Gene-Specific Epigenetic Regulation in Serious Infections with Systemic Inflammation

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Key Words

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Abstract

Inflammation is a fundamental biologic process that is evolutionally conserved by a germ line code. The interplay between epigenetics and environment directs the code into temporally distinct inflammatory responses, which can be acute or chronic. Here, we discuss the epigenetic processes of innate immune cells during serious infections with systemic inflammation in four stages: homeostasis, incitement, evolution, and resolution. We describe feed-forward loops of serious infections with systemic inflammation that create gene-specific silent facultative heterochromatin and active euchromatin according to gene function, and speculate on the role of epigenetics in survival.

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Introduction

The typical features of inflammation, both good and bad, were recorded in the earliest human writing [1], which also recognized that no matter what injury or in-

vading microorganism incited the process, an orderly sequence of events evolved. In fact, epigenetic changes written into the germ line explain how organisms generate different biologic responses to varying environmental factors and stress – in embryonic development, adult cell renewal, and specific diseases, such as cancer [2]. The importance of the interplay of germ line DNA and epigenetics in inflammation is still emerging [3].

The phases of inflammation differ contextually and temporally to generate the distinct host responses and outcomes that distinguish inflammatory diseases. For example, the systemic inflammatory response syndrome (SIRS) proceeds in a continuum from mild to severe forms of sepsis (or noninfectious causes) with vascular shock and/or multiorgan failure [4]. Serious infections kill millions yearly, with an unyielding mortality rate of 20–70% [5]. We refer to the phenotypes of serious infections as *severe systemic inflammation* (SSI). Here, we review the epigenetic gene-specific reprogramming, in the context of the unique pathophysiology of SSI. Readers are referred to a recent detailed review of transcription events controlling inflammatory genes, which alludes to epigenetics [6].

Several thousand innate immunity and inflammation genes may participate directly or indirectly in acute and chronic inflammatory processes [7]. In the basal (or homeostatic) state, they are repressed until a diverse recep-

Fig. 1. Gene reprogramming during serious infection with SSI. Gene expression during SSI is depicted as occurring in four temporally distinct phases: poised; incitement; evolution; resolution. Following derepression of poised promoters, induction of rapid-response proinflammatory genes incites an acute proinflammatory phase, which is rapidly terminated by posttranslational processes. The evolving phase is characterized by activated or repressed gene expression, according to function: rapid response proinflammatory genes that incited SSI are transcriptionally repressed by formation of facultative heterochromatin, and anti-inflammatory and antimicrobial genes are activated from a euchromatin state. A return to homeostasis with reversal of facultative heterochromatin occurs during the resolving phase.

tor system, primarily the Toll-like receptors (TLRs), senses a threat [8]. It signals threatened cells to rapidly transcribe a set of poised proinflammatory genes to incite the acute phase of inflammation [9]. If the threat is limited, the acute proinflammatory genes return to the basal state within hours, and physiologic resolution follows [3]. If the threat is severe, the incitement phase is replaced within 4–6 h by a gene-specific epigenetic reprogram that may last from days to weeks; we observed it in humans for up to 21 days [10]. During this stage, certain gene modules (e.g. rapid-response, acute proinflammatory) are transcriptionally silenced, while others (e.g. anti-inflammatory, antimicrobial) remain activated [11]. This predictable and sustained epigenetic paradigm produces a distinct SSI clinical phenotype and predicts poor outcome [12].

The silencing reflects the phenomenon of *endotoxin tolerance* that correlates with high mortality and morbidity, but may or may not be directly causal for this. Its rapid development probably explains the failure of multiple anti-inflammatory therapies in human sepsis, which

were given after the inciting phase [3], since epigenetics provides quick, natural protection against the excessive response and autotoxicity of acute pro-inflammatory gene products disseminated by the circulation. Note that the epigenetic silencing state apparently does not exist or, at least, persist during chronic inflammations like rheumatoid arthritis or regional enteritis [3], which, unlike SSI, benefit from anti-inflammatory therapies [13]. The reason for this paradox is unknown, but it has important therapeutic implications. We propose that distinct epigenetic mechanisms regulate acute and chronic inflammation.

Both clinical experience and systems biology provide useful contextual and temporal models for the gene reprogramming associated with SSI. We used both approaches to generate a scheme to elucidate SSI epigenetics (fig. 1). It reflects feed-forward loops directed by nuclear factor κ B (NF κ B) members p65 and RelB, discussed in detail below. Our SSI model is divided into four phases: (1) basal homeostatic control, or *poise*; (2) release of the poised state with concomitant transcription of rapid-response genes that induce SSI, or *incitement*; (3) gene-specific epigenetic reprogramming that silences facultative heterochromatin while maintaining modules of activated euchromatin, or *evolution*, and (4) reversing gene-selective reprogramming and returning to homeostasis, or *resolution*. We acknowledge its simplistic nature. For example, it excludes the posttranscriptional events that control mRNA processing and protein synthesis, both of which are also directed by epigenetic events that control expression of RNA binding proteins and inhibitory RNAs [14]. It is still useful for correlating epigenetic processes with distinct chromatin modifications. Across this spectrum, sets of genes are silenced, expressed, or unperturbed, irrespective of inducing receptors and signaling pathways, emphasizing the ultimate control of specificity by chromatin [3, 15].

Three Concepts Are Fundamental to Understanding SSI: Homeostasis, Feed-Forward Loops and Cell Survival Strategies

Homeostasis

SSI is an extreme deviation from homeostasis, almost suggesting that evolution made a serious error. However, the evolutionary intent of all inflammation, including SSI, is to defend against attack and to return to a peaceful state. No one better articulated the homeostatic disaster of SSI than Lewis Thomas in his essay on *The Lives of a*

Cell [16]: 'It is the information carried by germs that we cannot abide. Macromolecules are read by our tissues as the very worst of bad news. We are likely to turn on every defense at our disposal; we will bomb, defoliate, blockade, seal off, and destroy all the tissues of the area. It is shambles.'

Since homeostasis, as defined is dynamic, it is not surprising that opposing forces influence inflammation. Although this review focuses on nuclear-based epigenetic processes accompanying SSI, the coupling of cells to the environment during all types of inflammation depends on positive and negative regulatory processes in a variety of cell types [17]. During all phases of SSI, both positive and negative balancing mechanisms involve extra- and intracellular sensors, cytosolic and nuclear signaling pathways, and chromatin structural modifications. Extracellular controls are not covered in this review.

Feed-Forward Loops

Both inductive systems biology and reductive reasoning emphasize the importance of *feed-forward loops* linked to the germ line and epigenetic codes of biologic processes, including inflammation. Systems biology identifies feed-forward loops using a hypothesis-generating approach [18], a tactic that unexpectedly identified activated transcription factor 3 (ATF3) in an NFκB-dependent repression loop in TLR-stimulated macrophages [19]. Feed-forward loops occur in several forms [20]. Using reductive research, we identified two epigenetic loops in SSI. One is an *incoherent feed-forward loop* in which an *activator* generates a primary *target* and its repressor. In SSI, p65 activator targets tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β, representing a larger module) and feeds forward to induce RelB as a target repressor [21]. The other is a *coherent feed-forward loop* in which an activator generates a primary target module as well as an activator of a distinct target. In SSI, the activator p65 targets TNFα and IL-1β and feeds forward to induce RelB as an activator of a distinct target module, e.g. IκB inhibitor-α (IκBα) [22]. Thus, these two feed-forward loops from p65 generate RelB as a dual-function mediator of distinct targets with different functions and provide one essential mechanism for gene-specific programming that requires integrating transcription factors with epigenetic histone and DNA modifiers. In this context, the incitement phase generates both the cytokine burst and the epigenetic-dependent and gene-specific feature of evolving SSI. Resolution occurs when unknown forces reverse these loops. Other distinct loops undoubtedly exist, coupled or not to the p65-to-RelB

loops. Systems biology provides an excellent strategy to identify them [23].

Survival

The evolutionary intent of life is survival; as a fundamental biologic process, inflammation must aim to support survival. How rescue loops link SSI epigenetics to survival is unknown, but the resolution state correlates with survival and a return to homeostasis. We speculate that the balance between death (apoptosis, necrosis) and life in cells and tissues during SSI links epigenetics with cell energy and nutrition in the attempt to repair injury and survive the shambles.

The Basal State of SSI-Inducing Genes Is Epigenetically Silent but Poised

As is schematically shown in figure 2, distinct combinations of protein, histone and DNA modifiers acting on proximal promoters direct the phenotypic phases of SSI.

Most rapid-response primary-response genes constitutively bind RNA polymerase II at their proximal promoters and, in some cases, must elongate or splice mRNA to initiate a higher level of transcription [24, 25]. Recent information indicates that innate immunity genes are epigenetically repressed in the basal state but poised to respond rapidly to threats [26]. This homeostatic readiness involves a DNA complex that constitutively recognizes transcription factors *c-jun* or NFκB p50 as homodimers. They couple to nuclear receptor corepressor (NCoR), silencing retinoid acid mediators and thyroid hormone receptors (SMRT) [9].

In studies of TNFα, IL-1β, and IκBα, we added an epigenetic layer to the poised-promoter concept [22, 27]. Like most promoters of rapid-response, NFκB-dependent genes, the TNFα and IL-1β promoters constitutively bind NFκB p50 homodimers at NFκB proximal promoter sites. A detailed analysis of the TNFα gene showed concomitantly bound histone H3 lysine 9-methyltransferase G9a, high-mobility box B1 (HMGB1) chromatin structural protein, heterochromatin protein 1 (HP1), and DNA cytosine methyl transferase DNT3a/b [27]. This functional complex directs dimethylation of histone H3 lysine 9 (H3K9) (a histone code silencing mark) and repressive hypermethylation of the adjacent DNA cytosine plus guanine (CpG) sites. Conspicuous by its absence is histone H1 linker binding, which is a mark of more highly condensed chromatin [28]. Further, we find that in the basal state, a nucleosome covers the most active NFκB

Fig. 2. Distinct combinations of protein, histone and DNA modifiers acting on proximal promoters direct the phenotypic phases of SSI (component details are provided in the text). Poised: a complex of p50 homodimers, transcription corepressors and epigenetic histone-silencing modifications combine in a checkpoint to prevent constitutive transcription. Inciting: TLR-dependent processes derepress the promoters of rapid-response genes, leading to p65/p50 transactive heterodimer formation and competent transcription. Not shown is the rapid disassembly of the p65/p50 heterodimer by p65 proteasome degradation (see fig. 1 and text). Evolving: A p65-dependent feed-forward loop induces RelB de novo, after which it both initiates gene-selective facultative heterochromatin formation to silence acute proinflammatory gene promoters and activates anti-inflammatory gene promoters. Resolution is not shown but includes diminishing RelB production and reversal of chromatin modifications.

Fig. 3. Nucleosomes reposition at the TNFα promoter during SSI. The proximal promoter, about -500 bp, is depicted, showing the K2 and K3 (active) κB regulatory *cis* elements. During the poised state, nucleosome 1 covers K3. The LPS TLR4 inciting phase directs p65 binding and repositions nucleosomes 1 and 2 by a Nap1 histone 2A.Z chaperone. The RelB-initiated evolving phase repositions nucleosome 2 over the K3 κB site, where it remains after TL4 stimulation (endotoxin tolerance). The interdependent, multicomponent silencing complex that condenses chromatin by histone and DNA methylation is sustained as facultative heterochromatin during SSI evolution. RelB removal by siRNA repositions nucleosome 2 by ATP-dependent BAF complex. It is not known how naturally resolving SSI repositions nucleosomes to generate a competent promoter.

site (K3) [29], but its position differs in the evolution phase (fig. 3, discussed below).

Together, data support the conclusion that the poised state of the rapid-response genes responsible for inciting inflammation is epigenetically regulated homeostasis.

These gene-specific promoters are likely germ line encoded and emerge epigenetically during cell development and differentiation. For example, the TNFα promoter is highly CG methylated and inaccessible to NFκB activation in 3T3 murine embryo-derived fibroblasts, but environmen-

tal changes can manipulate it to become responsive to lipopolysaccharide [30]. Thus, epigenetic check points for inflammation are specific to cell-type and environment.

The Phase That Incites SSI Releases the Poised and Silent Epigenetically Controlled Basal State to Produce a Damaging Cytokine Storm That Is Rapidly Muted by the Disruption of Transcription

This phase of SSI (fig. 2) has received the most attention since the discovery of the TLR innate immunity sensor system. Highly vigilant pattern recognition receptors, such as the TLR family, rapidly deliver bad news through a network of cytosolic and nuclear signals of limited specificity, as the ultimate specificity of the response is gene-dependent and epigenetically controlled [3, 31]. As incitement begins, poised and epigenetically silenced acute proinflammatory genes derepress, allowing *trans*-acting factors to couple to cognate *cis* elements [9]. Any TLR that senses a threat can modify chromatin to link the poised promoters to mediator complex and RNA polymerase II. During this phase, the repressor complex is removed, and the active euchromatin state of demethylated H3K9, phosphorylated H3S10, and hypomethylated CpG promoter DNA occurs [27, 32]. The precise mechanisms controlling derepression remain unclear but include ubiquitination and proteasome degradation [33, 34].

Whether SSI or the milder SIRS follows the infection-induced incitement phase is ultimately determined by the magnitude of the threat and the resulting cellular and tissue-determined gene expression level [35]. All TLR sensors can probably induce SSI [36].

In the animal kingdom, the NF κ B family is the master switch for inciting acute and chronic inflammation [37]. Among these members – RelA/p65, RelB, c-rel, p105/p50, p100/p52 – RelA/p65 (p65) is the commander-in-chief. Virtually all external (e.g. extracellular pathogens or alarmins) or internal (e.g. DNA breaks or intracellular pathogens) threats, once sensed, pass through the p65/p50/I κ B cytoplasmic checkpoint, although other pathways (e.g. stress kinases and phosphatases) contribute. After cytoplasmic release by I κ B α proteasome degradation, cooperative mediators specifically modify p65 into its bound, transactive state by site-specific phosphorylation, acetylation, and methylation. An H3 serine kinase phosphorylates H3S10 to support chromatin opening and start-site competence [2]. Our unpublished observations support mitogen-activated stress kinase (MSK), I κ B kinase- α (IKK α), and/or Aurora B as

possible regulators of this H3S10 phosphorylation. Whether chromatin opening first involves p65, the histone H3S10 kinase, or another process, such as H3K4 trimethylation and H3K9 demethylation, is unclear. In any event, the epigenetic ‘histone code’ switches from transcription-negative H3K9 dimethylation to transcription-positive H3S10 phosphorylation, and poised promoters eject corepressors NCoR, SMRT, G9a, HP1, HMGB1, and DNMT3a.

The ultimate magnitude of gene expression, including protein production, is also encoded in processes that degrade mRNA – virtually all rapid-response genes are rapidly degraded and contain 3'-UTR AU-rich sequences – and/or regulate translation [38]. These mechanisms use RNA-binding proteins and micro-RNAs, whose expression is probably epigenetically controlled.

During the incitement phase of SSI, p65-dependent transcription of autotoxic TNF α and IL-1 β genes, among others, peaks within 1–3 h and then rapidly dissipates, as p65 disappears from the promoter [39]. Gene-encoded proteins also peak and wane. Termination of NF κ B-dependent gene transcription requires ubiquitination and proteasome-directed degradation of DNA-bound p65 [33]. Degradation is preceded by deacetylation by NAD-dependent sirtuin protein 1 [40] coupled to a complex containing copper metabolism Murr1 domain-containing proteins (COMMs) bound to Cullin-RING ubiquitin ligase [41]. Tight regulation is assured by rapid, p65-dependent I κ B α transcription and translation in a p65-driven feed-forward loop, providing enough newly synthesized I κ B α to resequester p65 and p50. The I κ B α promoter as a housekeeping gene is constitutively open in the homeostatic state and further activated during this phase [22]. These cooperative events quickly stop transcription of the acute proinflammatory gene module, producing the precipitous drop in the first-phase curve noted in figure 1. If the early removal of p65 is disrupted, transcription is prolonged, and the overall magnitude of gene expression increases SSI risk. Thus, both the height and width of the gene expression curve during the inciting phase of SSI determine the severity of inflammation.

The Evolution Phase Epigenetically Generates Gene-Specific Facultative Heterochromatin to Silence Transcription Selectively and Reversibly

Before Lewis Thomas colorfully described SSI as ‘shambles’ [16], Paul Beeson described and presciently interpreted endotoxin tolerance [42], later identified as an

Table 1. Epigenetic reprogramming in human and murine models of evolving SSI

Component	THP1 human monocytes	Human SSI	Murine endotoxin tolerance
Transcription repressor/activator RelB	de novo synthesis and promoter binding	de novo synthesis and promoter binding	not reported
Histone code switch	H3S10 phosphorylation to H3K9 dimethylation	H3S10 phosphorylation to H3K9 dimethylation	supported by histone deacetylation and remodeling complex
DNA CpG methylation	induced by Dnmt3a/b on TNF α promoter	induced by Dnmt3a/b on TNF α promoter	not reported
Gene selection with concomitant repression and activation	yes	yes	yes

SSI feature, and now known to be epigenetically based. Comparing Beeson's observations then and now:

Then: requires an inciting pyrogenic inflammatory phase; *Now:* requires inciting TLR-dependent NF κ B p65 transactivation, which induces repressive RelB

Then: dose-dependent phenotypes; *Now:* gene-expression amplitude induces SIRS vs. SSI

Then: cell-mediated; *Now:* blood and tissue leukocyte-mediated

Then: can last for weeks but is reversible; *Now:* can last for a day (SIRS) or weeks (SSI) but is reversible

Then: a 'remarkable' process with 'no satisfactory mechanism'; *Now:* a remarkable and SSI-distinct, gene-specific, epigenetic reprogramming code

The first nuclear mechanism responsible for the endotoxin tolerance observed in SSI to be discovered was transcriptional repression of multiple genes in circulating blood leukocytes (neutrophils and monocytes/macrophages) [10, 43]. Another important discovery showed that cellular models of endotoxin tolerance simulate many molecular features of SSI [44, 45]. 'For over two decades, our studies of the THP1 human promonocytic leukemia cell line have accurately predicted molecular events also observed in SSI blood leukocytes' [3] (table 1).

All SSI phases can be studied in THP1 cells. The gene-specific nature of the SSI and endotoxin-tolerance phenotypes, with increased and sustained expression of anti-inflammatory genes coupled to repression of acute pro-inflammatory genes, clinically became known as the compensatory anti-inflammatory syndrome [4]. Its mechanisms are still emerging but involve both transcriptional and posttranscriptional processes. For example, we found mRNA stabilization and enhanced translation of specific genes to be responsible for increased in-

terleukin 1 receptor antagonist levels during SSI [46]. This path requires a TLR4-dependent activation of phosphatidylinositol 3 kinase. Our first clue that epigenetics played a part in SSI was recognizing a nuclear NF κ B paradox: as SSI evolves, cytosolic and nuclear activation dissociate [39]. Many animal and human studies reported increased cytosolic NF κ B activation with p65 nuclear accumulation during SSI, which correlated with poor prognosis [3, 47]. This finding seemed contradictory since NF κ B-dependent, acute proinflammatory genes are repressed during SSI. To resolve the inconsistency, we analyzed p65 and p50 binding at the IL-1 β promoter during the transcriptionally silent evolving phase of SSI. We found that transcriptionally repressed leukocytes remained responsive to TLR4 stimulation by endotoxin in THP1 cells and human SSI [48], as shown by rapid degradation of cytosolic I κ B α and nuclear translocation and accumulation of p65 transcription factor. The change in cytosol signals remains unexplained. We found disrupted p65 promoter binding to IL-1 β in the silenced phenotype in both endotoxin-tolerant THP1 cells and human SSI blood leukocytes [39]. In contrast, IL-1 β and TNF α promoter binding of p50 occurs in the basal state (discussed above) and during silencing, supporting the conclusion that p50 homodimers participate in silencing acute pro-inflammatory gene expression during endotoxin tolerance and SSI [11, 49].

Our study of disrupted p65 promoter binding prompted epigenetic analysis of the evolving phase of SSI associated with endotoxin tolerance. We found that an activated histone code for H310 phosphorylation switches to repressive H3K9 dimethylation during the incitement phase [39]. This was the first hint that an epigenetic paradigm plays a crucial role in regulating gene expression during SSI.

We later implicated RelB as a negative regulator both necessary and sufficient for silencing acute proinflammatory IL-1 β and TNF α during endotoxin tolerance and human SSI [48]. This unique NF κ B family member was first characterized as an NF κ B repressor and then as a positive transcription factor that primarily heterodimerizes with NF κ B p100 or p52 [50]. RelB-deficient mice have generalized multiorgan inflammation [51]. RelB protein, which is not expressed in the basal homeostatic phase in innate immunity leukocytes, is induced as SSI evolves. Once it accumulates in the nucleus, RelB acts in a p65-dependent feed-forward loop to repress specific gene sets (fig. 2). It binds coincident retained p50 on the IL-1 β and TNF α proximal promoters in a presumed dimer switch with p65 [52], but also accumulates with promoter p50 to activate I κ B α transcription. RelB removal by siRNA reverses facultative heterochromatin and restores IL-1 β transcription in both THP1 cells and human SSI leukocytes but inhibits I κ B α transcription [22, 53]. Transfection analysis of wild-type RelB into naïve THP1 cells further establishes it as a crucially important dual regulator. The transfected RelB requires a TLR4 signal to move to promoters.

RelB *initiates* silent facultative heterochromatin at the IL-1 β and TNF α promoters by forming a complex of HP1 and G9a H3K9 dimethyl transferase [21]. The Rel homology domain directly interacts with the N terminal of G9a, which generates the histone H3K9 dimethylated silent state. HP1 binds to methylated sites, and siRNA RelB knockdown prevents formation of the facultative heterochromatin complex and transcription silencing. Together, our data support the conclusion that two feed-forward loops involving p65 and RelB target both TNF α and IL-1 β as a silenced module and I κ B α as part of an activated module (fig. 2).

We identified more components of the silencing chromatin complex associated with evolving SSI. HP1 recruitment also links to DNA methyl transferase Dnmt3a/b, which de novo methylates TNF α promoter CpG [27]. Other reports indicate that Dnmt3a/b associates with SUV39H1 histone methyltransferase, which trimethylates H3K27 and recruits polycomb group proteins to propagate condensed chromatin [2]. We have not studied polycomb group proteins, but SUV39H1 is recruited to the TNF α promoter after G9a and HP1 [27].

Nuclear structural proteins also play an important role in epigenetic silencing and formation of facultative heterochromatin. Histone 1 (H1) linker protein accumulates at repressed promoters, targeting the DNA entry site [54]. Both H1 and high-mobility group B1

(HMGB1) histone structural protein bind to linker DNA near the nucleosome dyad. In some studies, these two structural proteins have opposing effects on the nucleosome: H1 stabilizes it by ‘sealing’ two turns of DNA around the histone octamer, and HMGB1 destabilizes it, probably by bending the adjacent DNA. We find both H1 and HMGB1 in condensed TNF α chromatin during the SSI evolving phase [53]. We also made the unexpected observation that HMGB1 acts as a repressor. If HMGB1 and H1 are removed by siRNA, RelB does not bind to the TNF α promoter and facultative heterochromatin does not form. Thus, these two structural determinants play a crucial role in initiating RelB function and may place a temporary lock on facultative heterochromatin through H1. Interestingly, we do not find H1 at the poised basal promoter. Interestingly, as a cooperative nuclear repressor in SSI, HMGB1 acts as both a negative and positive regulator of inflammation when located extracellularly.

Together, our data support the conclusion that RelB and G9a combine to foster a large, interdependent, multi-molecular epigenetic complex that includes histone modifiers, DNA methyl transferases, and chromatin structural proteins (fig. 2). Other TLR-induced transcription repressor proteins induced during the evolving SSI phase that might directly participate in the epigenetic silencing have been identified. These include inducible cAMP early repressor (ICER) that binds to CRE sites [55], B cell lymphoma 3 (BCL3) that binds to p50 to block its proteasome degradation [56], and ATF3 that negatively regulates CRE sites [19]. No reports to our knowledge have performed detailed epigenetic analysis linked to the repressors, nor whether they are a part of the RelB program.

The SSI Resolution Phase Correlates with Disappearance of the Feed-Forward Loops and Resumption of the p65-Dependent Incitement Phase

Little is understood about epigenetic events in the resolution stage of SSI, except how important they are. Reversal of facultative heterochromatin to euchromatin portends rescue and survival – the end of shambles and endotoxin tolerance. Building an understanding of gene-specific reprogramming in cell models, animal models, and human SSI is therefore critical.

The course of epigenetic, gene-specific silencing varies. Humans given a low dose of intravenous endotoxin generate endotoxin tolerance, which is sustained for

about 24 h [57], when blood leukocytes again respond to TLR signals. This appears typical of SIRS. Gene-selective reprogramming can last much longer, up to several weeks in human SSI [10]. This ‘memory’ supports but does not prove that vertically passed epigenetics occurs in short-lived blood and tissue innate immune cells (e.g. neutrophils). The duration of the silencing phase in humans or animals with SSI probably varies with the magnitude of the threat, which informs chromatin how long to sustain the gene-specific facultative heterochromatin. The type of infecting organism has little effect on the stereotypic SSI process [5].

The resolution phase in the THP1 model provides an excellent way to follow the return of facultative heterochromatin to poised euchromatin. Our preliminary data support the conclusion that after 72–96 h, RelB protein levels wane, and RelB promoter binding diminishes. The cells regain their ability to bind p65 and incite transcription, although it cannot be reinduced without endotoxin stimulation of TLR4.

Understanding the Concepts Preventing versus Reversing Endotoxin Tolerance/SSI Epigenetic Gene-Selective Reprogramming Is Critical to Improving SSI Therapy

Facultative heterochromatin, by definition, is a reversible structure, unlike pericentric heterochromatin [58]. A number of reports propose reversing endotoxin tolerance. However, this interpretation is often flawed: gene silencing/tolerance were prevented, not *reversed*. Since silencing requires a p65-dependent inciting phase, any manipulation that disrupts NF κ B cytosolic or nuclear processes will prevent it. Moreover, removing a constitutive negative homeostatic regulator prior to the inciting phase increases transcription magnitude, systemic inflammation, and, consequently, mortality in animal models. This prevention paradigm likely applies to all *de novo* repressors implicated in endotoxin tolerance, including SH2 domain polyinositol-5-phosphatase-1, IL-1 receptor-associated kinase 1-M, and A20 zinc finger editor of ubiquitin RelB, ICER, BCL3, and ATF3 [3, 17]. Whether removal of these proteins *after* chromatin condenses alters or reverses silencing is a very different question.

We do find that facultative heterochromatin can be reversed after it is condensed. For example, RelB siRNA delivered during the silencing phase and the protein synthesis inhibitor cycloheximide (which produces RelB protein degradation) reverse the facultative heterochro-

matin state and restore p65-dependent transcription [submitted for publication]. However, reversing silencing to induce resolution is not simple. Inhibition of ‘classical’ class 1 and 2 (HDAC) by trichostatin enhances the incitement phase but can neither block the development of tolerance nor restore endotoxin sensitivity in the evolution phase [59]. In contrast, the protein phosphatase 1 inhibitor okadaic acid stabilizes IL-1 β mRNA to increase protein production in transcriptionally silenced cells but cannot activate transcription [60], supporting the conclusions that both transcription and posttranscription controls regulate gene expression during SSI. More work is needed to discover ways to reverse SSI gene-specific repression.

Nucleosomes Reposition during Each SSI Phase

Promoter nucleosomes adopt certain positions to regulate transcription factor binding and are displaced from promoter DNA by promoter-binding transcription factors, chromatin-remodeling complexes, and histone chaperones [61]. The selective nucleosomal organization observed in many systems provides different access to chromatin structure and different transcriptional potential between active and inactive promoters.

We recently mapped the nucleosomes on the silenced TNF α proximal promoter in the endotoxin-tolerant THP1 SSI model [29]. Two proximal nucleosomes reposition from the basal poised state to a permissive location during the lipopolysaccharide-inciting stage, and move to repressive locations in the evolution stage (fig. 3). This sequence prevents, allows, and then again prevents access to p65 during the distinct phases. Moreover, ectopic expression of RelB induction during the evolution phase promotes nucleosome repositioning to repressive locations, while RelB inhibition in tolerant cells moves the same nucleosomes to permissive locations (fig. 3). This shifting supports reformation of a poised state during resolution, although the nucleosomes’ exact locations may differ from basal homeostasis.

We also found that the ATP-independent chromatin remodeler NAP1 promotes nucleosome positioning during incitement [29]. In contrast, nucleosome repositioning during the formation of facultative heterochromatin requires the ATP-dependent BAF member of the SWI/SNF remodeling complex. Together, these results support the conclusion that TNF α promoter nucleosomes selectively and dynamically remodel during SSI phases.

The Epigenetic Human SSI Paradigm Occurs in Mice

Our investigations of SSI epigenetics exclusively involve human cells. An elegant study using macrophages derived from murine bone-marrow confirms and extends our understanding of epigenetic gene-specific regulation [15]. Using an in vitro system of lipopolysaccharide tolerance, two functionally distinct classes of TLR4-induced genes required distinct patterns of TLR-induced chromatin modifications consistent with our studies in THP1 cells and human SSI. Putative facultative heterochromatin was coupled with transient silencing of acute proinflammatory mediators and activation of antimicrobial and anti-inflammatory mediators. The functions of RelB in initiating repression and activating transcription were not investigated. Table 1 compares gene reprogramming features of THP1 cells, murine macrophages, and human SSI. It is suspected, but not proven, that murine SSI in vivo will simulate the epigenetic paradigm.

Perspective

Our understanding of epigenetic regulation of SSI is emerging, and further defining its features will inform basic biology, translational science, and clinical medicine. Observations and related questions include:

- (1) *New approaches to SSI intervention are needed.* For example, (1) Will reversing facultative heterochromatin during the evolution stage alter the outcome? (2) Do the two interventions (anti-HMGB1 and ethyl pyruvate) found to improve outcomes when administered during evolution modify gene reprogramming?
- (2) *The new signaling networks generated by gene reprogramming must be determined.* What alternative TLR4-dependent paths activate cytosolic NF κ B during the evolution phase?
- (3) *The spatial and temporal features of chronic and acute inflammation vary.* How does the epigenetic process of chronic inflammation, which does not disrupt p65 binding to silence acute proinflammatory genes, differ from SSI?
- (4) *NF κ B nuclear disruption with gene silencing may differ among compartments (e.g. liver vs. lung).* Do such compartments have tissue-specific gene reprogramming?
- (5) *SSI represses and activates genes.* What global changes in transcription and translation couple with the two RelB-dependent feed-forward loops? Do other proteins that act to negatively regulate transcription at the level of DNA (e.g. ATF3, ICER, Bcl3) act with or dis-

tinct from RelB? What epigenetic processes sustain activation? What proximal signaling networks influence each phase of SSI?

- (6) *ATP depletion and mitochondrial damage occur during the SSI incitement phase.* Is cellular energy linked to epigenetic regulation?
- (7) *Micro RNAs help to regulate inflammation.* Do they influence SSI phases?
- (8) *SSI resolution correlates with the reversal of silencing facultative heterochromatin.* What processes restore poised euchromatin?

Conclusions

An epigenetic dynamic between proteins and chromatin histones and DNA instructs gene transcription during the inciting, evolving, and resolving phases of inflammation. In serious infections with SSI, the inciting phase is like any other, except for its magnitude: TLR-dependent signals use the NF κ B master regulator to rapidly derepress, activate and quickly deactivate the epigenetically poised promoters of acute proinflammatory genes. The evolving phase of SSI, however, is distinct: feed-forward loops generated by the inciting phase epigenetically silence or activate selective genes, according to function. To silence acute proinflammatory gene transcription – a state of endotoxin tolerance – de novo induction of RelB recruits an interdependent multicomponent facultative heterochromatin complex that repositions proximal promoter nucleosomes. To activate anti-inflammatory gene transcription, dual functioning RelB recruits to responsive euchromatin. These epigenetic modifications revert as SSI resolves and homeostasis returns. Thus, epigenetics phenotypically delineate the clinical features of SSI.

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