel). (D) Time courses of the fraction of bound receptors in simulations of HeLa cells with continuous TNF treatment at the indicated concentration (1, 10 and 100 ng/mL TNF, light, medium and dark red respectively; left). Time courses of the fraction of bound receptors in simulations of HeLa cells exposed to a brief TNF pulse at 100 ng/mL (middle) or 10 ng/mL (right). Simulations were run for three different pulse durations (10, 30 and 60 sec, light, medium and dark blue respectively).
Figure 2.3 (Continued)

A Determining a transcription-inducing nuclear RelA fold-change threshold

B 100 ng/ml TNF data

C 10 ng/ml TNF data

D Simulations TNF-TNFR1 binding

Continuous treatment

Pulse treatment
Figure 2.4 Same-cell nuclear EGFP-RelA translocation and transcript number data allow classification of cells as TNF treatment ‘responders’ or ‘non-responders’.

(A&B) Histograms of the transcript number distributions in untreated (top) and TNF-treated (10 ng/mL; bottom) EGFP-RelA HeLa cells for *IL8* (A) and *TNFAIP3* (B). Data re-analysed from Lee et al. (2014) (Lee et al., 2014). The light gray zones indicate a threshold for the ‘baseline’ number of transcripts expected to be observed in untreated (or ‘non-responsive’) HeLa cells (< 8 for *IL8* and < 35 for *TNFAIP3*). Distributions were generated from data collected on \(n = 50\) to 156 cells from at least two independent experiments.
2.3.3 The duration of TNF exposure required for NF-κB activation is concentration-dependent

We next assessed EGFP-RelA nuclear translocation in response to different TNF pulse durations (Figure 2.3B,C). We found that only 22% and 14% of cells were non-responsive when exposed to 100 ng/mL TNF for 10-sec or 30-sec respectively, versus 6% for continuous treatment. Still, fold-change distributions for a 30-sec pulse or continuous treatment with 100 ng/ml TNF were not significantly different (Figure 2.3B). At 10 ng/ml TNF, cells required a longer pulse for transcription-inducing EGFP-RelA translocation; over 67% of cells were non-responsive after a 30-sec pulse (Figure 2.3C). This percentage dropped to 11% for a 60-sec pulse and to 2% for continuously treated cells and in this scenario, the nuclear EGFP-RelA fold-change distributions were still significantly different. A similar concentration-dependence was recently reported for the treatment duration required for LPS-induced NF-κB activation and PDGF-induced PDGFR phosphorylation (Kellogg et al., 2015; Ng et al., 2015).

In contrast to a short TNF pulse, distributions of nuclear EGFP-RelA fold change observed under continuous treatment with 10 ng/mL vs. 100 ng/mL TNF are similar (Figure 2.3B,C; p = 0.30, unpaired Kolmogorov-Smirnov test). However, others have reported that TNF concentration impacts both the fraction of cells responding as well as certain response parameters such as the lag before RelA nuclear entry (Tay et al., 2010a; Turner et al., 2010). Applying our threshold for transcription-inducing EGFP-RelA translocation, we found that when we reduced the TNF concentration to 1 or 0.1 ng/mL, fewer cells responded (Figure 2.5A). Moreover, the average nuclear EGFP-RelA fold change in responding cells decreased with TNF concentration (Figure 2.5). This suggests that for continuously exposed cells, TNF concentration
is reflected both in the percentage of responding cells and in the NF-κB pathway response amplitude although this response saturates by 10 ng/mL TNF.
Figure 2.5 Fraction of responding cells and their average nuclear FP-RelA fold change are TNF dose-dependent.

(A) Dose-response curve of the EGFP-RelA HeLa cells treated with TNF, as expressed as the percentage of ‘responder’ cells (with nEGFP-RelA fold change > 1.22) observed at each TNF concentration. (B) Bar graph of the average fold change in nuclear EGFP-RelA in ‘responder’ cells observed when treating EGFP-RelA Hela cells continuously with the indicated TNF concentrations. Error bars represent the standard error of the mean (S.E.M.). For both panels we re-analyzed a dataset originally published in (Lee et al., 2014); this dataset combined data from three to six independent experiments, resulting in between 78 and 198 total cells per condition.
To understand how ligand-receptor interactions vary with TNF concentration and pulse duration, we simulated TNF-TNFR1 reversible binding kinetics as a general bimolecular surface reaction (Lauffenburger and Linderman, 1993). Simulations using previously determined binding constants for $^{125}$I-labeled TNF with TNFR1 in HeLa cells (Grell et al., 1998) showed that receptors could be saturated within 30-60 sec with 100 ng/mL TNF, but only after 5-8 min with 10 ng/mL TNF (Figure 2.3D on page 30). Only ~50% of receptors may be bound after a 60-sec pulse at 10 ng/mL TNF (Figure 2.3D), explaining the observed concentration-dependence of the minimal TNF pulse duration for NF-κB activation. This result is not sensitive to receptor number and is robust to rather large variation in parameter values (Figure 2.6 and Figure 2.7). Notably, the dissociation of TNF from TNFR1 is slow, suggesting that complexes should persist long after a pulse (Figure 2.3D). Because internalization of TNF-bound receptors begins as early as several minutes after exposure to TNF (Micheau and Tschopp, 2003; Schneider-Brachert et al., 2004), our simulations predict that a short TNF pulse could be sufficient to activate later signaling events such as the assembly of pro-apoptotic Complex II.
Figure 2.6 The abundance of TNFR1 and TNFR2 shows little impact on simulated bound receptor fraction.

Plots show the simulated relationship between initial TNFR1 (top panel) or TNFR2 (bottom panel) concentration and maximal bound TNFR1, TNFR2 and total receptors (top, middle and bottom rows respectively) following TNF stimulation of the indicated durations. Receptor concentration was uniformly sampled in the exponent for values between 102 to 107 proteins per cell; all other parameters were set at their default value. Vertical bars represent the measured concentrations for TNFR1 (3,000 molecules/cell for HeLa and Kym-1) and TNFR2 (30,000 molecules/cell for Kym-1)(Grell et al., 1998). Maximal bound total receptors vary because as TNFR1 or TNFR2 abundance is varied, the ratio or TNFR1:TNFR2 varies.
Figure 2.6 (Continued)
Figure 2.7 Binding and unbinding rates of TNF to TNFR1 non-linearly impact the maximal fraction of TNFR1 bound.

Heatmaps of the maximum fraction (%) of TNFR1 bound to TNF in 2-hr simulations where $k_r$ and $k_f$ were varied linearly over the exponent. $k_f$ and $k_r$ are the association (1.833x10^7 M^{-1}s^{-1}) and dissociation rate (3.5x10^{-4} s^{-1}) constants for TNF-TNFR1. The fraction bound is also dependent on duration of the TNF pulse (rows) and TNF concentration (columns). Vertical black lines indicate the default $k_r$ value (Grell et al., 1998); horizontal black lines indicate the default $k_f$ value (Grell et al., 1998); dark red numbers are the values for fraction of TNFR1 bound at their intersection.
Figure 2.7 (Continued)
2.3.4  Apoptosis occurs in response to a short pulse of TNF

To monitor caspase activity in single TNF-treated cells, we imaged HeLa cells expressing a FRET-based initiator caspase reporter (IC-RP; (Albeck et al., 2008b)) and quantified IC-RP cleavage by the CFP/YFP ratio. Cells were pre-treated with interferon-γ (IFNγ) then TNF-treated (Figure 2.8A); IFNγ is another cytokine that sensitizes many cancer cell lines to TNF-induced apoptosis, partly via increased initiator caspase-8 expression (De Ambrosis et al., 2007; Fulda and Debatin, 2002; Langaas et al., 2001; Li et al., 2002; Ruiz-Ruiz et al., 2000). IC-RP cleavage accumulation varied cell-to-cell, likely, at least in part, because of natural variation in the abundance of apoptotic signaling molecules (Bhola and Simon, 2009; Flusberg et al., 2013; Gaudet et al., 2012; Spencer et al., 2009). For cells that underwent TNF-induced apoptosis, cleaved IC-RP accumulated sharply and the CFP/YFP peak corresponded with apoptotic morphology before cell detachment, revealing the ‘time of death’. In cells that survived, CFP/YFP fluctuations were small (Figure 2.8A). In continuous TNF treatment, we observed significantly more apoptosis at 100 ng/mL than at 10 ng/mL (Figure 2.9A) and therefore focused on 100 ng/mL TNF for our analysis of how extent and timing of cell death responses vary with pulse duration. Although the serum TNF concentrations are much lower than 100 ng/mL, because cytokine secretion is effectively captured by neighboring cells (Han et al., 2010; Lauffenburger et al., 1998), cells exposed to TNF in vivo, in a tissue or tumor, should experience a “puff” of TNF at local concentrations that far exceed those measured in blood.
Figure 2.8 A short TNF pulse induces caspase activity and cell death in HeLa and Kym-1 cells.

(A) Time-lapse images of five IC-RP expressing HeLa cells treated continuously with 100 ng/mL TNF (left). Single-cell time courses of caspase activity are quantified by the CFP/YFP ratio (right). (B) Single-cell time course of caspase activity for HeLa cells exposed to a 100 ng/mL TNF pulse of indicated duration (n = 67 to 93 cells in each condition, data from one of five independent replicate experiments). (C) Average percentages of HeLa cells that die within the indicated time period after a 100 ng/mL TNF pulse of indicated duration. Error bars designate the S.E.M. for five independent replicate experiments, with cell numbers between 50 and 375 in each condition for each experiment. P-values are reported indicating that percentages of total cell death are not significantly different between 1-min pulse and continuous treatment (p = 0.80; paired two-tailed t-test) and that the percentage of cell death after a 60-min pulse is significantly lower than during continuous treatment (p < 0.004; paired one-tailed t-test) but not significantly lower than after a 1-min pulse (p = 0.07; paired one-tailed t-test). (D) Time-lapse images of four Kym-1 cells treated continuously with 1 ng/mL TNF. Time of cell death was noted as the first frame in which the cell took on a rounded morphology with membrane blebbing (arrowheads). (E) Average percentages of Kym-1 cells that die within 10 hours of exposure to a pulse of indicated duration with 1 ng/mL TNF (left) or 100 ng/mL TNF (right). Error bars designate the S.E.M. for n = 3 (1 ng/ml) or n = 4 (100 ng/ml) independent replicate experiments (between 25 and 100 cells were tracked in each condition for each experiment). All of the 1 ng/mL pulse durations, except the 30-min pulse (p = 0.07), induce significantly less cell death than continuous treatment (p < 0.015; paired one-tailed t-tests). For 100 ng/mL TNF, all of the pulse treatments as much cell death as continuous treatment (p > 0.25 for all; paired two-tailed t-tests).
Figure 2.9 Apoptosis timing shows subtle but statistically significant differences when comparing a TNF pulse to continuous treatment in IFNγ-pre-treated cells.

(A) Bar graph of the percentages of apoptotic cells in IC-RP HeLa cells continuously treated with the indicated TNF concentration for 10 hours. Error bars represent the standard deviation from three biological replicates; p-value for an unpaired two-tailed t-test, n = 67 to 100 cells per replicate per condition. (B) Bar graph of the mean cell death time of IC-RP HeLa cells treated with 100 ng/mL TNF for the indicated duration. Error bars represent the standard error of the mean from n = 5 independent replicate experiments. P-value shows significantly shorter mean cell death time as determined by a paired one-tailed t-test.
Because TNF-induced apoptosis occurs over many hours, we increased experimental throughput by performing wash-out experiments in 96-well plates. We reproducibly achieved a pulse of 30-sec or longer and quantified dynamics of IC-RP cleavage after a TNF pulse ranging from 30-sec to 60-min and for cells treated continuously. Consistently, we observed that a 60-min pulse of TNF induced less cell death than 10-hr continuous TNF treatment (Figure 2.8B,C; p < 0.004, paired one-tailed t-test). By contrast, a 1-min pulse was at least as effective at killing cells as continuous TNF treatment (Figure 2.8B,C). Indeed, for the durations tested, cell death was maximal after a 1-min pulse (Figure 2.8C), although this difference did not reach statistical significance. This suggests that shortening exposure to high-concentration TNF does not necessarily decrease its pro-apoptotic effect. In addition, cells that died in response to a pulse of up to 30 min of TNF died earlier on average than continuously treated cells (Figure 2.8C and Figure 2.9B). TNF-induced signaling pathways may therefore be distinctly coordinated in at least a subpopulation of cells exposed to a TNF pulse. We found subtle, but statistically significant differences in EGFP-RelA dynamics and endogenous RelA distributions for IFNγ-pre-treated cells then treated with a 1-min TNF pulse versus cells exposed to TNF continuously (Figure 2.10). Nevertheless, additional distinctions likely also lie elsewhere in the TNF-induced signaling network.
Figure 2.10 RelA translocation shows subtle but statistically significant differences when comparing a 1-min TNF pulse to continuous treatment in IFNγ-pre-treated cells.

(A) Violin plots of the relative frequency distributions of maximum fold change in nuclear EGFP-RelA (nEGFP-RelA) in EGFP-RelA treated with 100 ng/mL TNF for the indicated duration and imaged for 60 min by live-cell microscopy. Above the 1.22 fold change threshold (blue line) cells were classified as ‘responders’ (blue), and below as ‘non-responders’ (gray). The median (thick black line) and upper and lower quartiles (thin black lines) are marked. P-value of a Kolmogorov-Smirnov test of the equality of the distributions is reported. Data from two independent experiments were combined, n = 158 cells for 1-min pulse and n = 99 cells for continuous and violin plots were generated in MatLab from smoothened histograms with normal kernel. (B) Bar graphs of the fold change of the mean nuclear RelA (mean nRelA at t = 60 min divided by mean nRelA at t = 0 min) observed by live-cell imaging in EFGP-RelA HeLa (left) and by immunofluorescence using a mouse monoclonal antibody against RelA (Santa Cruz Biotechnologies) in IC-RP HeLa (right). Error bars represent the 95% confidence interval determined by bootstrapping; n = 84 and 48 cells for replicate 1, n = 74 and 51 cells for replicate 2 for EGFP-RelA HeLa and n = 4996 cells (t = 0 min), n = 2161 cells (t = 60 min, 1-min pulse) and n = 1183 cells (t = 60 min, continuous treatment) for IC-RP HeLa.
Our simulations of TNF-TNFR1 binding suggest that at low TNF concentrations much longer exposures might be required to achieve binding of TNF to a large fraction of TNFR1 (Figure 2.3D on page 30). Therefore, we sought to test whether a cell line sensitive to low TNF concentrations, the Kym-1 human rhabdomyosarcoma cell line, would exhibit duration-dependent cell death at a low TNF concentration. Indeed, we found that at 1 ng/ml TNF, the fraction of Kym-1 cells that die increased with exposure duration (Figure 2.8D,E on page 42). At 100 ng/ml TNF, a 30-sec exposure to TNF induced as much cell death as continuous treatment (Figure 2.8E). As for the timing of cell death, our data suggests that a short pulse of high-concentration TNF induced cells to die significantly earlier than continuous treatment (Figure 2.11). Kym-1 cells may be more sensitive to TNF because they express both TNFR1 and TNFR2 (3,000 TNFR1s vs 30,000 TNFR2s; (Grell et al., 1998)). Even if only TNFR1 induces extrinsic apoptosis, more abundant TNFR2s still capture TNF (albeit with lower affinity (Grell et al., 1998)) and, after a low-concentration TNF pulse, ligand released from TNFR2 may re-bind to TNFR1, leading to apoptosis via slow accumulation of internalized TNF-TNFR1 in certain Kym-1 cells. In contrast, for a high-concentration TNF pulse, a large fraction of TNFR1 can be bound by TNF simultaneously (Figure 2.3D on page 30) and cell death is efficiently induced (Figure 2.8E on page 42).
Figure 2.11 Cell death occurs on average earlier in Kym-1 treated with a short TNF pulse than in those treated continuously.

(A & B) Violin plots of the relative frequency distributions of cell death times for Kym-1 cells treated with 100 ng/mL (A) or 1 ng/mL (B) TNF for the indicated duration. The median (red line) and upper and lower quartiles (black lines) are marked and average percentages of dead cells at t = 10 hr are indicated in heat maps below the graphs. To obtain median and quartile values, surviving cells were assigned a death time of 25 hr and therefore lower quartile, median and upper quartile are indicated only if there is more than 25%, 50% or 75% cell death, respectively. Kolmogorov-Smirnov tests of the equality of the distributions show that cell death time distributions are different from that obtained with continuous treatment at the same concentration, except for the 30-sec pulse treatment. Data from four (100 ng/mL TNF; A) or three (1 ng/mL TNF; B) independent experiments were combined for a total of between 131 and 278 cells per condition; violin plots were generated in MatLab from histograms without smoothing, bin size of 7.5 min. (C) Bar graph showing the percentages of cell death at t = 2 hr for Kym-1 cells treated with 100 ng/mL TNF for the indicated duration. One-tailed t-tests show that only a pulse of 1 min, 5 min or 10 min results in significantly more cell death within the first 2 hours after TNF treatment than continuous treatment (nonsignificant results not marked).
Figure 2.11 (Continued)

A 100 ng/mL TNF

B 1 ng/mL TNF

C 100 ng/mL TNF
2.4 Discussion

Based on the current understanding of TNF-induced signaling, cell-to-cell differences in the formation of Complex I and Complex II should alter the relative strength of pro-survival and pro-apoptotic signals. TNF dissociates slowly from TNFR1 (Grell et al., 1998 and Figure 2.3D on page 30), over a timescale longer than that of internalization of TNF-bound receptor complexes (Neumeyer et al., 2006; Schneider-Brachert et al., 2004; Schutze et al., 1999). Considering this slow dissociation, two aspects of our results are surprising: 1) that cell death timing differed following a short pulse vs. continuous treatment and 2) that fewer HeLa cells died after a 60-min pulse than after a shorter pulse or continuous treatment. However, the TNF-induced cell fate decision may integrate other pathways downstream of Complex I and Complex II. Specifically, TNF-induced autocrine signals are known to contribute to the fate decision (Janes et al., 2006) and these secreted pro-survival and pro-apoptotic factors are likely diluted during flow and wash-out experiments. Analogously, the flow of blood and interstitial fluids could influence the extent and timing of TNF-induced cell death in vivo.

Overall, our results show that treatment duration is an important mediator of the TNF-induced cell death decision and that in certain contexts reaching the highest fractional kill may not require maximizing the duration of exposure to a pro-death stimulus. These findings meet a growing body of work showing that signaling dynamics as well as the timing and sequence of drug addiction can all influence cell fate decisions (Albeck et al., 2013; Fulda and Debatin, 2002; Kubota et al., 2012; Lee et al., 2012). It will be interesting in the future to examine the effect of exposure duration, and the interplay with ligand-receptor-affinity, in other cellular signaling networks.
2.5 Materials and methods

2.5.1 Cell culture and treatment of cells with TNF

HeLa cells (ATCC) stably expressing EGFP-RelA (described in (Lee et al., 2014)) and HeLa stably expressing IC-RP (described in (Albeck et al., 2008a)) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2 mM L-glutamine (Invitrogen) at 37°C and 5% CO₂. Kym-1 cells (Sekiguchi et al., 1985) were cultured in RPMI1640 supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2 mM L-glutamine (Invitrogen) at 37°C and 5% CO₂. For experiments in microfluidic devices, TNF treatments are described below. For experiments in 96-well imaging plates (BD Biosciences), on day 1, HeLa cells were seeded at ~4000 cells/well; Kym-1 cells were seeded at ~6000 cells/well. On day 2, culture medium was replaced with medium with 200 U/mL IFNγ (Roche). On day 3, two hours prior to TNF treatment, culture medium was replaced (with IFNγ) and 24 hrs after IFNγ treatment, complete medium with or without TNF was spiked into wells to yield the indicated final TNF concentrations. After the specified duration, TNF-containing medium was removed; cells were rapidly washed three times and then incubated in the appropriate medium without TNF for the duration of the experiment. Care was taken to use only conditioned and warmed medium for all the washes and media changes during the experiment, to minimize disturbances to the cells.
2.5.2 Device fabrication and operation

Using conventional soft-lithography techniques (Duffy et al., 1998), microfluidic chips with two inlets and three chambers for cell culture (illustrated in Figure 2.1A on page 25) were made in PDMS (Sylgard 184, Dow Corning Corporation) and bonded to a No. 1.5 glass coverslip (ThermoScientific). Pipet tips (200 μL) were inserted into 1/16” tygon tubing connected to steel tubes (16-gauge) into punched inlets and outlets to act as fluid reservoirs.

To prepare for experiments, devices were sterilized with 70% ethanol then abundantly flushed with sterile PBS. Channel surfaces were pacified by flushing the devices with complete culture medium and incubating >12 hrs. Cells were then seeded at appropriate density and allowed to adhere for 12 hrs under no-flow conditions (all reservoirs at equal height). Medium was replaced then and again 24 hrs and 4 hrs before an experiment.

For treatment with a pulse of TNF, medium with TNF at the final desired concentration was prepared with 1μg/ml Alexa Fluor® 647 conjugated-BSA (Life Technologies). The device was securely mounted on a custom stage for a BD Pathway 855 BioImager and flow allowed to reach a steady state in the pre-pulse mode (Figure 2.1A) while monitoring by imaging the Alexa-647-BSA epifluorescence signal. To pulse cells with TNF, the ‘medium + TNF’ reservoir was temporarily raised manually (Figure 2.1A). Pulse duration was verified by imaging the Alexa-647-BSA epifluorescence signal.
2.5.3 Live-cell imaging and analysis

For all live-cell experiments, HeLa cells stably expressing EGFP-RelA or IC-RP were imaged for 30 min before addition of TNF. Wide-field epifluorescence and transmitted light live-cell imaging was done in an environmentally controlled chamber (37°C, 5% CO₂) on the BD Pathway 855 BioImager using a UAPO/340 20x objective (0.75 NA; Olympus), capturing images at 3 min intervals for EGFP-RelA HeLa and at 10 min intervals for IC-RP HeLa. Data was extracted from flat-field and background corrected time-lapse images using ImageJ. Mean fluorescence intensity (MFI) of nuclear EGFP-RelA was collected for each cell at each time point using custom scripts. For caspase activity measurements, the ratios of MFI in the CFP and YFP channels were calculated. For Kym-1 cells, the time of cell death was determined by finding for each cell the first frame where membrane blebbing, a hallmark of apoptosis, was evident. For all experiments, cells that overlapped, left the imaging field or divided within an hour of TNF treatment were excluded from analysis.

2.5.4 Simulations of liquid flow in the devices

Three-dimensional simulations were carried out using COMSOL Multiphysics 3.5 (COMSOL, Inc., USA), modeling the actual shape and dimensions of the device. Simulations solved Navier–Stokes equations with the different boundaries conditions and the following assumptions: 1) fluids similar to water (incompressible Newtonian fluid with a density of 998.2 kg·m⁻³, and a dynamic viscosity of 0.001 N·s·m⁻²), 2) no-slip boundary conditions on the channel walls and 3) steady-state conditions were reached. The hydrostatic pressures at each inlet were calculated from on its height relative that of the outlet reservoir.
2.5.5 Model of bimolecular surface reaction

Binding of TNF to TNFR1 and TNFR2 was modeled as independent reversible receptor-ligand interactions with constant ligand concentration (Lauffenburger and Linderman, 1993):

\[
\frac{\partial C_1}{\partial t} = k_{f1} L_0 (R1 - C_1) - k_{r1} C_1
\]

\[
\frac{\partial C_2}{\partial t} = k_{f2} L_0 (R2 - C_2) - k_{r2} C_2
\]

\( C_1 \) and \( C_2 \) the concentration of TNF-bound TNFR1 and TNFR2, respectively. \( L_0 \) is the concentration of TNF. \( R1 \) and \( R2 \) are the total concentration of receptors (assuming 3,000 TNFR1 molecules per cell for HeLa and Kym-1, and 30,000 TNFR2 molecules per cell for Kym-1). \( k_{f1} \) and \( k_{r1} \) are the association \((1.833 \times 10^7 \text{ M}^{-1}\text{s}^{-1})\) and dissociation rate \((3.5 \times 10^{-4} \text{s}^{-1})\) constants for TNF-TNFR1. \( k_{f2} \) and \( k_{r2} \) are the association \((2.5 \times 10^7 \text{ M}^{-1}\text{s}^{-1})\) and dissociation rate \((0.011 \text{s}^{-1})\) constants for TNF-TNFR2 (as reported in a previous study (Grell et al., 1998)). We surmise that constant ligand concentration is an appropriate approximation as with a very high medium:cell volume ratio, the number of ligand molecules vastly surpasses that of receptors. Simulations were carried out in MatLab (MathWorks) using the ode45s solver.
Chapter 3 NF-κB-dependent transcription exhibits robustness to changes in cell shape
Contributions:

For this project, I benefitted from the assistance of Hunter L. Elliott of the Harvard Image and Data Analysis Core for setting up image analysis pipelines for smFISH data as well as from help from Joe N. Paulson, a postdoctoral researcher in the Center for Computational Cancer Genomics at the Dana-Farber Cancer Institute, for some of the statistical analyses.

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The authors declare that there are no conflicts of interest.
3.1 Abstract

Cell shape and the cellular microenvironment were recently reported to correlate with the subcellular distribution of NF-κB when measured at single time points, pre-treatment and one hour post-TNF treatment. However, little is known of how cell shape affects NF-κB dynamics and NF-κB-dependent transcription. To study the effects of cell shape, I used a two-pronged approach. Initially, I used live-cell imaging and single-molecule fluorescence in situ hybridization (smFISH) to examine whether descriptors of cell shape correlate with NF-κB dynamics and NF-κB-dependent transcription in unperturbed populations of single cells. Next, I used surface micro-patterning to impose different geometry and degrees of spreading on cells and examine NF-κB-dependent transcription in these cells. I found that descriptors that quantify cell spreading, such as cell area and nuclear area, correlate with NF-κB dynamics and NF-κB-dependent transcription. In addition, imposing bigger amount of spreading on cells increased the transcript abundance for two NF-κB-dependent genes, A20 and IκBα. In contrast, the relationships between geometry-related cell shape descriptors and NF-κB-dependent transcription are more subtle and complex. Importantly, despite observing the correlations between cell spreading and NF-κB activity, I found that the effects of cell shape on NF-κB dynamics and on NF-κB-dependent transcription were small. Together, my results suggest that NF-κB dynamics and NF-κB-dependent transcription can be quite robust to changes in cell shape.
3.2 Introduction

Cell-to-cell variability has been observed in many aspects of biology. One particularly interesting aspect is signal transduction. Even cells from an isogenic population treated with the same stimuli respond differently. This cell-to-cell variability has been observed in both immediate signaling events and eventual cell fate decisions resulting from signal transduction (reviewed in (Snijder and Pelkmans, 2011; Xia et al., 2014)). For example, TNF-induced NF-κB dynamics and TNF-induced NF-κB-dependent transcription vary from cell to cell (Lee et al., 2014). Another example is TRAIL-induced caspase activation and TRAIL-induced cell death (Spencer et al., 2009).

Although the components of signaling pathways have been explored as sources of variability, how factors outside a pathway affect its activity is less clear. One set of factors that may have been overlooked is that cells in our body adopt different shapes, e.g. different amount of spreading and different geometry. Pathologists have long been using cell shape as a marker for cancer progression. One of the key marker for high-grade cancer is the high variability in cell shape and nuclear to cytoplasmic ratio (Baba A, 2007). In addition, cultured cancer cells also adopt different, and variable, cell shapes. Although an important role for cell shape has been suggested for survival and differentiation (Chen et al., 1997; McBeath et al., 2004), much less is known about whether variability in cell shape can explain variability in signal transduction. Furthermore, it is not known if changes in cell shape are sufficient to change signal transduction.

In a recent study, Sero and colleagues have reported correlations between certain descriptors of cell shape and static measures of NF-κB sub-cellular distribution in either unstimulated or TNF-stimulated breast cancer cells (Sero et al., 2015). NF-κB is a transcription
factor that is actively shuttled to the cytoplasm in most unstimulated cells. When a cell is stimulated by TNF, activated signals result in the degradation of inhibitor of κB (IκB) and NF-κB is allowed to accumulate into the nucleus, activating the transcription of hundreds of genes (reviewed in (Gilmore, 2006)). While the study by Sero and colleagues suggests that the activity in this important pathway may be influenced by cell shape, it left many questions unanswered. Are the dynamics of NF-κB nuclear translocation correlated to cell shape? Is the NF-κB-dependent transcriptional output also correlated to descriptors of cell shape? What’s more, if there are correlations between these signaling events and cell shape, are these due to a causal link between cell shape and the NF-κB response? In other words, would imposed changes in cell shape be enough to cause changes in NF-κB pathway activity? Finally, what are the strengths of these effects? Are cell shapes effects large enough to drive biologically relevant changes in NF-κB-driven gene transcription?

To address these questions, I first performed a high-content imaging analysis to examine the relationships between NF-κB dynamics and certain cell shape descriptors under unstimulated and TNF-stimulated conditions. I also examined whether NF-κB-dependent transcription is affected by cell shape by measuring cell shape descriptors and single-cell transcript numbers of two NF-κB-dependent genes, A20 and IκBα. Finally, to test whether there are causal relationships between cell shape and NF-κB-dependent transcription, I used micro-patterning to force cells to adopt specific geometries and spreading then measured the transcript abundance. Overall, I found that descriptors that quantify cell spreading, such as cell area and nuclear area, correlate with NF-κB nuclear translocation and NF-κB-dependent transcription. In addition, imposing bigger amount of spreading on cells increased the transcript abundance for NF-κB-dependent genes. In contrast, the relationships between geometry-related cell shape descriptors
and NF-κB-dependent transcription are more subtle and complex. Importantly, despite observing the correlations between cell shape and NF-κB activity, I found that the effects of cell shape on NF-κB dynamics and on NF-κB-dependent transcription were small. Taken together, my investigations suggest NF-κB dynamics and NF-κB-dependent transcription are quite robust to changes in cell shape.
3.3 Results

3.3.1 TNF-induced NF-κB dynamics and cell shape vary in individual cancer cells

To monitor NF-κB dynamics in single cells, I generated HeLa cells stably expressing mVenus-RelA, the RelA protein which I tagged at its N-terminus to an improved yellow fluorescent protein named "mVenus" ((Kremers et al., 2011); Figure 3.1A). In addition, for the purpose of automatic image analysis, I engineered these cells to express a nuclear marker, iRFP-NLS. This cell line, stably expressing mVenus-RelA and iRFP-NLS will be referred to as mVenus-RelA HeLa hereafter. Using these cells, I measured the cellular and nuclear amounts of mVenus-RelA by time-lapse imaging before and after TNF treatment. Upon TNF treatment, single cells showed variable and transient nuclear translocation of mVenus-RelA (Figure 3.1B,C). Specifically, I found that the intensity and timing of nuclear translocation varied from cell to cell, consistent with what others have observed in HeLa cells and other cell types (Ashall et al., 2009; Hughey et al., 2015; Nelson et al., 2004). To quantify the cell-to-cell variability, I focused on three quantitative descriptors of RelA nuclear translocation and two quantitative descriptors of RelA abundance in individual cells (Figure 3.1C,D). The descriptors of RelA nuclear translocation included: 1) the baseline nuclear mean intensity, a measure of the concentration of RelA in the nucleus before treatment; 2) the max nuclear mean intensity, a measure of the maximal concentration of RelA in the nucleus over the time tracked; and 3) the max nuclear fold change, a measure of maximal fold change of RelA concentration in the nucleus over the time tracked, calculated as the ratio of nuclear baseline mean intensity to nuclear max mean intensity. The descriptors of RelA abundance included: 1) the cellular
integrated intensity, a measure of the total abundance of mVenus-RelA in the cell and 2) the cellular mean intensity, a measure of the average concentration of RelA in the cell, calculated as ratio of cellular integrated intensity to cell area. I found that the coefficients of variation (CVs) for all the descriptors showed similar values near 30% (Figure 3.1C,D) indicating that upon TNF treatment, single cells have a variable response in the NF-κB dynamics with cells on the lower end of the distribution having a descriptor value between two- and three-fold lower than the cells in the high value range.

One observation from my images was that not only were the NF-κB dynamics variable from cell to cell, but that cells also markedly varied in their shapes (Figure 3.1B,E). To measure this variability, I focused on three quantitative descriptors of spreading and two quantitative descriptors of cell geometry. More specifically, the spreading-related descriptors included: 1) cell area, an absolute measure of the amount of spreading of each cell; 2) nuclear area, an indicator of the size of the nucleus; and 3) the ratio of nuclear to cell area, a relative measure of cell spreading. Geometry-related descriptors included cell and nuclear roundness. These take values between 0 and 1, with a value of 1 indicating a perfect circular shape and values approaching 0 indicating an increasingly elongated polygon. I found that cell area, cell roundness, and the ratio of nuclear to cell area had a high degree of variability with CV values of 44%, 43%, and 35% respectively while nuclear area and nuclear roundness had a CV of only 23% and 10% respectively (Figure 3.1F).
Figure 3.1 TNF-induced NF-κB dynamics and cell shapes vary in live cells.

(A) Schematic of live-cell reporter constructs. Both reporters are under the regulation of the constitutive EF1a promoter. The live-cell reporter for RelA expresses full-length RelA N-terminally fused to the fluorescent protein mVenus (top). The live-cell nuclear marker expresses the infrared fluorescent protein (iRFP) C-terminally fused to a nuclear localization sequence (NLS; bottom). (B) Time-lapse images of HeLa cells stably expressing mVenus-RelA iRFP-NLS (mVenus-RelA HeLa). Cells were treated with 100ng/ml TNF and images were acquired at indicated time post TNF treatment. Only images from the mVenus channel are shown. (C) Single-cell mVenus-RelA nuclear mean intensity time course quantified from cells in B. Nuclear mean intensity is represented as three-frame running average. (D) Mean coefficients of variation for select time course descriptors of mVenus-RelA. Dots represent the CV values from individual biological replicate experiments. (E) Representative images of the mVenus channel (top) and iRFP channel (bottom) of same field of view for untreated mVenus-RelA HeLa demonstrating segmentation of cell contour and nuclear contour outlined in cyan. (F) Mean coefficients of variation for select cell shape descriptors. Dots represent the CV values from individual biological replicate experiments.
Figure 3.1 (Continued)
3.3.2 TNF-induced NF-κB dynamics are subtly correlated with cell shape in individual cancer cells

Given the variability in cell shape and in TNF-induced NF-κB dynamics, I was interested to investigate if correlations exist between descriptors of cell shape and descriptors of NF-κB dynamics. Using time-lapse images of mVenus-RelA HeLa cells collected pre- and post-TNF treatment, I extracted values for these quantitative descriptors for each cell. Cell shape descriptors were calculated for the images at t = 0 min, just before TNF addition. I found that in general there were few strong linear correlations between cell shape and NF-κB dynamics (Figure 3.2). Two exceptions were the cell area and ratio of nuclear area to cell area which appear to have moderately strong, positive or negative, correlations with two descriptors of NF-κB dynamics: cellular integrated intensity and cellular baseline mean intensity. Importantly, these are the two descriptors of NF-κB dynamics which measured the RelA abundance in the cell, the descriptors measuring the nuclear translocation of RelA did not show any strong correlations with cell shape descriptors.
Figure 3.2 Correlations between descriptors of TNF-induced NF-κB dynamics and descriptors of cell shape.

Time course images from mVenus-RelA HeLa cells treated with 100 ng/mL TNF were used to calculate descriptor values of NF-κB dynamics and cell shape (see materials and methods for definition). Scatter plots of log transformed descriptors with NF-κB dynamics on the Y-axes and cell shape descriptors on the X-axes where each dot represents data from a single cell. Red line is the best-fit line assuming a linear correlation. n = 44 cells in total, combined from two biological replicate experiments.
Figure 3.2 (Continued)
Because all the relationships between descriptors of NF-κB dynamics and descriptors of cell shape seemed monotonic but non-linear, I calculated the Spearman correlation coefficients (r) for each scatterplot in Figure 3.2 as shown in the corresponding grid in Figure 3.3. From these, I observed two interesting trends. First, comparing across different descriptors of NF-κB dynamics, I found that descriptors of RelA abundance were more strongly affected by cell shape than the descriptors of RelA nuclear translocation (correlation strength defined as the absolute value of the Spearman correlation coefficient, i.e., |r|; Figure 3.3). For example, the cellular integrated intensity has weak to strong correlation strengths (|r|=0.3 - 0.6) with all the cell shape descriptors whereas the nuclear max fold change is only weakly correlated to cell area (|r|=0.3) and to the ratio of nuclear area to cellular area (|r|=0.4). This suggests that in mVenus-RelA HeLa cells, RelA nuclear translocation was not strongly affected by cell shape – but that cell shape correlated with RelA abundance.

A second trend is apparent when we compare across descriptors of cell shape: spreading-related descriptors appear to have stronger impacts on the NF-κB dynamics than the geometry-related descriptors. For example, cell area, a spreading-related descriptor, had weak to strong correlation strengths (|r|=0.3 - 0.7) with all of the descriptors of NF-κB dynamics. In contrast, nuclear roundness, a geometry-related feature, only had weak to moderate correlation strength (|r| = 0.4) with cellular integrated intensity. Overall, this suggests that certain descriptors of cell shape, those that describe cellular spreading have more influence on NF-κB dynamics than the others.
Figure 3.3 Quantification of correlations between TNF-induced NF-κB dynamics and cell shape.

Matrix depicts Spearman correlation strength and statistical significance between descriptors of NF-κB dynamics and descriptors of cell shape with a darker color and a narrower ellipse indicate a stronger correlation. The color and the tilt show the direction of correlation (red and a tilt from upper left towards lower right indicates negative correlation and vice versa). The Spearman's rank correlation (r) and statistical significance of the correlation (p) are given for each combination of NF-κB dynamics and cell shape descriptor. Statistical significance assessment was controlled for multiple hypotheses testing with a Holms-Bonferroni correction. * means $P \leq 0.05$; ** means $P \leq 0.01$; *** means $P \leq 0.001$; **** means $P \leq 0.0001$. n = 44 cells in total, combined from two biological replicate experiments.
Figure 3.3 (Continued)

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3.3.3 RelA-dependent transcription varies in both untreated and TNF-treated individual cancer cells

At both the cell population and single-cell level, differences in NF-κB dynamics have been shown to predict different transcription profiles of NF-κB-dependent genes (Ashall et al., 2009; Lee et al., 2014). Given my observation of correlations between certain descriptors of cell shape and descriptors of NF-κB dynamics, I wanted to investigate whether cell shape also affects RelA-dependent transcription. I wanted to determine whether any correlations exist between cell shape and both baseline (i.e. unstimulated), and TNF-induced, RelA-dependent transcription. To achieve the former, I performed single-molecule RNA FISH (smFISH) experiments in unstimulated mVenus-RelA HeLa cells and HCC1143 cells. I used probes targeting transcripts coding for IκBα which is RelA-dependent and constitutively expressed. To measure TNF-induced RelA-dependent transcription, I performed smFISH experiments in mVenus-RelA HeLa cells and HCC1143 cells after 1-hr TNF treatment at a saturating dose of 100 ng/ml TNF. In this case, I used probes targeting transcripts coding for A20. A20 was a particularly appropriate target for investigating TNF-induced transcription because cells have very few if any A20 transcripts before TNF treatment but have abundant A20 transcripts after 1-hr TNF treatment (Lee et al., 2014).

The smFISH approach was particularly appropriate for these experiments because it allows counting of individual mRNA molecules in single cells. Therefore, it is possible to quantify baseline and TNF-induced RelA-dependent transcriptional output by counting IκBα and A20 mRNA transcripts respectively, in single cells (Figure 3.4A). Similar to what I observed for NF-κB dynamics, I found that both mVenus-RelA HeLa and HCC1143 cells showed substantial
cell-to-cell variability in the transcript numbers of A20 and IkBα (Figure 3.4B), with CVs of 45% and 33%, respectively in mVenus-RelA HeLa cells, and 58% and 43%, respectively in HCC1143 cells. This is consistent with what our group had previously observed in EGFP-RelA HeLa cells (Lee et al., 2014).
Figure 3.4 RelA-dependent transcription varies from cell to cell in both untreated and TNF-treated cells.

(A) Representative fluorescence images of HCC1143 cells treated with 0ng/ml or 100ng/ml TNF for one hour and stained by smFISH to quantify the transcripts of IκBα and A20, respectively. Yellow circle and arrowhead indicate single mRNA transcript. (B) Quantification of A20 and IκBα mRNA transcripts per cell in mVenus-RelA HeLa and HCC1143 cells. Data presented as mean +/- inter-quartile region (IQR), each dot represents a transcript number from an individual cell. n = 55 - 91 cells in total, combined from two biological replicate experiments.
Figure 3.4 (Continued)

A

HCC1143.A20

HCC1143.IkBq

B

Median +/- IQR

Log (number of transcripts per cell)
3.3.4 Transcript numbers of RelA-dependent genes are subtly correlated with cell shape in individual cancer cells

As mentioned above (Section 3.3.3), at both the cell population and single-cell level, NF-κB dynamics are quantitatively predictive of the transcription profiles of NF-κB-dependent genes (Ashall et al., 2009; Lee et al., 2014). Given the correlations between cell shape and NF-κB dynamics I described in Section 3.3.2, I hypothesized that cell shape may also affect RelA-dependent transcription in single cells. Thus, I examined the correlation between descriptors of cell shape and the A20 and IκBα transcript numbers measured in untreated or TNF-treated cells. I found that RelA-dependent transcription was affected by cell shape in ways that were similar to the NF-κB dynamics. First of all, there were generally no strong correlations between descriptors of cell shape and IκBα and A20 transcript number (all |r| < 0.50; Figure 3.5 and Figure 3.6). Second, I found that spreading-related descriptors appeared to have the strongest impact on the IκBα and A20 transcript numbers (with |r| = 0.01 – 0.49; Figure 3.5 and Figure 3.6). Geometry-related descriptors, on the other hand, had relatively weaker impact (|r| = 0.01 – 0.28; Figure 3.5 and Figure 3.6). Notably however, only a few of the correlations were statistically significant; this is possibly due to the small sample size but also likely reflects the fact that many correlations are weak. Despite the lack of statistical power and significance, the fact that we observed a similar pattern for the correlation of cell shape with both NF-κB dynamics and RelA-dependent transcription suggests that these effects of cell shape, while relatively small, are potentially biologically relevant.

When I compared the smFISH data across the two different gene targets in both cell lines, it appeared that not all gene expression was equally affected by cell shape. For example, IκBα
transcript number in untreated HCC1143 cells showed the strongest correlation with cell area and nuclear area ($|r| = 0.42$ and $0.49$, respectively) while A20 transcript number in TNF-stimulated HCC1143 cells had the weakest ($|r| = 0.20$ and $0.23$, respectively; Figure 3.5 and Figure 3.6). In contrast, when considering the data from mVenus-RelA HeLa cells, the same set of correlations were more similar to each other ($|r| = 0.20$ and $0.38$ for IκBα vs. $|r| = 0.33$ and $0.36$ for A20), suggesting that the impact of cell shape on RelA-dependent transcription may be both cell line- and gene-specific.
Figure 3.5 Specific descriptors of cell shape correlate with RelA-dependent transcription.

Scatter plots of descriptors of cell shape and number of A20 or IκBα transcripts in mVenus-RelA HeLa or HCC1143 single cells. The log-transformed numbers of transcripts in single cells are plotted on the Y-axes and descriptors of cell shape in the same cells are on the X-axes. Each dot represents data from a single cell. Red line is the best-fit line assuming a linear correlation. n = 55 - 91 cells in total, combined from two biological replicate experiments.
Figure 3.6 Quantification of correlations between cell shape and RelA-dependent transcription.

Matrix depicts Spearman correlation strength and statistical significance between descriptors of cell shape and A20 or IκBα transcript number in mVenus-RelA HeLa or HCC1143 cells. A darker color and a narrower ellipse indicate a stronger correlation. The color and the tilt show the direction of correlation (red and a tilt from upper left towards lower right indicates negative correlation and vice versa). The Spearman's rank correlation (r) and statistical significance of the correlation (p) are given for each combination of cell shape descriptor and A20 or IκBα transcript number in mVenus-RelA HeLa or HCC1143 cells. Statistical significance assessment was controlled for multiple hypotheses testing with a Holms-Bonferroni correction. * means P ≤ 0.05; ** means P ≤ 0.01; *** means P ≤ 0.001; **** means P ≤ 0.0001. n = 55 - 91 cells in total, combined from two biological replicate experiments.
Figure 3.6 (Continued)

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3.3.5 Effects of neighbor contacts may be mediated by spreading

Contact with neighboring cells could influence how much a cell can, or cannot spread. Thus, I hypothesized that in general, cells without contact with neighboring cells would tend to be less constrained and therefore spread to a greater cell area. Because I found that cell area correlates with NF-κB dynamics and NF-κB-dependent transcription (Figure 3.2, Figure 3.3, Figure 3.5, and Figure 3.6 on page 66, 69, 77, and 78, respectively), my hypothesis implies that neighbor contacts should also affect NF-κB dynamics and NF-κB-dependent transcription. Indeed, the results presented by Sero et al. suggest that neighbor contacts could affect RelA sub-cellular distribution in both untreated and treated HCC1143 cells (Sero et al., 2015).

To investigate whether neighbor contacts could affect the RelA-dependent transcription in HCC1143 cells, I compared the distributions of A20 transcript numbers in TNF-stimulated HCC1143 cells with and without neighbors. Cells without neighbors appeared to have higher A20 transcript numbers on average than those with neighbors (Figure 3.7), suggesting that neighbor contacts can affect RelA-dependent transcription. Additionally, for this particular population of TNF-stimulated HCC1143 cells, cells without neighbors had, on average, larger cell area than the ones with neighbors, suggesting that neighbor contacts affected both cell area and the RelA-dependent transcription in HCC1143 cells. Because even within a sample containing only cells without neighbors (i.e. cells on isolated micro-patterns), I observed that cell area positively correlated with the transcript numbers of A20 (Figure 3.5 and Figure 3.6), the simplest explanation is that neighbor contacts do limit cell spreading, and that in turn, cell spreading affects TNF-induced and RelA-dependent transcription of A20.
Interestingly, when I further analyzed the transcript numbers of IκBα in untreated HCC1143 cells, I noticed that, possibly due to small sample size, this particular subset of cells without neighbors had, on average, a smaller cell area than those with neighbors, although the difference was not statistically significant (Figure 3.7). For this particular population of untreated HCC1143 cells, the trend between IκBα transcript numbers and neighbor contacts was reversed from what I observed for TNF-induced A20 transcript numbers: cells without neighbors had lower IκBα transcript number than those with neighbors. This supports my working model: that although neighbor contacts can affect RelA-dependent transcription, these effects are most likely to be mediated by how neighbors limit spreading as measured by cell area.
Figure 3.7 Effects of neighbor contacts may be mediated by cell spreading.

(A) Quantification of the number of A20 and IkBa transcripts in individual HCC1143 cells (log scale). (B) Scatter plots of log-transformed cell area for the cells in (A). Horizontal lines overlaid on each distribution denote the median +/- inter-quartile region (IQR). n = 29 and n = 44 for HCC143.A20 without neighbors and with neighbors, respectively. n = 29 and n = 32 for HCC143.IkBα without neighbors and with neighbors, respectively. Statistical significance (p) was assessed using an unpaired, non-parametric, rank-comparing, Mann-Whitney test.
3.3.6 Modulation of cell spreading by micro-patterning has a small effect on RelA-dependent transcription

Given the consistent correlations between spreading and NF-κB dynamics descriptors as well as RelA-dependent transcription, I next wanted to test whether imposed differences in spreading and geometry can cause changes in RelA-dependent transcription. To achieve fine control of cell spreading and geometry, I seeded cells into a commercially available 96-well CYTOO™ plate where cells could only attach to regularly arrayed micro-patterns coated with molecules driving cell adhesion, such as fibronectin. Micro-patterns were adhesive islands of defined size and geometry for attachment of one single cell per island. These adhesive islands were separated by non-adhesive regions. Therefore, the geometry and amount of spreading that the cells could adopt was dictated by the geometry and size of the adhesive islands, with 3000-5000 islands of the same geometry and size in each well (Table 3.1 and Figure 3.8). For each of five different geometries directed by a micro-pattern, I tested two different amount of spreading, allowing the cells to spread to 700 μm² (small size) or 1100 μm² (large size). As a control condition, some wells were fully coated with fibronectin, leaving the cells free to adopt various geometries and various degrees of spreading.

In addition to fine control of cell spreading and geometry, micro-patterning can also allow us to overcome one important obstacle in studying cell shape. One feature of cancer cells is that they can change their cell shapes constantly and quickly. HeLa cells, for example, could change their cell shapes within a few minutes, much more quickly than the time it takes TNF stimulation to take effect (data not shown). This feature of cancer cells makes it much more
difficult for us to decide both at what time point to measure the cell shape and to know on which
time scale of cytokine stimulation this observed cell shape is relevant. Micro-patterning is an
effective way to solve this problem, by forcing cells to adopt a specific shape of interest
throughout the duration of the experiment.
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<th>Crossbow (Large)</th>
<th>Crossbow (Small)</th>
<th>H (Large)</th>
<th>H (Small)</th>
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Figure 3.8 Micro-patterning reduces variability in spreading and geometry of cells.

(A) Scatter plots of log transformed cell area measured from mVenus-RelA HeLa and HCC1143 cells grown on fully coated surfaces, small size micro-patterns, or large size micro-patterns. CV value for each distribution is displayed in the tables above the scatter plots. (B) Fluorescent images of mVenus-RelA HeLa cells in mVenus channel of cells grown on fully coated surface (left) or on micro-patterns of disc geometry and small size (right). (C) Transmitted image of HCC1143 cells on fully coated surface (left) or on micro-patterns of disc geometry and small size (right). The black line in the image on the right is an artificial border generated by the microscope during montage imaging mode.
Figure 3.8 (Continued)

A. 

<table>
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</tr>
</tbody>
</table>

Log (cell area measured in pixels)

Full (700μm²) | Small (700μm²) | Large (1100μm²)

B. 

- mVenus-RelA HeLa on fully-coated surface
- mVenus-RelA HeLa on small disc micro-patterns

C. 

- HCC1143 on fully-coated surface
- HCC1143 on small disc micro-patterns
Before testing whether differences in spreading can cause changes in RelA-dependent transcription, I first wanted to demonstrate the ability of micro-patterning to effectively control cell spreading. To evaluate this, I compared the cell area for cells on the fully coated surface, the small-size micro-patterns and the large-size micro-patterns (Figure 3.8A). As expected, cells grown on the small-size and the large-size micro-patterns had a cell area that matched the micro-pattern-predicted sizes of 700 μm² and 1100 μm², respectively. Moreover, compared to the fully coated surface, micro-patterning markedly decreased the variability in spreading as indicated by a dramatic decrease in the CVs (Figure 3.8A), suggesting that cells on micro-patterns have much more controlled and uniform spreading.
Having demonstrated that micro-patterning can effectively control spreading, I next wanted to test whether the correlation between spreading and RelA-dependent transcription is causal. Once cells were fully attached on micro-patterns or fully coated surface, I measured RelA-dependent baseline and TNF-induced transcription using smFISH, as described in Section 3.3.3 (page 71). Briefly, to examine RelA-dependent transcription at baseline, I performed smFISH experiments in unstimulated mVenus-RelA HeLa cells and HCC1143 cells using probes targeting constitutively expressed IκBα transcripts. To examine TNF-induced RelA-dependent transcription, I performed smFISH experiments in mVenus-RelA HeLa cells and HCC1143 cells after 1-hr TNF treatment at a saturating dose of 100 ng/ml, using probes targeting A20 transcripts.

To test whether differences in spreading can cause changes in RelA-dependent transcription, I examined the transcript numbers of A20 and IκBα in cells grown on small-size micro-patterns compared to those that were grown on large-size micro-patterns. I observed that cells grown on larger micro-patterns had higher transcription levels on average (Figure 3.9). However, the effect size is relatively small: for an increase of nearly 60% in cell area, there was only a 14-22% increase in median transcript abundance. Even though the effect size was small, it was consistently observed across both genes and cell lines tested. In addition to the effect being small, it can be non-linear. For IκBα transcription in mVenus-RelA HeLa cells grown on fully coated surface, there was no increase when comparing to cells on large-size micro-patterns, although the cells grown on the fully coated surface had, on average, a much larger cell area (Figure 3.8A and Figure 3.9). Overall this suggests that although more spreading causes more RelA-dependent transcription, this effect is non-linear and plateaus at the top of the range of cell
spreading. Once cells reached a certain amount of spreading, the effects of spreading were diminished.
Figure 3.9 Cells on larger micro-patterns have greater RelA-dependent transcription.

Boxplots of quantification of A20 and IxBα mRNA transcripts per cell in mVenus-RelA HeLa and HCC1143 cells. Middle horizontal bar and notches indicate median and 95% confidence interval of the median, respectively. Horizontal bars at the top and bottom indicate median +/- interquartile region (IQR). The ends of vertical lines indicate median +/- 1.5 IQR. Values outside this range are represented as individual points in the boxplots.
Figure 3.9 (Continued)

**Comparative Analysis:**

- **HeLa A20**
  - Full
  - Large (1100\(\mu\)m\(^2\))
  - Small (700\(\mu\)m\(^2\))

- **HeLa IkB\(\alpha\)**
  - Full
  - Large (1100\(\mu\)m\(^2\))
  - Small (700\(\mu\)m\(^2\))

- **HCC1143 A20**
  - Full
  - Large (1100\(\mu\)m\(^2\))
  - Small (700\(\mu\)m\(^2\))

- **HCC1143 IkB\(\alpha\)**
  - Full
  - Large (1100\(\mu\)m\(^2\))
  - Small (700\(\mu\)m\(^2\))
To examine whether spreading also correlated with RelA-dependent transcription in cells grown on micro-patterns of different geometries, I analyzed the transcripts numbers of A20 and IκBα in cells of different geometries (Figure 3.10). For micro-patterns of any given geometry, cells on larger micro-patterns had higher number of transcripts than those on smaller micro-patterns. The positive correlation between spreading and RelA-dependent transcripts appeared to be consistent throughout all geometries. When I examined the data more closely, I observed that the differences were more pronounced for some micro-patterns (e.g. micro-patterns of crossbow geometry for IκBα transcription in HCC1143 cells) while less so for others (e.g. micro-patterns of Y geometry for IκBα transcription in HCC1143 cells). Although the effects were in general subtle, and only a few of the differences were statistically significant, the fact that the effect was consistent throughout the dataset strongly suggests that spreading may indeed impact transcript number, albeit with a weak effect size.
Figure 3.10 For certain geometries, cells on larger micro-patterns have statistically significant higher RelA-dependent transcription.

Boxplots of A20 and IκBα mRNA transcripts per cell from mVenus-RelA HeLa and HCC1143 cells. Middle horizontal bar and notches indicate median and 95% confidence interval of the median, respectively. Horizontal bars at the top and bottom indicate median +/- interquartile region (IQR). The ends of vertical lines indicate median +/- 1.5 IQR. Values outside this range are represented as individual points in the boxplots. Data for fully coated surface control is in left column followed by pairs of large and small micro-patterns of different geometry. For each cell line and each gene target (e.g. HCC1143.A20), pair-wise differences between the mean of each distribution and all other distributions were assessed by one-way ANOVA test. Post-ANOVA, a Tukey’s test was used to correct for multiple hypothesis testing. The family-wise significance level used was 0.05. Only differences with an adjusted p-value less than or equal to 0.05 are shown at the top of the graph. For each boxplot, data for n = 49 to n = 91 cells from two biological replicate experiments were combined and shown here.
Figure 3.10 (Continued)
To make sure that the small observed effects of spreading were not due to a technical bias in detecting smFISH spots in cells of different degrees of spreading, I analyzed the detectability of individual smFISH spots for cells grown on different-size micro-patterns. I quantified the strength of the signal of individual smFISH spots, calculating for each cell the average signal-to-noise ratio (SNR) of all transcripts detected (Figure 3.11). Unlike the consistent trend between spreading and transcript numbers across different geometries, there was no consistent trend between spreading and SNRs across different geometries, and none of the SNR distributions were statistically significant different from the others (Figure 3.11). Therefore, my analysis suggests that the small observed effects of spreading on transcription are unlikely to be due to the technical challenges of detecting and counting smFISH spots in cells with smaller cell area and therefore likely to be true differences in transcript numbers.
Figure 3.11 The detectability of smFISH signals is not affected by micro-pattern sizes.

Boxplots of the mean signal-to-noise ratio (SNR) of all IκBα smFISH spots in individual HCC1143 cells. First, for each smFISH spot in the cell, its SNR value was calculated. Then, for each cell, we calculate the mean spot SNR. Y-axis values represent the mean of all signal-to-noise ratios for all smFISH spots of IκBα transcripts for a given cell. For each boxplot, the middle horizontal bar and notches indicate the median and 95% confidence interval, respectively. Horizontal bars at the top and bottom indicate median +/- interquartile region (IQR). The ends of the vertical lines at the top and bottom indicate median +/- 1.5 IQR. Values outside this range are represented as individual points. Data for micro-patterns of the same geometry but of different sizes are grouped next to each other. Pair-wise differences between the mean of each boxplot and all other boxplots were assessed by a one-way ANOVA test. Post-ANOVA, Tukey’s test was used to correct for multiple hypothesis testing. The family-wise significance and confidence level was 0.05; none of the pair-wise comparisons had an adjusted p-value larger than 0.05, and therefore no adjusted p-values are shown. For each boxplot, data from n = 84 to n = 90 cells from two biological replicate experiments were combined and shown.
3.3.7 Modulation of cell geometry by micro-patterning has a small effect on RelA-dependent transcription

In addition to spreading, changes in geometry of the cell might also cause differences in RelA-dependent transcription. In Section 3.3.4, I examined the correlations between descriptors of cell shape and RelA-dependent transcription for cells on fully coated surface, focusing on two geometry-related cell-shape descriptors, cell and nuclear roundness (Section 3.3.4; Figure 3.5 and Figure 3.6 on page 77 and 78, respectively). However, because I did not investigate other aspects of cell geometry, such as symmetry or polarization, I was limited in my ability to generalize and draw comprehensive conclusions about the potential effects of cell geometry on RelA-dependent transcription. To test whether changes in geometry of the cell may also cause differences in RelA-dependent transcription, cells were grown on micro-patterns of the five following geometries: Disc, Crossbow, H, Y and L (Table 3.1 on page 86).

Before testing whether differences in geometry might cause changes in RelA-dependent transcription, I first wanted to determine if micro-patterning could effectively control the geometry of the cells. To evaluate this, I examined the geometry for cells on the fully coated surface and the micro-patterns. As expected, cells on micro-patterns adopted the geometry as specified by the micro-patterns (Figure 3.8B,C shows an example for the disc geometry). Moreover, compared to the fully coated surface, micro-patterning markedly decreased the variability in geometry, suggesting micro-patterning enables precise control of uniformed geometry.

Having demonstrated that micro-patterning effectively controlled the cell geometry, I next wanted to test whether changes in cell geometry, while maintaining the same amount of cell
spreading, were sufficient to cause differences in RelA-dependent transcription. To examine the effects of geometry, the dataset first presented in Figure 3.10 was reorganized to allow examination of any trends across cells of different geometries by grouping together the data for micro-patterns of the same size but of different geometries (Figure 3.12, the top and bottom panels show data for small-size and large-size micro-patterns respectively). Furthermore, datasets in each panel were ranked according to the median number of transcripts.

Several interesting trends can be observed. First, when comparing across both cell lines and both genes tested, the ranking of the geometries differed for small vs. large micro-patterns. For example, for HCC1143 cells grown on small-size micro-patterns, the highest IκBα median transcript numbers was found in the L-geometry and the lowest was in the crossbow-geometry. This is in contrast, to the cells grown on large-size micro-patterns where cells adopting the crossbow-geometry actually had the highest IκBα transcript numbers; the cells adopting the Y-geometry had the lowest IκBα transcript numbers (Figure 3.12). The inconsistent ranking of geometries for different size micro-patterns suggests that spreading may play a role in defining the relationship between cell geometry and RelA-dependent transcription.

A second trend that I observed was that the rankings of geometries shared more similarity for the same gene across the two cell lines than for the two different genes assayed in the same cell line. For example, the rankings for HeLa and HCC1143 A20 transcript numbers were more similar than those for HeLa A20 vs. HeLa IκBα transcript numbers.

Finally, if we compared the data for A20 vs. that for IκBα different genes, the rankings of different cell geometries showed more consistency across cell lines for A20 than for IκBα transcript numbers. For example, for A20, the rank-order for different geometries were similar in both mVenus-RelA HeLa and HCC1143. This similarity was observed for both the small-size
and large-size micro-patterns. However, for IκBα transcript numbers, the rank-order was found to be similar between mVenus-RelA HeLa and HCC1143 cells only for the small-size micro-patterns, the rank-order is quite different for large-size micro-patterns.

Overall, these results suggest that the relationship between RelA-dependent transcription and geometry are likely to be complex. Furthermore, it is clear that the effects of geometry on transcription are quite small; only a few transcript number distributions are statistically significantly different from each other (Figure 3.12). These results lead me to conclude that RelA-dependent transcription, both TNF-induced and at baseline in untreated cells, can be quite robust to changes in cell geometry.
Figure 3.12 The relationship between the geometry of a cell and its RelA-dependent transcription is complex.

Boxplots of A20 and IκBα mRNA transcripts per cell for HeLa mVenus-p65 iRFP-NLS and HCC1143 cells. Middle horizontal bar and notches indicate the median and 95% confidence interval, respectively. Horizontal bars at the top and bottom indicate median +/- interquartile region (IQR). The ends of the vertical lines indicate median +/- 1.5 IQR. Values outside this range are represented as individual points. Data for a particular gene and cell line are presented in the same column. For each column, data for small micro-patterns are presented in the upper half while data for large micro-patterns are presented in the lower half. For each cell line and each gene target (e.g. HCC1143.A20), pair-wise differences between the mean of each boxplot and all other boxplots were assessed by a one-way ANOVA test. Post ANOVA, Tukey’s test was used to correct for multiple hypothesis testing. The family-wise significance and confidence level was set to 0.05. Only differences with an adjusted p-value less than or equal to 0.05 are indicated above the boxplots. For each boxplot, data for n = 49 to n = 91 cells from two biological replicate experiments were combined and shown.
3.3.8 Micro-patterning reduces the variability in cell shape without reducing the variability in RelA-dependent transcription

In additional support of my conclusion that the effects of cell spreading and geometry on RelA-dependent baseline and TNF-induced transcription are small, I found that reducing the variability in cell shape by micro-patterning did not significantly reduce variability in transcript numbers of A20 and IκBα (Figure 3.8 on page 86 and Figure 3.13). Indeed, cells grown on micro-patterns had a dramatic reduction in their variability of spreading and micro-patterns effectively controlled their geometry (Figure 3.8). Nevertheless, the coefficients of variation of the transcript number distributions were similar to those for cells grown on fully coated surfaces. This held true across all geometries, for both micro-pattern sizes, both cell lines and both RelA target genes assayed (Figure 3.13), despite the fact that HCC1143 cells showed overall greater variability in transcript number and that for both cell lines baseline IκBα transcript numbers were less variable than TNF-induced A20 transcript numbers.
Figure 3.13 Standardized geometry and spreading of cells does not significantly reduce the variability in RelA-dependent transcription.

Bar graph of the coefficients of variation (CV) for A20 and IκBα transcription in mVenus-RelA HeLa and HCC1143 cells. For each bar, data for n = 49 - 91 cells combined from two biological replicate experiments were combined.
3.4 Discussion

Cell shape and the cellular microenvironment were recently reported to correlate with the subcellular distribution of NF-κB when measured at single time points, pre-treatment and one hour post-TNF treatment (Sero et al., 2015). Here, I examined whether key descriptors of NF-κB pathway activity correlate with cell shape using both live-cell imaging of RelA nuclear translocation dynamics and smFISH to measure single-cell transcript abundances. I also tested whether imposing a cell shape is sufficient to quantitatively dictate NF-κB-dependent transcriptional output. Overall, I found only weak effects of cell shape on RelA nuclear translocation dynamics and the abundance of transcripts from two NF-κB-dependent genes.

One surprising outcome is that all five of the descriptors of NF-κB dynamics that I measured in mVenus-RelA HeLa cells were only weakly, if at all, correlated with nuclear roundness, which was previously implicated in determining NF-κB subcellular distribution (Sero et al., 2015). It is possible that these results are cell-line-dependent, or are dependent on particular forms of data normalization used in the image analysis. However, consistent with a lack of strong correlation between nuclear and cell roundness and NF-κB dynamics, I further observed that nuclear and cell roundness correlate only weakly with NF-κB-dependent transcription, if at all, in mVenus-RelA HeLa cells and HCC1143 cells. This latter cell line is one of the cell lines analyzed by Sero and colleagues (Sero et al., 2015).

As an additional test of the impact of cell geometry on NF-κB activity, I measured the effects of imposing specific geometries on NF-κB-dependent transcription. Again, I found only weak effects and these were not consistent across two NF-κB target genes and between cell lines. Interestingly, the five imposed geometries introduce different symmetries, different cytoskeletal
and organelle organization (Théry et al., 2006a; Théry et al., 2006b), and presented different amounts of fibronectin molecules for adhesion and mechanical force on cells (Albert and Schwarz, 2014). Overall, these results suggest that NF-κB dynamics and RelA-dependent transcription can be quite robust to variation in these factors.

Cell shape descriptors that did correlate with NF-κB dynamics descriptors were cell area, nuclear area and the ratio of nuclear area to cell area. For example, the spearman correlation coefficient between cell area and cellular baseline mean intensity of mVenus-RelA is -0.67 (Figure 3.3 on page 69). Nuclear area was observed to be linearly correlated with cell area for cells on fully coated surfaces ($r^2 = 0.31; p$ (two-tailed) $< 0.0001; $data not shown). This is consistent with the findings of Jain and colleagues, who showed that increasing cell area by micro-patterning also increased nuclear area (Jain et al., 2013), suggesting that nuclear area, cell area, and the ratio of nuclear area to cell area are all tightly associated with spreading.

The mechanism connecting spreading to NF-κB dynamics and NF-κB-dependent transcription awaits further investigation. However, several recent studies on how spreading can control various cellular functions may help shed some light on this. By using micro-patterning, Dupont et al. showed that the amount of spreading can control the sub-cellular localization of the transcription factors YAP and TAZ and that these two mechanosensitive transcriptional regulators are required to mediate the effects of spreading on survival and differentiation (Dupont et al., 2011). Thus, it may be tempting to postulate that the effects of spreading on NF-κB pathway activity are also mediated by YAP/TAZ although the link between YAP/TAZ and the NF-κB pathway needs further investigation. In addition to affecting YAP and TAZ activity, spreading has also been implicated in regulating focal adhesion kinase (FAK) activity, which in turn regulates the lipid composition of the cell membrane (Frechin et al., 2015). Through this,
spreading is likely to have a broad impact on signaling in the cell membrane, such as through the TNFR1 receptor. It has been reported that the lipid composition of the plasma membrane, such as the abundance of lipid rafts, affects TNFR1-induced signaling, with a higher lipid raft content associated with a higher level of signaling complex formation (Legler et al., 2003). Therefore, spreading may affect TNFR1 signaling via regulation of FAK and membrane lipid composition.

Together, our results show that, in cancer cells, NF-κB dynamics and NF-κB-dependent transcription are weakly affected by cell spreading although, overall, cell shape is not an important quantitative determinant of NF-κB-dependent transcription.
3.5 Materials and methods

3.5.1 Reporter constructs

**mVenus-RelA:** I constructed a plasmid for the expression of full-length human RelA fused at its N-terminus to the fluorescent protein mVenus (mVenus-RelA) under the constitutive promoter for EF1a. The EF1a promoter sequence was amplified from pEBA-XIAP (Addgene Plasmid #11558) and cloned into the BglII and XhoI sites in pMSCV-puro (Clonetech) to generate pMSCV-EF1a-puro. Full length human RelA was amplified from a p65-mCherry construct (kind gift of Inna Lavrik (Neumann et al., 2010) with primers Forward: CTCTGGATCCGACGAACTGTTCCCCCTCATC and Reverse: AGAGGCAGGCGCTTAGGAGCTGTACTGACTCAG) then and initially inserted into the vector pLPCX-Ntag-mVenus (kind gift from John Albeck) using BamHI and NotI. The resulting fusion protein of mVenus-p65 was amplified by PCR (Forward: GAGACTCGAGGCCACCATGGTGAGCAAGGGCGAGG and Reverse: AGAGGCAGGCGCTTAGGAGCTGTACTGACTCAG) and cloned into pMSCV-EF1a-puro using XhoI and NotI.

**iRFP-NLS:** To construct the plasmid expressing an infra-Red Fluorescent Protein (iRFP) with a C-terminal nuclear localization sequence (NLS), iRFP was amplified by PCR from pShuttle-CMV-iRFP (Addgene Plasmid #31856; primers Forward: GTGTGTGTGCAGCGATCCACCAGAGTGGCGGAAG; Reverse: GTGTGTAGATCTTCTTCCATCACGCGATCTGC). An NLS tag was designed by two rounds of primer annealing and amplification to create a tag with a triple NLS sequence (first
round primers: Forward

GCATGGACGAGCTGTACAAGGATCCAAAAAGAAAGGAAGGTA
GACCCGAAGAA AAAGAG

AAAGAG

Reverse: Second round primers:

CTCTTTTTCTTCGGGTCTAacctcctcctctcttttttGATCCTTGTAAGACTCGTCCAT

GC: Reverse complement:

GAAGAGGAAGGTAAGACCCGAAGAAAGGAAGAAAGTCAAGC
GACCCAGAAAAGAGAAAGAAAGAAAGAAAG

The iRFP PCR product and the 3XNLS coding sequences were ligated into pMSCV (SalI and NotI). Finally, the resulting iRFP-NLS fusion protein sequence was then amplified by PCR using these primers (Forward: GTGTGTGTAGGTAGCTCCCGTAGTCGATCCT; Reverse: GTGTGTCTGAGACCCAGACACCTGAAAACTGAAAGAAAGAAAAACTTTGAAC) and inserted into the vector of pBabe-EF1a-MCS-Hygro (using SalI and BspE1), allowing insertion but breaking the XhoI and AgeI sites in the process of the ligation. The pBabe-EF1a-MCS-Hygro vector was generated by adding the EF1a promoter (described above, here inserted between XhoI and AgeI) and an enhanced multiple cloning site (inserted into BamHI; Forward: GATCAGCTGAGATCTAACCCTGTGACGGTACGGGTAGAG; Reverse: GATCAGCTTACCTGATCAAGGTACCCGTTAGGATTCGTGAGCT) into pBabe-Hygro (Addgene Plasmid #1765).

The sequence fidelity of all constructs was verified by sequencing at the Dana-Farber/Harvard Cancer Center DNA Resource Core (Boston, MA, USA).
3.5.2 Generation and validation of mVenus-RelA iRFP-NLS HeLa cells

For expression of mVenus-p65 in HeLa cells (ATCC), retroviral infection of HeLa cells was performed as follows. On Day 1, I plated 1-2 x10^6 HEK293T cells onto 10-cm dish in 10 mL DMEM with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2 mM L-glutamine (Invitrogen). On Day 2, after 18-20 hours, HEK293T cells were transfected with 6 µg of pMSCV mVenus-RelA DNA and 6 µg of pCLAmpho DNA (Naviaux et al., 1996) using FuGENE-6 according to directions of the manufacturer (Roche). On Day 3, medium was changed then virus-containing culture supernatants were collected at 48 and 72 hours post-transfection. After filtration through a 0.45-µm syringe filter, viral supernatant was added to HeLa cells seeded in 10-cm dish 24 hours before the first infection (1-2 x10^6 cells seeded) in the presence of 8 µg/mL polybrene. Around 24 hours after the second infection, the HeLa cells were passaged and cells having stably integrated pMSCV-puro-mVenus-RelA were selected in medium with 1 µg/mL puromycin (Invitrogen) for a week (changing medium every two days).

Colonies derived from single cells were isolated and specific clonal cell lines were selected based on mVenus-RelA expression and responsiveness to TNF. TNF-induced translocation of mVenus-RelA in the selected clone displayed similar behavior to that of endogenous p65 based on fixed cell immunofluorescence data from the wild-type strain. The selected clone exhibits persistent cell-to-cell variability in mVenus-RelA expression: when cells were plated at very low density, cell-to-cell variability within sub-clones recapitulated the variable expression of the parental clone. This suggests that variation of mVenus-RelA expression level between individual cells is not predominantly due to contamination from multiple clonal lineages.
3.5.3 Cell lines and cell culture

HEK293T (ATCC) and mVenus-RelA HeLa were cultured in Dulbecco’s Modified Eagle Medium (DMEM) whereas HCC1143 cells (ATCC) were cultured in RPMI Medium 1640 at 37°C and 5% CO₂. All media were supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.2 mM L-glutamine (Invitrogen).

3.5.4 Micro-patterning and TNF treatment

Micro-patterned 96-well plate were designed and produced by CYTOO (https://cytoo.com/micro-pattern-products/plates/cytooplates%E2%84%A2-96-rw-starter). Activated micro-patterns were coated with 20 µg/mL bovine plasma fibronectin (Sigma F4759) in PBS for 2 hr at room temperature. Cells were then seeded at 2000 cells/well and allow to spread for 6 hr and then treated with 0 ng/mL or 100 ng/mL TNF (Peprotech). For all experiments, TNF was diluted into serum free media to appropriate concentrations so that 10 µL of the diluted TNF mixture was spiked into wells containing 190 µL of growth media resulting in final concentrations of TNF as indicated.

3.5.5 Live-cell imaging

For live-cell experiments, mVenus-RelA iRFP-NLS HeLa cells seeded on fibronectin were imaged for 0.5 to 1 hour before TNF addition. For experiments that included smFISH, cells were fixed immediately after live-cell image acquisition (see below). Wide-field epifluorescence and transmitted light live-cell imaging was carried out in an environmentally controlled chamber (37°C, 5% CO₂) on a BD Pathway 855 BioImager using a 20x objective (0.75 NA; UPlanSApo;
Olympus). Time-lapse images were collected over multiple fields in each well with a temporal resolution of 6~10 minutes per frame during both pre-TNF and post-TNF imaging periods.

3.5.6 **Live-cell image analysis and dynamics descriptors extraction**

Images were flat-field corrected and background was subtracted using ImageJ. Using a customized pipeline in CellProfiler (Carpenter et al., 2006), nuclei were segmented in the iRFP-NLS images then mean fluorescence intensity of nuclear mVenus-RelA was collected for each cell at each time point from time-lapse movies. Baseline nuclear mean intensity was defined as the average of nuclear mean fluorescence intensity before TNF addition. Max nuclear mean intensity was defined as the maximum of nuclear mean fluorescence intensity during the 1-hour TNF treatment. Max nuclear fold change was calculated for each cell (nucleus) as the ratio of its max nuclear mean intensity divided by its baseline nuclear mean intensity.

For some experiments, the cell integrated fluorescence intensity and cell mean fluorescence intensity of mVenus-p65 were also collected for each cell at the initial time point ($t = 0$) by manually tracing the outline of the cells.

For all experiments, cells that overlapped, cells that occupied the same micro-pattern, and cells that left the imaging field or divided within an hour of TNF treatment were not analyzed.

3.5.7 **Cell segmentation and cell shape descriptors extraction**

For live-cell NF-κB dynamics data, cells and nuclei were segmented by manually tracing the outlines of each cell in YFP channel and of its nucleus in the iRFP channel. Shape descriptors were extracted using ImageJ. These included: cell area, nuclear area, the ratio of nuclear to cell
area, cell roundness and nuclear roundness (both of which are calculated as $4\pi \times (\text{area}/\text{perimeter}^2)$).

For smFISH data, cells were segmented by manually tracing the outlines of cells using the transmitted light image and nuclei were segmented automatically using the Hoechst channel in CellProfiler. Shape descriptors associated with cells (cell area, nuclear area, ratio of nuclear to cell area, cell roundness and nuclear roundness) were extracted using ImageJ or CellProfiler.

### 3.5.8 smFISH probe design

Probe sets targeting TNFAIP3/A20 and NFKBIA/IκBα (Stellaris™ Probes, Biosearch Technologies) were described previously (Lee et al., 2014). Briefly, the probe sets were designed using online software (http://biosearchtech.com) and the following strategies: 1) maximize the number of 20-mer probes, up to 45 per target gene, 2) include a minimum spacing of 2-bp between probes, 3) target the open reading frame of the target mRNA first with as many high quality probes as possible then target 5’ and 3’ untranslated regions, 4) minimize differences from 45% GC content for each individual probe and 5) eliminate any probe with significant homology to other regions of the human genome. The TNFAIP3/A20 and NFKBIA/IκBα probe sets were ordered as CAL Fluor Red 610 (ex/em = 590/610 nm) conjugates (a single label per probe). The final probe sets consisted of 44 and 38 individual probes targeting TNFAIP3/A20 and NFKBIA/IκBα respectively; sequences are listed in the following table.
Table 3.2 List of *TNFAIP3* and *NFKBIA* probe sequences

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<th>Probe (5’-3’)</th>
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3.5.9 smFISH

Following TNF-treatment, cells in 96-well CYTOO starter plates were fixed in 4% paraformaldehyde for 10 minutes at room temperature, rinsed once with PBS using RNase free reagents (Ambion). Cells then were hybridized overnight with smFISH probes as described previously (Raj et al., 2008), with optimized probe-set specific conditions (as described in (Lee et al., 2014): 1) for CAL Fluor Red 610-labeled *TNFAIP3* probes, at 37°C with 50 nM pooled probes in 2XSSC with 10% formamide and 8% dextran sulfate; and 2) for CAL Fluor Red 610-labeled *NFKBIA*, at 37°C with 100 nM pooled probes in 2XSSC with 12% formamide, 10% dextran sulfate. Labeled cells were counterstained with Hoechst.

3.5.10 smFISH microscopy

Hybridized cells were imaged on the BD Pathway 855 BioImager using a 100x objective (0.75 NA; UPlanSApo; Olympus) using temperature-matched oil (n = 1.516, 23°C, from GE). For each field of view, I acquired an image in transmitted light, an image in the Hoechst channel, and a z-stack of 15-50 images with 0.3 µm intervals in the CAL Fluor Red 610 channel.

3.5.11 smFISH image analysis

Cells were first masked by low-pass Gaussian filtering (σ= 5) followed by thresholding at a manually determined, fixed value, where the same threshold was used on all images of a stack. smFISH punctae were then detected within this cell mask using a 3D generalization of the
algorithm presented in (Aguet et al., 2013). We estimated the Gaussian PSF-approximation sigma from initial sample data by allowing the XY sigma and Z sigma to vary during the 3D Gaussian fitting process, and taking the mode of the resulting sigma distributions. These PSF parameters were then fixed in all subsequent detections. We used $\sigma = 0.05$ during the detection and mixture-model fitting was enabled. To remove false positives, detected points were removed if their fitted amplitude was below the 95th percentile of the fitted amplitudes in a negative control cell with no smFISH puncta present. Per-spot signal-to noise ratios (SNRs) were estimated as the ratio between the fitted spot amplitude and the standard deviation of the fit residuals.

3.5.12 Statistical analysis

All analyses were performed using R version 3.2.0 and GraphPad Prism version 6.00 (GraphPad Software, La Jolla California USA, www.graphpad.com).

Using R, we calculated Pearson and Spearman correlations between descriptors of cell shape and of NF-κB dynamics as well as between descriptors of cell shape and NF-κB-dependent transcript numbers. Because data distributions were observed to be log-normal, all data was first log-transformed. Multiple testing hypotheses were controlled with a Holms-Bonferroni correction. We compared the average number of transcripts on micro-pattern shapes using ANOVA following log-transformation to determine whether there were significant differences in group means. However, Post-ANOVA, we applied Tukey’s test for multiple comparisons to more rigorously determine differences amongst groups.

To compare the average number of A20 or IκBα transcripts in HeLa and HCC1143 cells on fully coated surfaces, we first used ANOVA following log-transformation to determine
whether there were significant differences in group means. Post ANOVA, we applied Sidak’s test for multiple comparisons in GraphPad Prism to rigorously determine differences amongst groups.
Chapter 4 Conclusion
The importance of how cells respond appropriately to external and internal cues becomes apparent when defective signal transduction leads to disease. For example, loss of the insulin responsiveness leads to type 2 diabetes. Thus, learning which factors affect cellular decisions not only impacts our appreciation of the working principles of signaling networks in normal physiology, but can also help us design effective therapies for when things go wrong. In my thesis work, using the signaling network induced by TNF as a model system, I revealed how the duration of a stimulus can coordinate cell fate decisions and I uncovered some of the complex relationships between cell shape and NF-κB signaling pathway activity. My findings support a model where the duration of a TNF stimulus encodes information. Additionally, my results suggest that, at least in cancer cells, certain signaling pathway activities are robust to changes in cell shape. Taken together, these studies provide valuable insights into complex and dynamic cellular decision-making processes and advance our understanding of the various factors that can, or cannot, influence these decisions.
4.1 The duration of a stimulus encodes information

In Chapter 1, I discussed how two opposing pathways are activated by TNF binding to its receptor TNFR1: the pro-survival NF-κB pathway and the pro-apoptosis caspase pathway. Because caspase activation and subsequent cell death had been shown to happen only after internalization of TNFR1 (Micheau and Tschopp, 2003), I hypothesized that the duration of TNF exposure governs the relative strength of activation of these two pathways. Could TNF treatment duration influence the choice between cell survival and apoptosis, or more specifically, does a longer duration of TNF treatment lead to TNF-induced apoptosis?

In contrast to what I expected, my colleagues and I showed in Chapter 2 that a single 1-min TNF pulse can be as effective at killing human cancer cells as continuous TNF treatment (up to 10 hours, the duration of our experiments). At the TNF concentrations required for TNF-induced apoptosis, a 1-min pulse also induced as strong a pulse of NF-κB nuclear translocation as continuous treatment. This suggests that mechanisms other than duration also play a role in determining the strength of activation of these two opposing pathways. Our simulations of ligand-receptor binding suggest that, instead of exposure duration, the fraction of TNF-bound receptors at the cell surface at any given time may determine whether there is efficient subsequent activation of caspases.

Indeed, based on structural work on TNF, TNF receptors, and the death domain-containing adaptor proteins that form the intracellular TNF-induced signaling complexes, it is thought that TNF binding may lead to TNFR1 clustering (reviewed in (Li et al., 2013; Wu, 2013)). The fraction of TNF-bound receptor may therefore affect the efficiency with which
TNFR1 clusters can be formed in the plasma membrane, and therefore the efficiency with which downstream signaling, in particular, internalization-dependent caspase activation, is induced. In the future, this hypothesis could be tested by single-molecule imaging of TNF and TNFR1 on the cell surface, to quantify both the fraction of TNF-bound receptors and the clustering of TNF-bound TNFR1 while examining whether these are a determinant of whether individual cells undergo TNF-induced apoptosis. Such an approach would address the question: Does apoptosis follow a threshold rule whereby it only occurs if a cell shows a certain fraction of TNF-bound receptor or a certain fraction of clustered TNFR1?

In the context of the response of cells to a pulse of TNF, prior work by others suggested that, at least for the NF-κB pathway, cells respond in a digital, or “all-or-none” fashion. Indeed, in 2010, Tay and colleagues showed that for cultured 3T3 mouse fibroblast cells, if a single cell had TNF-induced NF-κB nuclear translocation, certain descriptors of NF-κB dynamics, such as the area of the first peak of nuclear translocation in an NF-κB time course, remained relatively constant over the entire range of concentrations (0.005-100 ng/ml) (Tay et al., 2010b). This suggests that, at the single-cell level, each cell responds to TNF in a digital, or “all-or-none” fashion. At the population level, the fraction of responding cells positively correlates with TNF concentration (Tay et al., 2010b). More recently, characterizing the NF-κB response of cultured 3T3 mouse fibroblast cells to a short pulse of LPS, they suggested that the response is digital at the single-cell level, but follows an “area-rule” at the population level (Kellogg et al., 2015). Under this area-rule, the fraction of cells that activate the NF-κB pathway is determined by the area under the TNF concentration vs. treatment duration curve, and a large fraction of responding cells can be achieved either by a short, high-concentration treatment, or a long, low-concentration treatment. However, we found that in a population of HeLa cells, a 1-min pulse of
TNF is actually more effective at killing than a 1-hr pulse of the same TNF concentration, a clear exception to an “area-rule”.

What could be factors that allow the cell death decision to break the area-rule? For one, TNF-induced autocrine signals are known to contribute to the fate decision (Janes et al., 2006) and these secreted pro-survival and pro-apoptotic factors are likely to be diluted during flow and wash-out experiments: removal of TNF at the end of the pulse would have led to removal of the autocrine factors. In vivo, although there is no “wash-out” per say, the flow of blood and interstitial fluids could analogously influence the extent and timing of TNF-induced cell death.

To understand how much TNF-induced autocrine factors contribute to the cell death decision in the context of a TNF pulse stimulation, one first step would be to compare cellular outcomes when the autocrine factors are removed (in a wash-out), to when they are allowed to remain in the culture. One interesting way in which this could be achieved would be to take advantage of the many existing TNF neutralizing reagents as a way to “inactivate” the TNF in the culture media instead of removing it. TNF neutralizing factors have been used clinically to treat inflammatory diseases such as rheumatoid arthritis, where part of the pathology is due to chronically elevated TNF levels. One example is a humanized mouse monoclonal antibody against TNF, marketed as Infliximab (reviewed in (Mpofu et al., 2005)). Another is a recombinant soluble TNF receptor coupled to a portion of a monoclonal human antibody, marketed as Etanercept (Mpofu et al., 2005). Indeed, cells can secrete their own TNF neutralizing factor in the form of soluble TNFR1, which binds TNF before it can bind to the transmembrane form of TNFR1 at the cell surface. Using such tools, one will be able to examine whether TNF-induced autocrine and paracrine signals affect cell fate decisions when cells are exposed to transient TNF stimulation.
In addition to the non-linearity in the relationship between the duration of TNF treatment and amount of cell death in a population, our work with TNF pulse treatment also showed that there is a minimum duration of treatment required to activate TNF-induced NF-κB signaling and cell death. Although our study has focused on cellular responses to TNF, similar observations have recently been made in cells treated with a cell wall component of some gram-negative bacteria – lipopolysaccharides or LPS (Kellogg et al., 2015). In that study, Kellogg and colleagues found that a 1-min, high-dose (500 ng/mL), exposure of LPS is required to activate NF-κB in 100% of cells in a population of cultured 3T3 mouse fibroblast cells. Interestingly, both LPS and TNF are pro-inflammatory signals. We know that unwarranted over- or under-activation of responses to pro-inflammatory signals can lead to undesired outcomes (inflammatory diseases, or insufficient immune response to a pathogen, respectively), yet these signals are known to fluctuate in the microenvironment (Altan-Bonnet and Germain, 2005; Gottschalk and Hathorn, 2012; Miskov-Zivanov et al., 2013). Thus, we can speculate that the requirement for a minimum duration of stimulation could be a generalized mechanism that cells use as a “filter”, to discriminate between real signals and spurious environmental fluctuations.

Although some of the mechanisms governing the response to a specific duration of TNF treatment remain to be discovered, together, our results suggest that the duration of treatment does encode information. This adds to a growing body of studies on how inputs that vary in time can affect cellular behaviors (Ashall et al., 2009; Kellogg and Tay, 2015; Lee et al., 2012; Zambrano et al., 2016). Furthermore, our observation that a short pulse of TNF can induce as much cell death as continuous treatment (for hours) also suggests that new therapeutic possibilities may exist where achieving the maximal effects on cells does not require maximizing the duration of exposure to a stimulus, or a drug. It will be interesting to see if these results can
be extended to other inducers of cancer cell death, but it certainly implies that to design effective therapy, one may need to take into consideration the dynamic profile of drug concentration.
4.2 Certain signaling pathway activities in cancer cells can be robust to changes in cell shape

In Chapter 1, I also discussed cell-to-cell variability in cellular responses to TNF, and in particular variability in TNF-induced NF-κB dynamics. One potential source of variability that was underexplored is heterogeneity in cell shape. One of my key results in Chapter 3, is that reducing heterogeneity in cell shape by micro-patterning did not significantly reduce the variability in transcript numbers for A20 and IκBα, two TNF-induced NF-κB target genes. This result strongly supports the conclusion that the effects of cell shape on NF-κB dynamics and on NF-κB-dependent transcription in cancer cells are actually quite small.

The fact that the effects of cell shape on TNF-induced signaling were small was somewhat surprising in light of recent studies linking cell geometry to several signaling outputs. In one of these studies, using mathematical modeling, the geometry of cells was reported to cause changes in the curvatures of the plasma membrane and, as a consequence, to change the distribution of activated receptors in the plasma membrane in general (Rangamani et al., 2013). For example, the model showed that the distribution of ligand bound receptors in spherical cells was homogeneous whereas that of ellipsoidal cells was inhomogeneous with a higher concentration at the tip of the cell than at the body of the cell in the case of extracellular ligand stimulation. Using micro-patterning techniques, monkey kidney tissue derived COS-7 cells were forced to take on either round or elliptical geometries. The distribution of EGFR before and after addition of EGF was monitored using fluorescently tagged EGFR in transiently transfected cells, and in both circular and elliptical geometries the EGFR distribution was consistent with the
modeling predictions. Although in this study, the authors acknowledged that differences in the distribution of activated receptors observed at the plasma membrane were small, they hypothesized that the small differences can be propagated down a signaling pathway and result in substantial differences in the downstream activities of many signaling pathways (Rangamani et al., 2013). My results show that their hypothesis cannot be generalized to all signaling that arises from ligand-receptor binding at the plasma membrane. However, my study was focused on the effects of cell shape on TNF-induced signaling in cancer cells. It is possible that stronger effects could be observed if the same experiments were carried out with primary cells as cancer cells do not always have the same responses to environmental cues as normal, healthy cells. For example, some cancer cells can survive anoikis, a programmed cell death process activated by the loss of attachment to the extra-cellular matrix (reviewed in (Gilmore, 2005; Guadamillas et al., 2011)). Therefore, it will be interesting in the future to investigate whether cell shape can play a bigger role in TNF-induced NF-κB signaling in normal healthy cells.

If heterogeneity in cell shape can only explain very little of the variability in TNF-induced NF-κB pathway activity in cancer cells, what are the other sources of variability? One approach that could be used to systematically address this question is to investigate the correlation of TNF-induced NF-κB dynamics in sister cells. If variability is primarily caused by genetic or epigenetic differences, we would expect sister cells to have nearly identical NF-κB dynamics as they should stably inherit the same genetic and epigenetic elements. Conversely, if variability is caused entirely by stochastic processes, sister cells should be no more similar to each other than random pairs of cells because any similarity in inherited factors would not govern their response to TNF. Thus, variability arising from differences in the abundance of proteins and signaling molecules (or in their activity or modification state) should produce a
distinctive form of inheritance in which the dynamics of newly born sister cells are very similar but then diverge in “older” pairs of sister cells. In these older sister cells, more proteins have turned over, and more new reactants have been made or modified and the sister cells have had a chance to diverge in their biochemical state. A study of a similar design examined the origin of variability in TRAIL-induced apoptosis and showed that variability in cell fate, and in the time to cell death for cells that die is primarily caused by differences in the abundance of proteins and signaling molecules. Furthermore, computational modeling and additional experiments showed that the contributions to this variability are multi-factorial and cannot be pinpointed to a specific protein (Aldridge et al., 2011; Gaudet et al., 2012). I suspect this may also be true for TNF-induced apoptosis because TNF and TRAIL share several of the same pathways activating apoptosis. Indeed, my preliminary results have shown that both the variability in TNF-induced cell fate and the variability in TNF-induced NF-κB dynamics are not due to purely stochastic factors (data not shown).

This study is one example of how cues from the physical environment, in addition to chemical cues, can affect cellular behavior in cancer cells (reviewed in (Charras and Sahai, 2014; Lu et al., 2012; Pickup et al., 2014; Wirtz et al., 2011)). This is of particular interests for understanding tumorigenesis and in devising effective therapies because of the growing appreciation of the differences in the physical environments of tumors compared to normal cells. For example, it has been reported that the extra-cellular matrix surrounding tumor cells can be abnormally stiff. This stiffness correlates with the malignancy of tumor cells (Mouw et al., 2014).
References


