TNF gene expression in macrophage activation and endotoxin tolerance

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TNF gene expression in macrophage activation and endotoxin tolerance

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TNF gene expression in macrophage activation and endotoxin tolerance

Abstract

TNF is an inflammatory cytokine that plays a critical role in the acute phase response to infection, and its dysregulation has been implicated in the pathology of several inflammatory and autoimmune disorders. *TNF* gene expression is regulated in a cell type- and inducer-specific manner that involves chromatin alterations at both the *TNF* promoter and distal DNase I hypersensitive (DH) sites within the *TNF/LT* locus. While the mechanisms underlying *TNF* gene activation in monocytes/macrophages and T cells have been studied intensively, the mechanisms of enhanced, repressed, and restored *TNF* gene expression in the context of classical macrophage activation and endotoxin tolerance remain largely unknown. We set out to understand how *TNF* gene expression is modulated during these biological processes by characterizing the chromatin environment of the *TNF/LT* locus.

In the context of classical macrophage activation, IFN-γ primes both circulating monocytes and tissue-resident macrophages so that, upon microbial recognition, there is an enhanced anti-microbial response; importantly, this results in enhanced *TNF* transcription. The mechanisms involved in priming are unclear; the IFN-γ-inducible transcription factors IRF1 and STAT1 have been implicated, but functional roles for these factors have not been determined. Here, we show in primary human monocyte-derived macrophages (MDMs) that IFN-γ poises the *TNF/LT* locus for enhanced *TNF* transcription by exposing the distal enhancer element hHS-8, and that the ability of this element to promote *TNF* gene expression in response to priming depends on its recruitment of IRF1. We also demonstrate that IFN-γ poises hHS-8 for enhancer function by promoting H3K27me3 enrichment, and that subsequent LPS stimulation triggers hHS-8 enhancer activation through demethylation of H3K27me3 followed by acetylation of H3K27. These experiments provide a mechanistic

explanation of how IFN-γ poises the *TNF/LT* locus for enhanced *TNF* transcription upon LPS stimulation, while at the same time providing potential targets for selective manipulation of TNF expression in primed macrophages.

In the context of endotoxin tolerance, repeated or prolonged exposure to LPS results in the reprogramming of monocytes and macrophages such that inflammatory responses, especially induction of TNF, are down-regulated; in this regard, the cell is considered tolerant or immunosuppressed. Notably, IFN-γ priming has been shown to abrogate endotoxin tolerance, thereby restoring responses to LPS stimulation. The mechanism of *TNF* gene repression during endotoxin tolerance and its restoration during IFN-γ-mediated abrogation remains unknown. Here, we have characterized the *TNF/LT* locus in endotoxin-tolerant monocytes and macrophages and found the *TNF* promoter to be nuclease accessible and enriched for H3K27me3 during repressed transcription. We have also found that IFN-γ priming restores *TNF* gene expression in an IRF1-independent manner, suggesting that IFN-γ primes non-tolerant and endotoxin-tolerant monocytes and macrophages by two distinct mechanisms; furthermore, we observed enrichment of H3K27ac at the *TNF* promoter and the appearance of a DH site ~4kb upstream of the *TNF* TSS in response to IFN-γ treatment of endotoxin-tolerant cells. These results suggest that chromatin modifications at the *TNF* promoter and distal DNA elements play an important role in IFN-γ-mediated abrogation of endotoxin tolerance.

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Chapter 1

Introduction

1.1 Tumor Necrosis Factor (TNF)

The effects of TNF have been documented since the early 1890s when patients with malignant tumors were observed to undergo spontaneous tumor regression following acute bacterial infections [1]. In 1962, it was suggested that bacterial lipopolysaccharide (LPS)-induced tumor necrosis requires additional factors, as evidenced by the fact that LPS treatment did not kill tumor cells *in vitro* [2]. Finally, in 1975, Carswell and co-workers from Lloyd Old's group formally identified TNF in the serum of *Mycobacterium (M.) bovis* strain Bacillus Calmette-Guérin (BCG)-immunized mice treated with LPS [3]. They demonstrated that TNF-positive serum inhibited the growth of two tumor cell lines: Meth A sarcoma and L929. These results were later confirmed when recombinant TNF (rTNF) was shown to induce hemorrhagic necrosis of transplanted Meth A sarcomas in mice [4]. Soon after, rTNF was administered to tumor-bearing patients, but side effects were severe and reminiscent of symptoms associated with sepsis; patients were reported to experience headache, nausea, vomiting, fever, rigors, anorexia, and diarrhea—all signs of systemic bacterial infection [5]. These findings marked the beginning of a new stage in TNF research, with focus on TNF as primarily a mediator of host defense and inflammation.

1.1.1 TNF in infectious disease

TNF, in the context of infectious disease, elicits an array of cellular responses to bacterial, parasitic, and viral pathogens. It is a potent activator of endothelial cells, which line the surfaces of the skin, respiratory tract, gastrointestinal tract, oral cavity, genitourinary tract, and eye. During infection, TNF triggers endothelial cells to secrete soluble (e.g., IL-8, MCP-1, IP-10) [6–8] and cell surface molecules (e.g., ICAM-1, VCAM-1) [9, 10] that promote the accumulation of leukocytes and inflammatory mediators to sites of infection. By increasing local blood flow and vascular permeability, TNF consequently induces the four cardinal signs of inflammation: heat, pain, redness, and swelling [11]. In addition to its local effects, TNF is an endogenous pyrogen that induces fever via its effects on the hypothalamus [12]. Notably, TNF induces the expression of IL-1, which is a significantly more potent pyrogen than TNF [12, 13].

The creation of mice deficient for TNF signaling led to further insights into TNF biology. For example, studies using mice with a disruption in the *TNF* gene (*Tnf*-/-) have demonstrated a critical role for TNF in granuloma formation during *M. tuberculosis* infection. Granulomas are organized structures consisting of macrophages, epithelioid cells, foam cells, and multinucleated giant cells that are surrounded by a rim of lymphocytes; the granuloma's primary function is to contain the bacteria, thereby preventing their dissemination [14]. In *Tnf*-/- mice, T cells were found to migrate to the lung but failed to localize in infected alveoli and participate in granuloma formation. In addition, *Tnf*-/- mice succumbed to a low dose of aerosolized *M. tuberculosis* that did not cause mortality in wild-type mice [15].

Additional studies have demonstrated that mice deficient in the TNF receptor (*Tnfr1*-/-) were severely impaired in clearing the intracellular bacteria *Listeria (L.) monocytogenes* and *Salmonella typhimurium*, and readily succumbed to infection [16, 17]. TNF signaling is also critical for control of non-bacterial pathogens such as the intracellular parasite *Leishmania*

(L.) major; *Tnfr1*-/- mice inoculated with *L. major* were able to clear infection but failed to resolve lesions, suggesting a key role for TNF in healing lesions [18]. Finally, herpes simplex virus-1 (HSV-1) infection was lethal in *Tnfr1*-/- mice, and, compared to wild-type animals, *Tnfr1*-/- mice had significantly higher viral loads in the brain, suggesting a role for TNF signaling in controlling HSV-1 replication in the central nervous system [19]. In conclusion, multiple studies using mice deficient in TNF signaling have demonstrated the importance of TNF in host responses to bacterial, parasitic, and viral infections.

1.1.2   TNF dysregulation and its clinical implications

When over-expressed, TNF is a major mediator of the pathogenesis associated with several inflammatory and autoimmune disorders; these include rheumatoid arthritis (RA), inflammatory bowel disease (IBD), multiple sclerosis, plaque psoriasis, ankylosing spondylitis, and sepsis [20]. Although the pathogenic cause of most chronic inflammatory diseases is unknown, the classic inflammatory responses promoted by TNF are thought to account for chronic inflammatory pathology and tissue damage. For example, RA is characterized by lymphocyte activation in the peripheral blood, synovium, and synovial fluid; this causes inflammation in the joints and surrounding tissues. Examination of synovial fluid from symptomatic joints of RA patients showed the presence of active TNF [21], and stimulation of synovial fibroblasts with TNF resulted in the production of collagenase and prostaglandin E2, which are known to mediate bone resorption by osteoclasts [22]. In addition, chronic over-production of TNF in mutant mice lacking the TNF AU-rich elements (TNF$^{\Delta ARE}$) corresponds with inflammatory arthritis. AU-rich elements are sequences that help regulate TNF mRNA degradation and translational repression in hematopoietic and stromal cells [23]. Joints of TNF$^{\Delta ARE}$ mice exhibited features of chronic symmetrical inflammatory polyarthritis; indeed, both early and later stages of disease displayed characteristics comparable to human rheumatoid arthritis [24].

Anti-TNF therapy has been in clinical use since 1998 and has been overwhelmingly successful for the treatment of inflammatory diseases, particularly RA and a type of IBD known as Crohn's disease (CD). Two anti-TNF antibodies (infliximab and adalimumab) [25, 26] and a soluble TNF receptor fusion protein (etanercept) [27] bind and neutralize both soluble and membrane-bound TNF. These three biologics fuel a major part of the $20+ billion global market for TNF inhibitors and have been used to treat more than 2 million patients worldwide. Two novel TNF inhibitors, certolizumab pegol [28] and golimumab [29] have recently entered the market and have been approved for the treatment of CD and RA, respectively [30].

CD is the other major inflammatory disorder treated with TNF inhibitors; it is a type of IBD, along with ulcerative colitis [31]. CD is a chronic gastrointestinal disorder characterized by persistent disturbances between the commensal bacteria of the normal microbiome and the gut lumen, resulting in the induction of mucosal inflammation. TNF was first linked to the inflammatory responses of CD when higher levels of TNF were found in the mucosa and stools of CD patients as compared to those of healthy individuals [32]. In addition, TNF-positive macrophages were significantly increased in the lamina propria of CD intestinal specimens than that of control specimens [33]. These and other studies led to the 1998 US FDA approval of infliximab as the fist monoclonal antibody for CD treatment [34].

Although anti-TNF therapy has been extraordinarily successful in the last two decades, there are certainly adverse effects associated with global ablation of such a critical, non-redundant cytokine. Patients receiving TNF inhibitors have an increased risk for intracellular bacterial infections, including Mycobacteria [35], Listeria, Salmonella, and Legionella [36]. In fact, patients are required to undergo latent tuberculosis (TB) screening before initiating anti-TNF therapy; this is done as an effort to reduce the risk of TB reactivation [37].

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In an effort to address the complications following systematic and continual TNF inhibition, recent studies have investigated the effects of selectively inhibiting TNF secreted from specific cell types [38]. The motivation is to primarily inhibit "pathogenic TNF" while sparing TNF that is essential for effective host defense. One study using mice deficient for TNF expression only in myeloid cells (i.e., monocytes, macrophages, neutrophils) showed that TNF produced by myeloid cells has a critical, non-redundant role in the early stages of *M. tuberculosis* infection but becomes redundant in the later stages of infection [39]. These mice were able to control chronic infection by recruiting activated, TNF-producing CD4$^+$ and CD8$^+$ T cells. In contrast, mice deficient for TNF expression only in CD4$^+$ and CD8$^+$ T cells were able to contain early *M. tuberculosis* infection but not persistent infection. Although granulomas were established, mice deficient for TNF in T cells were unable to efficiently recruit effector T cells to granulomas, thereby impairing the structural and functional integrity of granulomas during chronic infection. The authors conclude that TNF expressed from myeloid cells is primarily responsible for early immune function and that TNF expressed from T cells is essential to sustain protection against chronic TB infection. Thus, inhibition of TNF derived from T cells during latent *M. tuberculosis* infection may promote TB reactivation, suggesting that novel therapies for autoimmune diseases that are directed against myeloid-derived TNF would be preferable in latently infected TB patients.

In addition to directly suppressing TNF that is secreted from specific cellular sources, intracellular signaling mechanisms responsible for TNF production are also attractive targets for efforts aimed at developing more specific and less toxic anti-TNF therapies. In this regard, it is important to understand the mechanisms involved in regulation of TNF expression in various cell types at the level of both signal transduction pathways and chromatin, which was the overarching goal of this work.

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1.2 Role of IFN-*γ* in macrophage function

A series of studies in the 1960s that examined *L. monocytogenes* infection in BCG-immunized mice introduced the theory that macrophage activation requires a humoral factor released by lymphocytes in the presence of a specific antigen [40]. This unknown factor was later named IFN-*γ* [41]. In the 1980s, it was established that IFN-*γ* and macrophage-activating factor (MAF) are one and the same when IFN-*γ* was shown to prime murine peritoneal macrophages for enhanced antiviral activity and LPS-induced tumor cell killing [42–44]. Since then, IFN-*γ* priming has been shown to enhance various monocyte and macrophage functions, including pathogen recognition, antigen presentation, microbial killing, receptor-mediated phagocytosis, and promotion of CD4$^+$ T cell differentiation toward a Th1 phenotype [45].

1.2.1 IFN-*γ* priming

Classical macrophage activation

Natural Killer (NK) cells, Th1 lymphocytes, and CD8$^+$ cytotoxic lymphocytes were originally thought to be the exclusive sources of IFN-*γ*; however, subsequent studies have demonstrated IFN-*γ* secretion from other cell types, including B cells, NKT cells, and antigen-presenting cells (APCs) [46–50]. In the early stages of bacterial infection, macrophages secrete chemokines and cytokines (i.e., IL-12 and IL-18) that both attract NK cells to the site of inflammation and induce their production of IFN-*γ* [51]. In addition, IL-12 and IL-18 drive Th1 differentiation of antigen-specific naïve T cells, which also go on to secrete IFN-*γ* [52]. IFN-*γ* subsequently converts resting macrophages into potent microbial killers, a process known as classical macrophage activation. Many TLR agonists, like LPS, are known to effectively activate macrophages without the assistance of IFN-*γ* [53], but the action of IFN-*γ* priming is essential for robust innate immunity characterized by potent macrophage

activity. Most notably, IFN-γ primes monocyte and macrophage responses to TLR agonists, thereby augmenting the transcriptional expression of critical inflammatory mediators such as TNF [54], IL-12 [55, 56], IL-6 [57], and NO [58]. In one study, relatively increased TNF mRNA levels were detected in LPS-stimulated primary human monocytes primed with IFN-γ as compared to unprimed cells [59]. The authors also showed that pretreatment of monocytes with IFN-γ for 2h was sufficient for augmented TNF mRNA levels, and that the effects of IFN-γ lasted for up to 40h after treatment. Similar findings were reported for IL-12 p35 mRNA levels in human PBMCs [60], IL-6 mRNA levels in the human monocytic cell line THP-1 [57], and NO mRNA levels in a mouse macrophage cell line [58]. A major goal of this work was to determine the mechanism responsible for IFN-γ priming, which, until now, has been unclear.

The importance of IFN-γ for innate immunity is evidenced by the clinical outcomes of patients lacking IFN-γ, its receptor, or its key signaling molecules. It has been observed in clinical studies that patients with disrupted IFN-γ signaling show increased susceptibility to mycobacterial infection; in particular, a deletion of nucleotide 131 in the gene for IFN-γ receptor 1 (*IFNGR1*) that results in a frame-shift and loss of IFNGR1 surface expression was found in a child with fatal idiopathic disseminated BCG infection [61]. In a similar study, a mutation (395A>C) in *IFNGR1* was associated with severe mycobacterial infections in four Maltese children [62]. This mutation led to decreased surface expression of IFNGR1 in PBMCs. The authors speculated that increased susceptibility to mycobacterial infection in the affected patients was primarily due to macrophage impairment, particularly impaired macrophage activation. Notably, PBMCs from the affected patients were stimulated with IFN-γ and LPS, and IFN-γ was unable to enhance TNF levels in patient PBMCs, as compared to control cells. Other children with disseminated atypical mycobacterial infection, who also had impaired IFN-γ production, exhibited defective TNF production in response to IFN-γ and LPS [63]. In conclusion, understanding the mechanisms responsible

for IFN-γ-induced macrophage activation has clinical implications for host defense against mycobacteria and a number of other intracellular organisms.

Endotoxin tolerance and its abrogation

Formally, endotoxin tolerance is the phenomenon in which immune cells, primarily monocytes and macrophages, transiently become hyporesponsive or "tolerant" upon repeated or prolonged exposure of LPS [64]. Physiologically, endotoxin tolerance is considered to be a negative feedback response that protects the host against uncontrolled inflammation, as seen in sepsis and severe tissue injury. One of the earliest documentations of endotoxin tolerance reported reduced fever responses in rabbits undergoing daily injections of typhoid vaccine [65–67]. This phenomenon of endotoxin tolerance was characterized as transient, evidenced by the finding that 3 weeks without injections rendered the rabbits responsive again. Decades later, induction of endotoxin tolerance in mice was shown to be a macrophage-mediated response [68]. Specifically, when LPS-resistant mice (later found to be defective for the LPS signaling receptor) were given LPS-sensitive macrophages from a wild-type mouse, they became susceptible to lethal challenges of LPS. In addition, when a group of these transplanted mice were first challenged with non-lethal doses of LPS and then subjected to a lethal dose of LPS, all mice survived, thereby demonstrating that macrophages are the primary mediators of endotoxin tolerance. Subsequent *in vitro* studies have shown induction of endotoxin tolerance in human monocytes and macrophages with reduced TNF expression as the primary readout [69–71].

Though endotoxin-tolerant monocytes and macrophages are often characterized as un- or hyporesponsive, these cells do adopt "active" mechanisms of control as well. In particular, gene expression of anti-inflammatory cytokines like IL-10, TGF-β, and IL-1RA was shown to be enhanced in monocytes from sepsis patients, as compared to those from healthy donors [72]. Another study observed increased internalization of *Escherichia coli* cells in

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endotoxin-tolerant monocytes as compared to non-tolerant monocytes; additionally, higher levels of the surface receptor CD64, a well-known marker for phagocytosis, were found on endotoxin-tolerant monocytes as compared to non-tolerant monocytes [71]. Together, these results discredit the picture of endotoxin tolerance as macrophage "immunoparalysis".

Notably, IFN-γ has been shown to prevent the induction of endotoxin tolerance in human monocytes and macrophages. Co-treatment with recombinant IFN-γ during an initial challenge of LPS was shown to prevent endotoxin tolerance in Mono-Mac-6 cells, as evidenced by a restored induction of TNF mRNA levels upon a second challenge of LPS; notably, this was not observed in response to IFN-α and IFN-β priming [73]. Whether IFN-γ priming has a similar effect in both resting and endotoxin-tolerant monocytes is unknown. Indeed, the mechanisms responsible for endotoxin tolerance and IFN-γ-mediated abrogation remain unclear, and a major goal of the work described herein was to elucidate molecular mechanisms involved in these processes.

1.2.2 Crosstalk between IFN-γ and LPS signaling pathways

Components of the IFN-γ and LPS signaling pathways interact in various ways to enhance macrophage responses, as seen in resting monocytes and macrophages primed by IFN-γ [74]. The individual IFN-γ and LPS signaling pathways, and the interplay between components of the two signaling pathways, are described below.

IFN-γ signaling pathway

Signal transducer and activator of transcription-1 (STAT1) is the primary mediator of IFN-γ signaling [75]. IFN-γ binds to the IFN-γ receptor (IFNGR1), leading to the trans-phosphorylation and activation of receptor-associated janus kinase (JAK) 1 and JAK2 [76] (Figure 1.2.1). JAK1 then phosphorylates a subunit of IFNGR1 at tyrosine residue 440 (Y440) [78], thereby creating a docking site for STAT1. Via its SH2 domain, STAT1 binds

9
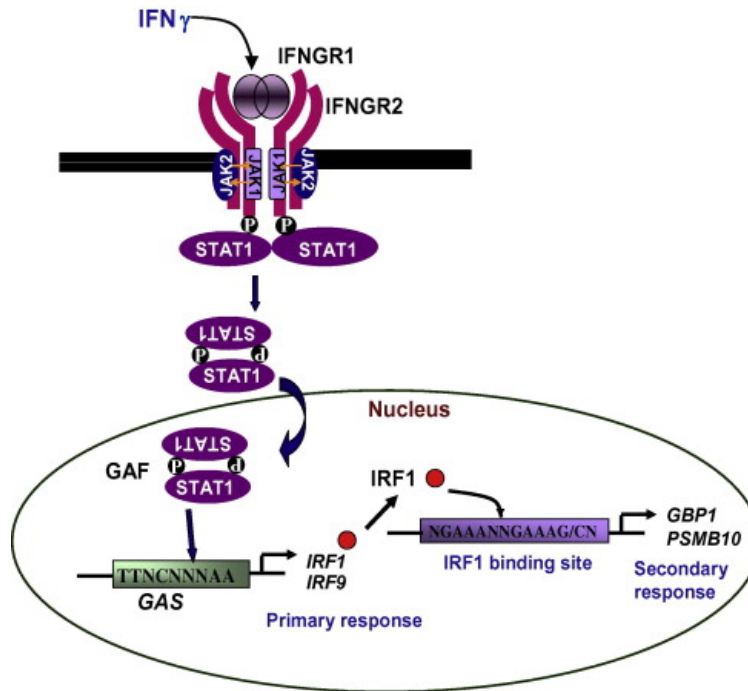
Figure 1.2.1: The IFN-γ signaling pathway. IFN-γ signaling activates the classical JAK/STAT pathway leading to STAT1 homodimerization and nuclear translocation. STAT1 binds to gamma-activated sequence (GAS) and activates the transcription of several primary response genes, including *IRF1*. Courtesy of (Saha, 2010) [77]

to the receptor and is then activated by phosphorylation of its tyrosine residue 701 (Y701) [79, 80]. Phosphorylated STAT1 homodimerizes, translocates to the nucleus, binds to a DNA element called gamma-activated sequence (*GAS*), and activates transcription of several primary response genes [77]. Notably, Stat1-/- and Ifng-/- mice have similar phenotypes in that they are non-responsive to both IFN-α and IFN-γ and are highly susceptible to microbial infection [75, 81].

One major primary response gene that is activated by IFN-γ signaling encodes Interferon Regulatory Factor 1 (IRF1). The IRF family consists of nine transcription factors that play pivotal roles in IFN-mediated immune responses [82]. IRF1 mediates the activation of several IFN-γ-inducible genes, including *NOS2*, *CASP1*, *COX2*, and *CIITA*. Interestingly, *Irf1-/-* mice exhibit impaired production of IL-12, which is an inducer of IFN-γ in T lymphocytes and NK cells [83]. Indeed, IRF1 recruitment to the *IL12A* promoter was shown

10
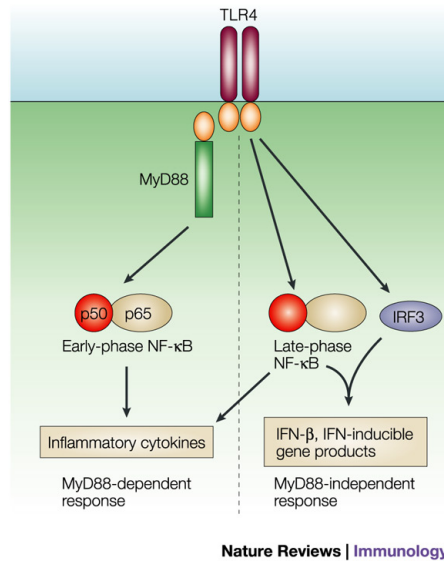
Figure 1.2.2: The TLR4/LPS signaling pathway. LPS signals through the TLR4 receptor and activates a MyD88-dependent or MyD88-independent pathway. The MyD88-dependent pathway involves NF-κB activation while the MyD88-independent pathway involves IRF3 activation and IFN-β expression. Courtesy of (Akira, 2004) [90]

to be necessary for enhanced IL-12 expression in monocytes primed by IFN-γ [84]. Thus, IFN-γ induces a positive feedback loop that is mediated by IRF1.

TLR4/LPS signaling pathway

With the help of several molecules, LPS binds to the extracellular domain of TLR4 and induces a signal transduction cascade that ultimately promotes (and suppresses) the expression of hundreds of genes [85]. Efficient recognition of LPS by TLR4 requires the co-receptor CD14 [86], LPS-binding protein (LBP) [87], and the accessory molecule MD-2 [88]. Signaling events downstream of TLR4 occur in either a MyD88-dependent or independent manner [89] (Figure 1.2.2). Importantly, production of TNF, IL-1β, and IL-6 in response to LPS was completely abolished in MyD88-deficient mice, highlighting the importance of this adaptor protein for expression of a subset of inflammatory genes [91]. Once MyD88 associates with TLR4, it recruits members of the IRAK family such as IRAK1, IRAK2, and IRAK4 [92]. IRAK4 phosphorylates IRAK1 [93], leading to their dissociation

from the receptor. IRAK1 binds TRAF6 [94] inducing the formation of a membrane-bound complex comprising IRAK1, TRAF6, TAK1, TAB1, and TAB2. TAK1 then activates IκB kinases (IKKs), which then form a complex, phosphorylate, and degrade IκB, an inhibitor of the transcription factor NF-κB. This results in the activation and nuclear translocation of NF-κB [95]. There are five subunits of the NF-κB family, but the subunits p50 and p65 associate to form the canonical NF-κB heterodimer, in which p50 binds to cognate DNA sequences and p65 activates transcription [96]. In addition to NF-κB, other important transcription factors like AP-1 and IRF5 are activated in a MyD88-dependent fashion [97].

Interestingly, there is a subset of LPS-inducible genes whose expression is not impaired in MyD88-deficient macrophages; these genes are induced via a MyD88-independent mechanism that requires IFN-β [98]. Upon LPS binding, TRAM is recruited to TLR4, resulting in the recruitment of TRIF [99, 100]. Subsequent events involving either RIP1 or TRAF6 lead to activation of NF-κB and AP-1 while events involving TBK1 lead to IRF3 activation by phosphorylation [90]. IRF3 and NF-κB assemble at the *IFNB* enhancer with other transcription factors and coactivators, thereby mediating the induction of *IFNB* gene expression. IFN-β, in turn, activates STAT1, which, as outlined above, leads to the induction of various inflammatory genes.

IFN-γ regulates TLR4/LPS signaling components

IFN-γ upregulates the expression of several TLR4/LPS signaling components. In human monocytes and macrophages, IFN-γ treatment was shown to augment TLR4 mRNA and TLR4 surface expression, as well as expression of crucial components of the receptor complex (i.e., MD-2 and MyD88) [101, 102]. In addition, LPS-induced phosphorylation of the downstream adaptor protein IRAK1 was significantly increased in endotoxin-tolerant monocytes treated with IFN-γ as compared to cells that were not treated with IFN-γ [103]. One study showed that treatment of monocytes with IFN-γ promoted IκB degradation and,

in turn, enhanced the DNA binding activity of NF-κB via EMSA analysis [104]; however, in another study, it was reported that IFN-γ does not affect IκB degradation but instead increases the expression of the NF-κB subunit p65 [105]. In any case, these combined data indicate that IFN-γ sensitizes the macrophage to LPS by upregulating crucial components for the receptor complex and by enhancing the activity of downstream signaling components.

In addition to enhancing TLR4/LPS signaling, IFN-γ suppresses feedback inhibitory mechanisms induced by TLR4/LPS signaling. IL-10 is a major anti-inflammatory cytokine that inhibits the activity of monocytes and macrophages, particularly the LPS-induced production of inflammatory cytokines [106]. IFN-γ has been shown to repress IL-10 production by inhibiting the transcription factor CREB, a critical component for *IL10* transcription [107]. Specifically, IFN-γ promotes phosphorylation and subsequent activation of glycogen synthase kinase 3β (GSK3β), a kinase that inhibits the function of AP-1 and CREB. The mechanism responsible for IFN-γ-mediated phosphorylation of GSK3β remains unclear.

Another mechanism by which IFN-γ inhibits anti-inflammatory responses to LPS stimulation involves the disruption of TGF-β signaling. Smad3 is an essential mediator of TGF-β signaling and induction of TGF-β-inducible genes. IFN-γ has been shown to impair Smad3 activation by (i) inhibiting its phosphorylation and subsequent nuclear translocation and (ii) inducing Smad7, an antagonistic Smad that prevents Smad3 interaction with the TGF-β receptor [108]. In conclusion, IFN-γ enhances innate immune activation by LPS via mechanisms that both promote inflammatory signaling processes and inhibit anti-inflammatory responses [109].

LPS regulates IFN-γ signaling components

As outlined above, IFN-γ augments LPS-induced signaling and the inflammatory response. At the same time, LPS has been shown to enhance the effects of IFN-γ stimulation

by increasing STAT1 phosphorylation at serine 727 (S727). Specifically, LPS stimulation in murine macropahges was shown to dramatically increase STAT1 phosphorylation at S727 at levels comparable to macrophages stimulated with IFN-γ [110, 111]. Again, STAT1 goes on to bind *GAS* and activate transcription of numerous IFN-γ-inducible genes. In addition to STAT1, LPS also activates the transcription factor PU.1 by phosphorylating its serine residue 148 (S148). PU.1 is a member of the Ets family of transcription factors and plays a pivotal role in macrophage maturation. Notably, PU.1 associates with IRF8, thereby forming a complex that binds *GAS* and enhances the expression of some IFN-γ-inducible genes [112].

1.3   Role of chromatin in inflammatory gene regulation

Identifying and characterizing signal transduction pathways is undoubtedly an essential process for defining regulatory mechanisms of the inflammatory response; however, recent studies have argued that chromatin status must be considered when explaining the selective nature of these mechanisms. In most instances, pathogen recognition by innate immune components results in the simultaneous transcriptional induction and inhibition of hundreds of genes whose chromatin environments are unique at each individual locus. Thus, it is critical to understand the many factors that comprise chromatin and regulate its conformational status in order to fully define the mechanisms responsible for inflammatory gene expression. When characterizing chromatin, several factors must be considered. These include DNA accessibility, transcription factor occupancy, histone modifications, DNA methylation, and higher-order organization.

1.3.1   Chromatin accessibility

In eukaryotes, DNA is packaged into chromatin, whose basic repeating unit is the nucleosome [113, 114]. Each nucleosome consists of ~146bp of DNA wrapped around an octamer

of four core histones [115]. When packaged into chromatin, DNA is relatively inaccessible to the TATA-binding protein and other factors that help mediate transcription; thus, nucleosomal DNA is considered to be repressive to gene expression. Importantly, regions of accessibility are created by the loss or remodeling of nucleosomes, which, in turn, provide docking sites for transcription factors and coregulators that bind DNA. Consequently, chromatin accessibility marks regions of DNA that function as regulatory elements such as promoters, enhancers, silencers, insulators, and locus control regions [116].

To detect regions of DNA accessibility in chromatin, investigators have traditionally relied on methods derived from the early finding that genomic regions that are accessible to transcription factors have a pronounced sensitivity to nuclease digestion, especially DNase I digestion [117, 118]. In fact, these regions are typically two orders of magnitude more sensitive to DNase I than nucleosomal DNA [116]. Pairing DNase I digestion with assays like Southern blotting, quantitative-PCR (qPCR), or DNA sequencing allows for the identification of regions that are accessible or hypersensitive to DNase I. These regions are termed DNase I hypersensitive (DH) sites and are usually markers of regulatory DNA elements [119, 120].

Within a given cell type, DH sites can either be constitutive or inducible [116]. Constitutive DH sites, as their name suggests, are independent of gene expression. Generally, these sites comprise promoter regions and are considered to be poised for transcriptional induction. In contrast, inducible DH sites appear prior to transcription and may persist long after the inducing agent is removed. One of the first inducible DH sites to be identified is in the *IL2* locus, which encodes IL-2, a pleiotropic cytokine produced by activated T cells [121]; human peripheral blood T cells were stimulated with mitogen, and an additional DH site appeared within the *IL2* promoter region. Since then, inducible DH sites have been found to not only be associated with transcriptional activation but also with transcriptional repression, demonstrating the diverse regulatory functions that individual DH sites can serve.

Recent studies have attempted to identify all DH sites in a given tissue or cell type with the aim of understanding cell type- or disease-specific regulatory networks. The Encyclopedia of DNA Elements (ENCODE) project has identified ~2.9 million unique, non-overlapping DH sites within the human genome [122]. A small proportion (~5%) of these sites localize to regions within 2.5kb of a transcriptional start site (TSS), while the majority of sites localize to regions greater than 2.5kb but within 250kb of a TSS. When classifying sites into either promoter or distal DH sites, the ENCODE authors designated any site found to be within 1kb upstream of a TSS as a promoter DH site. In addition, ~34% of these sites were specific to a single cell type while ~14% were detected in all 29 cell types examined. Interestingly, promoter DH sites generally were accessible across cell types in contrast to distal DH sites, which were found to be more limited to specific cell types. The differences in the degree of cell type specificity between promoter and distal DH sites highlight the importance of distal regulatory elements in the control of cell type-specific gene expression.

Notably, a vast majority of the distal DH sites exhibited chromatin features specific to enhancers. By definition, an enhancer is a small segment of DNA that augments transcription driven by its target promoter, which can be anywhere from several to hundreds of kilobases away [123]. For example, the limb bud enhancer for the mouse Sonic hedgehog (Shh) gene is located more than 1Mb from the *Shh* promoter [124]. Given the extreme variability among known enhancers in distance, orientation, and position relative to the target promoter, it is difficult to propose a single mechanism for enhancer function.

A representative example of enhancer complexity is found at the *IFNB* locus. Gene expression of IFN-β, an anti-viral cytokine primarily involved in innate immunity, utilizes a virus-inducible enhancer that is one of the best-characterized eukaryotic enhancers [125]. Proximal to the *IFNB* promoter, the enhancer is a 50bp element located ~100bp upstream of the *IFNB* TSS. The enhancer is subdivided into four positive regulatory domains (PRDs) designated PRDI, PRDII, PRDIII, and PRDIV; together, in response to appropriate stimula-
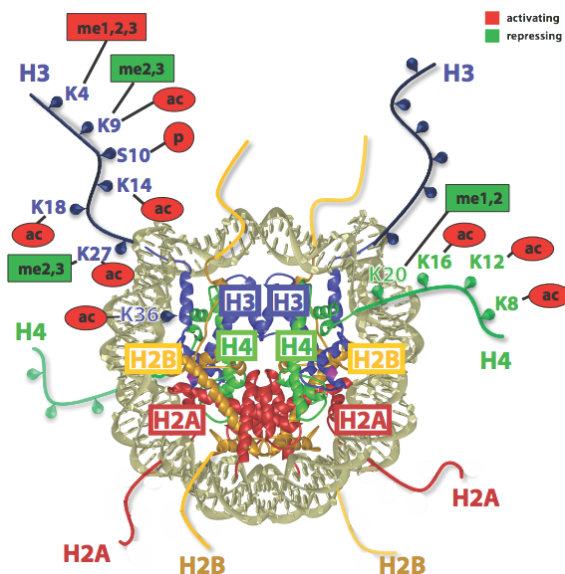
Figure 1.3.1: Structure of the canonical nucleosome. The histone octamer, illustrating the position of the N-terminal histone tails that are targets of post-translational modifications. Histones H3, H4, H2A, and H2B are shown in blue, green, yellow, and red, respectively. Diagram of a 2.8Å resolution structure [128, 129]. (Protein Data Bank code 1AOI) Kindly provided by Karolin Luger.

tion, these elements recruit a combination of factors that cooperatively bind and ultimately increase chromatin accessibility at the TATA box [126]. The *IFNB* enhancer provides one model of how chromatin accessibility is increased by a regulatory element; indeed, there are a variety of mechanisms that can be used to remodel chromatin.

1.3.2 Chromatin remodeling

Histone modifications

Considering the importance of chromatin accessibility for gene activation, many studies attempt to answer how chromatin is remodeled. Chromatin remodeling can be defined as any event that alters nuclease sensitivity of a region of chromatin [127]; one such event is the covalent modification of histones. There are five major families of histones: H1, H2A, H2B, H3, and H4. Typically, a nucleosome comprises two H2A/H2B heterodimers and an H3/H4 tetramer (Figure 1.3.1). Between each nucleosome core lies a stretch of linker DNA

ranging from 10 to 90bp that is either naked or bound by histone H1. Extending from the core domains are histone tails, which are often subjected to post-translational modifications like acetylation, methylation, ubiquitination, and phosphorylation; in fact, over 100 distinct histone modifications have been described [130].

Reversible histone acetylation was first proposed to be associated with transcriptional activity in eukaryotic cells in 1964 [131, 132]. This process locally relaxes chromatin by removing the positive charge on histone tails, thereby decreasing their affinity for the negatively charged phosphate groups of DNA. Consequently, histone acetylation promotes access to DNA for transcriptional regulatory factors. Histone acetyltransferases (HATs) transfer an acetyl group to the lysine residues of N-terminal histone tails, while histone deacetylases (HDACs) remove the acetyl group[133].

Histone acetylation has an important role in the activation of inflammatory genes. One such example is the gene *CSF2* encoding granulocyte-macrophage colony-stimulating factor (GM-CSF), which is an inducible cytokine that functions to promote a Th1-biased immune response. Increased *CSF2* gene expression involves acetylation of lysine 8 and lysine 12 of histone 4 (H4K8ac and H4K12ac, respectively) at the human *CSF2* promoter [134]. Importantly, a specific stimulus can induce gene expression at different loci through distinct patterns of histone acetylation. For example, infection of human endothelial cells with *L. monocytogenes* was shown to upregulate both *IL8* and *IFNG* gene expression; however, increased H3ac and H4ac levels were only observed at the *IL8* promoter and not at the *IFNG* promoter [135].

Unlike acetylation, histone methylation does not chemically alter the charge of histone tails; rather, the effects of mono-, di-, and trimethylation of lysine and arginine residues are thought to indirectly affect gene regulation. Methylation of particular lysine residues is associated with gene activation (H3K4) and repression (H3K9 and H3K27) [136]. In the case of H3K9, trimethylation appears to enhance nucleosome stability and promote

heterochromatin formation by increasing the affinity of adapter proteins to the histone tail. Specifically, Swi6, a yeast ortholog of heterochromatin protein 1, forms a complex with H3K9me3-modified tails that ultimately facilitates heterochromatin spreading [137]. In the case of H3K27, trimethylation increases the affinity of the chromodomain of the Drosophila Polycomb protein for histone tails, which is implicated in the maintenance of gene silencing [138, 139]. The precise mechanisms of Polycomb-mediated repression remain unknown.

The balance of histone methylation is maintained by the interplay between histone methyltransferases and demethylases. EZH2, a subunit of Polycomb repressor complex 2, is a histone methyltransferase that has been shown to catalyze methylation of H3K27 [140–142]. JumonjiD3 (JMJD3) and UTX, two related histone demethylases specific to H3K27me3 [143–146], were shown to regulate inflammatory responses in the context of macrophage activation [147]. Inhibiting both JMJD3 and UTX activity in primary human macrophages resulted in reduced inflammatory cytokine levels upon LPS stimulation. In addition, JMJD3 and UTX inhibition prevented both the LPS-induced loss of H3K27me3 at, and the recruitment of RNA polymerase (Pol) II to, the *TNF* promoter [147].

Findings from recent studies have helped support the theory of "histone crosstalk" where different histone modifications "communicate" in elaborate combinations to remodel chromatin [148]. Within a population of numerous and distinct histone modifications, the order of adding and removing neighboring modifications has been shown to affect transcriptional readouts. For example, in the context of T cell activation, it has been found that H3K4me3 promotes H3K9ac and H4K16ac enrichment [149]. Specifically, when human CD4$^+$ T cells were treated with HDAC inhibitors, a subset of gene promoters that were enriched for H3K4me3 also became enriched for H3K9ac and H4K16ac; in contrast, promoters lacking H3K4me3 did not become enriched for H3K9ac or H4K16ac. Notably, the increase in histone acetylation corresponded to increased Pol II recruitment but not transcription initiation. Interestingly, a subunit essential for methylation of H3K4 has been shown to associate with

an acetyltransferase that recognizes H4K16 [150, 151]. These data indicate that H3K4me3 poises a subset of silent genes in resting T cells for histone acetylation, suggesting crosstalk among these histone modifications.

In addition to the covalent modification of histones, nucleosomes can be modified by deposition of histone variants in a replication-independent manner [152]. Histone variants are defined as non-allelic isoforms of canonical histones that differ in DNA sequence and, in some cases, amino acid sequence [153]. Replacement of canonical histones with histone variants can alter the packaging structure of nucleosomes, often leading to a less stable nucleosome [154]; indeed, histone variants H2A.Z and H3.3 have been shown to preferentially localize near transcriptionally active genes, particularly at promoters and enhancers [155–157]. For example, in one study IFN-β stimulation triggered extensive and prolonged H3.3 deposition at the 3'-coding regions of IFN-inducible genes like *Stat1* [158]. Specifically, IFN-β-induced transcription was significantly reduced in mouse embryonic fibroblasts where H3.3 was knocked down by shRNA. This data supports the finding that histone variants play a major role in chromatin remodeling and transcription.

Chromatin-remodeling enzymes

Chromatin-remodeling enzymes can be defined as transcriptional regulators that promote transcription factor binding by altering nucleosome packaging [127]. Importantly, they lack DNA-binding specificity and must be recruited by other mechanisms, these involve interactions with transcription factors, histone modifications, and non-coding RNAs [159, 160]. Generally, chromatin-remodeling enzymes fall into one of three categories: i) ATP-dependent remodeling enzymes; ii) histone-modifying enzymes; and iii) DNA-modifying enzymes [161].

The ATP-dependent SWI/SNF complex was one of the first chromatin-remodeling complexes to be analyzed in the context of inflammatory gene regulation. Upon ionomycin and

PMA stimulation, SWI/SNF was found to rapidly associate with chromatin, as evidenced by nuclear localization, in murine lymphocytes [162]. Subsequent studies have examined the role for SWI/SNF in macrophages activated by LPS and have found that SWI/SNF discriminates between secondary and primary response genes [163]. Specifically, secondary response genes and primary response genes with delayed kinetics were found to be SWI/SNF-dependent while rapidly induced primary response genes were found to be SWI/SNF-independent. Furthermore, the promoters of SWI/SNF-dependent genes were found to be relatively inaccessible prior to LPS stimulation as compared to the promoters of SWI/SNF-independent genes, which were nuclease accessible, suggesting a poised promoter state exists for rapidly induced primary genes.

In addition to activating gene expression, chromatin-remodeling enzymes are known to repress gene expression by promoting chromatin inaccessibility. For example, nuclear receptor complex (NCoR) is an essential component of chromatin-remodeling complexes that repress genes through a combination of HDAC and other enzymatic activities. During macrophage activation, dissociation of NCoR from a subset of inflammatory genes was shown to correlate with gene activation, suggesting NCoR and its accompanying complex function as active repressors of gene activation in resting macrophages [164]. Notably, the transcription factor c-Jun was found to be required for NCoR association with DNA, and LPS-induced phosphorylation of c-Jun led to disassociation of both c-Jun and the NCoR complex at activated genes [164]. Together, these data suggest a mechanism of LPS-induced gene activation that involves the removal of a repressive chromatin-remodeling complex.

Pioneer transcription factors

It is poorly understood how transcription factors, chromatin-remodeling enzymes, and histone variants are initially recruited to sites of repressed and/or inaccessible chromatin.

21

One possibility involves pioneer factors, a special class of transcription factors that can bind to relatively inaccessible DNA prior to the binding of other factors and gene activation [165]. Notably, the DNA binding domain of the pioneer factor FOXA1 was shown to resemble that of linker histone [166], which is known to bind nonspecifically to nucleosomes [167]. Pioneer factors are thought to either directly initiate chromatin remodeling events or to mediate the recruitment of chromatin remodeling factors to a particular region of DNA. A recent study examining the pioneer factor PU.1 in murine macrophages argued that pioneer factors may mark enhancers that will later become activated by developmental or environmental cues [168]. The authors looked at global PU.1 and p300 recruitment in both resting and LPS-treated murine macrophages and found PU.1 recruitment to be similar in both conditions while p300 recruitment was expectedly enriched at distal DNA elements upon LPS stimulation. Importantly, approximately half of the sites where inducible p300 recruitment occurred overlapped with sites of constitutive PU.1 binding. Thus, PU.1 appeared to constitutively mark distal DNA elements for LPS-induced p300 binding [168].

1.3.3 Chromatin looping

With the advent of chromosome conformation capture (3C) technology, the functional implications of chromatin looping have been more thoroughly examined. Chromatin looping has long been proposed as a mechanism underlying the function of eukaryotic enhancers, allowing for the enhancer to be brought into close proximity with its target promoter while simultaneously looping out intervening DNA [169]. Although DNA looping has been powerfully demonstrated by electron microscopy, microscopy does not allow for systematic studies of DNA topology at a high resolution [170]. Thus, 3C and its derivative assays have allowed for *in vivo* population-based analyses of nuclear organization at an unprecedented resolution.

In 3C-based assays, cells are fixed with formaldehyde, as is done in chromatin im-

munoprecipitation, to cross-link DNA/DNA, DNA/protein, and protein/protein interactions [171]. Chromatin is then digested, ligated under dilute conditions, and analyzed by PCR for chromatin interactions. Importantly, dilute conditions promote ligation of DNA ends that are in close proximity to one another, which in turn favors the ligation of junctions between cross-linked DNA fragments. The frequency of interactions is measured by the abundance of amplified PCR product and is compared to the random frequency with which various loci interact. Additional 3C-based assays exist that examine the role of a specific protein of choice or allow for genome-wide investigations of intra- and/or interchromosomal interactions [172].

A major finding using 3C technology is that, over an extent of 120kb, the promoters for the genes encoding T_H2 cytokines (i.e., IL-4, IL-5, and IL-13) are in close spatial proximity to one another [173]. This configuration was found in both CD4$^+$ T lymphocytes and NK cells, but was found to be absent in both B cells and fibroblasts. Essentially, a chromatin hub of interacting elements at the T_H2 cytokine locus forms in a cell type-specific manner. Another major study utilized 3C-based methods with data from the ENCODE consortium to comprehensively examine long-range interactions between TSSs and distal DH sites characterized as enhancers; in human cell lines, more than 1000 interactions were observed [174]. Importantly, significant correlations were found between promoter-enhancer interactions and gene expression. This is perhaps the most comprehensive study to have examined both the chromatin organization of regulatory elements and their functional relationships. In conclusion, 3C-based methods have greatly expanded our understanding of genome structure and mechanisms of action for distal regulatory elements such as enhancers.

1.4 *TNF/LT* locus and its transcriptional regulation

The *TNF* promoter and its distal DH sites have been shown to control transcriptional activation in response to multiple stimuli in a variety of cell types [175]. From a drug discovery

perspective, defining the mechanisms involved in the cell type-specific control of *TNF* gene expression in the context of the *TNF/LT* locus and its chromatin environment is critical for the identification of novel targets for selective therapeutic modulation of TNF.

1.4.1 *TNF/LT* locus

The *TNF/LT* locus comprises the *TNF*, *LTA*, and *LTB* genes; their coding regions occupy ~12kb on human chromosome 6p21 in a region of the major histocompatibility complex (MHC) between class I, B (*HLA-B*) and class II, DR (*HLA-DR*) [176]. In addition to the *TNF* promoter, the *TNF/LT* locus contains multiple DH sites [177–179]. Recent studies by our laboratory have identified multiple constitutive and inducible DH sites that exhibit both shared, and cell type specificity between primary human monocytes and CD4$^+$ T cells (unpublished results). The Goldfeld lab has labeled the distal DH sites based on their distance from the *TNF* TSS. For example, human hypersensitive site -12 (hHS-12) is ~12kb upstream of the *TNF* TSS.

hHS-12 is the most distal DH site in the *TNF/LT* locus and is predicted to be ~200 bp in length. It is located ~12kb upstream of the *TNF* TSS. Examination of the site in primary human monocytes and CD4$^+$ T cells showed the presence of hHS-12 in monocytes but not in CD4$^+$ T cells. The sequence is A/T rich (65% AT).

hHS-8 (also identified as DHS44500 [178]) is located ~8kb upstream of the *TNF* TSS and is predicted to be ~1.3kb in length. One study using ENCODE data that surveyed genome-wide DNase I sensitivity predicted that the average non-promoter DH site is ~200 to 300bp in length [122]; thus, hHS-8 is an unusually large non-promoter DH site. In contrast to hHS-12, hHS-8 was found in both human monocytes and CD4$^+$ T cells. In mice, hHS-8 is known as HSS-9 [177].

hHS-5 is located ~5kb upstream of the *TNF* TSS and is predicted to be ~300bp in length. Like hHS-12, hHS-5 is AT-rich (59% AT) and is monocyte-specific [180]. In mice, hHS-5

is known as HSS-7.

hHS-4 is located ~4 kb upstream of the *TNF* TSS and is predicted to be ~120bp in length. Importantly, hHS-4 is ~500bp upstream of the *LTA* TSS. Examination of monocytes and CD4$^+$ T cells showed hHS-4 to be present in CD4$^+$ T cells and not in monocytes, supporting the finding that LT-α is minimally expressed in resting and activated monocytes and is substantially expressed in activated CD4$^+$ T cells [181]. In mice, hHS-4 is known as HSS-4 [177].

hHS+6 is located ~6kb downstream of the *TNF* TSS and is predicted to be ~200bp in length. DHA analysis showed the site to be within the coding region of the *LTB* gene; however, it is more likely to be within a non-coding region proximal to the *LTB* gene. Similar to hHS-8, hHS+6 was found in both monocytes and CD4$^+$ T cells.

hHS+8 is located ~8kb downstream of the *TNF* TSS and is predicted to be ~130bp in length. Importantly, hHS+8 is ~1kb upstream of the *LTB* TSS. hHS+8 is similar to hHS-4 in that it was found to be present in CD4$^+$ T cells and not in monocytes, also supporting the finding that LT-β is minimally expressed in resting and activated monocytes and is substantially expressed in CD4$^+$ T cells [181–183].

Although most of the DH sites found in the human *TNF/LT* locus are also found within the mouse *Tnf/Lt* locus, there are a few differences. For instance, hHS-5 (murine HSS-7) is found within the human locus but is more prominent in the murine locus, suggesting that a smaller percentage of human cells as compared to mouse cells, on average, contain this site. Another major difference between the murine and human locus is that one site, HSS+3, is murine-specific. HSS+3 is ~600bp in length and is found immediately downstream of the *TNF* gene; it is present in both murine monocytes and CD4$^+$ T cells [177, 180].

1.4.2 *TNF* promoter and its transcriptional regulation

Originally defined as a product of monocytes and macrophages, TNF expression has been observed in CD4$^+$ T cells [46], B cells [184], NK cells [48], mast cells [49], dendritic cells [50], fibroblasts [185], and neurons [186]. In addition, *TNF* transcription is induced by a number of stimuli [175], including pathogens such as bacterial LPS, viruses, and parasites; mitogens; cytokines; and antigen binding by B cells and CD4$^+$ T cells. Indeed, as described below, *TNF* transcription is regulated in a cell type- and stimulus-specific manner.

At the level of the *TNF* promoter, specific sets of transcription factors and coactivators assemble to form nucleoprotein complexes known as enhanceosomes [187–189]. Both protein-protein interactions and the conformational changes induced upon initial protein-DNA interactions facilitate the cooperative binding of additional factors to adjacent and overlapping sites. Additionally, coactivators that associate with multiple transcription factors can serve as "scaffold" proteins, thereby encouraging enhanceosome formation [190]. The first enhanceosome was described during the study of *IFNB* gene activation, and was shown to include at least three distinct transcription factors and the high mobility group protein HMG I(Y), which function in synergy to drive transcription [191, 192]. Each of the transcription factors recruited to the *IFNB* promoter has been shown to interact with CBP/p300, a histone acetyltransferase known to acetylate histones and non-histone proteins. In addition to acetylating histones within the *IFNB* promoter, CBP/p300 is thought to function as a scaffold protein, facilitating enhanceosome assembly [193].

Enhanceosome formation at the *TNF* promoter is particularly complex by comparison in that it has been shown to occur in a cell type- and inducer-specific fashion. The proximal *TNF* promoter maps to a highly conserved region ~200bp proximal to the *TNF* TSS, and is sufficient for transcriptional activation in response to multiple stimuli [189, 194–196]. Within the *TNF* promoter, there is a TATA box and multiple transcription factor binding sites, including six nuclear factor of activated T cells (NFAT) sites, four Ets/Elk-1 sites, two

Sp1 sites, an Egr site, and an ATF-2/c-Jun site [175] (Figure 1.4.1). In T cells, enhanceo-some formation was found to be inducer-specific; ionomycin, which increases intracellular $Ca2^+$ levels, was shown to promote NFAT binding to all six NFAT sites whereas Sendai virus infection was shown to promote less NFAT binding than ionomycin [187]. Differential NFAT binding at the promoter was partly due to differences in regional NFAT concentration, as more NFAT translocates into the nucleus upon ionomycin stimulation than after viral infection [187]. In addition, Sp1 binding was observed to a much larger extent at the -55NFAT/Sp1 site after Sendai virus infection as compared to ionomycin stimulation, and was shown to synergize with NFAT and ATF-2/c-Jun [198]. Importantly, the -55NFAT/Sp1 site is a fairly weak Sp1 site; thus Sp1 is outcompeted in scenarios of high NFAT levels like ionomycin stimulation. In conclusion, *TNF* transcription in T cells provides a striking example of stimulus-specific gene regulation that encompasses both regional concentration of specific transcription factors and affinities of binding sites.

In the case of monocytes and macrophages, LPS stimulation results in the formation of an enhanceosome that is distinct from that found in T cells in response to ionomycin treatment or Sendai virus infection. In particular, two Sp1 sites, four Ets/Elk-1 sites, an ATF-2/c-Jun site, and an Egr binding site were found to be critical for LPS induction of *TNF* transcription in macrophages [189]. Notably, Ets and Elk-1 proteins were found in place of NFAT at the *TNF* promoter upon LPS stimulation. Thus, the presence of overlapping DNA motifs that can serve as both NFAT and Ets/Elk-1 binding sites allows for a broad spectrum of distinct enhanceosomes to form at the *TNF* promoter depending on cell type and the signaling pathways triggered. Interestingly, *M. tuberculosis* induction of *TNF* transcription in macrophages involves an enhanceosome of similar composition to that of LPS induction [195].

In the case of LPS stimulation in monocytes and macrophages, some initial studies suggested a role for the transcription factor NF-κB, which is now known to play a key role in

A

-180–NFAT/Ets   upSp1   Egr   -149–NFAT

ATGCTTGTGTGTCCCCAAC **TTTCC** AAAT **CCCGC** **CCCC** GCGA **T** **GGAGAAGAAA** CCGAGACAGAAGGT

-200   -168   -164 -161   -134

-117–NFAT/Ets   CRE   κ3–NFAT   -84–Ets   -76–NFAT/Ets

GCAGGGCCCACTACCG **CTTCC** TCCAGA **TGAGCTCA** T **GGGTTTCTCC** ACCAAGGAAG **TTTTCC** GCTGG

-133   -67

-55–NFAT/Sp1   TATA box

TTGAATGATTC **TTTCCCCGCCC** CCTCTCGCCCCAGGGACA **TATAAA** GGCAGTTGTTGGCACACCCA

-66   -9   +1

B

		-180–NFAT/Ets	upSp1	Egr	-149–NFAT	-117–NFAT/Ets	CRE	κ3–NFAT	-84–Ets	-76–NFAT/Ets	-55–NFAT/Sp1
Cell Type	Stimulus										
T cell	α-CD3, lo				NFATp	NFATp	ATF-2/c-jun	(NFATp)₂		NFATp	NFATp
T cell	virus	Ets-1, Elk-1	Sp1			Ets-1, Elk-1	ATF-2/c-jun	(NFATp)₂	Ets-1, Elk-1	Ets-1, Elk-1	Sp1
B cell	IgM, PMA/lo	NFATp?				ATF-2/c-jun				NFATp	
mΦ	LPS/MTb	Ets-1, Elk-1	Sp1	Egr-1		Ets-1, Elk-1	ATF-2/c-jun		Ets-1, Elk-1	Ets-1, Elk-1	Sp1

C

T cells — high NFAT (TCR / Ca2+) — low NFAT (Virus)

Monocytes / Macrophages — very low / no NFAT (LPS / MTb)

Figure 1.4.1: Cell type- and inducer-specific enhanceosome formation at the proximal *TNF* promoter. (a) Sequence of human proximal *TNF* promoter showing the positions of transcription factor binding sites. (b) Transcription factors that bind at sites in the indicated cell types and in response to the indicated stimuli. (c) Model of inducer-specific enhanceosome formation at the proximal *TNF* promoter in T cells and monocytes. [175, 195, 197].

TLR signaling. Multiple NF-κB sites lie upstream of the *TNF* TSS, but these are generally not conserved between mice and humans and lie outside of the proximal promoter [196, 199, 200]. The one NF-κB-like site present in the proximal promoter, originally designated κ3 [196], is bound by NFAT proteins and functions as a calcineurin-dependent composite element within the adjacent CRE site. This site binds ATF-2/c-Jun heterodimer, and is required for *TNF* transcription in response to multiple stimuli [187, 189, 194, 195, 198, 201–206]. Through EMSA and quantitative DNase I footprinting analysis, recombinant p50/p65 was shown not to bind specifically to κ3 or any other site within the proximal *TNF* promoter. In addition, assays targeting NF-κB activity in monocytes and macrophages indicated a post-transcriptional induction of NF-κB in response to LPS for maintaining TNF mRNA levels in these cells, rather than a direct role in initiation of *TNF* transcription [207].

As stated previously, a primary function of transcription factors is to promote the recruitment of nucleosome remodeling enzymes to regulatory regions, thereby promoting chromatin accessibility and subsequent binding of RNA Pol II. Importantly, CBP/p300 has been shown to play a major role in *TNF* transcription in both macrophages and T cells [188, 189, 195]. Inhibition of CBP/p300 impairs *TNF* transcription induced by LPS in murine macrophages [188]. Strikingly, examination of splenic T cells from *Cbp*$^{+/-}$ mice showed that induction of *TNF* transcription by TCR engagement was dramatically reduced as compared to what is found in cells isolated from wild-type or *p300*$^{+/-}$ mice, indicating a novel inducer-specific role for CBP in TNF enhanceosome formation in activated T cells [188]. This conclusion was indeed supported by later findings in conditional knockout mice [208]. Together, these studies demonstrate the importance for CBP/p300 in *TNF* transcriptional activation and support a model where CBP/p300 acts as a "scaffold" protein during enhanceosome formation at the *TNF* promoter.

1.4.3 Distal regulatory elements at the *TNF/LT* locus

As stated previously, multiple DH sites distal to the *TNF* promoter lie within the *TNF/LT* locus. Importantly, many of the identified DH sites coincide with regions of highly conserved DNA, which suggests regulatory function. From an evolutionary perspective, functionally relevant sequences are typically subjected to negative selection, whereas non-functional sequences are subject to genetic drift and become increasingly different between species with increasing phylogenetic distance [209]. Thus, highly conserved non-coding DNA sequences are predicted to be regulatory elements. Extending the findings of conserved functional noncoding DNA sequences in the *TNF* promoter [197, 199, 210], other DH sites in the *TNF/LT* locus, including hHS-8, were found to be strongly conserved between mice and humans, as well as a number of other species [178].

DH sites within the murine *Tnf/Lt* locus have been shown to undergo inducible intrachromosomal interactions while functioning as enhancers. Increased levels of H3ac and H4ac were found at both HSS-9 and HSS+3 in murine T cells in response to anti-CD3/CD28 activation, suggesting inducible chromatin remodeling at these distal sites in the murine *Tnf/Lt* locus [177]. In addition, both HSS-9 and HSS+3 were shown to enhance *Tnf* promoter activity in a murine T cell line in an NFAT-dependent manner. Finally, it was demonstrated through 3C analysis that an inducible intrachromosomal interaction between the *Tnf* promoter, HSS-9, and HSS+3 occurred upon activation of murine T cells. Thus, the interplay of histone modifications, transcription factor binding, and intrachromosomal interactions orchestrate inducible enhancer function at the murine *Tnf/Lt* locus, allowing for rapid and potent activation of this critical immunological factor during innate immune responses.

1.5   Summary of Aims

Macrophages are fundamental cellular components of the innate immune response. Although the genetic programs and signal transduction pathways that contribute to macrophage activity in response to microbial infection have been thoroughly investigated, much less is known about the chromatin-remodeling factors and related chromatin environment that orchestrate these cellular processes. The overarching goal of this thesis project was to identify and dissect mechanisms of chromatin remodeling that control classical macrophage activation and endotoxin tolerance. To achieve this goal we used the *TNF/LT* locus as our model system. This locus encodes TNF, the archetypical inflammatory cytokine whose enhancement and repression is representative of classical macrophage activation and endotoxin tolerance, respectively.

Our first aim was to test the hypothesis that potent *TNF* gene expression in response to classical macrophage activation, where a priming signal by IFN-γ results in augmentation of *TNF* gene expression upon LPS stimulation, is regulated, at least in part, by IFN-γ-mediated chromatin modifications at the *TNF/LT* locus. Findings from experiments designed to address this hypothesis are described in Chapter 2. Our second aim was to test the hypothesis that chromatin modifications at the *TNF/LT* locus are also critical for the modulation of *TNF* gene expression during the induction of endotoxin tolerance in macrophages, and that the ability of IFN-γ to restore LPS-induced *TNF* gene expression in endotoxin-tolerant macrophages depends on similar chromatin changes as those induced by IFN-γ at the *TNF/LT* locus during macrophage priming. In Chapter 3, we present findings from experiments performed to address this hypothesis.

A diverse array of experimental techniques were employed to achieve the overall goal of this project, including DNase I hypersensitivity analysis, DNase I footprinting, chromatin immunoprecipitation (ChIP), and genetic knockdown and mouse knockout systems. The

experiments were performed, when possible, using primary human macrophages in order to obtain data of clinical relevance. The findings described in this dissertation provide novel insights into mechanisms of chromatin remodeling at play during macrophage priming, induction of endotoxin tolerance, and abrogation of endotoxin tolerance. We expect these findings to be broadly applicable to other primary response genes that are similarly affected by these biological processes. In addition, we anticipate that the findings described herein will provide a foundation for future research efforts aimed at defining and manipulating cellular targets that are critical for the regulation of *TNF* gene expression in the context of i) an innate immune response, and ii) the excessive inflammation found in autoimmune diseases.

Chapter 2

IFN-*γ* priming of LPS-mediated *TNF* gene expression occurs via inducible IRF1 binding and histone methylation at a highly conserved distal enhancer

2.1   Introduction

During infection, antigen-presenting cells secrete IL-12, which helps link innate and adaptive immunities by promoting the differentiation of naïve CD4$^+$ T cells toward a Th1 phenotype and by inducing IFN-*γ* secretion from NK cells and activated CD4$^+$ T cells [211]. Accordingly, IFN-*γ* sensitizes surrounding macrophages to microbial recognition and, most notably, augments the production of TNF [44, 59]. This phenomenon of IFN-*γ*-mediated macrophage sensitization is known as IFN-*γ* priming. *In vitro*, maximum effects of IFN-*γ* priming have been shown to occur within two hours of IFN-*γ* pre-treatment, resulting in enhanced mRNA levels of TNF [212] and of other cytokines and inflammatory molecules such as IL-12 [60], IL-6 [57], and NO [58] upon LPS stimulation.

The mechanisms involved in priming are unclear; the IFN-*γ*-inducible transcription factors IRF1 and STAT1 have been implicated [84, 213], but functional roles for these factors have not been determined. One study examining the effect of IFN-*γ* on chromatin modifica-

tions at the *TNF* promoter observed increased levels of H4ac in primary human monocytes treated with IFN-γ as compared to resting cells [214]. In addition, a recent study has shown H3K27ac enrichment at both the *TNF* promoter and a distal DH site during IFN-γ priming [213]. In the context of IFN-γ priming, functional roles for distal DH sites in the *TNF/LT* locus have not been demonstrated.

Here, we show that IFN-γ priming of *TNF* transcription requires functional binding of IRF1 to the distal *TNF* enhancer element hHS-8. IFN-γ also poises hHS-8 for enhancer function by increasing levels of H3K27me3. Subsequent LPS stimulation triggers hHS-8 enhancer activation by first demethylating and then acetylating H3K27. These experiments provide a mechanistic explanation for IFN-γ priming of the LPS response using TNF as a model system, while at the same time providing potential targets for selective manipulation of TNF overexpression in primed macrophages.

2.2   Results

2.2.1   IFN-γ primes monocytes and macrophages for enhanced *TNF* gene expression.

IFN-γ priming dramatically enhances *TNF* gene expression in LPS-induced monocyte and macrophage activation. We first observed the effect of IFN-γ priming in THP-1 cells, a human monocytic cell line, by pretreating cells with IFN-γ 2h before LPS stimulation and found a two-fold increase in TNF mRNA levels as compared to that of LPS alone (Figure 2.2.1a). To verify that enhanced *TNF* gene expression results in enhanced protein production, we measured TNF protein levels in supernatant and again found a two-fold increase in cells pretreated with IFN-γ as compared to cells treated with LPS alone (Figure 2.2.1b). We also verified that IFN-γ priming is not LPS specific by showing enhanced TNF expression in monocytes activated with whole lysate from *M. tuberculosis* strain H37Rv (Figure 2.2.1c). We next confirmed this phenomenon in primary human MDMs activated by LPS

(Figure 2.2.1d). Despite significant differences in morphology and function between mono-cytes and macrophages, IFN-γ synergizes with LPS in both cell types to enhance *TNF* gene expression at the transcriptional level, suggesting that IFN-γ affects the chromatin environment at the *TNF/LT* locus in a similar mechanism for both cell types. When proposing a mechanism for IFN-γ priming, we take note that both monocytes and macrophages treated with IFN-γ alone induced minimal levels of both TNF mRNA and protein and were comparable to untreated samples; thus, we hypothesized that although IFN-γ does not induce *TNF* transcription, it poises the *TNF/LT* locus for enhanced transcription in response to LPS stimulation.

2.2.2   IFN-γ promotes chromatin accessibility at hHS-8 in the *TNF/LT* locus.

We next looked at the ability of IFN-γ to remodel the *TNF/LT* locus by performing DNase I hypersensitivity assays (DHAs), and found that IFN-γ promotes DNase I cleavage at the human hypersensitive site, hHS-8. DHAs utilize the ability of DNase I to distinguish and cleave accessible DNA in packaged chromatin. DNA from both THP-1 cells and primary human MDMs stimulated with IFN-γ were partially digested with DNase I. BamHI and ScaI digestion allowed for visualization of the *TNF* promoter and hHS-8, respectively (Figure 2.2.2a). When comparing untreated and IFN-γ treated THP-1 cells, minimal differences in DNase I accessibility at the *TNF* promoter were observed. This is evidenced by similar band intensities between untreated and IFN-γ treated samples (Figure A.0.1a, lane 4 compared to lane 8). However, we detected a strongly enhanced band corresponding to hHS-8 from cells treated with IFN-γ as compared to untreated cells (Figure A.0.1b, lane 4 compared to lane 8). Results were repeated in primary human MDMs for both the *TNF* promoter and hHS-8 (Figures 2.2.2b, lane 3 compared to lane 6; 2.2.2c, lane 3 compared to lane 6). Thus, when thinking of a population of monocytes or macrophages, hHS-8 is constitutively present in a given percentage of cells, and, upon IFN-γ stimulation, the percentage of cells exhibiting
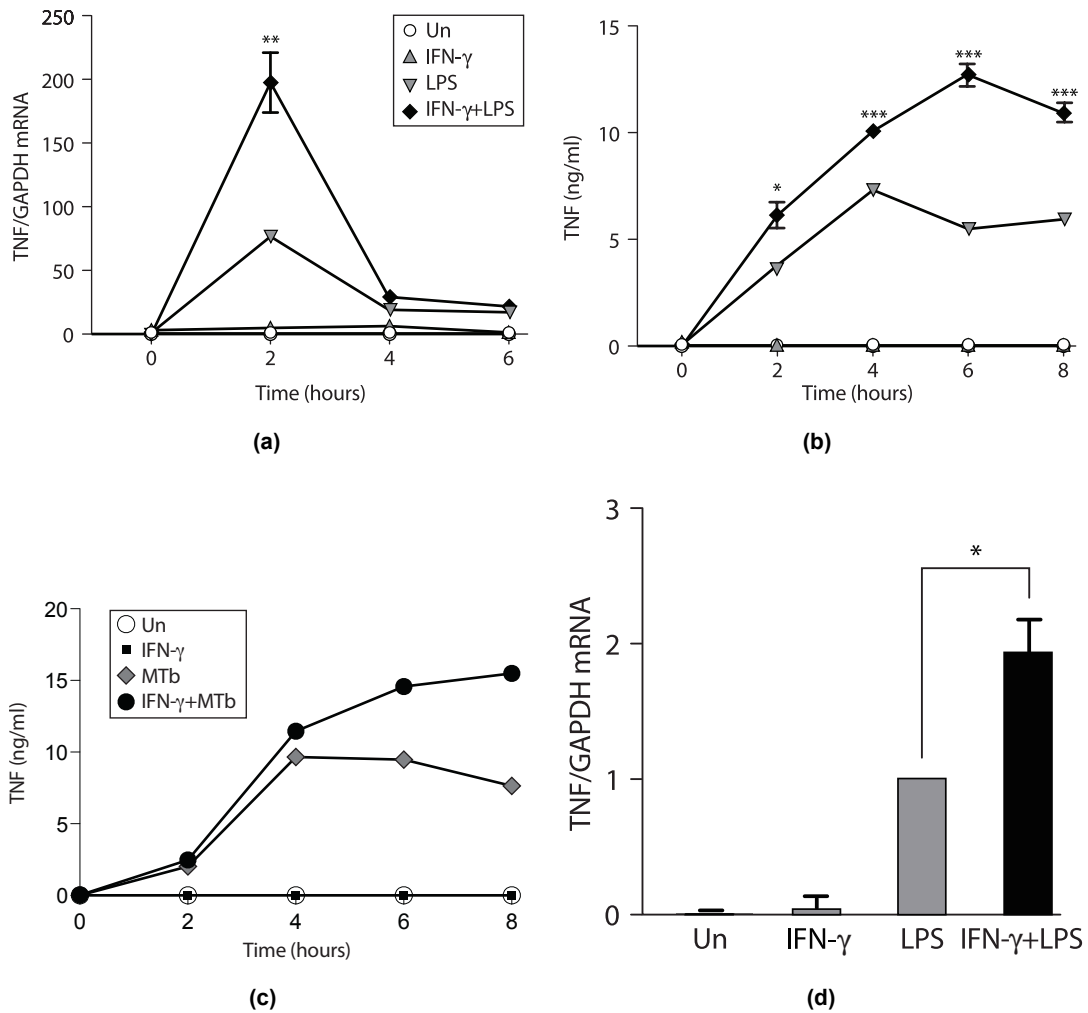
Figure 2.2.1: IFN-γ primes monocytes and macrophages for enhanced *TNF* gene expression. (a) THP-1 cells were stimulated with IFN-γ alone for 3h, LPS alone for 1h, and both IFN-γ and LPS (IFN-γ pretreatment for 2h followed by LPS treatment for 1h), and TNF mRNA levels were measured post LPS stimulation. (b) THP-1 cells were stimulated as in (a), and TNF protein levels in supernatants were measured by ELISA post LPS stimulation. (c) THP-1 cells were stimulated with IFN-γ alone for 3h, MTb lysate alone for 1h, and both IFN-γ and MTb lysate (IFN-γ pretreatment for 2h followed by MTb lysate treatment for 1h), and TNF protein levels in supernatants were measured as in (b). Data from 1 experiment. (d) Primary human MDMs were treated as in (a), and TNF mRNA levels were measured 1h post LPS stimulation. Data from 3 donors. (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$.

accessibility at hHS-8 significantly increases in the population.

In addition to DNase I accessibility, we analyzed nucleosome occupancy at the *TNF* promoter and hHS-8 by measuring total H3 levels by ChIP. As expected, we observed a significant loss in total H3 levels at the promoter in response to LPS stimulation (Figures 2.2.2d, 2.2.2e). We also observed a significant loss in total H3 levels at hHS-8 in response to IFN-*γ* and LPS stimulation (Figure 2.2.2d). Results were repeated in primary human MDMs isolated from a representative donor (Figure 2.2.2e). This data correlates with our finding that increased DNase I cleavage occurs at hHS-8 in response to IFN-*γ* treatment. Together, we show that IFN-*γ* poises the *TNF/LT* locus by exposing hHS-8, a hypersensitive site distal to the *TNF* promoter.

2.2.3   IRF1 binds to the *TNF* promoter and hHS-8 in an IFN-*γ*-inducible manner.

Increased chromatin accessibility often predicts binding of transcription factors and/or coactivators. We next sought to identify factors binding to the *TNF/LT* locus in an IFN-*γ*-inducible manner. Given that IFN-*γ* is a potent inducer of the transcription factor IRF1 [215] (Figure A.0.2), we investigated whether IRF1 binds to specific sequences within the *TNF/LT* locus, particularly at known DH sites. We scanned all DH sites for potential IRF binding sites similar to its consequence sequence (5'-AANNGAAANGAA-3') [82] and verified by both *in vitro* and *in vivo* methods that IRF1 binds to both the *TNF* promoter and hHS-8. Past studies have shown cell type- and inducer-specific recruitment of specific sets of transcription factors and coactivators to the proximal region of the *TNF* promoter (Figure 2.2.3a).

Quantitative DNase I footprinting analysis reveals where factors bind to DNA with the resolution of a single nucleotide. We observed that recombinant IRF1 (rIRF1) binds to the *TNF* promoter in a region ~50bp in length (Figure 2.2.3b). Notably, this is the same region where transcription factors Sp1, Egr1, and NFAT have been shown to bind, which further
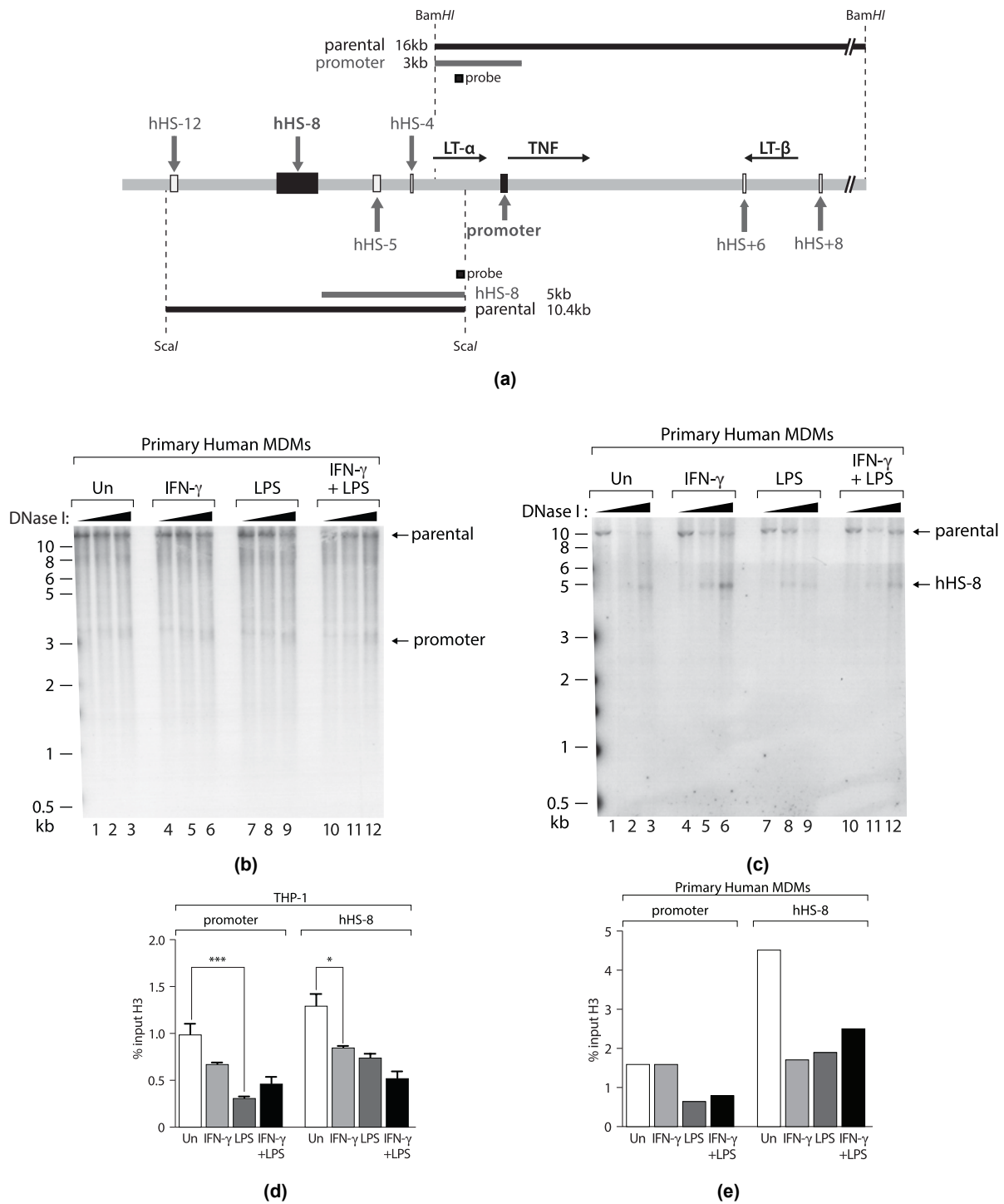
Figure 2.2.2: IFN-γ promotes chromatin accessibility at hHS-8 in the *TNF/LT* locus. (a) Map of the human *TNF/LT* locus. DH sites and positions and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. Positions of the parental ScaI, parental BamHI, and DNase I digestion products for the DHAs are indicated; fragment representing DNase I accessibility at the *TNF* promoter and hHS-8 is 3kb and 5kb in length, respectively. (b) IFN-γ does not affect DNase I cleavage at the *TNF* promoter. DHA using primary human MDMs. (c) IFN-γ strongly increases DNase I cleavage at hHS-8. (d) IFN-γ and LPS decrease nucleosome occupancy at the *TNF* promoter and hHS-8. ChIP using THP-1 cells (d) and primary human MDMs, 1 donor (e) measures total H3 levels. (*) $p \leq 0.05$ and (***) $p \leq 0.001$.

emphasizes the complexity of cell-type and inducer-specific regulation of *TNF* transcription (Figure 1.4.1). In addition to the *TNF* promoter, we observed that rIRF1 occupies a region in hHS-8 that is also ~50bp in length and contains four -GAAA- motifs, suggesting multiple tandem IRF1 binding sites (Figure 2.2.3c). Other *in vitro* assays using human T cells have shown binding of NFAT to regions near the newly identified IRF1 binding site (Figure 2.2.3a).

We next investigated whether IRF1 is recruited to the promoter and hHS-8 in an IFN-γ inducible manner. ChIP studies using primary human MDMs confirmed that upon IFN-γ stimulation, IRF1 is massively recruited to hHS-8 within the same region as identified by footprinting analysis and, to a lesser extent, to the *TNF* promoter (Figure 2.2.3d). Thus, upon IFN-γ stimulation, hHS-8 has a higher affinity for IRF1 than the *TNF* promoter. We speculated that IRF1 recruitment to both the *TNF* promoter and hHS-8 is important for the ability of IFN-γ to prime both monocytes and macrophages.

2.2.4   IRF1 is required for enhanced TNF expression in IFN-γ-primed monocytes and macrophages.

We next tested whether IRF1 plays a functional role in IFN-γ-induced enhancement of *TNF* gene expression. Using bone marrow-derived macrophages (BMDMs) from IRF1-deficient (*Irf1-/-*) mice, we show that IRF1 is required for enhanced *TNF* gene expression in murine macrophages primed with IFN-γ. We isolated BMDMs from wild-type and *Irf1-/-* mice and measured TNF protein levels in response to IFN-γ and LPS. We observed no significant differences in TNF protein levels between wild-type and *Irf1-/-* mice stimulated with IFN-γ and LPS alone. As seen in THP-1 cells and primary human MDMs, wild-type BMDMs secreted significantly higher levels of TNF protein when primed with IFN-γ than when treated with LPS alone (Figure 2.2.4a). However, we did not observe enhancement in *Irf1-/-* BMDMs (Figure 2.2.4b); thus, we conclude that IRF1 is required for IFN-γ-induced enhancement of
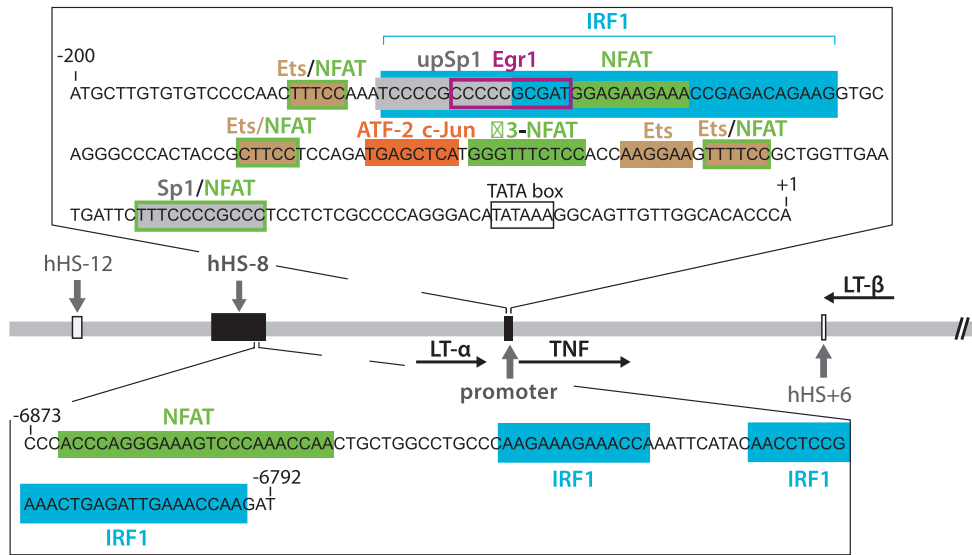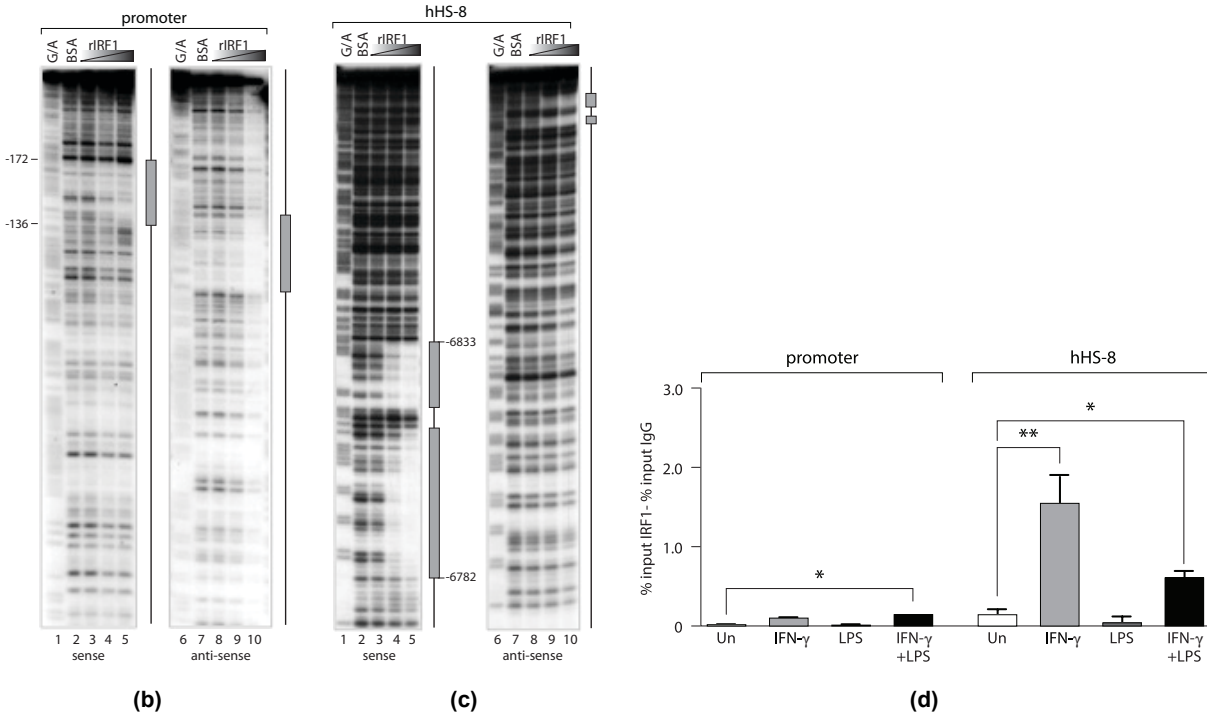
Figure 2.2.3: IRF1 binds to the *TNF* promoter and hHS-8 in an IFN-γ-inducible manner. (a) Human *TNF/LT* locus with partial sequences of both the *TNF* promoter and hHS-8 and positions of transcription factor binding sites. (b) rIRF1 binds to the *TNF* promoter. Quantitative DNase I footprinting analysis of the *TNF* promoter (-200 to +1) and increasing concentrations of rIRF1. Sense and anti-sense strand with G/A ladder and BSA control. Bars mark areas of rIRF1 binding at -172 to -136. (c) rIRF1 binds to hHS-8. Analysis of hHS-8 (-7031 to -6782); bars mark areas of rIRF1 binding at -6833 to -6782. (d) IRF1 is recruited to both the *TNF* promoter and hHS-8 in an IFN-γ-inducible manner. ChIP using primary human MDMs and analyzing IRF1 recruitment, data from 3 separate donors. (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

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TNF gene expression.

In addition to murine BMDMs, we looked at the ability of IFN-*γ* priming to enhance *TNF* gene expression in human monocytes treated with shRNA targeting IRF1. THP-1 cells were transfected with either shRNA targeting human IRF1 or control shRNA encoding a scrambled sequence. Sufficient knockdown of IRF1 mRNA was observed when cells were treated with IFN-*γ* for 3h (Figure 2.2.4c), which we concluded as sufficient knockdown of IRF1 protein considering its extreme instability (half-life ~30min) [216, 217]. As expected, we observed significant enhancement of TNF mRNA levels in IFN-*γ* primed THP-1 cells expressing control shRNA. In contrast, enhancement of TNF mRNA levels was lost in primed THP-1 cells expressing IRF1 shRNA (Figure 2.2.4d). These data indicate that IRF1, which we have shown to be massively recruited to hHS-8 upon IFN-*γ* stimulation, is necessary for enhanced *TNF* gene expression in both murine macrophages and human monocytes primed with IFN-*γ*. Indeed, the IRF family comprises nine transcription factors that all share similar consensus sequences [82]; however, this data suggests that other IRFs are not functionally redundant to IRF1 in the context of IFN-*γ* priming.

2.2.5 hHS-8 functions as an IFN-*γ*-inducible, IRF1-dependent enhancer of *TNF* transcription.

We speculated that hHS-8 might function as an IFN-*γ*-inducible regulatory element during IFN-*γ* priming of monocyte and macrophage activation. Specifically, we hypothesized that hHS-8 functions as an IFN-*γ*-inducible enhancer of *TNF* transcription, and that IRF1 plays a role in the ability of hHS-8 to function as an enhancer. We first analyzed sequence conservation at the IRF1 binding sites within hHS-8 and found these sites to be highly conserved among the great apes, small apes, old world monkeys, and new world monkeys. High sequence conservation within non-coding regions of DNA infers regulatory function [209]. We compared the human sequence to those of non-human primate species and sub-
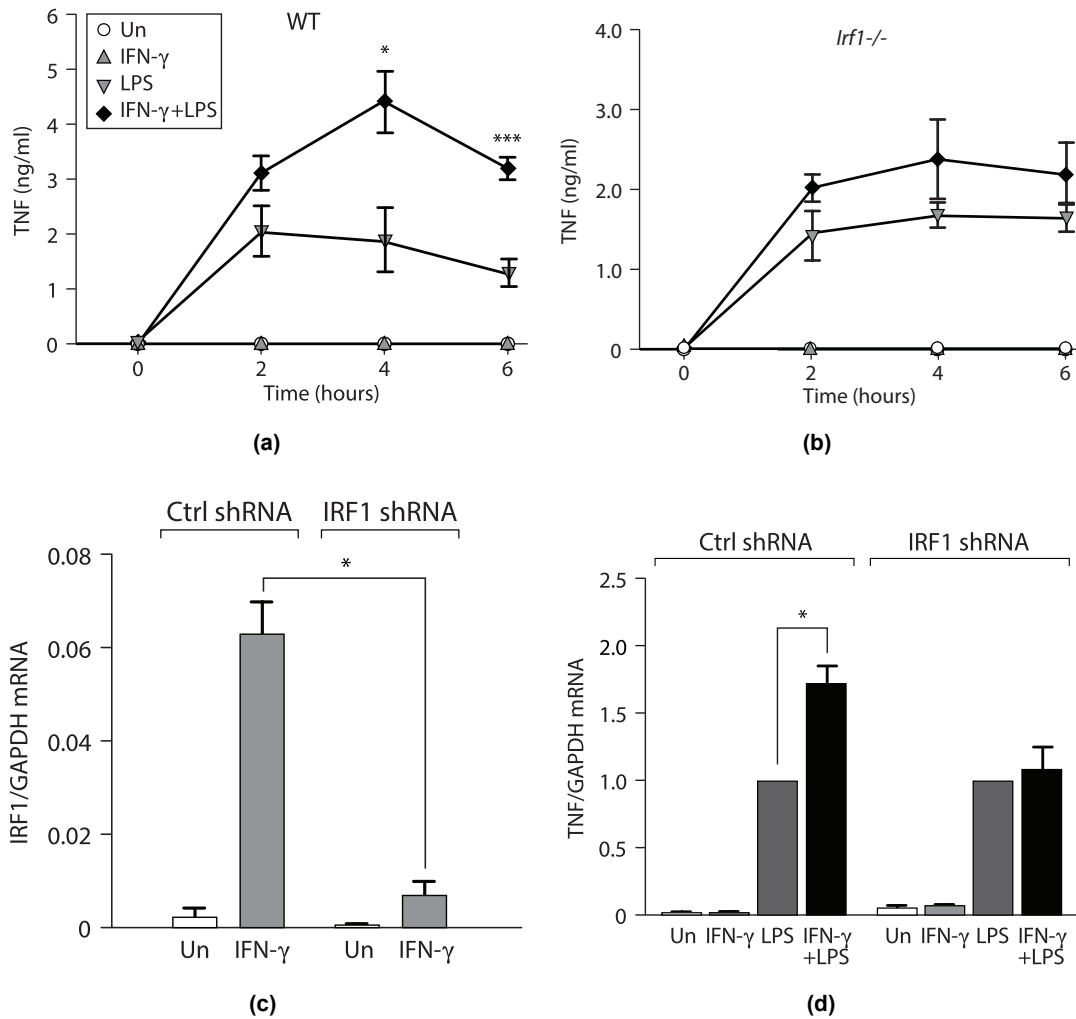
Figure 2.2.4: IRF1 is required for enhanced TNF expression in IFN-γ-primed monocytes and macrophages. (a,b) Enhanced *TNF* gene expression induced by IFN-γ priming is abrogated in IRF1-deficient murine BMDMs. Wild-type (a) and Irf1-/- (b) BMDMs were stimulated, and TNF protein levels in supernatants were measured by ELISA post LPS stimulation. Data from 3 separate experiments each with $N = 3$. (c) THP-1 cells that constitutively express lentivirally-delivered shRNA targeting IRF1 or control shRNA encoding a scrambled sequence were stimulated with IFN-γ alone for 3h, and IRF1 mRNA levels were measured. (d) Enhanced *TNF* gene expression induced by IFN-γ priming is abrogated in human monocytes where IRF1 expression is silenced. THP-1 cells expressing IRF1 and control shRNA were stimulated, and TNF mRNA levels were measured (shown relative to LPS values). (*) $p \leq 0.05$ and (***) $p \leq 0.001$.

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species including multiple individuals representing species of the great apes [chimpanzee (*Pan troglodytes*), gorilla (*Gorilla*), and orangutan (*Pongo*)], the small apes [two genera of gibbon (*Hylobatidae* and *Nomascus*)], the old world monkeys [macaque (*Macaca*), *Chlorocebus*, and *Papio*] and the new world monkeys [*Callithrix* and *Lagothrix*]. We found that the core -GAAA- motifs of the IRF1 binding sites in hHS-8 are completely conserved among human and all non-human primate species analyzed (Figure 2.2.5a), which suggests the presence of phylogenetic footprints in hHS-8. High sequence conservation among primates is similarly seen in the *TNF* promoter where multiple binding sites for transcription factors and coactivators are involved in highly coordinated cell type- and inducer-specific enhanceosome formation [197, 210]. Notably, analysis of IRF1 binding sites in HSS-9 (murine hHS-8) showed two differences in the core -GAAA- motifs (Figure 2.2.5a). Through EMSA analysis, we demonstrated that the differences in the mouse sequence do not impact IRF1 binding (Figure A.0.3, lane 2 compared to lane 4).

In order to test whether hHS-8 functions as an inducible enhancer of *TNF* transcription, we cloned the hHS-8 sequence (Figure A.0.4) into a reporter vector where luciferase expression is driven by the *TNF* promoter. We transiently co-transfected the constructs with pRL-TK (Renilla luciferase, internal control) into the murine macrophage cell line J774 and stimulated cells accordingly. Luciferase activity was measured by a dual-luciferase reporter assay. In macrophages primed with IFN-γ, the presence of hHS-8 significantly enhanced the ability of the *TNF* promoter to drive luciferase expression in response to LPS, confirming that hHS-8 functions as an IFN-γ-inducible enhancer.

In order to assess the importance of IRF1 in hHS-8 enhancer function, we also mutated all four highly conserved IRF1 -GAAA- motifs to -GAGG-, which we showed by EMSA analysis to completely disrupt IRF1 binding (Figure 2.2.5b). Mutation of the IRF1 binding sites in hHS-8 abolished its ability to enhance *TNF* promoter activity in macrophages primed by IFN-γ and induced by LPS (Figure A.0.5); thus, we conclude that hHS-8 functions as an

IRF1-dependent inducible enhancer of *TNF* transcription in macrophages primed by IFN-*γ*. Understanding the functional importance of IRF1 binding to the *TNF* promoter is complicated by the fact that LPS induced *TNF* transcription requires intact Sp1 and Egr binding sites [189] that were found to coincide with the IRF1 binding sites (Figure 2.2.3a). Thus, we only examined the functional importance of IRF1 binding to hHS-8.

2.2.6   H3K27me3 and H3K27ac play a critical role in IFN-*γ* priming.

H3K27me3 and H3K27ac have been shown to distinguish active and inactive enhancers in mouse and human embryonic stem cells [218–220]. We hypothesized that IFN-*γ* priming and LPS stimulation affect H3K27me3 and H3K27ac levels at hHS-8. ChIP studies using THP-1 cells showed that upon IFN-*γ* stimulation, levels of H3K27me3 increased at hHS-8, whereas levels at the *TNF* promoter remained constant (Figure 2.2.6a). Cells that were primed with IFN-*γ* and then stimulated with LPS were not enriched for H3K27me3 at hHS-8, suggesting that H3K27me3 marks hHS-8 during priming and is lost upon *TNF* gene activation. Enrichment of H3K27ac was also shown to be inducible; levels of H3K27ac significantly increased at hHS-8 in cells primed by IFN-*γ* and stimulated with LPS (Figure 2.2.6b). Notably, H3K27ac enrichment at hHS-8 did not occur in cells treated with LPS alone, demonstrating a requirement of IFN-*γ* priming for H3K27ac enrichment at hHS-8. H3K27ac levels at the *TNF* promoter dramatically increased upon stimulation with LPS alone, which supports the finding of LPS-induced CBP/p300 recruitment to the *TNF* promoter in monocytes and macrophages [189].

Next, we investigated the reciprocal nature of H3K27me3 and H3K27ac at hHS-8 and whether the marks were important for enhanced *TNF* gene expression in the context of IFN-*γ* priming. To do this, we inhibited the H3K27me3-specific demethylases JMJD3 and UTX. THP-1 cells were first primed by IFN-*γ* and then treated with either GSK-J4 [147] (active inhibitor of JMJD3 and UTX) or GSK-J5 [147] (inactive inhibitor) 30min before the LPS

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Homo sapiens　　　　　　AAGAAAGAAACCAAATT–CATACAACCTCCGAAACTGAGATTGAAACCAA　49
Pan troglodytes versus　AAGAAAGAAACCAAATT–CATACAACCTCCGAAACTGAGATTGAAACCAA　49
Gorilla g gorilla　　　　AAGAAAGAAACCAAATT–CATACAACCTCCGAAACTGAGATTGAAACCAA　49
Pongo pygmaeus　　　　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Pongo abelii　　　　　　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Hylobates lar　　　　　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Nomascus leucogenys　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Macaca mulatta　　　　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Chlorocebus sabaeus　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Papio Hamadryas　　　AAGAAAGAAACCAAATT–CATACAACCTCCGAAACTGAGATTGAAACCAA　50
Lagothrix lagotricha　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Callithrix jacchus　　AAGAAAGAAACCAAATTTCATACAACCTCCGAAACTGAGATTGAAACCAA　50
Mus musculus　　　　AAGAGAGGAACCAAATT–CATACAACCTCCAAAACTGAGATTGAAACCAA　49
　　　　　　　　　　　**** ** ********* ************ *****************

(a)

(b)
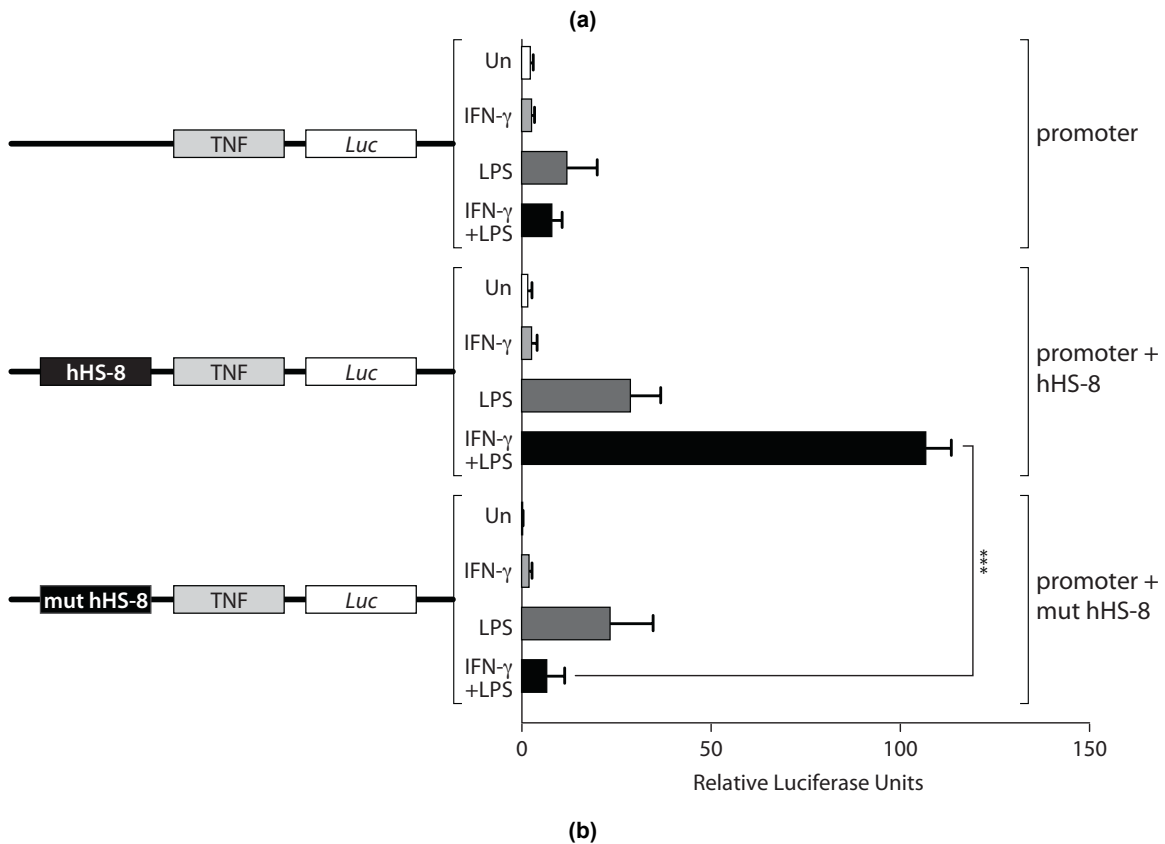
Figure 2.2.5: hHS-8 functions as an IFN-γ-inducible, IRF1-dependent enhancer of *TNF* transcription. (a) IRF1 binding sites (nucleotides -6833 to -6784) in hHS-8 are highly conserved among all primate species examined. Critical -GAAA- motifs for IRF1 binding are highlighted. (b) Disruption of IRF1 binding to hHS-8 abolishes inducible enhancer function and thus enhanced *TNF* transcription induced by IFN-γ priming. Constructs were transfected into J774 cells and stimulated with IFN-γ alone for 8h, LPS alone for 6h, and both IFN-γ and LPS (IFN-γ pretreatment for 2h followed by LPS treatment for 6h). "TNF" is the *TNF* promoter and "hHS-8" is the entire sequence of hHS-8 (1250bp in length), and "muthHS-8" is hHS-8 with mutations that disrupt IRF1 binding. (***) $p \leq 0.001$.

stimulations (Figure 2.2.6c). Notably, THP-1 cells are deficient in UTX expression [221] and thus serve as an effective model system for understanding the effects of specifically inhibiting JMJD3 with the compound. In cells treated with GSK-J5, we observed effective IFN-γ priming, evidenced by enhanced TNF mRNA levels upon LPS induction (Figure 2.2.6d). In contrast, IFN-γ priming failed to enhance TNF mRNA levels in cells treated with GSK-J4, demonstrating a critical role for H3K27me3 in IFN-γ priming and the ability to enhance *TNF* transcription. We hypothesize that IFN-γ poises hHS-8 for enhancer function through mechanisms that involve IRF1 recruitment and H3K27me3 enrichment. Subsequent LPS stimulation activates hHS-8 enhancer function through mechanisms that first demethylate and then acetylate H3K27.
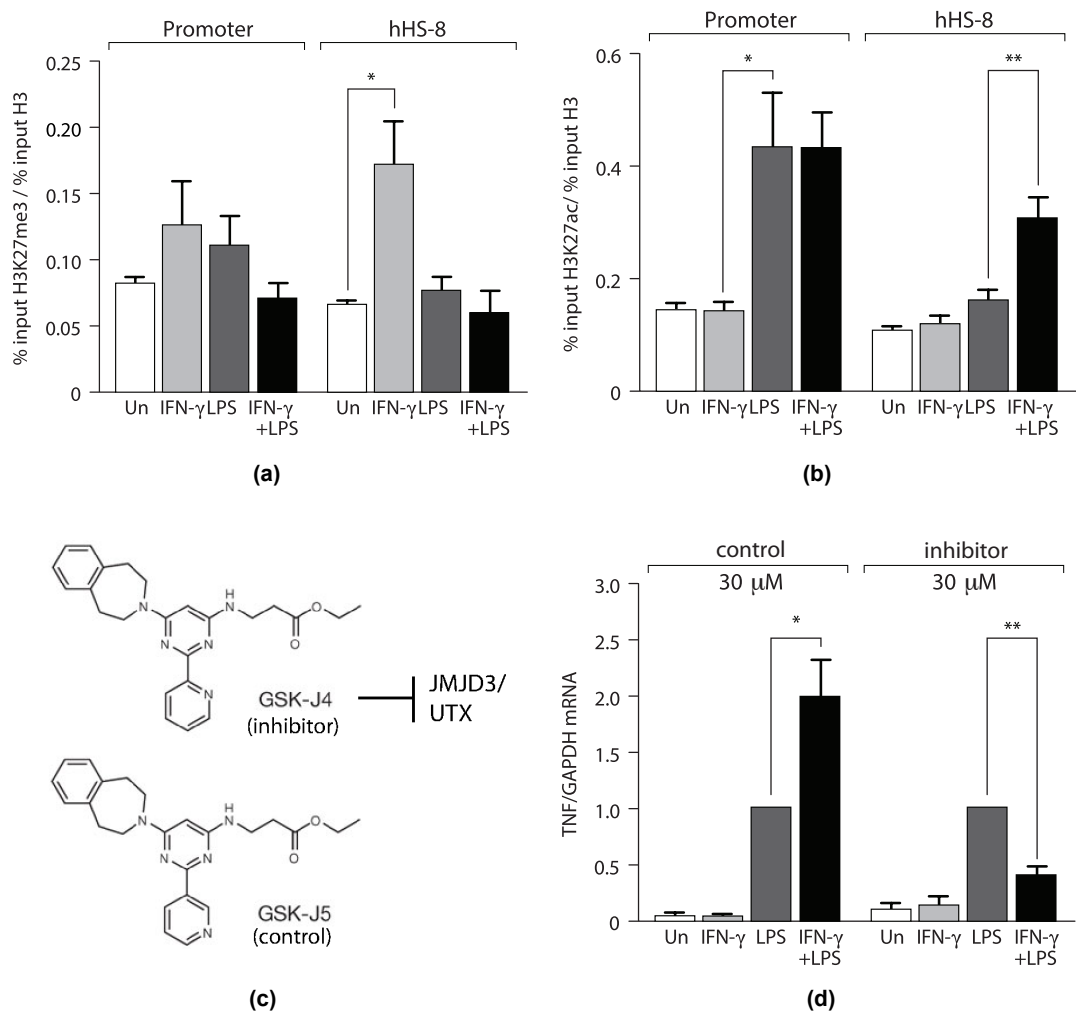
Figure 2.2.6: H3K27me3 and H3K27ac play a critical role in IFN-γ priming. (a) IFN-γ increases H3K27me3 prevalence at hHS-8. ChIP using THP-1 cells and analyzing H3K27me3 prevalence at the *TNF* promoter and hHS-8. (b) Activation of hHS-8 enhancer function corresponds with increased H3K27ac prevalence at hHS-8. ChIP using THP-1 cells and analyzing H2K37ac prevalence at the *TNF* promoter and hHS-8. (c) Chemical structures of the active JmjD3/UTX inhibitor (GSK-J4) and the inactive inhibitor (GSK-J5). (d) Inhibition of H3K27me3 demethylation prevents enhanced *TNF* gene expression induced by IFN-γ priming. THP-1 cells were treated with either the GSK-J5 or GSK-J4 compound (30uM) 30min before LPS stimulations, and TNF mRNA levels were measured (shown relative to LPS values). (*) $p \leq 0.05$ and (**) $p \leq 0.01$.

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2.3   Discussion

One of the most important functions of IFN-γ is to prime monocytes and macrophages thereby ensuring an enhanced response to microbial activation. The mechanism of IFN-γ priming has been investigated for IL-12 [84] and IL-6 [57], and in both cases, transcriptional regulation has been confined to the promoter. Considering the importance of TNF and its dysregulation in the pathology of several inflammatory disorders, we sought to determine the mechanism for IFN-γ-induced priming of *TNF* gene expression. Here, we observe transcriptional regulation by IFN-γ to be beyond the promoter by demonstrating a critical role for an inducible enhancer distal to the *TNF* TSS. In addition to showing a prominent role for IRF1 in enhancer function, we observe H3K27me3 marking a poised enhancer in an adult somatic cell in the context of immune signaling.

Spanning ~1.3kb, hHS-8 is nearly five times larger than the average non-promoter DH site [122], and we have shown functional IRF1 recruitment to its most highly conserved region. We do expect hHS-8 enhancer function to additionally involve the recruitment of coactivator proteins and multiple lineage-specific transcription factors. Indeed, studies in murine T cells have shown NFAT binding to HSS-9 [177]. Considering the necessity of IRF1 for enhanced *TNF* gene expression in primed monocytes and macrophages, we wonder whether IRF1 functions as a pioneer factor. First identified in yeast, pioneer factors are transcription factors that bind to DNA and recruit chromatin-remodeling complexes thereby promoting the recruitment of other factors to their corresponding DNA sequences that otherwise would have been inaccessible [165]. Additional binding studies in monocytes and macrophages are required in order to properly assess whether IRF1 functions as a pioneer factor. Indeed, IRF1 has been reported to interact with the histone acetyltransferase, CBP/p300 [222]. In addition, we have shown by ChIP analysis that p300 is recruited to hHS-8 upon LPS stimulation in primary human MDMs (Figure A.0.6). We can imagine

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a situation where IFN-γ utilizes IRF1 to recruit CBP/p300 to hHS-8; CBP/p300, along with the aid of other factors, could induce chromatin-remodeling events necessary for enhancer activation.

Enhancers are often characterized by chromatin signatures such as increased chromatin accessibility, enrichment of specific histone modifications like H3K4me1 and H3K27ac, prevalence of RNA Pol II, presence of histone variants H3.3 and H2A.Z, and increased prevalence of ATP-dependent chromatin remodeling complexes like CBP/p300 [223]. Recent studies in embryonic stem cells have shown that only a fraction of elements marked by H3K4me1 are actively engaged in enhancing transcription; thus, H3K4me1 does not distinguish between active and inactive/poised enhancers. The presence of H3K27ac has been extensively shown in both human and mouse embryonic stem cells to separate active from poised enhancers [218]. Specifically, active enhancers are enriched for H3K27ac while poised enhancers lack H3K27ac enrichment. In addition to lacking H3K27ac, poised enhancers have been shown, most often, in pluripotent cells to be enriched for H3K27me3 [219, 220]. Here, we show that in an adult somatic cell, IFN-γ marks a poised enhancer in the *TNF/LT* locus with H3K27me3 thereby supporting the finding that H3K37me3 enrichment at poised enhancers is not limited to pluripotent cells. We also show that upon LPS stimulation, levels of H3K27me3 are significantly decreased at hHS-8; this is followed by an enrichment of H3K27ac, which suggests enhancer activation.

What then is the mechanism for LPS-induced removal of H3K27me3 and addition of H3K27ac at hHS-8? We hypothesize that the same factor(s) responsible for demethylating H3K27 form a complex with the factor(s) responsible for acetylating H3K27. Interestingly, a study in *Drosophila* showed that UTX physically associated with CBP *in vivo* [224], suggesting that the individual activities of demethylating and acetylating H3K27 are functionally coupled. We hypothesize that upon LPS stimulation, complexes containing JMJD3 (or UTX) and CBP/p300 associate with hHS-8 and effectively demethylate and acetylate

H3K27 residues. This in turn is essential for hHS-8 enhancer activation.

In addition, investigating whether IRF1 recruitment to hHS-8 plays a functional role in the enrichment of H3K27me3 and H3K27ac may provide novel insights regarding the role of transcription factors in enhancer activation. ChIP analysis of H3K27me3 and H3K27ac in primed monocytes and macrophages where IRF1 expression is knocked down could provide insight into the many functions of IRF1. We would like to note that GSK-J4 targets global H3K27me3 demethylation and that it is difficult to understand the effects of this modification at one particular region of DNA. Thus experiments targeting the disruption of H3K27me3 demethylation specifically at hHS-8 would need to be employed.

In our model, we predict the formation of an IRF1-dependent intrachromosomal interaction at the *TNF/LT* locus between the promoter and hHS-8 upon TLR-induced activation in monoctyes and macrophages primed by IFN-γ. The mechanism(s) of action on promoters for enhancers has yet to be determined. The predominant model known as the "looping" model predicts a direct interaction between the promoter and enhancer resulting in the displacement or "looping out" of the intervening DNA [169]. Using chromosome conformation capture (3C) assay, an inducible intrachromosomal interaction between HSS-9, HSS+3, and the *TNF* promoter was demonstrated in murine T cells [177]. Unfortunately, we were unable to successfully perform the 3C assay using our model system. Other, less robust techniques suggesting a direct interaction between a promoter and enhancer involve Pol II occupancy at the enhancer. We have yet to investigate whether Pol II is recruited to hHS-8 upon TLR-activation in monoctyes or macrophages primed by IFN-γ. Other potential mechanisms of enhancer action include enhancer RNAs (eRNAs) [225]. eRNAs are transcripts that are products of transcription at Pol II-associated enhancers; they can either by polyadenylated or non-polyadenylated. The fact that not all enhancers are transcribed implies functional differences between transcribed and non-transcribed enhancers. It is thought that transcription of enhancers, similar to H3K27ac enrichment, might mark

active enhancers; however, the functional roles, if any, for eRNAs are unknown. It is of interest to investigate whether hHS-8 is transcribed.

In conclusion, our identification of critical protein-DNA interactions and inducible histone modifications during IFN-γ priming of LPS-induced *TNF* transcription not only provides a mechanism of IFN-γ priming but also provides potential therapeutic targets to control TNF dysregulation. Future studies will characterize additional factors and interactions that regulate this vital immune process.

2.4   Materials and Methods

Cell culture and stimulations

THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS. J774 cells were maintained in DMEM supplemented with 10% FBS. For primary human MDMs, enriched populations of human monocytes were isolated from healthy human donor buffy coats using a CD14$^+$ positive selection kit (Stemcell). MDMs were obtained after 6d of culture in RPMI-1640 medium supplemented with 5% human serum AB (Gemcell) and GM-CSF (50ng/ml; Peprotech). Cells were treated with IFN-γ (100ng/ml; R&D) and LPS (100ng/ml; Sigma E. coli O111:B4). GSK-J4 and GSK-J5 compounds [147] were generously supplied by GlaxoSmithKline, resuspended in DMSO, and used at a final concentration of 30μM.

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells with a QuickRNA Mini kit (Zymo) and treated with Turbo DNA-free kit (Invitrogen). cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen) and 20-residue oligo (dT) (Invitrogen). TNF mRNA levels were measured by the change-in-threshold ($\Delta\Delta$Ct) method based on quantitative real-time PCR in an iCycler iQ (Bio-rad) with SyberGreen Master Mix (Invitrogen) and primers rec-

Primer	Sequence
Human TNF exon 4 (F)	TCTTCTCGAACCCCGAGTGA
Human TNF exon 3 (R)	CCTCTGATGGCACCACCAG
Human GapdH (F)	GGTGGTCTCCTCTGACTTCAACA
Human GapdH (R)	GTTGCTGTAGCCAAATTCGTTGT
Human IRF1 (F)	GCCAGTCGACGAGGATGAGGAAGGG
Human IRF1 (R)	CCAGCGGCCGCCTGCTACGGTGCAC
ChIP IRF1, -244 to -82 (F)	GGGAGTGTGAGGGGTATCCT
ChIP IRF1, -244 to -82 (R)	CATTCAACCAGCGGAAAACT
ChIP IRF1, -6842 to -6737 (F)	GGCCTGCCCAAGAAAGAAACCAAA
ChIP IRF1, -6842 to -6737 (R)	ACGTGCATGTGAGATATGCGAAGG
ChIP TaqMan, *TNF* promoter (F)	GCGATGGAGAAGAAACCGAGACAGAA
ChIP TaqMan, *TNF* promoter (R)	GGGTGTGCCAACAACTGCCTTTAT
ChIP TaqMan, *TNF* promoter (probe)	/56-FAM/AGCTCATGG/ZEN/GTTTCTCCACCAAGGAA/3IABkFQ/
ChIP TaqMan, hHS-8 (F)	GGCCTGCCCAAGAAAGAAACCAAA
ChIP TaqMan, hHS-8 (R)	ACGTGCATGTGAGATATGCGAAGG
ChIP TaqMan, hHS-8 (probe)	/56-FAM/AACCAAGAT/ZEN/TGGCCCATCTCAAGGAGCA/3IABkFQ/

ognizing exon 4 and exon 3 of the human *TNF* gene, the human *GapdH* gene, and the human *IRF1* gene (Table 2.4.1).

Measurement of TNF production

TNF secretion was quantified by ELISA specific for murine and human TNF (R&D) using a VMax®Kinetic ELISA Microplate Reader.

DNase I Hypersensitivity Assay (DHA)

DHAs were performed using both THP-1 cells and primary human MDMs. MDMs were detached from culture surface using TrypLE™ (Life Technologies). Cells were harvested, washed with PBS, and resuspended in RSB buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, and 3mM MgCl2). Cells were lysed with lysis buffer (0.5% NP-40 in 1x RSB buffer) on ice for 5min. Resuspended nuclei in RSB buffer were treated with DNase I (40ng/ul) at

37C for 5min. DNase I activity was quenched upon addition of stop solution (0.6M NaCl, 20mM Tris-HCl pH 8.0, 10mM EDTA, and 1% SDS). Samples were treated with Proteinase K at 56C O/N. DNA was digested with ScaI and BamHI restriction enzymes and analyzed by Southern blotting using a radiolabeled P^{32} probe corresponding to the coding region of LT-α. 10ug of DNA was used for each lane.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with anti-IRF1 (H-205, Santa Cruz), Rb IgG (Diagenode), anti-H3K27me3 (C36B11, Cell Signaling), anti-H3K27ac (Active Motif), and anti-H3 (Abcam). THP-1 cells and primary human MDMs were treated (IFN-γ 100ng/ml, LPS 100ng/ml), fixed with 10% formaldehyde for 15min, treated with 2.5M glycine for 5min, harvested, washed with PBS, lysed with 0.25% Triton X-100 and 0.5% NP-40 for 5min, centrifuged at 1200RPM for 10min, resuspended in 1% SDS lysis buffer, and sonicated 5min for 4 cycles in a Biorupter. Sonicated DNA was set up for immunoprecipitation O/N and DNA-protein complexes were recovered by adding Protein A/G Plus Agarose Beads (Thermo Scientific) for 3h. Samples were washed 6 times with 1ml of wash buffer and treated with proteinase K at 65C O/N. Samples were treated with phenol/chloroform before O/N ethanol precipitation. DNA fragments for IRF1 recruitment were analyzed by quantitative real-time PCR with Sybergreen master mix (Invitrogen) and primer sets for regions -244 to -82 (promoter) relative to the *TNF* TSS and -6842 to -6737 (hHS-8) relative to the *TNF* TSS (Table 2.4.1). Rb IgG percent input values were subtracted from IRF1 percent input values. DNA fragments for H3K27me3 and H3K27ac analysis were analyzed by quantitative real-time PCR using Jumpstart™ Taq ReadyMix™ For Quantitative PCR (Sigma) and primer/probe sets for the *TNF* promoter and hHS-8 (Table 2.4.1). H3K27me3 and H3K27ac percent input values were normalized to H3 percent input values.

DNase I Footprinting Assay

Radiolabeled P^{32} fragments of the *TNF* promoter (-200 to +1) and hHS-8 (-7031 to -6782) regions were incubated with recombinant IRF1 protein (Abcam) and treated with diluted DNase I at RT for 5s before quenching enzyme activity with stop solution (0.13mM EDTA, 0.5% SDS, and tRNA). Samples were treated with phenol chloroform and DNA was precipitated O/N at -20C. G/A ladder was treated with 4% formic acid, radiolabeled with P^{32}, treated with 1M piperdine, and precipitated with n-butanol. DNA fragments were separated by electrophoresis on an 8% sequencing gel.

Mice

6-8 week old C57BL/6J mice and B6.129S2-Irf1^{tm1Mak}/J mice were purchased from Jackson Laboratories. Experimental procedures were done in accordance with the Institutional Animal Care and Use Committee (IACUC) and the Harvard Medical Area Standing Committee on Animals (HMA IACUC).

Isolation, culture, and stimulation of murine BMDMs

For the generation of murine bone marrow-derived macrophages (BMDMs), bone marrow cells of wild type or *Irf1-/-* mice (6-8wks) were cultured in DMEM medium supplemented with 10% FBS, 10% L929 cell conditioned medium (LCCM), 100U/ml penicillin, 100ug/ml streptomycin, and 2mM L-glutamine. Cells were fed on day 5 and media was changed on day 7, 3h before mIFN-γ (100ng/ml; R&D) and LPS (100ng/ml; Sigma E. coli O111:B4) treatment. Supernatant was collected 2, 4, and 6h after treatment.

Short hairpin RNA

The lentiviral plasmid PLKO.1 expressing shRNA targeting human IRF1 was purchased from the RNAi Consortium (TRC) Lentiviral shRNA library (Thermo Scientific). Clone

TRCN0000014668 with a target sequence of CGTGTGGATCTTGCCACATTT was validated in our laboratory. Control shRNA encodes a scrambled sequence. Lentiviruses encoding shRNA sequences were generated by transfecting the packaging cell line HEK-293T with the shRNA-encoding pLOK.1 plasmids in combination with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Effectene transfection reagent (Qiagen). Supernatants were collected 48h post-transfection, clarified by centrifugation, and stored at -80C. THP-1 cells were transduced with the lentiviral particles by culturing the cells with supernatants from the virus-producing cells in the presence of 8ug/ml polybrene (Millipore) and spinoculation for 2h at 2000RPM. Successfully transduced cells were selected and expanded by treatment with 0.8ug/ml puromycin.

Electrophoretic Mobility Shift Assay (EMSA)

Radiolabeled P^{32} oligonucleotides were added to THP-1 nuclear extracts or recombinant IRF1 protein (Abcam) in a binding buffer solution (10mM Tris-HCl pH 7.5, 53mM NaCl, 1mM DTT, 0.01% Nonidet-P40, 5% glycerol, and 0.05ug/ul of double-stranded poly(dI-dC)) at RT for 30min. In super-shift experiments, samples were incubated with 2ug of anti-IRF1 (H-205, Santa Cruz Biotechnology). Protein-DNA complexes were separated by electrophoresis on a 5% PAAG gel.

Sequencing

Procurement of cell lines and samples of blood or DNA from representative individuals of the primate species and subspecies was previously described [197, 210]. Genomic DNA was isolated using QIAamp DNA Blood kit (Qiagen). Sequence alignments were performed using ClustalW2 multiple sequence alignment provided by EMBL-EBI.

Plasmids

Construction of the *TNF* promoter-driven luciferase reporter was previously described [189]. The *TNF* promoter with hHS-8 plasmid was constructed by inserting nucleotides -7833 to -6583 relative to the *TNF* TSS into the *TNF* promoter-driven luciferase reporter construct using MluI and NheI restriction enzyme sites. The *TNF* promoter with mutated hHS-8 plasmid was constructed by circular site-directed mutagenesis.

Luciferase Reporter Assay

J774 cells were transfected with luciferase reporter constructs using an Effectene Transfection Reagent kit (Qiagen). Cells were treated with mIFN-γ (100ng/ml; R&D) and LPS (100ng/ml, Sigma E. coli O111:B4). Luciferase assays were performed 8h after treatment under the Dual Luciferase Reporter Assay System (Promega) using a Dynex luminometer and Renilla luciferase (pRL-TK) as a control.

Chapter 3

Characterization of chromatin alterations at the *TNF/LT* locus during endotoxin tolerance and its IFN-*γ*-mediated abrogation

3.1 Introduction

Sepsis is a major cause of death in modern intensive care units (ICUs) and accounts for a huge financial burden on public health care systems [226]. It is caused by severe infection and is classically characterized by an initial phase of uncontrolled systemic inflammation that is uniquely followed by a prolonged immunosuppressive state [227–230]. Indeed, this later, immunosuppressive phase frequently correlates with high risks of secondary infection and mortality during the patient's ICU stay [231]. Studies have demonstrated that blood monocytes isolated from sepsis patients who have entered the immunosuppressive phase share several characteristics with endotoxin-tolerant monocytes and macrophages [232–235]. Thus, an understanding of how monocytes and macrophages "switch" from inflammatory to immunosuppressive states could help clinicians more efficiently target the later, more severe stages of sepsis.

As stated previously, endotoxin tolerance is the phenomenon in which immune cells, primarily monocytes and macrophages, transiently become hyporesponsive or "tolerant" upon

repeated or prolonged exposure of LPS [64]. In the case of sepsis, monocytes isolated from sepsis patients and subsequently stimulated with LPS were shown to express significantly lower levels of inflammatory cytokines like Il-1β, IL-6, and especially TNF, as compared to LPS-induced monocytes from healthy individuals [232].

One study examining epigenetic modifications at individual promoters in murine macrophages demonstrated that chromatin modifications distinguish two distinct classes of genes during endotoxin tolerance."Class T" comprised genes that were tolerant upon repeated challenges of LPS while "class NT" comprised genes that remained inducible upon repeated challenges of LPS [236]. Importantly, "class T" promoters in contrast to "class NT" promoters remained primarily deacetylated and failed to recruit the chromatin remodeling protein Brg1. This and the work of others demonstrate the importance of examining chromatin modifications at individual promoters when determining mechanisms for selective modulation of inflammatory responses [237, 238].

Notably, IFN-γ priming has been shown to abrogate endotoxin tolerance, thereby restoring responses to LPS stimulation in monocytes and macrophages [73]. Using primary human monocytes, IFN-γ priming was shown to restore *IL6* transcription by promoting chromatin accessibility at the human *IL6* promoter [239]. Thus, the mechanisms responsible for the induction and abrogation of endotoxin tolerance involve chromatin remodeling. Few studies have focused on *TNF* gene regulation in the context of endotoxin tolerance [239–241] and none to our knowledge have looked beyond the promoter. Here, by examining the *TNF/LT* locus, we argue that alterations in chromatin modifications at the *TNF* promoter and distal DNA elements play a major role in the induction and IFN-γ-mediated abrogation of endotoxin tolerance. Specifically, we have shown that H3K27me3 marks the minimal *TNF* promoter in endotoxin-tolerant macrophages. In addition, we look beyond the *TNF* promoter and observe the appearance of an IFN-γ-inducible DH site that is ~4kb upstream of the *TNF* TSS.

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3.2 Results

3.2.1 IFN-*γ* restores *TNF* gene expression in endotoxin-tolerant monocytes and macrophages.

To induce endotoxin tolerance, we treated primary human MDMs with LPS (100ng/ml) for 24h followed by a second challenge of LPS (100ng/ml) (Figure A.1.1). From past *in vitro* studies, methods for inducing endotoxin tolerance vary with regards to the number of LPS challenges and the concentration of LPS used for each challenge. Despite these variations in experimental design, it is generally accepted that repeated or prolonged exposure of LPS induces endotoxin tolerance in monocytes and macrophages. We next measured TNF mRNA levels from both endotoxin-tolerant (T) and non-tolerant (LPS) MDMs stimulated with LPS. We observed a significant decrease in mRNA levels from endotoxin-tolerant MDMs as compared to non-tolerant MDMs, which suggests that the mechanism(s) of endotoxin tolerance targets *TNF* transcription (Figure 3.2.1a). We also observed the effects of IFN-*γ* priming by pretreating endotoxin-tolerant MDMs with IFN-*γ* 2h before the second challenge of LPS (Figure A.1.1). As reported in primary human monocytes [239], IFN-*γ* priming partially restores the TNF response in endotoxin-tolerant MDMs (Figure 3.2.1a). We also confirmed the induction and abrogation of endotoxin tolerance in THP-1 cells, a human monocytic cell line, using the same experimental design (Figure 3.2.1b).

3.2.2 IFN-*γ* abrogates endotoxin tolerance in an IRF1-independent manner.

Considering the mechanism that we have defined for IFN-*γ* priming in non-tolerant THP-1 cells and primary human MDMs, we sought to understand whether IRF1 plays a major role in the ability of IFN-*γ* to restore *TNF* gene expression in endotoxin-tolerant cells. THP-1 cells were transfected with either shRNA targeting human IRF1 or control shRNA encoding a scrambled sequence. Sufficient knockdown of IRF1 mRNA was observed when cells
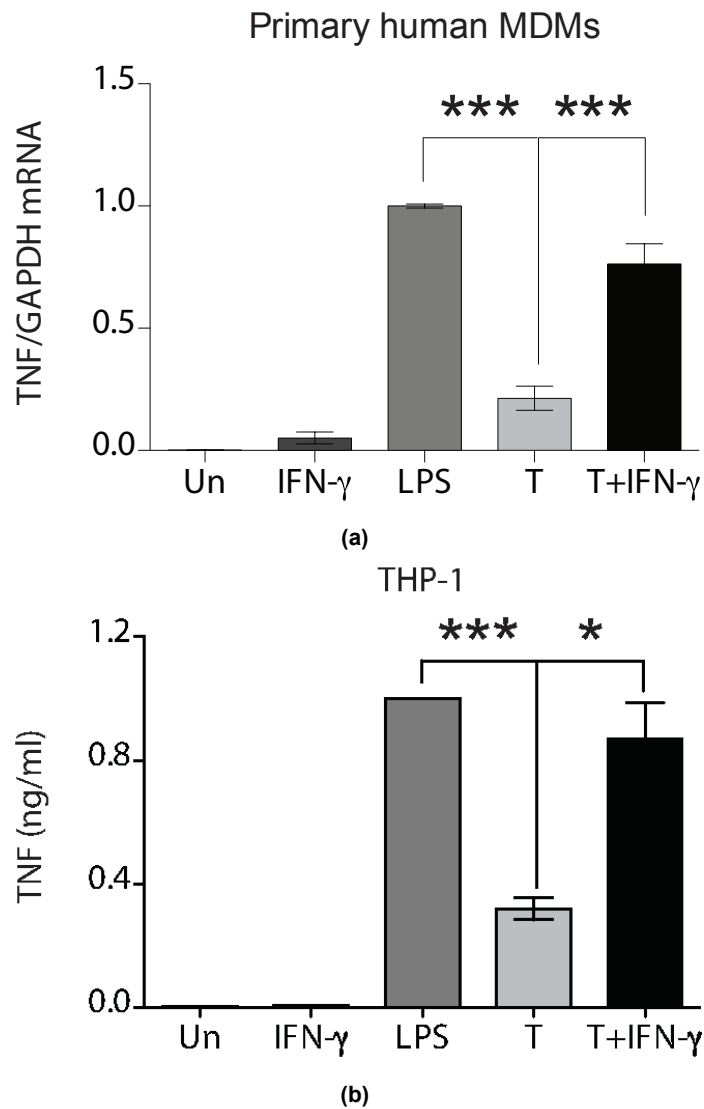
Figure 3.2.1: IFN-γ restores *TNF* gene expression in endotoxin-tolerant monocytes and macrophages. (a) Primary human MDMs were stimulated (experimental design Figure A.1.1), and TNF mRNA levels were measured. Data from 3 separate donors. (b) THP-1 cells were stimulated as in (a) and TNF protein levels in supernatants were measured by ELISA post LPS stimulation (shown relative to LPS values). (*) $p \leq 0.05$ and (***) $p \leq 0.001$.
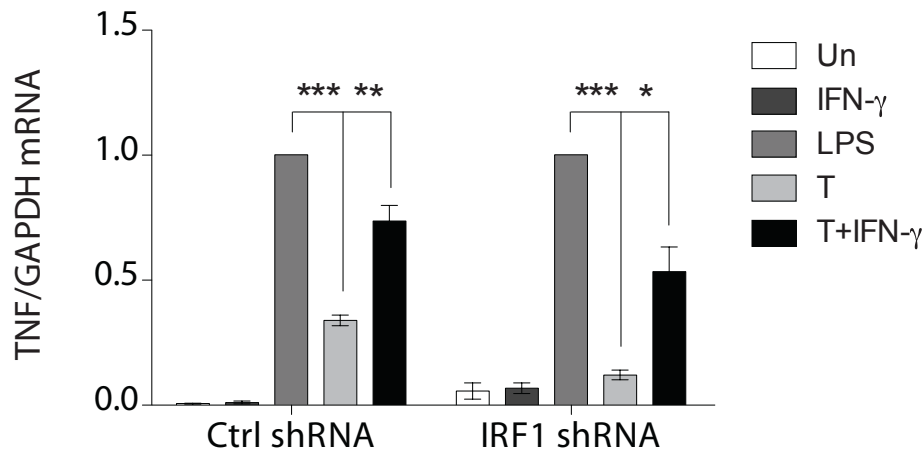
Figure 3.2.2: IFN-γ abrogates endotoxin tolerance in an IRF1-independent manner. THP-1 cells expressing IRF1 and control shRNA were stimulated, and TNF mRNA levels were measured (shown relative to LPS values). (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$.

were treated with IFN-γ for 3h (Figure 2.2.4b), which we concluded as sufficient knock-down of IRF1 protein considering its extreme instability (half-life ~30min) [215]. As expected, IFN-γ priming partially restored *TNF* gene expression in endotoxin-tolerant THP-1 cells expressing control shRNA (Figure 3.2.2). Importantly, partial restoration of *TNF* gene expression also occurred in endotoxin-tolerant THP-1 cells expressing IRF1 shRNA, demonstrating that IFN-γ does not require IRF1 to restore the TNF response during endotoxin tolerance. We hypothesize that two distinct mechanisms are responsible for the effects of IFN-γ in LPS responsive and non-responsive monocytes and macrophages.

3.2.3   IFN-γ promotes chromatin accessibility at the *TNF* promoter in endotoxin-tolerant monocytes.

We next examined changes in chromatin accessibility at the *TNF/LT* locus during the induction and abrogation of endotoxin tolerance by performing DNase I hypersensitivity assays (DHAs). We hypothesized that the induction of endotoxin tolerance results in decreased chromatin accessibility at the *TNF* promoter, and that IFN-γ-mediated abrogation of endotoxin tolerance results in the restoration of accessibility. DNA from endotoxin-tolerant and

non-tolerant THP-1 cells stimulated with IFN-γ was partially digested with DNase I. KpnI digestion allowed for visualization of DNase I hypersensitivity at the *TNF* promoter (Figure 3.2.3a). When comparing endotoxin-tolerant and non-tolerant monocytes stimulated with LPS, we observed minimal differences in DNase I hypersensitivity at the *TNF* promoter (Figure 3.2.3b, lane 6 compared to lane 8). We conclude that inhibition of TNF mRNA levels during endotoxin tolerance is not a result of decreased chromatin accessibility at the *TNF* promoter.

In fact, we observed increased chromatin accessibility, as evidenced by a slight widening of the band representing the *TNF* promoter (Figure 3.2.3b, lane 10). This suggests the appearance of an additional DH site, which may play a role in the mechanism by which IFN-γ priming restores *TNF* gene expression in endotoxin tolerance.

3.2.4 H3K27me3 and H3K27ac levels at the *TNF* promoter predict both the induction and abrogation of endotoxin tolerance in human monocytes and macrophages.

We next investigated histone modifications at the *TNF* promoter during the induction and abrogation of endotoxin tolerance, and identified two modifications that mark LPS responsive and non-responsive monocytes and macrophages. ChIP studies showed significant enrichment of H3K27me3 levels at the *TNF* promoter in endotoxin-tolerant THP-1 cells (Figure 3.2.4a). Conversely, endotoxin-tolerant cells primed by IFN-γ showed no enrichment for H3K27me3 levels at the *TNF* promoter. Results were repeated in primary human MDMs isolated from a representative donor (Figure 3.2.4b). We also observed a complementary response in H3K27ac levels at the *TNF* promoter in response to the induction and the abrogation of endotoxin tolerance. Specifically, IFN-γ priming resulted in a dramatic increase of H3K27ac levels at the *TNF* promoter in endotoxin-tolerant monocytes and macrophages (Figure 3.2.4c, 3.2.4d), which supports the previous finding of increased chromatin accessibility at the *TNF* promoter (Figure 3.2.3b). This data identifies distinct
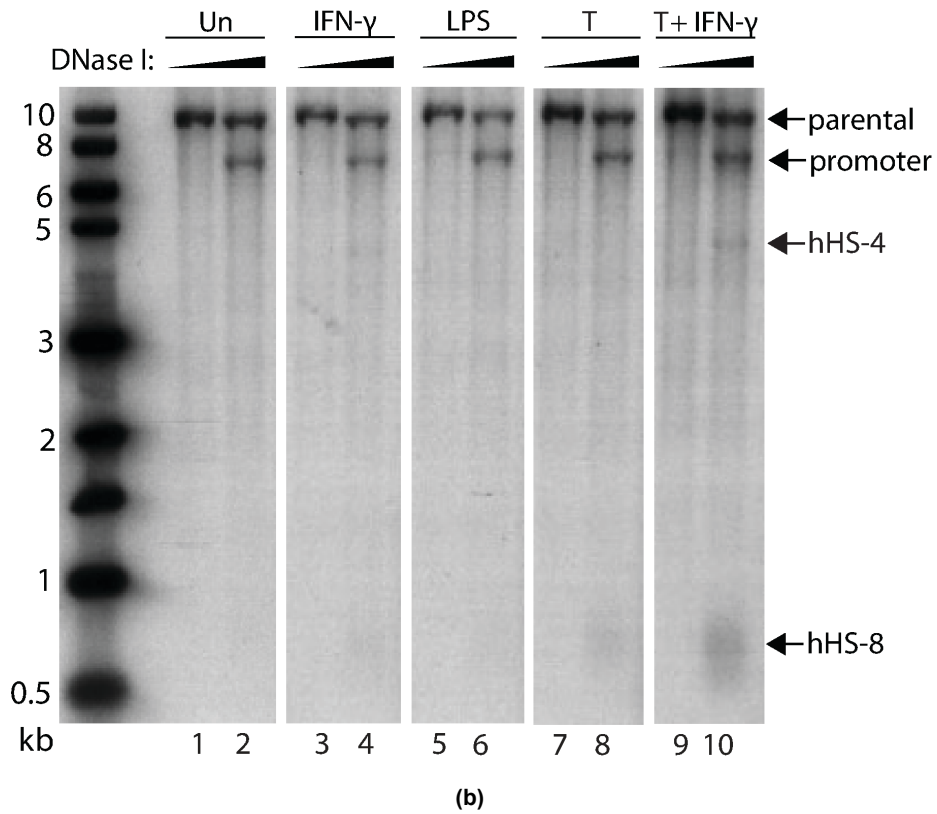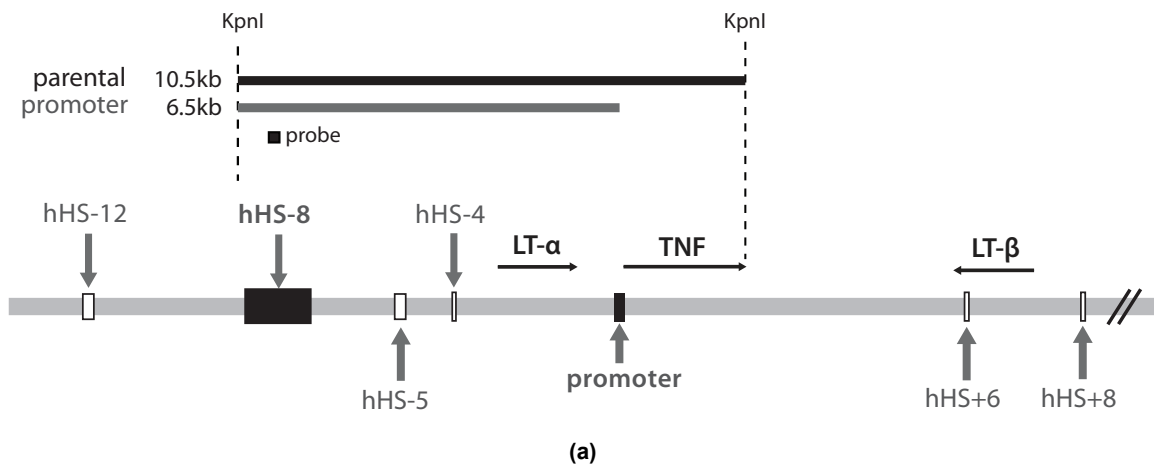
Figure 3.2.3: IFN-γ promotes chromatin accessibility at the *TNF* promoter in endotoxin-tolerant monocytes. (a) Map of the human *TNF/LT* locus. DH sites and positions and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. Positions of the parental KpnI and DNase I digestion products for the DHA are indicated. Fragment representing DNase I accessibility at the *TNF* promoter is 6.5kb in length. (b) IFN-γ increases DNase I cleavage at the *TNF* promoter in endotoxin-tolerant THP-1 cells.

histone modifications at the *TNF* promoter that associate with the induction and abrogation of endotoxin tolerance.

3.2.5 IFN-γ induces the formation of hHS-4 at the *TNF/LT* locus in endotoxin-tolerant human monocytes.

In addition to the *TNF* promoter, we observed chromatin alterations at distal DNA elements within the *TNF/LT* locus during the induction and abrogation of endotoxin tolerance. DNA from endotoxin-tolerant and non-tolerant THP-1 cells stimulated with and without IFN-γ was partially digested with DNase I. ScaI digestion allowed for visualization of both hHS-8 and hHS-4 (Figure 3.2.5a). We detected a strongly enhanced band corresponding to hHS-8 from endotoxin-tolerant cells as compared to non-tolerant cells stimulated with LPS (Figure 3.2.5b, lane 9 compared to lane 12). In addition, increased chromatin accessibility at hHS-8 was sustained in endotoxin-tolerant cells treated with IFN-γ. Past studies have shown that CBP/p300 is recruited to the *TNF* promoter in monocytes and macrophages activated by LPS [189]. We speculate that prolonged exposure and additional challenges of LPS induce continued CBP/p300 recruitment to the *TNF* promoter, thereby promoting DNase I cleavage at hHS-8.

Furthermore, IFN-γ priming in endotoxin-tolerant cells resulted in the formation of the DH site hHS-4 (Figure 3.2.5b, lane 15). This DH site is located ~4kb upstream of the *TNF* TSS and lies within the *LTA* promoter. Although hHS-4 (Figure A.1.2) is not constitutively present in monocytes and macrophages, studies from the Goldfeld lab have shown that hHS-4 is constitutively present in CD4$^+$ T cells (unpublished data). We also observed a slight increase in DNase I cleavage at hHS-4 in non-tolerant monocytes primed by IFN-γ. It is important to note that although non-tolerant and endotoxin-tolerant cells are primed by IFN-γ for the same duration (2h), induction of hHS-4 is significantly stronger in endotoxin-tolerant cells. This suggests that endotoxin tolerance provides an environment that promotes

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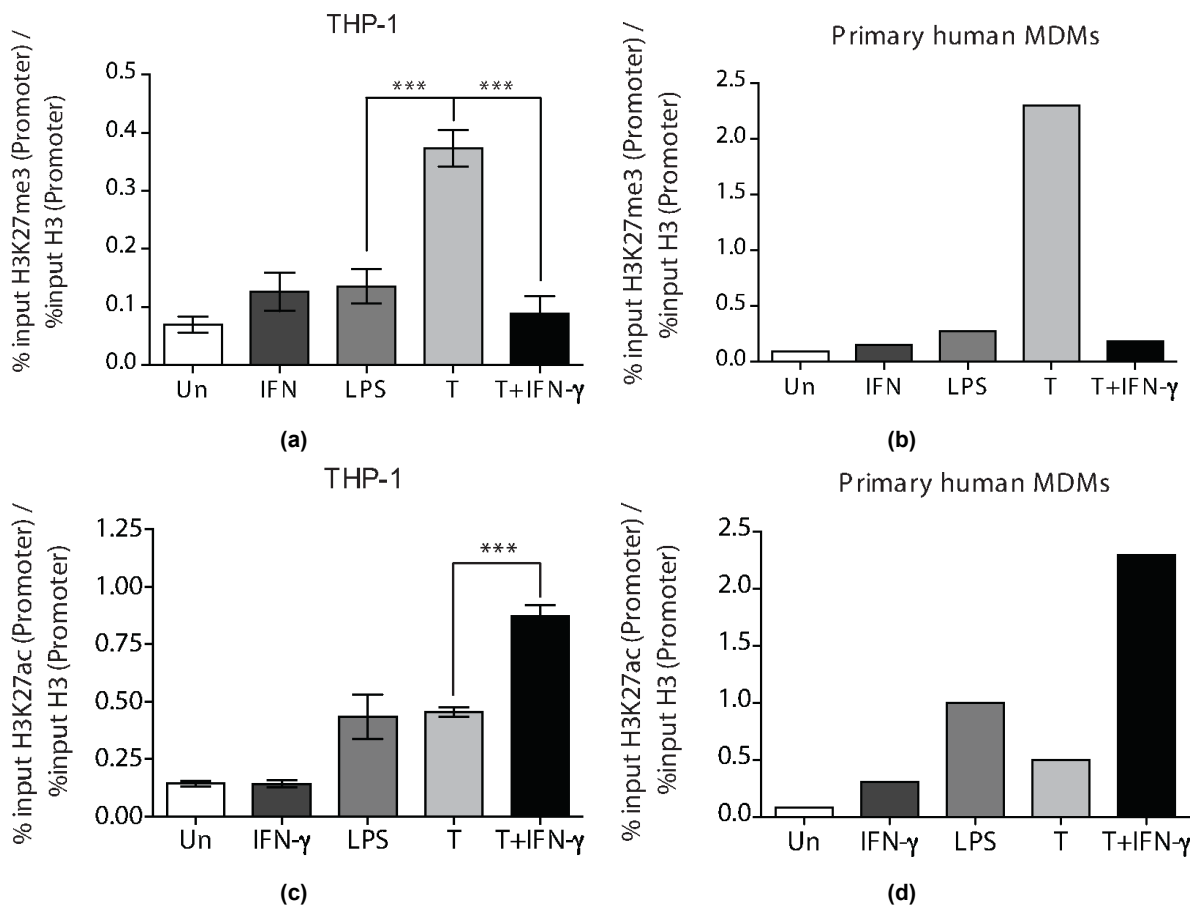
Figure 3.2.4: H3K27me3 and H3K27ac levels at the *TNF* promoter predict both the induction and abrogation of endotoxin tolerance in human monocytes and macrophages. (a) Induction of endotoxin tolerance correlates with an enrichment of H3K27me3 at the *TNF* promoter. ChIP using THP-1 cells and analyzing H3K27me3 prevalence. (b) ChIP as in (a) using primary human MDMs from a representative donor. (c) Abrogation of endotoxin tolerance correlates with an enrichment of H3K27ac at the *TNF* promoter. ChIP using THP-1 cells and analyzing H3K27ac prevalence. (d) ChIP as in (c) using primary human MDMs from a representative donor. (*) $p \leq 0.05$, (**) $p \leq 0.01$, and (***) $p \leq 0.001$.

chromatin accessibility at hHS-4.
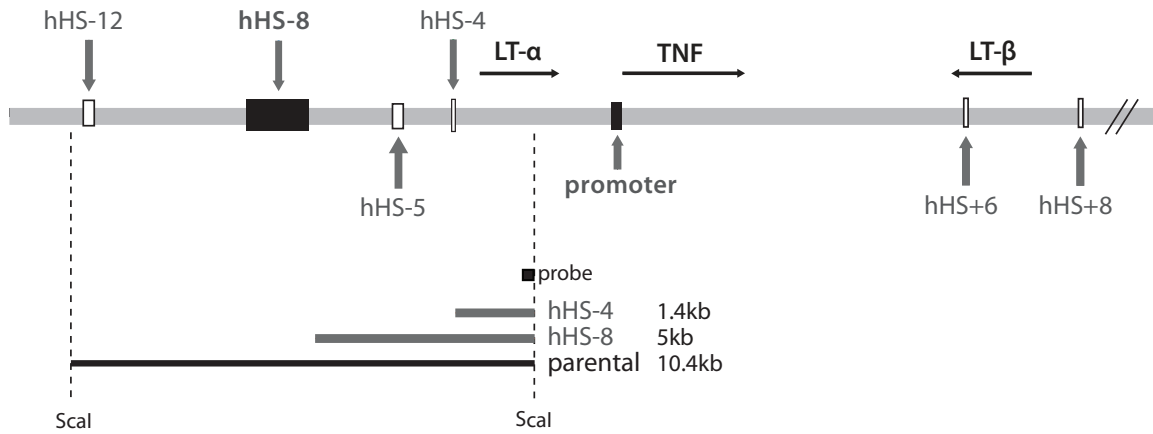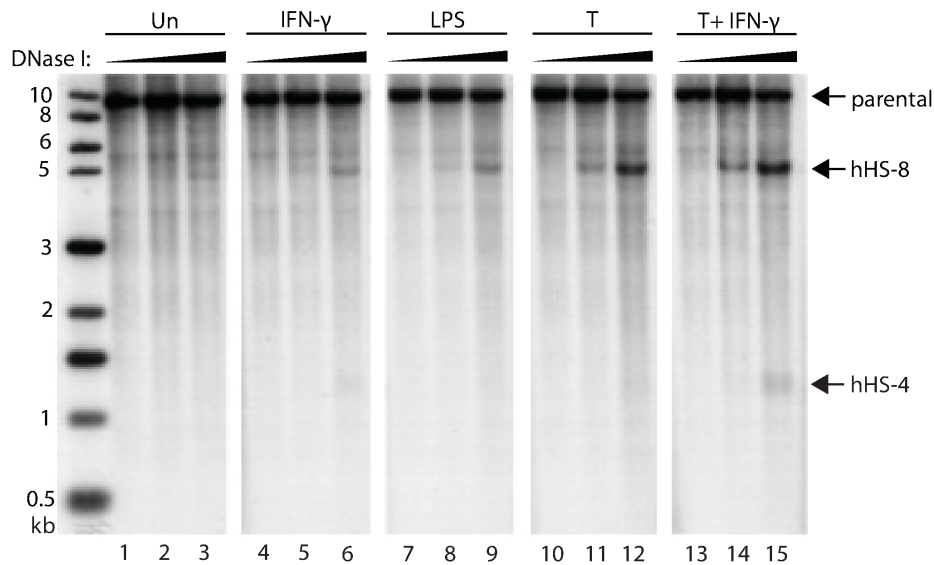
Figure 3.2.5: IFN-γ induces the formation of hHS-4 at the *TNF/LT* locus in endotoxin-tolerant human monocytes. (a) Map of the human *TNF/LT* locus. DH sites and positions and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. Positions of the parental ScaI and DNase I digestion products for the DHA are indicated. Fragment representing DNase I accessibility at the hHS-8 and hHS-4 is 5kb and 1.4kb in length, respectively. (b) IFN-γ dramatically increases DNase I cleavage at hHS-4 in endotoxin-tolerant THP-1 cells, as compared to non-tolerant THP-1 cells.

3.3   Discussion

Recent studies have highlighted the importance of examining chromatin and its environment when describing endotoxin tolerance and its IFN-γ-mediated abrogation. In this study, we have characterized the *TNF/LT* locus in endotoxin-tolerant monocytes and macrophages and found the *TNF* promoter to be nuclease accessible and enriched for H3K27me3 levels during repressed transcription. We have also found that IFN-γ priming restored *TNF* gene expression in an IRF1-indepenent manner; furthermore, we observed an enrichment of H3K27ac levels at the *TNF* promoter and the appearance of a DH site ~4kb upstream of the *TNF* TSS. These results support the finding that chromatin modifications at both the *TNF* promoter and distal DNA elements are critical for determining mechanisms for the induction of endotoxin tolerance and its IFN-γ-mediated abrogation.

We speculate that *TNF* and *IL6* genes are silenced by different mechanisms, and that mechanisms responsible for transcriptional silencing during endotoxin tolerance vary between genes and their respective loci. One study showed that LPS stimulation in endotoxin-tolerant murine macrophages resulted in decreased nuclease accessibility at the *IL6* promoter, providing evidence for a nucleosome barrier that blocks LPS-induced *IL6* gene expression [236]. Importantly, the authors noted that they did not detect enrichment of the normally repressive histone modification H3K27me3 at the *IL6* promoter. Another study confirmed these results and also demonstrated that priming human monocytes with IFN-γ prevents endotoxin tolerance, evidenced by restored IL-6 mRNA levels and chromatin accessibility at the *IL6* promoter [239]. In contrast, we observed comparable levels of nuclease accessibility at the *TNF* promoter in non-tolerant and endotoxin-tolerant monocytes stimulated with LPS. In addition, we observed an increase in H3K27me3 levels at the *TNF* promoter. Our data suggest that, unlike IL-6, *TNF* gene repression in endotoxin-tolerant monocytes does not involve alterations in chromatin accessibility at the promoter.

What then is the mechanism of *TNF* gene repression during endotoxin tolerance? As stated previously, H3K27me3 has been shown to increase the affinity of the chromodomain of the Drosophila Polycomb protein for histone tails, and this has been implicated in the maintenance of gene silencing [138, 139]. However, mechanisms of Polycomb mediated repression remain unknown. Notably, one study examining the *TNF* promoter in endotoxin-tolerant THP-1 cells observed an enrichment of H3K9me2 levels and an increase in DNA methylation, as compared to non-tolerant THP-1 cells stimulated with LPS [240]. They also show recruitment of both the H3K9 histone methyltransferase G9a and the DNA methyl-transferase Dnmt3a/b to the *TNF* promoter during endotoxin tolerance. Unfortunately, we were unable to repeat the H3K9me2 findings. Future experiments will have to explore the functional role of histone and DNA methylation in *TNF* gene repression during endotoxin tolerance.

We found chromatin modifications during IFN-*γ*-mediated abrogation of *TNF* gene repression to be similar to that of *IL6*; specifically, we observed an increase in nuclease accessibility and an enrichment of H3K27ac levels at the *TNF* promoter. Notably, past studies have prevented induction of endotoxin tolerance by pretreating cells with IFN-*γ* before the initial LPS challenge [239] whereas our work investigates the ability of IFN-*γ* to abrogate endotoxin tolerance after it has been induced. We find our work to be clinically more relevant when thinking of potential therapeutic targets for sepsis patients whose monocytes and macrophages have already entered an immunosuppressed state.

In addition, we have shown that IRF1 does not play a critical role in the ability of IFN-*γ* to restore *TNF* gene expression in endotoxin-tolerant cells, which suggests that IFN-*γ* priming employs distinct mechanisms for enhancing *TNF* transcription in resting cells as compared to endotoxin-tolerant cells. This we found unsurprising considering the fact that the chromatin environment of the *TNF/LT* locus is markedly different between resting and endotoxin-tolerant cells; thus, IFN-*γ* stimulation must employ distinct mechanisms to pro-

mote a chromatin environment that favors enhanced *TNF* gene expression. It will be important to identify transcription factors and chromatin remodeling enzymes essential for IFN-γ-mediated abrogation of endotoxin tolerance; STAT1 may play an important role considering its activation by IFN-γ signaling.

Finally, identification of hHS-4 as an IFN-γ-inducible DH site in endotoxin-tolerant monocytes suggests its role as a distal regulatory element. Although hHS-4 lies within or near the *LTA* promoter [242], preliminary experiments have shown that IFN-γ does not induce LT-α expression in endotoxin-tolerant monocytes or macrophages (data not shown). Notably, hHS-4 is constitutively present in CD4$^+$ T cells; thus, it may function as both a promoter in lymphocytes and an IFN-γ-inducible regulatory element in endotoxin-tolerant monocytes and macrophages. Future experiments will have to explore the functional role of hHS-4, particularly as an enhancer or insulator.

3.4   Materials and Methods

Cell culture and stimulations

THP-1 cells were maintained in RPMI-1640 supplemented with 10% FBS. For primary human MDMs, enriched populations of human monocytes were isolated from healthy human donor buffy coats using a CD14$^+$ positive selection kit (Stemcell). MDMs were obtained after 6d of culture in RPMI-1640 medium supplemented with 5% human serum AB (Gemcell) and GM-CSF (50ng/ml; Peprotech). Cells were treated with IFN-γ (100ng/ml; R&D) and LPS (100ng/ml; Sigma E. coli O111:B4).

RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted from cells with a QuickRNA Mini kit (Zymo) and treated with Turbo DNA-free kit (Invitrogen). cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen) and 20-residue oligo (dT) (Invitrogen). TNF mRNA levels

were measured by the change-in-threshold ($\Delta\Delta$Ct) method based on quantitative real-time PCR in an iCycler iQ (Bio-rad) with SyberGreen Master Mix (Invitrogen) and primers recognizing exon 4 and exon 3 of the human *TNF* gene and the human *GapdH* gene (Table 2.4.1).

DNase I Hypersensitivity Assay (DHA)

DHAs were performed using THP-1 cells. Cells were harvested, washed with PBS, and resuspended in RSB buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, and 3mM MgCl2). Cells were lysed with lysis buffer (0.5% NP-40 in 1x RSB buffer) on ice for 5min. Resuspended nuclei in RSB buffer were treated with DNase I (40ng/ul) at 37C for 5min. DNase I activity was quenched upon addition of stop solution (0.6M NaCl, 20mM Tris-HCl pH 8.0, 10mM EDTA, and 1% SDS). Samples were treated with Proteinase K at 56C O/N. For analysis of hHS-4, DNA was digested with either ScaI or KpnI and analyzed by Southern blotting using a radiolabeled P^{32} probe corresponding to the coding region of *LTA* or hHS-8, respectively. 10ug of DNA was used for each lane.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed with anti-H3K27me3 (C36B11, Cell Signaling), anti-H3K27ac (Active Motif), and anti-H3 (Abcam). THP-1 cells and primary human MDMs were treated (IFN-γ 100ng/ml, LPS 100ng/ml), fixed with 10% formaldehyde for 15min, treated with 2.5M glycine for 5min, harvested, washed with PBS, lysed with 0.25% Triton X-100 and 0.5% NP-40 for 5min, centrifuged at 1200RPM for 10min, resuspended in 1% SDS lysis buffer, and sonicated 5min for 4 cycles in a Biorupter. Sonicated DNA was set up for immunoprecipitation O/N and DNA-protein complexes were recovered by adding Protein A/G Plus Agarose Beads (Thermo Scientific) for 3h. Samples were washed 6 times with 1ml of wash buffer and treated with proteinase K at 65C O/N. Samples were treated

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with phenol/chloroform before O/N ethanol precipitation. DNA fragments for H3K27me3 and H3K27ac analysis were analyzed by quantitative real-time PCR using Jumpstart™ Taq ReadyMix™ For Quantitative PCR (Sigma) and a primer/probe set for the *TNF* promoter (Table 2.4.1). H3K27me3 and H3K27ac percent input values were normalized to H3 percent input values.

Short hairpin RNA

The lentiviral plasmid PLKO.1 expressing shRNA targeting human IRF1 was purchased from the RNAi Consortium (TRC) Lentiviral shRNA library (Thermo Scientific). Clone TRCN0000014668 with a target sequence of CGTGTGGATCTTGCCACATTT was validated in our laboratory. Control shRNA encodes a scrambled sequence. Lentiviruses encoding shRNA sequences were generated by transfecting the packaging cell line HEK-293T with the shRNA-encoding pLOK.1 plasmids in combination with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Effectene transfection reagent (Qiagen). Supernatants were collected 48h post-transfection, clarified by centrifugation, and stored at -80C. THP-1 cells were transduced with the lentiviral particles by culturing the cells with supernatants from the virus-producing cells in the presence of 8ug/ml polybrene (Millipore) and spinoculation for 2h at 2000RPM. Successfully transduced cells were selected and expanded by treatment with 0.8ug/ml puromycin.

Chapter 4

Concluding Remarks

The overarching goal of this thesis project was to use the *TNF/LT* locus as a model system to identify and dissect mechanisms of chromatin remodeling that control classical macrophage activation and endotoxin tolerance. In doing so, we have provided a mechanistic explanation of how IFN-γ poises the *TNF/LT* locus for enhanced *TNF* transcription upon LPS stimulation, while at the same time providing potential targets for selective manipulation of TNF expression in primed macrophages. We have also characterized the chromatin environment at the *TNF/LT* locus during the induction and IFN-γ-mediated abrogation of endotoxin tolerance. More generally, we have examined long-range distal DNA elements of the *TNF/LT* locus and demonstrated the importance of examining DNA elements beyond the promoter when investigating the elaborate nature of inflammatory responses and developing novel therapeutics for inflammatory diseases.

Importantly, distal DNA elements, particularly enhancers, are thought to be of primary importance when determining cell type-specific gene expression [122]. This is supported by the finding that enhancers have been observed to be largely cell selective, to be marked with cell type-specific histone modification patterns, and to actively function in a cell type-specific manner. Promoters, in contrast, have been observed to appear and function in a similar manner across cell types. In the *TNF/LT* locus, hHS-8 is present in the primary

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producers of TNF (i.e., monocytes, macrophages, and T cells); it is of interest to examine whether hHS-8 is present in other cells that are not primary producers of TNF.

As stated previously, studies using mice with cell type-specific deletions of the *TNF* gene have suggested that novel therapies for autoimmune diseases that specifically target myeloid-derived TNF would be preferable in latently infected TB patients [39]. In the case of our proposed model for IFN-γ priming, hHS-8 and its chromatin environment may provide potential targets for selective manipulation of TNF expression in monocytes/macrophages as compared to T cells. Although HSS-9 (murine hHS-8) was shown to function as an inducible enhancer in murine T cells [177], we hypothesize that transcription factors, chromatin-remodeling enzymes, and patterns of histone modifications will differ at hHS-8 between T cells and monocytes/macrophages. This is similarly seen at the *TNF* promoter when looking at enhanceosome formation in T cells and monocytes/macrophages [175].

In addition to demonstrating a high degree of cell type-specificity, distal DNA elements can be inducible and inducer-specific. Thus, it is critical to search for distal DNA elements in model systems that represent disease states. For example, in the *TNF/LT* locus, hHS-4 has been shown to be cell type-specific between resting primary monocytes and CD4^{+} T cells, and we have now shown hHS-4 to be inducer-specific within endotoxin-tolerant monocytes and macrophages. This finding demonstrates that selective modulation of individual genes of the inflammatory response requires a thorough understanding of inducible chromatin modifications for both the promoter and distal regulatory elements in different settings of pathology. In the case of TNF dysregulation, future experiments would ideally examine chromatin modifications at the *TNF/LT* locus in resting and IFN-γ-treated monocytes isolated from sepsis patients.

Lastly, we have identified hHS-8 and hHS-4 by DNase I hypersensitivity assays (DHAs) followed by Southern blotting. This method is feasible for identifying distal DNA elements $\leq$50kb from the target promoter; however, regulatory elements have been described as tens

to hundreds of kilobases away from their target promoter. Indeed, identifying distal DNA elements with respect to target genes can be quite challenging. Often times, distal DNA elements are assigned the closest gene as their target gene; unfortunately, there has been no report to date on the rate of false positives for this strategy. A recent study has applied chromosome conformation capture (3C) assay technology to identify interactions between transcription start sites (TSSs) and previously identified distal DNA elements throughout the human genome [174]. Considering the fact that enhancers are often found to be in close proximity with their target promoters, this method is thought to hold promise in identifying a percentage of target promoters. Ultimately, a greater understanding of how enhancers and other types of regulatory elements mechanistically function is required for high throughput methods of classifying regulatory elements with their target promoters.

Appendix A

Supplemental Figures

A.1   Supplemental Figures
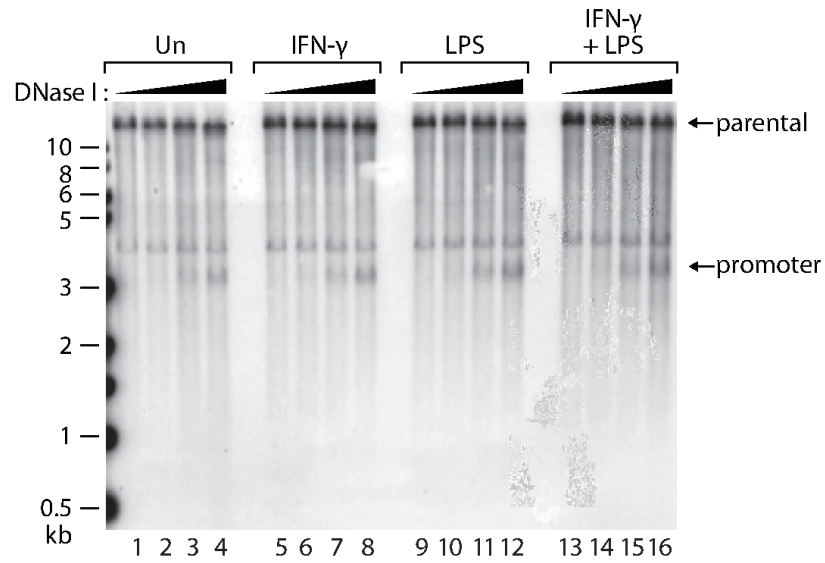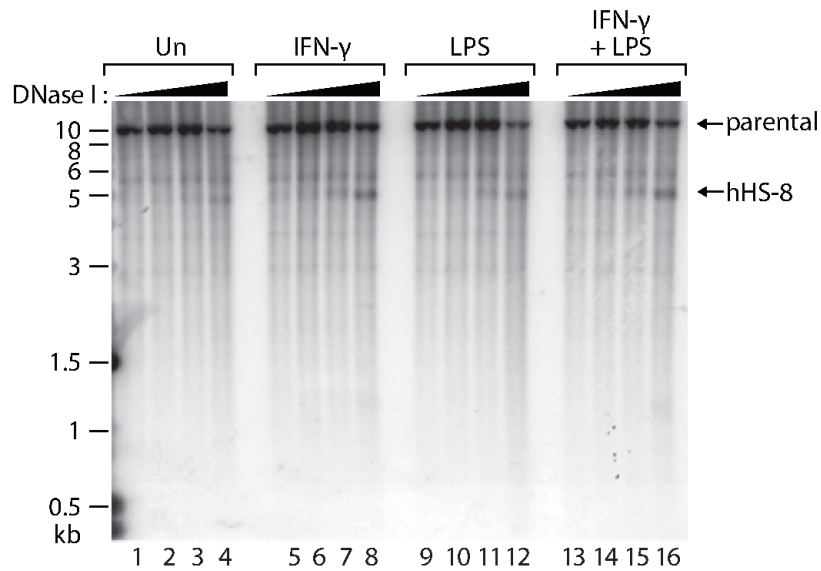
Figure A.0.1: IFN-γ promotes chromatin accessibility at hHS-8 in the *TNF/LT* locus. IFN-γ does not affect DNase I cleavage at the *TNF* promoter. DHA using THP-1 cells. DNase I digestion products for the DHAs are indicated; fragment representing DNase I accessibility at the *TNF* promoter and hHS-8 is ∼3kb and ∼5kb in length, respectively. (c) IFN-γ strongly increases DNase I cleavage at hHS-8.

Figure A.0.2: THP-1 cells were stimulated, and IRF1 mRNA levels were measured.


```
                -6838                                                    -6785
                  |                                                        |
Human  TGCCCAAGAAAGAAACCAAATT–CATACAACCTCCGAAACTGAGATTGAAACCAA
Mouse  TGCCCAAGAGGGAAACTGAATTTCACACAACCTCTAAAACTGAGATTGAAACCAA
```

Figure A.0.3: rIRF1 binds to hHS-8 in both the human and mouse *TNF/LT* locus. EMSA was performed with rIRF1 and human and mouse radiolabeled P^{32} oligonucleotides of sequences for positions -6838 to -6785.

```
Human GCTGGATGAAATCTAGAGGCTTTAC-CCTTCTTGCATGGCCCTGCATGACCTGGCCCCTGCCCTCCTCTCTGACCTCATC   79
Mouse GTTGAATAAAATGGAGATACTTTGTGTCTTC----------TCACATGGTCTGGC-CCTGCCTTCCTCTCCCACTGCATC   69
      * ** ** **** *** **** ****             **** ***** ****** ******* **  ****

Human TCCCACCCGCCTCCCAGTCTCTCTC-TCTGCTCCAGCCACACTGGCCTTCTGTTTGTCGTCAACACCCCCAGCTTGGTTC  158
Mouse TG--ACC--TCTTCAAGTGTCTCTGATGTGGTCAAGT-TCAGTTTCTTGCTGT---CCATCAGCATCCCTGGCTTCCTCC  141
      *    ***   ** * *** ***** * ** ** **  ** * ** ****   * *** ** ***  ****  * *

Human CGCCTTCCAGCCTTTGCAGTAGCTGCTCCCTTTACCTGAAATGCT----TTGCTCCCAAACTTTTACCTGGTCACTATTT  234
Mouse CTCCTCCCTACCTATCTGTCACCTGGGCCTTTT-CTT-CAGTGCTACCCTTGCTGAGAGTCACATTACTGAGTGCTGTAT  219
      * *** **  *** *    * *** ** *** ** *  * ****   *****   *  *   *** *   ** ** **

Human TTT-GTCATTTGGGTCTCAG------CTCCA--GTGCCACCCAAACACTCAAAGAGGATTTTGCTGACT-ACTGTATTTA  304
Mouse CCAAGAGTATTCTCTTCCACTCCCCACGCCACCCTGCCTTGTTATCCCTGTAGGACTGTTTTACAGATTCATTTTATTTT  299
         *    * **   *  **       * *** * ****      * * **  * **   **** * ** * * * *****

Human AAAG-----------TAGCTCCCTGCCACTCTTACAACATCAGCCTGTCTTATTTTCTCATAGTACCAATTTCTTCTTCA  373
Mouse ATGGCGATGCTGGGGATGGTCCCTGGGTCTCACTCACGCTAGGCAAGTGCTC-TATCTCTAA-GACACATCCCTGCTCTT  377
      *  *           ** ****** *** ** * ** ** * * **** * ** ** ** ** ** **

Human GTTTCTTTCTTTTCCGTCTGGCCTCACTGGAATACAAGCTCCACAGGTGCTGGTACCTTCTC--TGATCCCTTTGCCTCC  451
Mouse TGATGTGTCTTTTCCATCTGGCCTGGCTGGAACATGATCCCTAAGAGTAAAGG-ATTTACTCTTAGGATTCTTTGGCTCA  456
      * * ******** ******** ****** *   * * *   ** **   ** * * *** *       ***** ***

Human ATGTCCCTCCTGCCTTAGGACAATGCCCAGCATGTGTTAGGCACGCAGATACTCACTAAATGGAGGAATGGATGAATAAT  531
Mouse A----CTTTGTGCCACAGTATACTTCCTGCCTTG----------AACAAACTTCCTGGTTGGT--ACTCAGTAAAC---  516
      *     * *  ****  ** * * *  * **           * * ***  **   ***   * *     * **

Human TCATAAAGCAGGATAAAGTTCAACTTTAGGCTTGTGGCTCAGATTGGACTTACATTTAGAGTCAGATTTAAGTTTAGTGT  611
Mouse --------ATGTATTAAAAGCTGTTGGAGG-GTGCGG-TGAGGTAGGACA---AT----GTCCAGCTCTAATTTCTGGCT  579
              * ** ** *   *  * * *** ** ** * ** * **** **      *** * *** **  *  *

Human TAGGAGTGGTGGTAAACTGGTTTCAAGATTAGCCCTAGAAACAGGGTTGGGTTGGGGTAGAGGAGAAGTTTTATTTAGGG  691
Mouse CAG--------GTA---------GAAGTTCAACACCAGAGACAGGGTAA-GCTGTTTCACAGGAAGGAGTTTATTTGAGA  641
       **       ***         *** * * * *** ****** * **  * ** * **** *******  *

Human GGTTATTAATTGGGATGTGTTTAGATTTAAGGTTAGGGTTACAGTTGGGGTTGAGTTTGAGTTGTGATTTGGGTTGAGGT  771
Mouse GTCCTTTGAGGGGTATGTGCTTAGATTTGAGGTT------AC------------GTTTGAATTGTGATTTGGGTGAAGGT  703
      *   ** *  ** ***** ******** ****       **            ****** ************   ****

Human TAAATTTGGGTTAGGGTTGATGTTGGTATTAAATCCCAATTCAGGTTTTGAGGCTAAGTTCAAGTTTGAAGCTAATGTCA  851
Mouse TAAACTTGGGTAAAATCTGAGGCTA---AAAAA-------ACAGAGACTCACAGT-AG----TCTTTG--ACTACTGTCA  766
      **** ****** *   ** * *****   ***        *** * *  * **   ** *  ***   *** *****

Human TTTCAGTCTCATTTGGAGGCTTCAGAGATTTCACTAGTTTCTCCACAAAGACCACTATAAAGACTGTATTTCCCTGAGTC  931
Mouse GTTCAGCCTGGTTTGCAGGCTCCGGAGCTTTCTGTGGCCCTTCCTGAGAGT-GGTT--ATAGAC----------------  827
      ***** ** **** ***** * **** **** * ** * * ** * **  ***    * * ****

Human TGGGGCACAAGACTCC-AGTCATCAGCTCTCCCACCCAGGGAAAGTCCCAAACCAACTGCTGGCCTGCCCAAGAAAGAAA 1010
Mouse -GATGCACAAGACTCCTAGTCATCAGCCCTCCCCCCCGGGGAAAGTCCCAGA-CAGCTGTTGGCCTCCCCAAGAGAGAAA  905
       *  ************ ********** **** ***** *** ********* ** *** * ****** *** ***** ****

Human CCAAA-TTCATACAACCTCCGAAACTGAGATTGAAACCAAGATTGGCCCATCTCAAGGAGCATCCTTC-GCATATCTCAC 1088
Mouse CTGAATTTCACACAACCTCTAAAACTGAGATTGAAACCAAAATTGGCCCATGGCAAGGCATATCCGCCAGCGTGCTGCCA  985
      *  ** ** **** ******** ****************** ********** *  ****  * * *       *

Human ATGCACGTGACACTGAGCCTCAGCCCAGTCTTACCCTTCCTTCCTCTGTGTCTCTCATGTCT-----CCCCATCACCCTT 1163
Mouse GCTCCCGTGACACA--G-CTTAGCCAAGGCTTACCCTTCCTTCGTCTGTCTCCATCATCCCCTTATGCACCCTCTCCTTT 1062
      *  ********    * * ** ****  ** ************* ***** **  ****  *       * ** ** ** **

Human CT--TGCCT-------TCCCT---TTTTTGTCT--TTCAATGTCCCATTCTTCCTCTTT---AATTTAAATTTCTCTCTG 1226
Mouse CCTCTGCCCCCTCCAGGTCCCAGCCTTCTGCTCTGACTCAA-ATCTAATCCTCTCTCTGTCTCTCTTTCTGTCTCTCTCTG 1141
      *   ****       ****   ** *  ***  **** **  ** ** ****  ***  *       * ********

Human T------------GTCTCACTGTTA--ATTGCAA-TACC                                         1250
Mouse TCTCTGTCTCTGTCTTTCTCTGTCTCCGTCTCTCTGTCT                                         1180
      *            * ** ****   *  *      *
```

Figure A.0.4: hHS-8 is highly conserved between human and mouse. Alignment of human and mouse sequences of hHS-8 (~1250bp).

79

WT Mu

-6838   -6785

WT **TGCCCAAGAAAGAAACCAAATTCATACAACCTCCGAAACTGAGATTGAAACCAA**

Mu **TGCCCAAGAGGGAGGCCAAATTCATACAACCTCCGAGGCTGAGATTGAGGCCAA**

Free probe

1 2
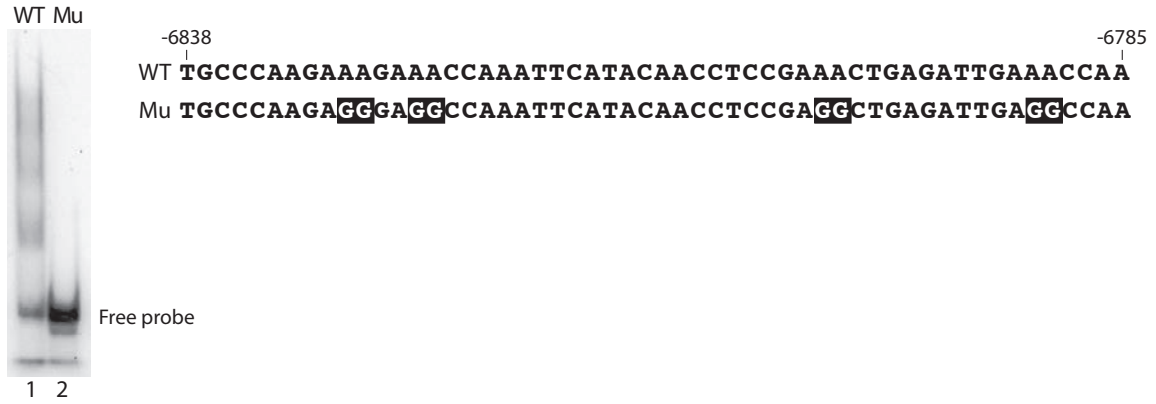
Figure A.0.5: Nucleotide changes in the critical -GAAA- motifs disrupt rIRF1 binding. EMSA was performed with rIRF1 and wildtype (wt) and mutant (mu) radiolabeled P^{32} oligonucleotides (sequences for positions -6838 to -6785).
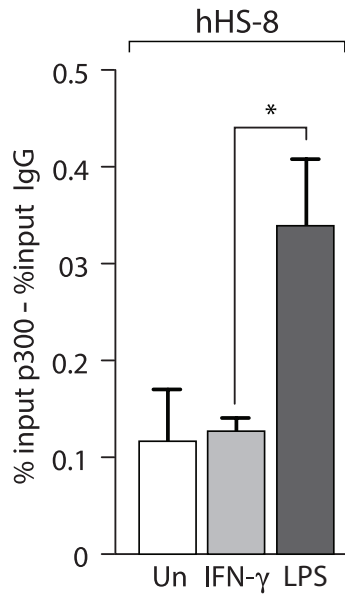

Figure A.0.6: p300 is recruited to hHS-8 upon LPS stimulation. ChIP using primary human MDMs and analyzing p300 recruitment to hHS-8. Stimulations were IFN-γ alone for 3h, LPS alone for 1h, and both IFN-γ and LPS (IFN-γ pretreatment for 2h followed by LPS treatment for 1h). (*) $p \leq 0.05$. Data from 3 separate donors.
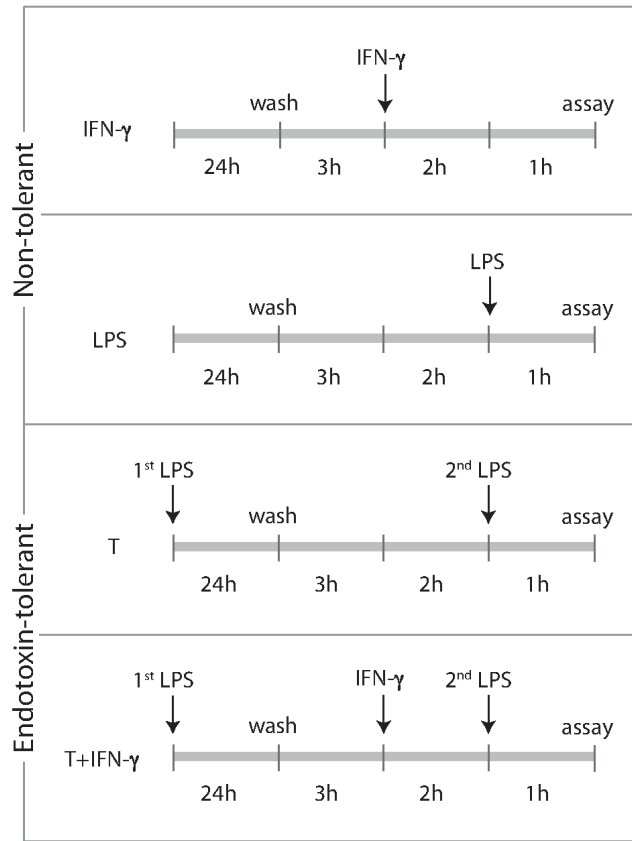
Figure A.1.1: Experimental design of endotoxin tolerance induction and IFN-γ-mediated abrogation of endotoxin tolerance.

```
Human  CCTCACCCATGTGGAATTCTG--------AAACTTCCTTTGTAGAAAACTTTGGAAGGTG  52
Mouse  -ATAAATGATGAGCAAATTTATATACAACAAAGTTATCTGTCATAAATATATGTAAGAAG  59
        *  *   *** * ** * *        *** **    *   * ***   * ** ***    *

Human  TCTGCCACATTGATCCTGGAATGTGTGTTTATTTGGGGTTATATAAATCTGTTCTGTG--  110
Mouse  TA------ATAAGTCCTTTG-TGTGTGTTTATGTGTGGTTATATGTATGTGTGCTGTGTG  112
        *        **    ****   ********** ** ********   ** *** *****

Human  --GAAGCCACCTGAAGTCA---  127
Mouse  TGGAAGCCA---GAGGACAACC  131
         *******    ** * **
```

Figure A.1.2: hHS-4 is highly conserved between human and mouse. Alignment of human and mouse sequences of hHS-4 (~120bp).

References

[1] P. Bruns. Die heilwirkung des erysipels auf geschwulste (the healing effect of erysipelas on tumors). *Beitr Klin Chir*, 3:443–446, 1868.

[2] Shear M.J O'Malley W.E, Achinstein B. Action of bacterial polysaccharide on tumors. ii: damage of sarcoma 37 by serum of mice treated with serratia marcescens polysaccharide and induced tolerance. *J. Natl Cancer Inst.*, 29:1169–1175, 1962.

[3] Kassel RL Green S Fiore N Williamson B Carswell EA, Old LJ. An endotoxin-induced serum factor that causes necrosis of tumors. *PNAS*, 72(9):3666–3670, 1975.

[4] D. Pennica, G. E. Nedwin, J. S. Hayflick, P. H. Seeburg, R. Derynck, M. A. Palladino, W. J. Kohr, B. B. Aggarwal, and D. V. Goeddel. Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. *Nature*, 312(5996):724–9, 1984. Pennica, D Nedwin, G E Hayflick, J S Seeburg, P H Derynck, R Palladino, M A Kohr, W J Aggarwal, B B Goeddel, D V Comparative Study England Nature Nature. 1984 Dec 20-1985 Jan 2;312(5996):724-9.

[5] H. R. Michie, D. R. Spriggs, K. R. Manogue, M. L. Sherman, A. Revhaug, S. T. O'Dwyer, K. Arthur, C. A. Dinarello, A. Cerami, S. M. Wolff, and et al. Tumor necrosis factor and endotoxin induce similar metabolic responses in human beings. *Surgery*, 104(2):280–6, 1988. Michie, H R Spriggs, D R Manogue, K R Sherman, M L Revhaug, A O'Dwyer, S T Arthur, K Dinarello, C A Cerami, A Wolff, S M 20-9299/PHS HHS/ A121359/PHS HHS/ P50 GM36428-03/GM/NIGMS NIH HHS/ Surgery. 1988 Aug;104(2):280-6.

[6] G. Davatelis, P. Tekamp-Olson, S. D. Wolpe, K. Hermsen, C. Luedke, C. Gallegos, D. Coit, J. Merryweather, and A. Cerami. Cloning and characterization of a cdna for murine macrophage inflammatory protein (mip), a novel monokine with inflammatory and chemokinetic properties. *J Exp Med*, 167(6):1939–44, 1988. Davatelis, G Tekamp-Olson, P Wolpe, S D Hermsen, K Luedke, C Gallegos, C Coit, D Merryweather, J Cerami, A J Exp Med. 1988 Jun 1;167(6):1939-44.

[7] C. G. Larsen, C. O. Zachariae, J. J. Oppenheim, and K. Matsushima. Production of monocyte chemotactic and activating factor (mcaf) by human dermal fibroblasts in response to interleukin 1 or tumor necrosis factor. *Biochem Biophys Res Commun*, 160(3):1403–8, 1989. Larsen, C G Zachariae, C O Oppenheim, J J Matsushima, K Biochem Biophys Res Commun. 1989 May 15;160(3):1403-8.

[8] M. Gomez-Chiarri, T. A. Hamilton, J. Egido, and S. N. Emancipator. Expression of ip-10, a lipopolysaccharide- and interferon-gamma-inducible protein, in murine mesangial cells in culture. *Am J Pathol*, 142(2):433–9, 1993. Gomez-Chiarri, M Hamilton, T A Egido, J Emancipator, S N AI 31230/AI/NIAID NIH HHS/ CA 39621/CA/NCI NIH HHS/ DK 38544/DK/NIDDK NIH HHS/ Am J Pathol. 1993 Feb;142(2):433-9.

[9] J. S. Pober. Effects of tumour necrosis factor and related cytokines on vascular endothelial cells. *Ciba Found Symp*, 131:170–84, 1987. Pober, J S HL-22602/HL/NHLBI NIH HHS/ HL-36003/HL/NHLBI NIH HHS/ NETHERLANDS Ciba Found Symp. 1987;131:170-84.

[10] L. Osborn, C. Hession, R. Tizard, C. Vassallo, S. Luhowskyj, G. Chi-Rosso, and R. Lobb. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell*, 59(6):1203–11, 1989. Osborn, L Hession, C Tizard, R Vassallo, C Luhowskyj, S Chi-Rosso, G Lobb, R Cell. 1989 Dec 22;59(6):1203-11.

[11] J. A. Royall, R. L. Berkow, J. S. Beckman, M. K. Cunningham, S. Matalon, and B. A. Freeman. Tumor necrosis factor and interleukin 1 alpha increase vascular endothelial permeability. *Am J Physiol*, 257(6 Pt 1):L399–410, 1989. Royall, J A Berkow, R L Beckman, J S Cunningham, M K Matalon, S Freeman, B A NS-24275/NS/NINDS NIH HHS/ Am J Physiol. 1989 Dec;257(6 Pt 1):L399-410.

[12] C. A. Dinarello, J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, Jr. Palladino, M. A., and J. V. O'Connor. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. *J Exp Med*, 163(6):1433–50, 1986. Dinarello, C A Cannon, J G Wolff, S M Bernheim, H A Beutler, B Cerami, A Figari, I S Palladino, M A Jr O'Connor, J V AI 15614/AI/NIAID NIH HHS/ J Exp Med. 1986 Jun 1;163(6):1433-50.

[13] J. Davidson, A. S. Milton, and D. Rotondo. A study of the pyrogenic actions of interleukin-1 alpha and interleukin-1 beta: interactions with a steroidal and a non-steroidal anti-inflammatory agent. *Br J Pharmacol*, 100(3):542–6, 1990. Davidson, J Milton, A S Rotondo, D Wellcome Trust/United Kingdom ENGLAND Br J Pharmacol. 1990 Jul;100(3):542-6.

[14] T. Ulrichs, G. A. Kosmiadi, V. Trusov, S. Jorg, L. Pradl, M. Titukhina, V. Mishenko, N. Gushina, and S. H. Kaufmann. Human tuberculous granulomas induce peripheral lymphoid follicle-like structures to orchestrate local host defence in the lung. *J Pathol*, 204(2):217–28, 2004. Ulrichs, Timo Kosmiadi, George A Trusov, Vsevolod Jorg, Sabine Pradl, Lydia Titukhina, Marina Mishenko, Vladimir Gushina, Nadya Kaufmann, Stefan H E England J Pathol. 2004 Oct;204(2):217-28.

[15] A. G. Bean, D. R. Roach, H. Briscoe, M. P. France, H. Korner, J. D. Sedgwick, and W. J. Britton. Structural deficiencies in granuloma formation in tnf gene-

targeted mice underlie the heightened susceptibility to aerosol mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. *J Immunol*, 162(6):3504–11, 1999. Bean, A G Roach, D R Briscoe, H France, M P Korner, H Sedgwick, J D Britton, W J Baltimore, Md. : 1950 J Immunol. 1999 Mar 15;162(6):3504-11.

[16] J. Rothe, W. Lesslauer, H. Lotscher, Y. Lang, P. Koebel, F. Kontgen, A. Althage, R. Zinkernagel, M. Steinmetz, and H. Bluethmann. Mice lacking the tumour necrosis factor receptor 1 are resistant to tnf-mediated toxicity but highly susceptible to infection by listeria monocytogenes. *Nature*, 364(6440):798–802, 1993. Rothe, J Lesslauer, W Lotscher, H Lang, Y Koebel, P Kontgen, F Althage, A Zinkernagel, R Steinmetz, M Bluethmann, H ENGLAND Nature. 1993 Aug 26;364(6440):798-802.

[17] P. Everest, M. Roberts, and G. Dougan. Susceptibility to salmonella typhimurium infection and effectiveness of vaccination in mice deficient in the tumor necrosis factor alpha p55 receptor. *Infect Immun*, 66(7):3355–64, 1998. Everest, P Roberts, M Dougan, G Wellcome Trust/United Kingdom Infect Immun. 1998 Jul;66(7):3355-64.

[18] M. Nashleanas, S. Kanaly, and P. Scott. Control of leishmania major infection in mice lacking tnf receptors. *J Immunol*, 160(11):5506–13, 1998. Nashleanas, M Kanaly, S Scott, P AI 35914/AI/NIAID NIH HHS/ AI 41880/AI/NIAID NIH HHS/ Baltimore, Md. : 1950 J Immunol. 1998 Jun 1;160(11):5506-13.

[19] M. C. Vilela, G. K. Lima, D. H. Rodrigues, N. Lacerda-Queiroz, D. S. Mansur, A. S. de Miranda, M. A. Rachid, E. G. Kroon, L. Q. Vieira, M. A. Campos, M. M. Teixeira, and A. L. Teixeira. Tnfr1 plays a critical role in the control of severe hsv-1 encephalitis. *Neurosci Lett*, 479(1):58–62, 2010. Vilela, Marcia Carvalho Lima, Graciela Kunrath Rodrigues, David Henrique Lacerda-Queiroz, Norinne Mansur, Daniel Santos de Miranda, Aline Silva Rachid, Milene Alvarenga Kroon, Erna Geessien Vieira, Leda Quercia Campos, Marco Antonio Teixeira, Mauro Martins Teixeira, Antonio Lucio Ireland Neurosci Lett. 2010 Jul 19;479(1):58-62. doi: 10.1016/j.neulet.2010.05.028. Epub 2010 May 15.

[20] E. C. Keystone and C. F. Ware. Tumor necrosis factor and anti-tumor necrosis factor therapies. *J Rheumatol Suppl*, 85:27–39, 2010. Keystone, Edward C Ware, Carl F Canada J Rheumatol Suppl. 2010 May;85:27-39. doi: 10.3899/jrheum.091463.

[21] G. Buchan, K. Barrett, M. Turner, D. Chantry, R. N. Maini, and M. Feldmann. Interleukin-1 and tumour necrosis factor mrna expression in rheumatoid arthritis: prolonged production of il-1 alpha. *Clin Exp Immunol*, 73(3):449–55, 1988. Buchan, G Barrett, K Turner, M Chantry, D Maini, R N Feldmann, M ENGLAND Clin Exp Immunol. 1988 Sep;73(3):449-55.

[22] J. M. Dayer, B. Beutler, and A. Cerami. Cachectin/tumor necrosis factor stimulates collagenase and prostaglandin e2 production by human synovial cells and dermal

fibroblasts. *J Exp Med*, 162(6):2163–8, 1985. Dayer, J M Beutler, B Cerami, A AM01314/AM/NIADDK NIH HHS/ J Exp Med. 1985 Dec 1;162(6):2163-8.

[23] C. Y. Chen and A. B. Shyu. Au-rich elements: characterization and importance in mrna degradation. *Trends Biochem Sci*, 20(11):465–70, 1995. Chen, C Y Shyu, A B GM-46454/GM/NIGMS NIH HHS/ ENGLAND Trends Biochem Sci. 1995 Nov;20(11):465-70.

[24] D. Kontoyiannis, M. Pasparakis, T. T. Pizarro, F. Cominelli, and G. Kollias. Impaired on/off regulation of tnf biosynthesis in mice lacking tnf au-rich elements: implications for joint and gut-associated immunopathologies. *Immunity*, 10(3):387–98, 1999. Kontoyiannis, D Pasparakis, M Pizarro, T T Cominelli, F Kollias, G DK42191/DK/NIDDK NIH HHS/ Immunity. 1999 Mar;10(3):387-98.

[25] M. J. Elliott, R. N. Maini, M. Feldmann, J. R. Kalden, C. Antoni, J. S. Smolen, B. Leeb, F. C. Breedveld, J. D. Macfarlane, H. Bijl, and et al. Randomised double-blind comparison of chimeric monoclonal antibody to tumour necrosis factor alpha (ca2) versus placebo in rheumatoid arthritis. *Lancet*, 344(8930):1105–10, 1994. Elliott, M J Maini, R N Feldmann, M Kalden, J R Antoni, C Smolen, J S Leeb, B Breedveld, F C Macfarlane, J D Bijl, H ENGLAND Lancet. 1994 Oct 22;344(8930):1105-10.

[26] A. den Broeder, L. van de Putte, R. Rau, M. Schattenkirchner, P. Van Riel, O. Sander, C. Binder, H. Fenner, Y. Bankmann, R. Velagapudi, J. Kempeni, and H. Kupper. A single dose, placebo controlled study of the fully human anti-tumor necrosis factor-alpha antibody adalimumab (d2e7) in patients with rheumatoid arthritis. *J Rheumatol*, 29(11):2288–98, 2002. den Broeder, Alfons van de Putte, Leo Rau, Rolf Schattenkirchner, Manfred Van Riel, Piet Sander, Oliver Binder, Christina Fenner, Helmut Bankmann, Yvonne Velagapudi, Raja Kempeni, Joachim Kupper, Hartmut Canada J Rheumatol. 2002 Nov;29(11):2288-98.

[27] L. W. Moreland, M. H. Schiff, S. W. Baumgartner, E. A. Tindall, R. M. Fleischmann, K. J. Bulpitt, A. L. Weaver, E. C. Keystone, D. E. Furst, P. J. Mease, E. M. Ruderman, D. A. Horwitz, D. G. Arkfeld, L. Garrison, D. J. Burge, C. M. Blosch, M. L. Lange, N. D. McDonnell, and M. E. Weinblatt. Etanercept therapy in rheumatoid arthritis. a randomized, controlled trial. *Ann Intern Med*, 130(6):478–86, 1999. Moreland, L W Schiff, M H Baumgartner, S W Tindall, E A Fleischmann, R M Bulpitt, K J Weaver, A L Keystone, E C Furst, D E Mease, P J Ruderman, E M Horwitz, D A Arkfeld, D G Garrison, L Burge, D J Blosch, C M Lange, M L McDonnell, N D Weinblatt, M E Ann Intern Med. 1999 Mar 16;130(6):478-86.

[28] E. H. Choy, B. Hazleman, M. Smith, K. Moss, L. Lisi, D. G. Scott, J. Patel, M. Sopwith, and D. A. Isenberg. Efficacy of a novel pegylated humanized anti-tnf fragment (cdp870) in patients with rheumatoid arthritis: a phase ii double-blinded, randomized, dose-escalating trial. *Rheumatology (Oxford)*, 41(10):1133–7, 2002. Choy, E

H S Hazleman, B Smith, M Moss, K Lisi, L Scott, D G I Patel, J Sopwith, M Isenberg, D A England Oxford, England Rheumatology (Oxford). 2002 Oct;41(10):1133-7.

[29] H. Zhou, H. Jang, R. M. Fleischmann, E. Bouman-Thio, Z. Xu, J. C. Marini, C. Pendley, Q. Jiao, G. Shankar, S. J. Marciniak, S. B. Cohen, M. U. Rahman, D. Baker, M. A. Mascelli, H. M. Davis, and D. E. Everitt. Pharmacokinetics and safety of golimumab, a fully human anti-tnf-alpha monoclonal antibody, in subjects with rheumatoid arthritis. *J Clin Pharmacol*, 47(3):383–96, 2007. Zhou, Honghui Jang, Haishan Fleischmann, Roy M Bouman-Thio, Esther Xu, Zhenhua Marini, Joseph C Pendley, Charles Jiao, Qun Shankar, Gopi Marciniak, Stanley J Cohen, Stanley B Rahman, Mahboob U Baker, Daniel Mascelli, Mary Ann Davis, Hugh M Everitt, Daniel E J Clin Pharmacol. 2007 Mar;47(3):383-96.

[30] M. P. Karampetsou, S. N. Liossis, and P. P. Sfikakis. Tnf-alpha antagonists beyond approved indications: stories of success and prospects for the future. *QJM*, 103(12):917–28, 2010. Karampetsou, M P Liossis, S-N C Sfikakis, P P England QJM. 2010 Dec;103(12):917-28. doi: 10.1093/qjmed/hcq152. Epub 2010 Aug 27.

[31] V. K. Denmark and L. Mayer. Current status of monoclonal antibody therapy for the treatment of inflammatory bowel disease: an update. *Expert Rev Clin Immunol*, 9(1):77–92, 2013. Denmark, Vera Kandror Mayer, Lloyd P01 DK072201/DK/NIDDK NIH HHS/ England Expert Rev Clin Immunol. 2013 Jan;9(1):77-92. doi: 10.1586/eci.12.91.

[32] C. P. Braegger, S. Nicholls, S. H. Murch, S. Stephens, and T. T. MacDonald. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet*, 339(8785):89–91, 1992. Braegger, C P Nicholls, S Murch, S H Stephens, S MacDonald, T T Wellcome Trust/United Kingdom ENGLAND Lancet. 1992 Jan 11;339(8785):89-91.

[33] S. H. Murch, C. P. Braegger, J. A. Walker-Smith, and T. T. MacDonald. Location of tumour necrosis factor alpha by immunohistochemistry in chronic inflammatory bowel disease. *Gut*, 34(12):1705–9, 1993. Murch, S H Braegger, C P Walker-Smith, J A MacDonald, T T Wellcome Trust/United Kingdom ENGLAND Gut. 1993 Dec;34(12):1705-9.

[34] H. M. van Dullemen, S. J. van Deventer, D. W. Hommes, H. A. Bijl, J. Jansen, G. N. Tytgat, and J. Woody. Treatment of crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (ca2). *Gastroenterology*, 109(1):129–35, 1995. van Dullemen, H M van Deventer, S J Hommes, D W Bijl, H A Jansen, J Tytgat, G N Woody, J Gastroenterology. 1995 Jul;109(1):129-35.

[35] J. Keane, S. Gershon, R. P. Wise, E. Mirabile-Levens, J. Kasznica, W. D. Schwieterman, J. N. Siegel, and M. M. Braun. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med*, 345(15):1098–104,

2001. Keane, J Gershon, S Wise, R P Mirabile-Levens, E Kasznica, J Schwieterman, W D Siegel, J N Braun, M M HL-03964/HL/NHLBI NIH HHS/ N Engl J Med. 2001 Oct 11;345(15):1098-104.

[36] W. G. Dixon, K. Watson, M. Lunt, K. L. Hyrich, A. J. Silman, and D. P. Symmons. Rates of serious infection, including site-specific and bacterial intracellular infection, in rheumatoid arthritis patients receiving anti-tumor necrosis factor therapy: results from the british society for rheumatology biologics register. *Arthritis Rheum*, 54(8):2368–76, 2006. Dixon, W G Watson, K Lunt, M Hyrich, K L Silman, A J Symmons, D P M British Society for Rheumatology Biologics Register Arthritis Rheum. 2006 Aug;54(8):2368-76.

[37] Y. Zhang. Persistent and dormant tubercle bacilli and latent tuberculosis. *Front Biosci*, 9:1136–56, 2004. Zhang, Ying AI-44063/AI/NIAID NIH HHS/ AI-49485/AI/NIAID NIH HHS/ Front Biosci. 2004 May 1;9:1136-56.

[38] A. A. Kruglov, A. Kuchmiy, S. I. Grivennikov, A. V. Tumanov, D. V. Kuprash, and S. A. Nedospasov. Physiological functions of tumor necrosis factor and the consequences of its pathologic overexpression or blockade: mouse models. *Cytokine Growth Factor Rev*, 19(3-4):231–44, 2008. Kruglov, Andrei A Kuchmiy, Anna Grivennikov, Sergei I Tumanov, Alexei V Kuprash, Dmitry V Nedospasov, Sergei A Howard Hughes Medical Institute/ England Cytokine Growth Factor Rev. 2008 Jun-Aug;19(3-4):231-44. doi: 10.1016/j.cytogfr.2008.04.010. Epub 2008 May 27.

[39] N. Allie, S. I. Grivennikov, R. Keeton, N. J. Hsu, M. L. Bourigault, N. Court, C. Fremond, V. Yeremeev, Y. Shebzukhov, B. Ryffel, S. A. Nedospasov, V. F. Quesniaux, and M. Jacobs. Prominent role for t cell-derived tumour necrosis factor for sustained control of mycobacterium tuberculosis infection. *Sci Rep*, 3:1809, 2013. Allie, Nasiema Grivennikov, Sergei I Keeton, Roanne Hsu, Nai-Jen Bourigault, Marie-Laure Court, Nathalie Fremond, Cecile Yeremeev, Vladimir Shebzukhov, Yuriy Ryffel, Bernhard Nedospasov, Sergei A Quesniaux, Valerie F J Jacobs, Muazzam England Sci Rep. 2013;3:1809. doi: 10.1038/srep01809.

[40] G. B. Mackaness. The influence of immunologically committed lymphoid cells on macrophage activity in vivo. *J Exp Med*, 129(5):973–92, 1969. Mackaness, G B J Exp Med. 1969 May 1;129(5):973-92.

[41] Interferon nomenclature. *Nature*, 286(5769):110, 1980. ENGLAND Nature. 1980 Jul 10;286(5769):110.

[42] C. F. Nathan, H. G. Remold, and J. R. David. Characterization of a lymphocyte factor which alters macrophage functions. *J Exp Med*, 137(2):275–90, 1973. Nathan, C F Remold, H G David, J R J Exp Med. 1973 Feb 1;137(2):275-90.

[43] W. K. Roberts and A. Vasil. Evidence for the identity of murine gamma interferon and macrophage activating factor. *J Interferon Res*, 2(4):519–32, 1982. Roberts, W K Vasil, A J Interferon Res. 1982;2(4):519-32.

[44] J. L. Pace, S. W. Russell, B. A. Torres, H. M. Johnson, and P. W. Gray. Recombinant mouse gamma interferon induces the priming step in macrophage activation for tumor cell killing. *J Immunol*, 130(5):2011–3, 1983. Pace, J L Russell, S W Torres, B A Johnson, H M Gray, P W AI 16428/AI/NIAID NIH HHS/ CA 31199/CA/NCI NIH HHS/ Baltimore, Md. : 1950 J Immunol. 1983 May;130(5):2011-3.

[45] K. Schroder, P. J. Hertzog, T. Ravasi, and D. A. Hume. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol*, 75(2):163–89, 2004. Schroder, Kate Hertzog, Paul J Ravasi, Timothy Hume, David A J Leukoc Biol. 2004 Feb;75(2):163-89. Epub 2003 Oct 2.

[46] M. C. Cuturi, M. Murphy, M. P. Costa-Giomi, R. Weinmann, B. Perussia, and G. Trinchieri. Independent regulation of tumor necrosis factor and lymphotoxin production by human peripheral blood lymphocytes. *J Exp Med*, 165(6):1581–94, 1987. Cuturi, M C Murphy, M Costa-Giomi, M P Weinmann, R Perussia, B Trinchieri, G CA 10815/CA/NCI NIH HHS/ CA 20833/CA/NCI NIH HHS/ CA 21124/CA/NCI NIH HHS/ etc. J Exp Med. 1987 Jun 1;165(6):1581-94.

[47] S. S. Sung, J. M. Bjorndahl, C. Y. Wang, H. T. Kao, and S. M. Fu. Production of tumor necrosis factor/cachectin by human t cell lines and peripheral blood t lymphocytes stimulated by phorbol myristate acetate and anti-cd3 antibody. *J Exp Med*, 167(3):937–53, 1988. Sung, S S Bjorndahl, J M Wang, C Y Kao, H T Fu, S M CA-34546/CA/NCI NIH HHS/ CA-37954/CA/NCI NIH HHS/ J Exp Med. 1988 Mar 1;167(3):937-53.

[48] S. E. Christmas, A. Meager, and M. Moore. Production of interferon and tumour necrosis factor by cloned human natural cytotoxic lymphocytes and t cells. *Clin Exp Immunol*, 69(2):441–50, 1987. Christmas, S E Meager, A Moore, M ENGLAND Clin Exp Immunol. 1987 Aug;69(2):441-50.

[49] J. R. Gordon and S. J. Galli. Mast cells as a source of both preformed and immunologically inducible tnf-alpha/cachectin. *Nature*, 346(6281):274–6, 1990. Gordon, J R Galli, S J ENGLAND Nature. 1990 Jul 19;346(6281):274-6.

[50] C. Caux, C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. Activation of human dendritic cells through cd40 cross-linking. *J Exp Med*, 180(4):1263–72, 1994. Caux, C Massacrier, C Vanbervliet, B Dubois, B Van Kooten, C Durand, I Banchereau, J J Exp Med. 1994 Oct 1;180(4):1263-72.

[51] U. Boehm, T. Klamp, M. Groot, and J. C. Howard. Cellular responses to interferon-gamma. *Annu Rev Immunol*, 15:749–95, 1997. Boehm, U Klamp, T Groot, M Howard, J C Annu Rev Immunol. 1997;15:749-95.

[52] K. Takeda, H. Tsutsui, T. Yoshimoto, O. Adachi, N. Yoshida, T. Kishimoto, H. Okamura, K. Nakanishi, and S. Akira. Defective nk cell activity and th1 response in

il-18-deficient mice. *Immunity*, 8(3):383–90, 1998. Takeda, K Tsutsui, H Yoshi-moto, T Adachi, O Yoshida, N Kishimoto, T Okamura, H Nakanishi, K Akira, S Immunity. 1998 Mar;8(3):383-90.

[53] H. M. Ogmundsdottir and D. M. Weir. Mechanisms of macrophage activation. *Clin Exp Immunol*, 40(2):223–34, 1980. Ogmundsdottir, H M Weir, D M ENGLAND Clin Exp Immunol. 1980 May;40(2):223-34.

[54] G. J. Jurkovich, W. J. Mileski, R. V. Maier, R. K. Winn, and C. L. Rice. Interferon gamma increases sensitivity to endotoxin. *J Surg Res*, 51(3):197–203, 1991. Ju-rkovich, G J Mileski, W J Maier, R V Winn, R K Rice, C L GM 07037/GM/NIGMS NIH HHS/ GM 42686/GM/NIGMS NIH HHS/ HL 43141/HL/NHLBI NIH HHS/ J Surg Res. 1991 Sep;51(3):197-203.

[55] M. P. Hayes, J. Wang, and M. A. Norcross. Regulation of interleukin-12 expression in human monocytes: selective priming by interferon-gamma of lipopolysaccharide-inducible p35 and p40 genes. *Blood*, 86(2):646–50, 1995. Hayes, M P Wang, J Norcross, M A Blood. 1995 Jul 15;86(2):646-50.

[56] M. J. Sweet, K. J. Stacey, D. K. Kakuda, D. Markovich, and D. A. Hume. Ifn-gamma primes macrophage responses to bacterial dna. *J Interferon Cytokine Res*, 18(4):263–71, 1998. Sweet, M J Stacey, K J Kakuda, D K Markovich, D Hume, D A J Interferon Cytokine Res. 1998 Apr;18(4):263-71.

[57] L. Faggioli, M. Merola, J. Hiscott, A. Furia, R. Monese, M. Tovey, and M. Palmieri. Molecular mechanisms regulating induction of interleukin-6 gene transcription by interferon-gamma. *Eur J Immunol*, 27(11):3022–30, 1997. Faggioli, L Merola, M Hiscott, J Furia, A Monese, R Tovey, M Palmieri, M GERMANY Eur J Immunol. 1997 Nov;27(11):3022-30.

[58] R. B. Lorsbach, W. J. Murphy, C. J. Lowenstein, S. H. Snyder, and S. W. Russell. Expression of the nitric oxide synthase gene in mouse macrophages activated for tumor cell killing. molecular basis for the synergy between interferon-gamma and lipopolysaccharide. *J Biol Chem*, 268(3):1908–13, 1993. Lorsbach, R B Murphy, W J Lowenstein, C J Snyder, S H Russell, S W P01 CA54474/CA/NCI NIH HHS/ R01 CA31199/CA/NCI NIH HHS/ J Biol Chem. 1993 Jan 25;268(3):1908-13.

[59] M. P. Hayes and K. C. Zoon. Priming of human monocytes for enhanced lipopolysac-charide responses: expression of alpha interferon, interferon regulatory factors, and tumor necrosis factor. *Infect Immun*, 61(8):3222–7, 1993. Hayes, M P Zoon, K C United states Infection and immunity Infect Immun. 1993 Aug;61(8):3222-7.

[60] X. Ma, J. M. Chow, G. Gri, G. Carra, F. Gerosa, S. F. Wolf, R. Dzialo, and G. Trinchieri. The interleukin 12 p40 gene promoter is primed by interferon gamma in monocytic cells. *J Exp Med*, 183(1):147–57, 1996. Ma, X Chow, J M Gri, G Carra, G Gerosa, F Wolf, S F Dzialo, R Trinchieri, G CA-10815/CA/NCI NIH HHS/

CA-20833/CA/NCI NIH HHS/ CA-32898/CA/NCI NIH HHS/ etc. J Exp Med. 1996 Jan 1;183(1):147-57.

[61] E. Jouanguy, F. Altare, S. Lamhamedi, P. Revy, J. F. Emile, M. Newport, M. Levin, S. Blanche, E. Seboun, A. Fischer, and J. L. Casanova. Interferon-gamma-receptor deficiency in an infant with fatal bacille calmette-guerin infection. *N Engl J Med*, 335(26):1956–61, 1996. Jouanguy, E Altare, F Lamhamedi, S Revy, P Emile, J F Newport, M Levin, M Blanche, S Seboun, E Fischer, A Casanova, J L N Engl J Med. 1996 Dec 26;335(26):1956-61.

[62] M. J. Newport, C. M. Huxley, S. Huston, C. M. Hawrylowicz, B. A. Oostra, R. Williamson, and M. Levin. A mutation in the interferon-gamma-receptor gene and susceptibility to mycobacterial infection. *N Engl J Med*, 335(26):1941–9, 1996. Newport, M J Huxley, C M Huston, S Hawrylowicz, C M Oostra, B A Williamson, R Levin, M N Engl J Med. 1996 Dec 26;335(26):1941-9.

[63] S. M. Holland, S. E. Dorman, A. Kwon, I. F. Pitha-Rowe, D. M. Frucht, S. M. Gerstberger, G. J. Noel, P. Vesterhus, M. R. Brown, and T. A. Fleisher. Abnormal regulation of interferon-gamma, interleukin-12, and tumor necrosis factor-alpha in human interferon-gamma receptor 1 deficiency. *J Infect Dis*, 178(4):1095–104, 1998. Holland, S M Dorman, S E Kwon, A Pitha-Rowe, I F Frucht, D M Gerstberger, S M Noel, G J Vesterhus, P Brown, M R Fleisher, T A J Infect Dis. 1998 Oct;178(4):1095-104.

[64] S. K. Biswas and E. Lopez-Collazo. Endotoxin tolerance: new mechanisms, molecules and clinical significance. *Trends Immunol*, 30(10):475–87, 2009. Biswas, Subhra K Lopez-Collazo, Eduardo England Trends Immunol. 2009 Oct;30(10):475-87. doi: 10.1016/j.it.2009.07.009. Epub 2009 Sep 24.

[65] P. B. Beeson. Tolerance to bacterial pyrogens : I. factors influencing its development. *J Exp Med*, 86(1):29–38, 1947. Beeson, P B Technical Assistance of Elizabeth Roberts J Exp Med. 1947 Jun 30;86(1):29-38.

[66] P. B. Beeson. Tolerance to bacterial pyrogens : Ii. role of the reticulo-endothelial system. *J Exp Med*, 86(1):39–44, 1947. Beeson, P B Technical Assistance of Elizabeth Roberts J Exp Med. 1947 Jun 30;86(1):39-44.

[67] H. R. Morgan. Tolerance to the toxic action of somatic antigens of enteric bacteria. *J Immunol*, 59(2):129–34, 1948. MORGAN, H R Not Available Baltimore, Md. : 1950 J Immunol. 1948 Jun;59(2):129-34.

[68] M. A. Freudenberg and C. Galanos. Induction of tolerance to lipopolysaccharide (lps)-d-galactosamine lethality by pretreatment with lps is mediated by macrophages. *Infect Immun*, 56(5):1352–7, 1988. Freudenberg, M A Galanos, C Infect Immun. 1988 May;56(5):1352-7.

[69] S. K. Biswas, P. Bist, M. K. Dhillon, T. Kajiji, C. Del Fresno, M. Yamamoto, E. Lopez-Collazo, S. Akira, and V. Tergaonkar. Role for myd88-independent, trif pathway in lipid a/tlr4-induced endotoxin tolerance. *J Immunol*, 179(6):4083–92, 2007. Biswas, Subhra K Bist, Pradeep Dhillon, Manprit Kaur Kajiji, Tasneem Del Fresno, Carlos Yamamoto, Masahiro Lopez-Collazo, Eduardo Akira, Shizuo Tergaonkar, Vinay Baltimore, Md. : 1950 J Immunol. 2007 Sep 15;179(6):4083-92.

[70] M. A. Dobrovolskaia and S. N. Vogel. Toll receptors, cd14, and macrophage activation and deactivation by lps. *Microbes Infect*, 4(9):903–14, 2002. Dobrovolskaia, Marina A Vogel, Stefanie N AI-18797/AI/NIAID NIH HHS/ AI-44936/AI/NIAID NIH HHS/ France Microbes Infect. 2002 Jul;4(9):903-14.

[71] C. del Fresno, F. Garcia-Rio, V. Gomez-Pina, A. Soares-Schanoski, I. Fernandez-Ruiz, T. Jurado, T. Kajiji, C. Shu, E. Marin, A. Gutierrez del Arroyo, C. Prados, F. Arnalich, P. Fuentes-Prior, S. K. Biswas, and E. Lopez-Collazo. Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients. *J Immunol*, 182(10):6494–507, 2009. del Fresno, Carlos Garcia-Rio, Francisco Gomez-Pina, Vanesa Soares-Schanoski, Alessandra Fernandez-Ruiz, Irene Jurado, Teresa Kajiji, Tasneem Shu, Chen Marin, Elvira Gutierrez del Arroyo, Ana Prados, Concepcion Arnalich, Francisco Fuentes-Prior, Pablo Biswas, Subhra K Lopez-Collazo, Eduardo Baltimore, Md. : 1950 J Immunol. 2009 May 15;182(10):6494-507. doi: 10.4049/jimmunol.0803350.

[72] M. Adib-Conquy, C. Adrie, C. Fitting, O. Gattolliat, R. Beyaert, and J. M. Cavaillon. Up-regulation of myd88s and sigirr, molecules inhibiting toll-like receptor signaling, in monocytes from septic patients. *Crit Care Med*, 34(9):2377–85, 2006. Adib-Conquy, Minou Adrie, Christophe Fitting, Catherine Gattolliat, Olivier Beyaert, Rudi Cavaillon, Jean-Marc Crit Care Med. 2006 Sep;34(9):2377-85.

[73] J. G. Haas, N. Meyer, G. Riethmuller, and H. W. Ziegler-Heitbrock. Inhibition of lipopolysaccharide-induced in vitro desensitization by interferon-gamma. *Eur J Immunol*, 20(5):1181–4, 1990. Haas, J G Meyer, N Riethmuller, G Ziegler-Heitbrock, H W GERMANY, WEST Eur J Immunol. 1990 May;20(5):1181-4.

[74] K. Schroder, M. J. Sweet, and D. A. Hume. Signal integration between ifngamma and tlr signalling pathways in macrophages. *Immunobiology*, 211(6-8):511–24, 2006. Schroder, Kate Sweet, Matthew J Hume, David A Review Germany Immunobiology Immunobiology. 2006;211(6-8):511-24. Epub 2006 Jul 5.

[75] M. A. Meraz, J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, and R. D. Schreiber. Targeted disruption of the stat1 gene in mice reveals unexpected physiologic specificity in the jak-stat signaling pathway. *Cell*, 84(3):431–42, 1996. Meraz, M A White, J M Sheehan, K C Bach, E A Rodig,

S J Dighe, A S Kaplan, D H Riley, J K Greenlund, A C Campbell, D Carver-Moore, K DuBois, R N Clark, R Aguet, M Schreiber, R D Cell. 1996 Feb 9;84(3):431-42.

[76] D. Watling, D. Guschin, M. Muller, O. Silvennoinen, B. A. Witthuhn, F. W. Quelle, N. C. Rogers, C. Schindler, G. R. Stark, J. N. Ihle, and et al. Complementation by the protein tyrosine kinase jak2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature*, 366(6451):166–70, 1993. Watling, D Guschin, D Muller, M Silvennoinen, O Witthuhn, B A Quelle, F W Rogers, N C Schindler, C Stark, G R Ihle, J N ENGLAND Nature. 1993 Nov 11;366(6451):166-70.

[77] B. Saha, S. Jyothi Prasanna, B. Chandrasekar, and D. Nandi. Gene modulation and immunoregulatory roles of interferon gamma. *Cytokine*, 50(1):1–14, 2010. Saha, Banishree Jyothi Prasanna, S Chandrasekar, Bhagawat Nandi, Dipankar Cytokine. 2010 Apr;50(1):1-14. doi: 10.1016/j.cyto.2009.11.021. Epub 2009 Dec 29.

[78] M. A. Farrar, J. D. Campbell, and R. D. Schreiber. Identification of a functionally important sequence in the c terminus of the interferon-gamma receptor. *Proc Natl Acad Sci U S A*, 89(24):11706–10, 1992. Farrar, M A Campbell, J D Schreiber, R D AI24854/AI/NIAID NIH HHS/ CA43059/CA/NCI NIH HHS/ Proc Natl Acad Sci U S A. 1992 Dec 15;89(24):11706-10.

[79] Z. Wen, Z. Zhong, and Jr. Darnell, J. E. Maximal activation of transcription by stat1 and stat3 requires both tyrosine and serine phosphorylation. *Cell*, 82(2):241–50, 1995. Wen, Z Zhong, Z Darnell, J E Jr AI32489/AI/NIAID NIH HHS/ Cell. 1995 Jul 28;82(2):241-50.

[80] L. Varinou, K. Ramsauer, M. Karaghiosoff, T. Kolbe, K. Pfeffer, M. Muller, and T. Decker. Phosphorylation of the stat1 transactivation domain is required for full-fledged ifn-gamma-dependent innate immunity. *Immunity*, 19(6):793–802, 2003. Varinou, Louisa Ramsauer, Katrin Karaghiosoff, Marina Kolbe, Thomas Pfeffer, Klaus Muller, Mathias Decker, Thomas Immunity. 2003 Dec;19(6):793-802.

[81] D. K. Dalton, S. Pitts-Meek, S. Keshav, I. S. Figari, A. Bradley, and T. A. Stewart. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science*, 259(5102):1739–42, 1993. Dalton, D K Pitts-Meek, S Keshav, S Figari, I S Bradley, A Stewart, T A New York, N.Y. Science. 1993 Mar 19;259(5102):1739-42.

[82] T. Tamura, H. Yanai, D. Savitsky, and T. Taniguchi. The irf family transcription factors in immunity and oncogenesis. *Annu Rev Immunol*, 26:535–84, 2008. Tamura, Tomohiko Yanai, Hideyuki Savitsky, David Taniguchi, Tadatsugu Annu Rev Immunol. 2008;26:535-84. doi: 10.1146/annurev.immunol.26.021607.090400.

[83] H. Negishi, Y. Fujita, H. Yanai, S. Sakaguchi, X. Ouyang, M. Shinohara, H. Takayanagi, Y. Ohba, T. Taniguchi, and K. Honda. Evidence for licensing of

ifn-gamma-induced ifn regulatory factor 1 transcription factor by myd88 in toll-like receptor-dependent gene induction program. *Proc Natl Acad Sci U S A*, 103(41):15136–41, 2006. Negishi, Hideo Fujita, Yasuyuki Yanai, Hideyuki Sakaguchi, Shinya Ouyang, Xinshou Shinohara, Masahiro Takayanagi, Hiroshi Ohba, Yusuke Taniguchi, Tadatsugu Honda, Kenya Proc Natl Acad Sci U S A. 2006 Oct 10;103(41):15136-41. Epub 2006 Oct 3.

[84] J. Liu, S. Cao, L. M. Herman, and X. Ma. Differential regulation of interleukin (il)-12 p35 and p40 gene expression and interferon (ifn)-gamma-primed il-12 production by ifn regulatory factor 1. *J Exp Med*, 198(8):1265–76, 2003. Liu, Jianguo Cao, Shanjin Herman, Lisa M Ma, Xiaojing AI-045899/AI/NIAID NIH HHS/ CA-79772/CA/NCI NIH HHS/ J Exp Med. 2003 Oct 20;198(8):1265-76.

[85] C. M. Rosenberger, M. G. Scott, M. R. Gold, R. E. Hancock, and B. B. Finlay. Salmonella typhimurium infection and lipopolysaccharide stimulation induce similar changes in macrophage gene expression. *J Immunol*, 164(11):5894–904, 2000. Rosenberger, C M Scott, M G Gold, M R Hancock, R E Finlay, B B Baltimore, Md. : 1950 J Immunol. 2000 Jun 1;164(11):5894-904.

[86] S. D. Wright, R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison. Cd14, a receptor for complexes of lipopolysaccharide (lps) and lps binding protein. *Science*, 249(4975):1431–3, 1990. Wright, S D Ramos, R A Tobias, P S Ulevitch, R J Mathison, J C AI 15136/AI/NIAID NIH HHS/ AI 22003/AI/NIAID NIH HHS/ AI 24775/AI/NIAID NIH HHS/ New York, N.Y. Science. 1990 Sep 21;249(4975):1431-3.

[87] S. D. Wright, P. S. Tobias, R. J. Ulevitch, and R. A. Ramos. Lipopolysaccharide (lps) binding protein opsonizes lps-bearing particles for recognition by a novel receptor on macrophages. *J Exp Med*, 170(4):1231–41, 1989. Wright, S D Tobias, P S Ulevitch, R J Ramos, R A AI-22003/AI/NIAID NIH HHS/ AI-24775/AI/NIAID NIH HHS/ AI-25563/AI/NIAID NIH HHS/ etc. J Exp Med. 1989 Oct 1;170(4):1231-41.

[88] R. Shimazu, S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. Md-2, a molecule that confers lipopolysaccharide responsiveness on toll-like receptor 4. *J Exp Med*, 189(11):1777–82, 1999. Shimazu, R Akashi, S Ogata, H Nagai, Y Fukudome, K Miyake, K Kimoto, M J Exp Med. 1999 Jun 7;189(11):1777-82.

[89] Y. C. Lu, W. C. Yeh, and P. S. Ohashi. Lps/tlr4 signal transduction pathway. *Cytokine*, 42(2):145–51, 2008. Lu, Yong-Chen Yeh, Wen-Chen Ohashi, Pamela S Cytokine. 2008 May;42(2):145-51. doi: 10.1016/j.cyto.2008.01.006. Epub 2008 Mar 4.

[90] S. Akira and K. Takeda. Toll-like receptor signalling. *Nat Rev Immunol*, 4(7):499–511, 2004. Akira, Shizuo Takeda, Kiyoshi England Nat Rev Immunol. 2004 Jul;4(7):499-511.

[91] T. Kawai, O. Adachi, T. Ogawa, K. Takeda, and S. Akira. Unresponsiveness of myd88-deficient mice to endotoxin. *Immunity*, 11(1):115–22, 1999. Kawai, T Adachi, O Ogawa, T Takeda, K Akira, S Immunity. 1999 Jul;11(1):115-22.

[92] S. N. Vogel, K. A. Fitzgerald, and M. J. Fenton. Tlrs: differential adapter utilization by toll-like receptors mediates tlr-specific patterns of gene expression. *Mol Interv*, 3(8):466–77, 2003. Vogel, Stefanie N Fitzgerald, Katherine A Fenton, Matthew J AI-18797/AI/NIAID NIH HHS/ AI-47233/AI/NIAID NIH HHS/ Mol Interv. 2003 Dec;3(8):466-77.

[93] S. Li, A. Strelow, E. J. Fontana, and H. Wesche. Irak-4: a novel member of the irak family with the properties of an irak-kinase. *Proc Natl Acad Sci U S A*, 99(8):5567–72, 2002. Li, Shyun Strelow, Astrid Fontana, Elizabeth J Wesche, Holger Proc Natl Acad Sci U S A. 2002 Apr 16;99(8):5567-72.

[94] Z. Cao, J. Xiong, M. Takeuchi, T. Kurama, and D. V. Goeddel. Traf6 is a signal transducer for interleukin-1. *Nature*, 383(6599):443–6, 1996. Cao, Z Xiong, J Takeuchi, M Kurama, T Goeddel, D V ENGLAND Nature. 1996 Oct 3;383(6599):443-6.

[95] M. U. Martin and H. Wesche. Summary and comparison of the signaling mechanisms of the toll/interleukin-1 receptor family. *Biochim Biophys Acta*, 1592(3):265–80, 2002. Martin, Michael U Wesche, Holger Netherlands Biochim Biophys Acta. 2002 Nov 11;1592(3):265-80.

[96] J. Napetschnig and H. Wu. Molecular basis of nf-kappab signaling. *Annu Rev Biophys*, 42:443–68, 2013. Napetschnig, Johanna Wu, Hao R01 AI089882/AI/NIAID NIH HHS/ R01AI045937/AI/NIAID NIH HHS/ R01AI079260/AI/NIAID NIH HHS/ R01AI50872/AI/NIAID NIH HHS/ Annu Rev Biophys. 2013;42:443-68. doi: 10.1146/annurev-biophys-083012-130338. Epub 2013 Mar 11.

[97] M. Y. Balkhi, K. A. Fitzgerald, and P. M. Pitha. Functional regulation of myd88-activated interferon regulatory factor 5 by k63-linked polyubiquitination. *Mol Cell Biol*, 28(24):7296–308, 2008. Balkhi, Mumtaz Yaseen Fitzgerald, Katherine A Pitha, Paula M AI067497/AI/NIAID NIH HHS/ CA19737-22A1/CA/NCI NIH HHS/ R01 AI067632-02A1/AI/NIAID NIH HHS/ Mol Cell Biol. 2008 Dec;28(24):7296-308. doi: 10.1128/MCB.00662-08. Epub 2008 Sep 29.

[98] K. E. Thomas, C. L. Galligan, R. D. Newman, E. N. Fish, and S. N. Vogel. Contribution of interferon-beta to the murine macrophage response to the toll-like receptor 4 agonist, lipopolysaccharide. *J Biol Chem*, 281(41):31119–30, 2006. Thomas, Karen E Galligan, Carole L Newman, Raj Deonarain Fish, Eleanor N Vogel, Stefanie N AI18797/AI/NIAID NIH HHS/ J Biol Chem. 2006 Oct 13;281(41):31119-30. Epub 2006 Aug 15.

[99] K. A. Fitzgerald, D. C. Rowe, B. J. Barnes, D. R. Caffrey, A. Visintin, E. Latz, B. Monks, P. M. Pitha, and D. T. Golenbock. Lps-tlr4 signaling to irf-3/7 and

nf-kappab involves the toll adapters tram and trif. *J Exp Med*, 198(7):1043–55, 2003. Fitzgerald, Katherine A Rowe, Daniel C Barnes, Betsy J Caffrey, Daniel R Visintin, Alberto Latz, Eicke Monks, Brian Pitha, Paula M Golenbock, Douglas T 1R21 AI 0554537 01/AI/NIAID NIH HHS/ DK 50305/DK/NIDDK NIH HHS/ GM 54060/GM/NIGMS NIH HHS/ GM 63244/GM/NIGMS NIH HHS/ T32 AI 07349-14/AI/NIAID NIH HHS/ J Exp Med. 2003 Oct 6;198(7):1043-55. Epub 2003 Sep 29.

[100] M. Yamamoto, S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. Role of adaptor trif in the myd88-independent toll-like receptor signaling pathway. *Science*, 301(5633):640–3, 2003. Yamamoto, Masahiro Sato, Shintaro Hemmi, Hiroaki Hoshino, Katsuaki Kaisho, Tsuneyasu Sanjo, Hideki Takeuchi, Osamu Sugiyama, Masanaka Okabe, Masaru Takeda, Kiyoshi Akira, Shizuo New York, N.Y. Science. 2003 Aug 1;301(5633):640-3. Epub 2003 Jul 10.

[101] D. Bosisio, N. Polentarutti, M. Sironi, S. Bernasconi, K. Miyake, G. R. Webb, M. U. Martin, A. Mantovani, and M. Muzio. Stimulation of toll-like receptor 4 expression in human mononuclear phagocytes by interferon-gamma: a molecular basis for priming and synergism with bacterial lipopolysaccharide. *Blood*, 99(9):3427–31, 2002. Bosisio, Daniela Polentarutti, Nadia Sironi, Marina Bernasconi, Sergio Miyake, Kensuke Webb, Ginette R Martin, Michael U Mantovani, Alberto Muzio, Marta Blood. 2002 May 1;99(9):3427-31.

[102] R. Tamai, S. Sugawara, O. Takeuchi, S. Akira, and H. Takada. Synergistic effects of lipopolysaccharide and interferon-gamma in inducing interleukin-8 production in human monocytic thp-1 cells is accompanied by up-regulation of cd14, toll-like receptor 4, md-2 and myd88 expression. *J Endotoxin Res*, 9(3):145–53, 2003. Tamai, Riyoko Sugawara, Shunji Takeuchi, Osamu Akira, Shizuo Takada, Haruhiko England J Endotoxin Res. 2003;9(3):145-53.

[103] M. Adib-Conquy and J. M. Cavaillon. Gamma interferon and granulocyte/monocyte colony-stimulating factor prevent endotoxin tolerance in human monocytes by promoting interleukin-1 receptor-associated kinase expression and its association to myd88 and not by modulating tlr4 expression. *J Biol Chem*, 277(31):27927–34, 2002. Adib-Conquy, Minou Cavaillon, Jean-Marc J Biol Chem. 2002 Aug 2;277(31):27927-34. Epub 2002 May 24.

[104] T. K. Held, X. Weihua, L. Yuan, D. V. Kalvakolanu, and A. S. Cross. Gamma interferon augments macrophage activation by lipopolysaccharide by two distinct mechanisms, at the signal transduction level and via an autocrine mechanism involving tumor necrosis factor alpha and interleukin-1. *Infect Immun*, 67(1):206–12, 1999. Held, T K Weihua, X Yuan, L Kalvakolanu, D V Cross, A S Infect Immun. 1999 Jan;67(1):206-12.

[105] H. de Wit, D. Hoogstraten, R. M. Halie, and E. Vellenga. Interferon-gamma modulates the lipopolysaccharide-induced expression of ap-1 and nf-kappa b at the mrna and protein level in human monocytes. *Exp Hematol*, 24(2):228–35, 1996. de Wit, H Hoogstraten, D Halie, R M Vellenga, E Exp Hematol. 1996 Feb;24(2):228-35.

[106] R. de Waal Malefyt, J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. Interleukin 10(il-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of il-10 produced by monocytes. *J Exp Med*, 174(5):1209–20, 1991. de Waal Malefyt, R Abrams, J Bennett, B Figdor, C G de Vries, J E J Exp Med. 1991 Nov 1;174(5):1209-20.

[107] X. Hu, P. K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T. T. Lu, J. R. Woodgett, and L. B. Ivashkiv. Ifn-gamma suppresses il-10 production and synergizes with tlr2 by regulating gsk3 and creb/ap-1 proteins. *Immunity*, 24(5):563–74, 2006. Hu, Xiaoyu Paik, Paul K Chen, Janice Yarilina, Anna Kockeritz, Lisa Lu, Theresa T Woodgett, James R Ivashkiv, Lionel B C06-RR12538-01/RR/NCRR NIH HHS/ Immunity. 2006 May;24(5):563-74.

[108] L. Ulloa, J. Doody, and J. Massague. Inhibition of transforming growth factor-beta/smad signalling by the interferon-gamma/stat pathway. *Nature*, 397(6721):710–3, 1999. Ulloa, L Doody, J Massague, J ENGLAND Nature. 1999 Feb 25;397(6721):710-3.

[109] C. Herrero, X. Hu, W. P. Li, S. Samuels, M. N. Sharif, S. Kotenko, and L. B. Ivashkiv. Reprogramming of il-10 activity and signaling by ifn-gamma. *J Immunol*, 171(10):5034–41, 2003. Herrero, Carmen Hu, Xiaoyu Li, Wai Ping Samuels, Stuart Sharif, M Nusrat Kotenko, Sergei Ivashkiv, Lionel B AI51139/AI/NIAID NIH HHS/ Baltimore, Md. : 1950 J Immunol. 2003 Nov 15;171(10):5034-41.

[110] P. Kovarik, D. Stoiber, M. Novy, and T. Decker. Stat1 combines signals derived from ifn-gamma and lps receptors during macrophage activation. *EMBO J*, 17(13):3660–8, 1998. Kovarik, P Stoiber, D Novy, M Decker, T ENGLAND EMBO J. 1998 Jul 1;17(13):3660-8.

[111] L. A. Cramer, S. L. Nelson, and M. J. Klemsz. Synergistic induction of the tap-1 gene by ifn-gamma and lipopolysaccharide in macrophages is regulated by stat1. *J Immunol*, 165(6):3190–7, 2000. Cramer, L A Nelson, S L Klemsz, M J CA71384/CA/NCI NIH HHS/ Baltimore, Md. : 1950 J Immunol. 2000 Sep 15;165(6):3190-7.

[112] T. V. Pedchenko, G. Y. Park, M. Joo, T. S. Blackwell, and J. W. Christman. Inducible binding of pu.1 and interacting proteins to the toll-like receptor 4 promoter during endotoxemia. *Am J Physiol Lung Cell Mol Physiol*, 289(3):L429–37, 2005. Pedchenko, Tetyana V Park, Gye Young Joo, Myungsoo Blackwell, Timothy S Christman, John W Am J Physiol Lung Cell Mol Physiol. 2005 Sep;289(3):L429-37.

[113] M. Noll. Internal structure of the chromatin subunit. *Nucleic Acids Res*, 1(11):1573–8, 1974. Noll, M ENGLAND Nucleic Acids Res. 1974 Nov;1(11):1573-8.

[114] K. E. Van Holde, C. G. Sahasrabuddhe, and B. R. Shaw. A model for particulate structure in chromatin. *Nucleic Acids Res*, 1(11):1579–86, 1974. Van Holde, K E Sahasrabuddhe, C G Shaw, B R ENGLAND Nucleic Acids Res. 1974 Nov;1(11):1579-86.

[115] P. N. Cockerill. Structure and function of active chromatin and dnase i hypersensitive sites. *FEBS J*, 278(13):2182–210, 2011. Cockerill, Peter N Biotechnology and Biological Sciences Research Council/United Kingdom England FEBS J. 2011 Jul;278(13):2182-210. doi: 10.1111/j.1742-4658.2011.08128.x. Epub 2011 May 26.

[116] D. S. Gross and W. T. Garrard. Nuclease hypersensitive sites in chromatin. *Annu Rev Biochem*, 57:159–97, 1988. Gross, D S Garrard, W T GM22201/GM/NIGMS NIH HHS/ GM29935/GM/NIGMS NIH HHS/ GM31689/GM/NIGMS NIH HHS/ Annu Rev Biochem. 1988;57:159-97.

[117] J. D. McGhee, W. I. Wood, M. Dolan, J. D. Engel, and G. Felsenfeld. A 200 base pair region at the 5' end of the chicken adult beta-globin gene is accessible to nuclease digestion. *Cell*, 27(1 Pt 2):45–55, 1981. McGhee, J D Wood, W I Dolan, M Engel, J D Felsenfeld, G Cell. 1981 Nov;27(1 Pt 2):45-55.

[118] M. A. Keene, V. Corces, K. Lowenhaupt, and S. C. Elgin. Dnase i hypersensitive sites in drosophila chromatin occur at the 5' ends of regions of transcription. *Proc Natl Acad Sci U S A*, 78(1):143–6, 1981. Keene, M A Corces, V Lowenhaupt, K Elgin, S C GM 20779/GM/NIGMS NIH HHS/ Proc Natl Acad Sci U S A. 1981 Jan;78(1):143-6.

[119] A. J. Varshavsky, O. H. Sundin, and M. J. Bohn. Sv40 viral minichromosome: preferential exposure of the origin of replication as probed by restriction endonucleases. *Nucleic Acids Res*, 5(10):3469–77, 1978. Varshavsky, A J Sundin, O H Bohn, M J ENGLAND Nucleic Acids Res. 1978 Oct;5(10):3469-77.

[120] W. A. Scott and D. J. Wigmore. Sites in simian virus 40 chromatin which are preferentially cleaved by endonucleases. *Cell*, 15(4):1511–8, 1978. Scott, W A Wigmore, D J ENGLAND Cell. 1978 Dec;15(4):1511-8.

[121] U. Siebenlist, D. B. Durand, P. Bressler, N. J. Holbrook, C. A. Norris, M. Kamoun, J. A. Kant, and G. R. Crabtree. Promoter region of interleukin-2 gene undergoes chromatin structure changes and confers inducibility on chloramphenicol acetyltransferase gene during activation of t cells. *Mol Cell Biol*, 6(9):3042–9, 1986. Siebenlist, U Durand, D B Bressler, P Holbrook, N J Norris, C A Kamoun, M Kant, J A Crabtree, G R AI20846/AI/NIAID NIH HHS/ CA39612/CA/NCI NIH HHS/ HL33942/HL/NHLBI NIH HHS/ etc. Mol Cell Biol. 1986 Sep;6(9):3042-9.

[122] R. E. Thurman, E. Rynes, R. Humbert, J. Vierstra, M. T. Maurano, E. Haugen, N. C. Sheffield, A. B. Stergachis, H. Wang, B. Vernot, K. Garg, S. John, R. Sandstrom, D. Bates, L. Boatman, T. K. Canfield, M. Diegel, D. Dunn, A. K. Ebersol, T. Frum, E. Giste, A. K. Johnson, E. M. Johnson, T. Kutyavin, B. Lajoie, B. K. Lee, K. Lee, D. London, D. Lotakis, S. Neph, F. Neri, E. D. Nguyen, H. Qu, A. P. Reynolds, V. Roach, A. Safi, M. E. Sanchez, A. Sanyal, A. Shafer, J. M. Simon, L. Song, S. Vong, M. Weaver, Y. Yan, Z. Zhang, Z. Zhang, B. Lenhard, M. Tewari, M. O. Dorschner, R. S. Hansen, P. A. Navas, G. Stamatoyannopoulos, V. R. Iyer, J. D. Lieb, S. R. Sunyaev, J. M. Akey, P. J. Sabo, R. Kaul, T. S. Furey, J. Dekker, G. E. Crawford, and J. A. Stamatoyannopoulos. The accessible chromatin landscape of the human genome. *Nature*, 489(7414):75–82, 2012. Thurman, Robert E Rynes, Eric Humbert, Richard Vierstra, Jeff Maurano, Matthew T Haugen, Eric Sheffield, Nathan C Stergachis, Andrew B Wang, Hao Vernot, Benjamin Garg, Kavita John, Sam Sandstrom, Richard Bates, Daniel Boatman, Lisa Canfield, Theresa K Diegel, Morgan Dunn, Douglas Ebersol, Abigail K Frum, Tristan Giste, Erika Johnson, Audra K Johnson, Ericka M Kutyavin, Tanya Lajoie, Bryan Lee, Bum-Kyu Lee, Kristen London, Darin Lotakis, Dimitra Neph, Shane Neri, Fidencio Nguyen, Eric D Qu, Hongzhu Reynolds, Alex P Roach, Vaughn Safi, Alexias Sanchez, Minerva E Sanyal, Amartya Shafer, Anthony Simon, Jeremy M Song, Lingyun Vong, Shinny Weaver, Molly Yan, Yongqi Zhang, Zhancheng Zhang, Zhuzhu Lenhard, Boris Tewari, Muneesh Dorschner, Michael O Hansen, R Scott Navas, Patrick A Stamatoyannopoulos, George Iyer, Vishwanath R Lieb, Jason D Sunyaev, Shamil R Akey, Joshua M Sabo, Peter J Kaul, Rajinder Furey, Terrence S Dekker, Job Crawford, Gregory E Stamatoyannopoulos, John A GM076036/GM/NIGMS NIH HHS/ HG004563/HG/NHGRI NIH HHS/ HG004592/HG/NHGRI NIH HHS/ HHSN261200800001E/PHS HHS/ R01 HG003143/HG/NHGRI NIH HHS/ R01MH084676/MH/NIMH NIH HHS/ U54 HG004563/HG/NHGRI NIH HHS/ U54 HG004592/HG/NHGRI NIH HHS/ England Nature. 2012 Sep 6;489(7414):75-82. doi: 10.1038/nature11232.

[123] M. Bulger and M. Groudine. Functional and mechanistic diversity of distal transcription enhancers. *Cell*, 144(3):327–39, 2011. Bulger, Michael Groudine, Mark R01 DK070687/DK/NIDDK NIH HHS/ R01 HL065440/HL/NHLBI NIH HHS/ R01 HL065440-11/HL/NHLBI NIH HHS/ R01 HL065440-12/HL/NHLBI NIH HHS/ R01 HL065440-13/HL/NHLBI NIH HHS/ R01 HL65440/HL/NHLBI NIH HHS/ R37 DK044746/DK/NIDDK NIH HHS/ R37 DK44746/DK/NIDDK NIH HHS/ Cell. 2011 Feb 4;144(3):327-39. doi: 10.1016/j.cell.2011.01.024.

[124] T. Amano, T. Sagai, H. Tanabe, Y. Mizushina, H. Nakazawa, and T. Shiroishi. Chromosomal dynamics at the shh locus: limb bud-specific differential regulation of competence and active transcription. *Dev Cell*, 16(1):47–57, 2009. Amano, Takanori Sagai, Tomoko Tanabe, Hideyuki Mizushina, Yoichi Nakazawa, Hiromi Shiroishi, Toshihiko Dev Cell. 2009 Jan;16(1):47-57. doi: 10.1016/j.devcel.2008.11.011. Epub 2008 Dec 18.

[125] T. Fujita, H. Shibuya, H. Hotta, K. Yamanishi, and T. Taniguchi. Interferon-beta gene regulation: tandemly repeated sequences of a synthetic 6 bp oligomer function as a virus-inducible enhancer. *Cell*, 49(3):357–67, 1987. Fujita, T Shibuya, H Hotta, H Yamanishi, K Taniguchi, T Cell. 1987 May 8;49(3):357-67.

[126] E. Ford and D. Thanos. The transcriptional code of human ifn-beta gene expression. *Biochim Biophys Acta*, 1799(3-4):328–36, 2010. Ford, Ethan Thanos, Dimitris Netherlands Biochim Biophys Acta. 2010 Mar-Apr;1799(3-4):328-36. doi: 10.1016/j.bbagrm.2010.01.010. Epub 2010 Jan 30.

[127] J. D. Aalfs and R. E. Kingston. What does 'chromatin remodeling' mean? *Trends Biochem Sci*, 25(11):548–55, 2000. Aalfs, J D Kingston, R E ENGLAND Trends Biochem Sci. 2000 Nov;25(11):548-55.

[128] K. Luger, A. W. Mader, R. K. Richmond, D. F. Sargent, and T. J. Richmond. Crystal structure of the nucleosome core particle at 2.8 a resolution. *Nature*, 389(6648):251–60, 1997. Luger, K Mader, A W Richmond, R K Sargent, D F Richmond, T J ENGLAND Nature. 1997 Sep 18;389(6648):251-60.

[129] J. V. Falvo, L. D. Jasenosky, L. Kruidenier, and A. E. Goldfeld. Epigenetic control of cytokine gene expression: regulation of the tnf/lt locus and t helper cell differentiation. *Adv Immunol*, 118:37–128, 2013. Falvo, James V Jasenosky, Luke D Kruidenier, Laurens Goldfeld, Anne E GM076685/GM/NIGMS NIH HHS/ HL-059838/HL/NHLBI NIH HHS/ Adv Immunol. 2013;118:37-128. doi: 10.1016/B978-0-12-407708-9.00002-9.

[130] S. D. Taverna, H. Li, A. J. Ruthenburg, C. D. Allis, and D. J. Patel. How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nat Struct Mol Biol*, 14(11):1025–40, 2007. Taverna, Sean D Li, Haitao Ruthenburg, Alexander J Allis, C David Patel, Dinshaw J GM53512/GM/NIGMS NIH HHS/ GM63959/GM/NIGMS NIH HHS/ Nat Struct Mol Biol. 2007 Nov;14(11):1025-40. Epub 2007 Nov 5.

[131] V. G. Allfrey, R. Faulkner, and A. E. Mirsky. Acetylation and methylation of histones and their possible role in the regulation of rna synthesis. *Proc Natl Acad Sci U S A*, 51:786–94, 1964. ALLFREY, V G FAULKNER, R MIRSKY, A E Proc Natl Acad Sci U S A. 1964 May;51:786-94.

[132] B. G. Pogo, V. G. Allfrey, and A. E. Mirsky. Rna synthesis and histone acetylation during the course of gene activation in lymphocytes. *Proc Natl Acad Sci U S A*, 55(4):805–12, 1966. Pogo, B G Allfrey, V G Mirsky, A E Proc Natl Acad Sci U S A. 1966 Apr;55(4):805-12.

[133] J. E. Brownell, J. Zhou, T. Ranalli, R. Kobayashi, D. G. Edmondson, S. Y. Roth, and C. D. Allis. Tetrahymena histone acetyltransferase a: a homolog to yeast gcn5p linking histone acetylation to gene activation. *Cell*, 84(6):843–51, 1996.

Brownell, J E Zhou, J Ranalli, T Kobayashi, R Edmondson, D G Roth, S Y Allis, C D F32CA65098/CA/NCI NIH HHS/ GM51189/GM/NIGMS NIH HHS/ GM53512/GM/NIGMS NIH HHS/ etc. Cell. 1996 Mar 22;84(6):843-51.

[134] K. Ito, P. J. Barnes, and I. M. Adcock. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone h4 acetylation on lysines 8 and 12. *Mol Cell Biol*, 20(18):6891–903, 2000. Ito, K Barnes, P J Adcock, I M Mol Cell Biol. 2000 Sep;20(18):6891-903.

[135] B. Schmeck, W. Beermann, V. van Laak, J. Zahlten, B. Opitz, M. Witzenrath, A. C. Hocke, T. Chakraborty, M. Kracht, S. Rosseau, N. Suttorp, and S. Hippenstiel. Intracellular bacteria differentially regulated endothelial cytokine release by mapk-dependent histone modification. *J Immunol*, 175(5):2843–50, 2005. Schmeck, Bernd Beermann, Wiebke van Laak, Vincent Zahlten, Janine Opitz, Bastian Witzenrath, Martin Hocke, Andreas C Chakraborty, Trinad Kracht, Michael Rosseau, Simone Suttorp, Norbert Hippenstiel, Stefan Baltimore, Md. : 1950 J Immunol. 2005 Sep 1;175(5):2843-50.

[136] G. E. Zentner and S. Henikoff. Regulation of nucleosome dynamics by histone modifications. *Nat Struct Mol Biol*, 20(3):259–66, 2013. Zentner, Gabriel E Henikoff, Steven 5U01 HG004274/HG/NHGRI NIH HHS/ R01 ES020116/ES/NIEHS NIH HHS/ U54 CA143862/CA/NCI NIH HHS/ Howard Hughes Medical Institute/ Nat Struct Mol Biol. 2013 Mar;20(3):259-66. doi: 10.1038/nsmb.2470.

[137] D. Canzio, M. Liao, N. Naber, E. Pate, A. Larson, S. Wu, D. B. Marina, J. F. Garcia, H. D. Madhani, R. Cooke, P. Schuck, Y. Cheng, and G. J. Narlikar. A conformational switch in hp1 releases auto-inhibition to drive heterochromatin assembly. *Nature*, 496(7445):377–81, 2013. Canzio, Daniele Liao, Maofu Naber, Nariman Pate, Edward Larson, Adam Wu, Shenping Marina, Diana B Garcia, Jennifer F Madhani, Hiten D Cooke, Roger Schuck, Peter Cheng, Yifan Narlikar, Geeta J AR053720/AR/NIAMS NIH HHS/ R01GM071801/GM/NIGMS NIH HHS/ England Nature. 2013 Apr 18;496(7445):377-81. doi: 10.1038/nature12032. Epub 2013 Mar 13.

[138] W. Fischle, Y. Wang, S. A. Jacobs, Y. Kim, C. D. Allis, and S. Khorasanizadeh. Molecular basis for the discrimination of repressive methyl-lysine marks in histone h3 by polycomb and hp1 chromodomains. *Genes Dev*, 17(15):1870–81, 2003. Fischle, Wolfgang Wang, Yanming Jacobs, Steven A Kim, Youngchang Allis, C David Khorasanizadeh, Sepideh GM53512/GM/NIGMS NIH HHS/ GM63959/GM/NIGMS NIH HHS/ Genes Dev. 2003 Aug 1;17(15):1870-81.

[139] T. Swigut and J. Wysocka. H3k27 demethylases, at long last. *Cell*, 131(1):29–32, 2007. Swigut, Tomek Wysocka, Joanna Cell. 2007 Oct 5;131(1):29-32.

[140] R. Cao, L. Wang, H. Wang, L. Xia, H. Erdjument-Bromage, P. Tempst, R. S. Jones, and Y. Zhang. Role of histone h3 lysine 27 methylation in polycomb-group silencing.

Science, 298(5595):1039–43, 2002. Cao, Ru Wang, Liangjun Wang, Hengbin Xia, Li Erdjument-Bromage, Hediye Tempst, Paul Jones, Richard S Zhang, Yi New York, N.Y. Science. 2002 Nov 1;298(5595):1039-43. Epub 2002 Sep 26.

[141] A. Kuzmichev, K. Nishioka, H. Erdjument-Bromage, P. Tempst, and D. Reinberg. Histone methyltransferase activity associated with a human multiprotein complex containing the enhancer of zeste protein. *Genes Dev*, 16(22):2893–905, 2002. Kuzmichev, Andrei Nishioka, Kenichi Erdjument-Bromage, Hediye Tempst, Paul Reinberg, Danny GM-37120/GM/NIGMS NIH HHS/ P30 CA08748/CA/NCI NIH HHS/ Genes Dev. 2002 Nov 15;16(22):2893-905.

[142] B. Czermin, R. Melfi, D. McCabe, V. Seitz, A. Imhof, and V. Pirrotta. Drosophila enhancer of zeste/esc complexes have a histone h3 methyltransferase activity that marks chromosomal polycomb sites. *Cell*, 111(2):185–96, 2002. Czermin, Birgit Melfi, Raffaella McCabe, Donna Seitz, Volker Imhof, Axel Pirrotta, Vincenzo Cell. 2002 Oct 18;111(2):185-96.

[143] K. Agger, P. A. Cloos, J. Christensen, D. Pasini, S. Rose, J. Rappsilber, I. Issaeva, E. Canaani, A. E. Salcini, and K. Helin. Utx and jmjd3 are histone h3k27 demethy-lases involved in hox gene regulation and development. *Nature*, 449(7163):731–4, 2007. Agger, Karl Cloos, Paul A C Christensen, Jesper Pasini, Diego Rose, Simon Rappsilber, Juri Issaeva, Irina Canaani, Eli Salcini, Anna Elisabetta Helin, Kristian England Nature. 2007 Oct 11;449(7163):731-4. Epub 2007 Aug 22.

[144] F. De Santa, M. G. Totaro, E. Prosperini, S. Notarbartolo, G. Testa, and G. Na-toli. The histone h3 lysine-27 demethylase jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell*, 130(6):1083–94, 2007. De Santa, Francesca Totaro, Maria Grazia Prosperini, Elena Notarbartolo, Samuele Testa, Giuseppe Natoli, Gioacchino Cell. 2007 Sep 21;130(6):1083-94. Epub 2007 Sep 6.

[145] F. Lan, P. E. Bayliss, J. L. Rinn, J. R. Whetstine, J. K. Wang, S. Chen, S. Iwase, R. Alpatov, I. Issaeva, E. Canaani, T. M. Roberts, H. Y. Chang, and Y. Shi. A histone h3 lysine 27 demethylase regulates animal posterior development. *Na-ture*, 449(7163):689–94, 2007. Lan, Fei Bayliss, Peter E Rinn, John L Whetstine, Johnathan R Wang, Jordon K Chen, Shuzhen Iwase, Shigeki Alpatov, Roman Is-saeva, Irina Canaani, Eli Roberts, Thomas M Chang, Howard Y Shi, Yang England Nature. 2007 Oct 11;449(7163):689-94. Epub 2007 Sep 12.

[146] M. G. Lee, R. Villa, P. Trojer, J. Norman, K. P. Yan, D. Reinberg, L. Di Croce, and R. Shiekhattar. Demethylation of h3k27 regulates polycomb recruitment and h2a ubiquitination. *Science*, 318(5849):447–50, 2007. Lee, Min Gyu Villa, Raffaella Trojer, Patrick Norman, Jessica Yan, Kai-Ping Reinberg, Danny Di Croce, Luciano Shiekhattar, Ramin R01CA090758/CA/NCI NIH HHS/ New York, N.Y. Science. 2007 Oct 19;318(5849):447-50. Epub 2007 Aug 30.

[147] L. Kruidenier, C. W. Chung, Z. Cheng, J. Liddle, K. Che, G. Joberty, M. Bantscheff, C. Bountra, A. Bridges, H. Diallo, D. Eberhard, S. Hutchinson, E. Jones, R. Katso, M. Leveridge, P. K. Mander, J. Mosley, C. Ramirez-Molina, P. Rowland, C. J. Schofield, R. J. Sheppard, J. E. Smith, C. Swales, R. Tanner, P. Thomas, A. Tumber, G. Drewes, U. Oppermann, D. J. Patel, K. Lee, and D. M. Wilson. A selective jumonji h3k27 demethylase inhibitor modulates the proinflammatory macrophage response. *Nature*, 488(7411):404–8, 2012. Kruidenier, Laurens Chung, Chun-wa Cheng, Zhongjun Liddle, John Che, KaHing Joberty, Gerard Bantscheff, Marcus Bountra, Chas Bridges, Angela Diallo, Hawa Eberhard, Dirk Hutchinson, Sue Jones, Emma Katso, Roy Leveridge, Melanie Mander, Palwinder K Mosley, Julie Ramirez-Molina, Cesar Rowland, Paul Schofield, Christopher J Sheppard, Robert J Smith, Julia E Swales, Catherine Tanner, Robert Thomas, Pamela Tumber, Anthony Drewes, Gerard Oppermann, Udo Patel, Dinshaw J Lee, Kevin Wilson, David M Canadian Institutes of Health Research/Canada Wellcome Trust/United Kingdom England Nature. 2012 Aug 16;488(7411):404-8. doi: 10.1038/nature11262.

[148] J. S. Lee, E. Smith, and A. Shilatifard. The language of histone crosstalk. *Cell*, 142(5):682–5, 2010. Lee, Jung-Shin Smith, Edwin Shilatifard, Ali R01 CA089455/CA/NCI NIH HHS/ R01 CA150265/CA/NCI NIH HHS/ R01 CA150265-02/CA/NCI NIH HHS/ R01 GM069905/GM/NIGMS NIH HHS/ R01CA150265/CA/NCI NIH HHS/ R01CA89455/CA/NCI NIH HHS/ R01GM069905/GM/NIGMS NIH HHS/ Cell. 2010 Sep 3;142(5):682-5. doi: 10.1016/j.cell.2010.08.011.

[149] Z. Wang, C. Zang, K. Cui, D. E. Schones, A. Barski, W. Peng, and K. Zhao. Genome-wide mapping of hats and hdacs reveals distinct functions in active and inactive genes. *Cell*, 138(5):1019–31, 2009. Wang, Zhibin Zang, Chongzhi Cui, Kairong Schones, Dustin E Barski, Artem Peng, Weiqun Zhao, Keji Z01 HL005801-05/HL/NHLBI NIH HHS/ Cell. 2009 Sep 4;138(5):1019-31. doi: 10.1016/j.cell.2009.06.049. Epub 2009 Aug 20.

[150] X. Li, L. Wu, C. A. Corsa, S. Kunkel, and Y. Dou. Two mammalian mof complexes regulate transcription activation by distinct mechanisms. *Mol Cell*, 36(2):290–301, 2009. Li, Xiangzhi Wu, Lipeng Corsa, Callie Ann Sprunger Kunkel, Steve Dou, Yali R01 GM082856/GM/NIGMS NIH HHS/ R01 GM082856-01A2/GM/NIGMS NIH HHS/ R01 GM082856-04/GM/NIGMS NIH HHS/ R01GM082856/GM/NIGMS NIH HHS/ Mol Cell. 2009 Oct 23;36(2):290-301. doi: 10.1016/j.molcel.2009.07.031.

[151] Y. Cai, J. Jin, S. K. Swanson, M. D. Cole, S. H. Choi, L. Florens, M. P. Washburn, J. W. Conaway, and R. C. Conaway. Subunit composition and substrate specificity of a mof-containing histone acetyltransferase distinct from the male-specific lethal (msl) complex. *J Biol Chem*, 285(7):4268–72, 2010. Cai, Yong Jin, Jingji Swanson, Selene K Cole, Michael D Choi, Seung Hyuk Florens, Laurence Washburn, Michael P Conaway, Joan W Conaway, Ronald C P30 CA023108/CA/NCI NIH HHS/ R01

CA055248-17/CA/NCI NIH HHS/ R01 CA055248-18/CA/NCI NIH HHS/ R01 CA055248-19/CA/NCI NIH HHS/ R01 CA055248-20/CA/NCI NIH HHS/ R01 GM041628-24/GM/NIGMS NIH HHS/ R37 GM041628-22/GM/NIGMS NIH HHS/ R37 GM41628/GM/NIGMS NIH HHS/ J Biol Chem. 2010 Feb 12;285(7):4268-72. doi: 10.1074/jbc.C109.087981. Epub 2009 Dec 14.

[152] K. Ahmad and S. Henikoff. The histone variant h3.3 marks active chromatin by replication-independent nucleosome assembly. *Mol Cell*, 9(6):1191–200, 2002. Ahmad, Kami Henikoff, Steven Mol Cell. 2002 Jun;9(6):1191-200.

[153] H. S. Malik and S. Henikoff. Phylogenomics of the nucleosome. *Nat Struct Biol*, 10(11):882–91, 2003. Malik, Harmit S Henikoff, Steven Nat Struct Biol. 2003 Nov;10(11):882-91.

[154] C. Jin and G. Felsenfeld. Nucleosome stability mediated by histone variants h3.3 and h2a.z. *Genes Dev*, 21(12):1519–29, 2007. Jin, Chunyuan Felsenfeld, Gary Genes Dev. 2007 Jun 15;21(12):1519-29.

[155] E. McKittrick, P. R. Gafken, K. Ahmad, and S. Henikoff. Histone h3.3 is enriched in covalent modifications associated with active chromatin. *Proc Natl Acad Sci U S A*, 101(6):1525–30, 2004. McKittrick, Erin Gafken, Philip R Ahmad, Kami Henikoff, Steven Proc Natl Acad Sci U S A. 2004 Feb 10;101(6):1525-30. Epub 2004 Jan 19.

[156] Y. Mito, J. G. Henikoff, and S. Henikoff. Genome-scale profiling of histone h3.3 replacement patterns. *Nat Genet*, 37(10):1090–7, 2005. Mito, Yoshiko Henikoff, Jorja G Henikoff, Steven Nat Genet. 2005 Oct;37(10):1090-7. Epub 2005 Sep 11.

[157] G. Hu, K. Cui, D. Northrup, C. Liu, C. Wang, Q. Tang, K. Ge, D. Levens, C. Crane-Robinson, and K. Zhao. H2a.z facilitates access of active and repressive complexes to chromatin in embryonic stem cell self-renewal and differentiation. *Cell Stem Cell*, 12(2):180–92, 2013. Hu, Gangqing Cui, Kairong Northrup, Daniel Liu, Chengyu Wang, Chaochen Tang, Qingsong Ge, Kai Levens, David Crane-Robinson, Colyn Zhao, Keji Cell Stem Cell. 2013 Feb 7;12(2):180-92. doi: 10.1016/j.stem.2012.11.003. Epub 2012 Dec 20.

[158] T. Tamura, M. Smith, T. Kanno, H. Dasenbrock, A. Nishiyama, and K. Ozato. Inducible deposition of the histone variant h3.3 in interferon-stimulated genes. *J Biol Chem*, 284(18):12217–25, 2009. Tamura, Tomohiko Smith, Matthew Kanno, Tomohiko Dasenbrock, Hormuzdiyer Nishiyama, Akira Ozato, Keiko J Biol Chem. 2009 May 1;284(18):12217-25. doi: 10.1074/jbc.M805651200. Epub 2009 Feb 25.

[159] J. Wysocka, T. Swigut, H. Xiao, T. A. Milne, S. Y. Kwon, J. Landry, M. Kauer, A. J. Tackett, B. T. Chait, P. Badenhorst, C. Wu, and C. D. Allis. A phd finger of nurf couples histone h3 lysine 4 trimethylation with chromatin remodelling. *Nature*, 442(7098):86–90, 2006. Wysocka, Joanna Swigut, Tomek Xiao, Hua Milne, Thomas

A Kwon, So Yeon Landry, Joe Kauer, Monika Tackett, Alan J Chait, Brian T Baden-horst, Paul Wu, Carl Allis, C David England Nature. 2006 Jul 6;442(7098):86-90. Epub 2006 May 21.

[160] C. R. Clapier and B. R. Cairns. The biology of chromatin remodeling complexes. *Annu Rev Biochem*, 78:273–304, 2009. Clapier, Cedric R Cairns, Bradley R Howard Hughes Medical Institute/ Annu Rev Biochem. 2009;78:273-304. doi: 10.1146/annurev.biochem.77.062706.153223.

[161] A. L. Wurster and M. J. Pazin. Atp-dependent chromatin remodeling in t cells. *Biochem Cell Biol*, 90(1):1–13, 2012. Wurster, Andrea L Pazin, Michael J 1 Z01 AG000524/AG/NIA NIH HHS/ ZIA AG000524-07/AG/NIA NIH HHS/ Canada Biochem Cell Biol. 2012 Feb;90(1):1-13. doi: 10.1139/o11-042. Epub 2011 Oct 14.

[162] K. Zhao, W. Wang, O. J. Rando, Y. Xue, K. Swiderek, A. Kuo, and G. R. Crab-tree. Rapid and phosphoinositol-dependent binding of the swi/snf-like baf complex to chromatin after t lymphocyte receptor signaling. *Cell*, 95(5):625–36, 1998. Zhao, K Wang, W Rando, O J Xue, Y Swiderek, K Kuo, A Crabtree, G R Cell. 1998 Nov 25;95(5):625-36.

[163] V. R. Ramirez-Carrozzi, A. A. Nazarian, C. C. Li, S. L. Gore, R. Sridharan, A. N. Imbalzano, and S. T. Smale. Selective and antagonistic functions of swi/snf and mi-2beta nucleosome remodeling complexes during an inflammatory response. *Genes Dev*, 20(3):282–96, 2006. Ramirez-Carrozzi, Vladimir R Nazarian, Aaron A Li, Caiyi C Gore, Sarah L Sridharan, Rupa Imbalzano, Anthony N Smale, Stephen T CA009120/CA/NCI NIH HHS/ Genes Dev. 2006 Feb 1;20(3):282-96.

[164] S. Ghisletti, W. Huang, K. Jepsen, C. Benner, G. Hardiman, M. G. Rosenfeld, and C. K. Glass. Cooperative ncor/smrt interactions establish a corepressor-based strategy for integration of inflammatory and anti-inflammatory signaling pathways. *Genes Dev*, 23(6):681–93, 2009. Ghisletti, Serena Huang, Wendy Jepsen, Kristen Benner, Chris Hardiman, Gary Rosenfeld, Michael G Glass, Christopher K CA052599/CA/NCI NIH HHS/ DK063491/DK/NIDDK NIH HHS/ DK074868/DK/NIDDK NIH HHS/ DK3949/DK/NIDDK NIH HHS/ GM069338/GM/NIGMS NIH HHS/ HL065445/HL/NHLBI NIH HHS/ HL088093/HL/NHLBI NIH HHS/ Genes Dev. 2009 Mar 15;23(6):681-93. doi: 10.1101/gad.1773109.

[165] K. S. Zaret and J. S. Carroll. Pioneer transcription factors: establishing competence for gene expression. *Genes Dev*, 25(21):2227–41, 2011. Zaret, Kenneth S Carroll, Jason S R01DK082623/DK/NIDDK NIH HHS/ R37 GM036477-29/GM/NIGMS NIH HHS/ R37GM36477/GM/NIGMS NIH HHS/ Genes Dev. 2011 Nov 1;25(21):2227-41. doi: 10.1101/gad.176826.111.

[166] K. S. Zaret, J. M. Caravaca, A. Tulin, and T. Sekiya. Nuclear mobility and mi-totic chromosome binding: similarities between pioneer transcription factor foxa

and linker histone h1. *Cold Spring Harb Symp Quant Biol*, 75:219–26, 2010. Zaret, K S Caravaca, J M Tulin, A Sekiya, T R01-DK82623/DK/NIDDK NIH HHS/ R37-GM36477/GM/NIGMS NIH HHS/ Cold Spring Harb Symp Quant Biol. 2010;75:219-26. doi: 10.1101/sqb.2010.75.061. Epub 2011 Apr 18.

[167] Y. B. Zhou, S. E. Gerchman, V. Ramakrishnan, A. Travers, and S. Muyldermans. Position and orientation of the globular domain of linker histone h5 on the nucleosome. *Nature*, 395(6700):402–5, 1998. Zhou, Y B Gerchman, S E Ramakrishnan, V Travers, A Muyldermans, S ENGLAND Nature. 1998 Sep 24;395(6700):402-5.

[168] S. Ghisletti, I. Barozzi, F. Mietton, S. Polletti, F. De Santa, E. Venturini, L. Gregory, L. Lonie, A. Chew, C. L. Wei, J. Ragoussis, and G. Natoli. Identification and characterization of enhancers controlling the inflammatory gene expression program in macrophages. *Immunity*, 32(3):317–28, 2010. Ghisletti, Serena Barozzi, Iros Mietton, Flore Polletti, Sara De Santa, Francesca Venturini, Elisa Gregory, Lorna Lonie, Lorne Chew, Adeline Wei, Chia-Lin Ragoussis, Jiannis Natoli, Gioacchino 1R01HG003521-01/HG/NHGRI NIH HHS/ Immunity. 2010 Mar 26;32(3):317-28. doi: 10.1016/j.immuni.2010.02.008. Epub 2010 Mar 4.

[169] I. Krivega and A. Dean. Enhancer and promoter interactions-long distance calls. *Curr Opin Genet Dev*, 22(2):79–85, 2012. Krivega, Ivan Dean, Ann ZIA DK015508-23/DK/NIDDK NIH HHS/ ZIA DK075033-02/DK/NIDDK NIH HHS/ England Curr Opin Genet Dev. 2012 Apr;22(2):79-85. doi: 10.1016/j.gde.2011.11.001. Epub 2011 Dec 12.

[170] E. de Wit and W. de Laat. A decade of 3c technologies: insights into nuclear organization. *Genes Dev*, 26(1):11–24, 2012. de Wit, Elzo de Laat, Wouter Genes Dev. 2012 Jan 1;26(1):11-24. doi: 10.1101/gad.179804.111.

[171] J. Dekker, K. Rippe, M. Dekker, and N. Kleckner. Capturing chromosome conformation. *Science*, 295(5558):1306–11, 2002. Dekker, Job Rippe, Karsten Dekker, Martijn Kleckner, Nancy GM25326/GM/NIGMS NIH HHS/ GM44794/GM/NIGMS NIH HHS/ New York, N.Y. Science. 2002 Feb 15;295(5558):1306-11.

[172] A. Gavrilov, E. Eivazova, I. Priozhkova, M. Lipinski, S. Razin, and Y. Vassetzky. Chromosome conformation capture (from 3c to 5c) and its chip-based modification. *Methods Mol Biol*, 567:171–88, 2009. Gavrilov, Alexey Eivazova, Elvira Priozhkova, Iryna Lipinski, Marc Razin, Sergey Vassetzky, Yegor Clifton, N.J. Methods Mol Biol. 2009;567:171-88. doi: 10.1007/978-1-60327-414-2_12.

[173] C. G. Spilianakis and R. A. Flavell. Long-range intrachromosomal interactions in the t helper type 2 cytokine locus. *Nat Immunol*, 5(10):1017–27, 2004. Spilianakis, Charalampos G Flavell, Richard A Nat Immunol. 2004 Oct;5(10):1017-27. Epub 2004 Sep 19.

[174] A. Sanyal, B. R. Lajoie, G. Jain, and J. Dekker. The long-range interaction landscape of gene promoters. *Nature*, 489(7414):109–13, 2012. Sanyal, Amartya Lajoie, Bryan R Jain, Gaurav Dekker, Job HG003143/HG/NHGRI NIH HHS/ HG003143-06S1/HG/NHGRI NIH HHS/ R01 HG003143/HG/NHGRI NIH HHS/ England Nature. 2012 Sep 6;489(7414):109-13. doi: 10.1038/nature11279.

[175] J. V. Falvo, A. V. Tsytsykova, and A. E. Goldfeld. Transcriptional control of the tnf gene. *Curr Dir Autoimmun*, 11:27–60, 2010. Falvo, James V Tsytsykova, Alla V Goldfeld, Anne E R01 GM 076685/GM/NIGMS NIH HHS/ Switzerland Curr Dir Autoimmun. 2010;11:27-60. doi: 10.1159/000289196. Epub 2010 Feb 18.

[176] G. E. Nedwin, S. L. Naylor, A. Y. Sakaguchi, D. Smith, J. Jarrett-Nedwin, D. Pennica, D. V. Goeddel, and P. W. Gray. Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. *Nucleic Acids Res*, 13(17):6361–73, 1985. Nedwin, G E Naylor, S L Sakaguchi, A Y Smith, D Jarrett-Nedwin, J Pennica, D Goeddel, D V Gray, P W ENGLAND Nucleic Acids Res. 1985 Sep 11;13(17):6361-73.

[177] A. V. Tsytsykova, R. Rajsbaum, J. V. Falvo, F. Ligeiro, S. R. Neely, and A. E. Goldfeld. Activation-dependent intrachromosomal interactions formed by the tnf gene promoter and two distal enhancers. *Proc Natl Acad Sci U S A*, 104(43):16850–5, 2007. Tsytsykova, Alla V Rajsbaum, Ricardo Falvo, James V Ligeiro, Filipa Neely, Simon R Goldfeld, Anne E R01 GM 076685/GM/NIGMS NIH HHS/United States Research Support, N.I.H., Extramural United States Proceedings of the National Academy of Sciences of the United States of America Proc Natl Acad Sci U S A. 2007 Oct 23;104(43):16850-5. Epub 2007 Oct 16.

[178] J. M. Taylor, K. Wicks, C. Vandiedonck, and J. C. Knight. Chromatin profiling across the human tumour necrosis factor gene locus reveals a complex, cell type-specific landscape with novel regulatory elements. *Nucleic Acids Res*, 36(15):4845–62, 2008. Taylor, Jennifer M Wicks, Kate Vandiedonck, Claire Knight, Julian C 074318/Z/04/Z/Wellcome Trust/United Kingdom Research Support, Non-U.S. Gov't England Nucleic acids research Nucleic Acids Res. 2008 Sep;36(15):4845-62. Epub 2008 Jul 24.

[179] R. Barthel and A. E. Goldfeld. T cell-specific expression of the human tnf-alpha gene involves a functional and highly conserved chromatin signature in intron 3. *J Immunol*, 171(7):3612–9, 2003. Barthel, Robert Goldfeld, Anne E GM56492/GM/NIGMS NIH HHS/ HL59838/HL/NHLBI NIH HHS/ Baltimore, Md. : 1950 J Immunol. 2003 Oct 1;171(7):3612-9.

[180] S. Biglione, A. V. Tsytsykova, and A. E. Goldfeld. Monocyte-specific accessibility of a matrix attachment region in the tumor necrosis factor locus. *J Biol Chem*. Journal article The Journal of biological chemistry J Biol Chem. 2011 Oct 25.

[181] C. F. Ware, P. D. Crowe, M. H. Grayson, M. J. Androlewicz, and J. L. Browning. Expression of surface lymphotoxin and tumor necrosis factor on activated t, b, and natural killer cells. *J Immunol*, 149(12):3881–8, 1992. Ware, C F Crowe, P D Grayson, M H Androlewicz, M J Browning, J L DK-07310/DK/NIDDK NIH HHS/ Baltimore, Md. : 1950 J Immunol. 1992 Dec 15;149(12):3881-8.

[182] I. Millet and N. H. Ruddle. Differential regulation of lymphotoxin (lt), lymphotoxin-beta (lt-beta), and tnf-alpha in murine t cell clones activated through the tcr. *J Immunol*, 152(9):4336–46, 1994. Millet, I Ruddle, N H CA 16885/CA/NCI NIH HHS/ Baltimore, Md. : 1950 J Immunol. 1994 May 1;152(9):4336-46.

[183] J. L. Browning, I. D. Sizing, P. Lawton, P. R. Bourdon, P. D. Rennert, G. R. Majeau, C. M. Ambrose, C. Hession, K. Miatkowski, D. A. Griffiths, A. Ngam-ek, W. Meier, C. D. Benjamin, and P. S. Hochman. Characterization of lymphotoxin-alpha beta complexes on the surface of mouse lymphocytes. *J Immunol*, 159(7):3288–98, 1997. Browning, J L Sizing, I D Lawton, P Bourdon, P R Rennert, P D Majeau, G R Ambrose, C M Hession, C Miatkowski, K Griffiths, D A Ngam-ek, A Meier, W Benjamin, C D Hochman, P S Baltimore, Md. : 1950 J Immunol. 1997 Oct 1;159(7):3288-98.

[184] B. D. Williamson, E. A. Carswell, B. Y. Rubin, J. S. Prendergast, and L. J. Old. Human tumor necrosis factor produced by human b-cell lines: synergistic cytotoxic interaction with human interferon. *Proc Natl Acad Sci U S A*, 80(17):5397–401, 1983. Williamson, B D Carswell, E A Rubin, B Y Prendergast, J S Old, L J AI-17920/AI/NIAID NIH HHS/ CA-08748/CA/NCI NIH HHS/ Proc Natl Acad Sci U S A. 1983 Sep;80(17):5397-401.

[185] E. A. Havell and B. J. Rogerson. Endotoxin-induced tumor necrosis factor alpha synthesis in murine embryo fibroblasts. *Infect Immun*, 61(5):1630–5, 1993. Havell, E A Rogerson, B J AI-23544/AI/NIAID NIH HHS/ Infect Immun. 1993 May;61(5):1630-5.

[186] Y. L. Niu, Z. Guo, and R. H. Zhou. Up-regulation of tnf-alpha in neurons of dorsal root ganglia and spinal cord during coronary artery occlusion in rats. *Cytokine*, 47(1):23–9, 2009. Niu, Yan-Lan Guo, Zheng Zhou, Ri-Hua Cytokine. 2009 Jul;47(1):23-9. doi: 10.1016/j.cyto.2009.04.003. Epub 2009 Apr 26.

[187] J. V. Falvo, A. M. Uglialoro, B. M. Brinkman, M. Merika, B. S. Parekh, E. Y. Tsai, H. C. King, A. D. Morielli, E. G. Peralta, T. Maniatis, D. Thanos, and A. E. Goldfeld. Stimulus-specific assembly of enhancer complexes on the tumor necrosis factor alpha gene promoter. *Mol Cell Biol*, 20(6):2239–47, 2000. Falvo, J V Uglialoro, A M Brinkman, B M Merika, M Parekh, B S Tsai, E Y King, H C Morielli, A D Peralta, E G Maniatis, T Thanos, D Goldfeld, A E AI20642/AI/NIAID NIH HHS/United States GM54605/GM/NIGMS NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states Molecular and cellular biology Mol Cell Biol. 2000 Mar;20(6):2239-47.

[188] J. V. Falvo, B. M. Brinkman, A. V. Tsytsykova, E. Y. Tsai, T. P. Yao, A. L. Kung, and A. E. Goldfeld. A stimulus-specific role for creb-binding protein (cbp) in t cell receptor-activated tumor necrosis factor alpha gene expression. *Proc Natl Acad Sci U S A*, 97(8):3925–9, 2000. Falvo, J V Brinkman, B M Tsytsykova, A V Tsai, E Y Yao, T P Kung, A L Goldfeld, A E GM-56492/GM/NIGMS NIH HHS/ Proc Natl Acad Sci U S A. 2000 Apr 11;97(8):3925-9.

[189] E. Y. Tsai, J. V. Falvo, A. V. Tsytsykova, A. K. Barczak, A. M. Reimold, L. H. Glimcher, M. J. Fenton, D. C. Gordon, I. F. Dunn, and A. E. Goldfeld. A lipopolysaccharide-specific enhancer complex involving ets, elk-1, sp1, and creb binding protein and p300 is recruited to the tumor necrosis factor alpha promoter in vivo. *Mol Cell Biol*, 20(16):6084–94, 2000. Tsai, E Y Falvo, J V Tsytsykova, A V Barczak, A K Reimold, A M Glimcher, L H Fenton, M J Gordon, D C Dunn, I F Goldfeld, A E AI-32412/AI/NIAID NIH HHS/United States GM-56492/GM/NIGMS NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states Molecular and cellular biology Mol Cell Biol. 2000 Aug;20(16):6084-94.

[190] D. Panne. The enhanceosome. *Curr Opin Struct Biol*, 18(2):236–42, 2008. Panne, Daniel England Curr Opin Struct Biol. 2008 Apr;18(2):236-42. doi: 10.1016/j.sbi.2007.12.002. Epub 2008 Feb 21.

[191] D. Thanos and T. Maniatis. Virus induction of human ifn beta gene expression requires the assembly of an enhanceosome. *Cell*, 83(7):1091–100, 1995. Thanos, D Maniatis, T AI20642/AI/NIAID NIH HHS/ Cell. 1995 Dec 29;83(7):1091-100.

[192] J. V. Falvo, D. Thanos, and T. Maniatis. Reversal of intrinsic dna bends in the ifn beta gene enhancer by transcription factors and the architectural protein hmg i(y). *Cell*, 83(7):1101–11, 1995. Falvo, J V Thanos, D Maniatis, T AI 20642/AI/NIAID NIH HHS/ Cell. 1995 Dec 29;83(7):1101-11.

[193] M. Merika, A. J. Williams, G. Chen, T. Collins, and D. Thanos. Recruitment of cbp/p300 by the ifn beta enhanceosome is required for synergistic activation of transcription. *Mol Cell*, 1(2):277–87, 1998. Merika, M Williams, A J Chen, G Collins, T Thanos, D 1RO1GM54605/GM/NIGMS NIH HHS/ HL35716/HL/NHLBI NIH HHS/ HL45462/HL/NHLBI NIH HHS/ Mol Cell. 1998 Jan;1(2):277-87.

[194] A. E. Goldfeld, P. G. McCaffrey, J. L. Strominger, and A. Rao. Identification of a novel cyclosporin-sensitive element in the human tumor necrosis factor alpha gene promoter. *J Exp Med*, 178(4):1365–79, 1993. Goldfeld, A E McCaffrey, P G Strominger, J L Rao, A AI-00683/AI/NIAID NIH HHS/United States CA-42471/CA/NCI NIH HHS/United States CA-58735/CA/NCI NIH HHS/United States etc. Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states The Journal of experimental medicine J Exp Med. 1993 Oct 1;178(4):1365-79.

[195] R. Barthel, A. V. Tsytsykova, A. K. Barczak, E. Y. Tsai, C. C. Dascher, M. B. Brenner, and A. E. Goldfeld. Regulation of tumor necrosis factor alpha gene expression by mycobacteria involves the assembly of a unique enhanceosome dependent on the coactivator proteins cbp/p300. *Mol Cell Biol*, 23(2):526–33, 2003. Barthel, Robert Tsytsykova, Alla V Barczak, Amy K Tsai, Eunice Y Dascher, Christopher C Brenner, Michael B Goldfeld, Anne E GM56492/GM/NIGMS NIH HHS/United States HL59838/HL/NHLBI NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United States Molecular and cellular biology Mol Cell Biol. 2003 Jan;23(2):526-33.

[196] A. E. Goldfeld, C. Doyle, and T. Maniatis. Human tumor necrosis factor alpha gene regulation by virus and lipopolysaccharide. *Proc Natl Acad Sci U S A*, 87(24):9769–73, 1990. Goldfeld, A E Doyle, C Maniatis, T AI20642/AI/NIAID NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states Proceedings of the National Academy of Sciences of the United States of America Proc Natl Acad Sci U S A. 1990 Dec;87(24):9769-73.

[197] A. Baena, A. R. Mootnick, J. V. Falvo, A. V. Tsytskova, F. Ligeiro, O. M. Diop, C. Brieva, P. Gagneux, S. J. O'Brien, O. A. Ryder, and A. E. Goldfeld. Primate tnf promoters reveal markers of phylogeny and evolution of innate immunity. *PLoS One*, 2(7):e621, 2007. Baena, Andres Mootnick, Alan R Falvo, James V Tsytskova, Alla V Ligeiro, Filipa Diop, Ousmane M Brieva, Claudia Gagneux, Pascal O'Brien, Stephen J Ryder, Oliver A Goldfeld, Anne E GM076685/GM/NIGMS NIH HHS/ HL59838/HL/NHLBI NIH HHS/ PLoS One. 2007 Jul 18;2(7):e621.

[198] A. V. Tsytsykova and A. E. Goldfeld. Inducer-specific enhanceosome formation controls tumor necrosis factor alpha gene expression in t lymphocytes. *Mol Cell Biol*, 22(8):2620–31, 2002. Tsytsykova, Alla V Goldfeld, Anne E GM-56492/GM/NIGMS NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United States Molecular and cellular biology Mol Cell Biol. 2002 Apr;22(8):2620-31.

[199] D. V. Kuprash, I. A. Udalova, R. L. Turetskaya, D. Kwiatkowski, N. R. Rice, and S. A. Nedospasov. Similarities and differences between human and murine tnf promoters in their response to lipopolysaccharide. *J Immunol*, 162(7):4045–52, 1999. Kuprash, D V Udalova, I A Turetskaya, R L Kwiatkowski, D Rice, N R Nedospasov, S A N01-CO-56000/CO/NCI NIH HHS/ Baltimore, Md. : 1950 J Immunol. 1999 Apr 1;162(7):4045-52.

[200] I. A. Udalova, J. C. Knight, V. Vidal, S. A. Nedospasov, and D. Kwiatkowski. Complex nf-kappab interactions at the distal tumor necrosis factor promoter region in human monocytes. *J Biol Chem*, 273(33):21178–86, 1998. Udalova, I A Knight, J C Vidal, V Nedospasov, S A Kwiatkowski, D J Biol Chem. 1998 Aug 14;273(33):21178-86.

[201] A. E. Goldfeld, E. Tsai, R. Kincaid, P. J. Belshaw, S. L. Schrieber, J. L. Strominger, and A. Rao. Calcineurin mediates human tumor necrosis factor alpha gene induction in stimulated t and b cells. *J Exp Med*, 180(2):763–8, 1994. Goldfeld, A E Tsai, E Kincaid, R Belshaw, P J Schrieber, S L Strominger, J L Rao, A CA-42471/CA/NCI NIH HHS/ CA-58735/CA/NCI NIH HHS/ GM-38627/GM/NIGMS NIH HHS/ etc. J Exp Med. 1994 Aug 1;180(2):763-8.

[202] P. G. McCaffrey, A. E. Goldfeld, and A. Rao. The role of nfatp in cyclosporin a-sensitive tumor necrosis factor-alpha gene transcription. *J Biol Chem*, 269(48):30445–50, 1994. McCaffrey, P G Goldfeld, A E Rao, A CA-42471/CA/NCI NIH HHS/United States CA-58735/CA/NCI NIH HHS/United States GM-46227/GM/NIGMS NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states The Journal of biological chemistry J Biol Chem. 1994 Dec 2;269(48):30445-50.

[203] E. Y. Tsai, J. Jain, P. A. Pesavento, A. Rao, and A. E. Goldfeld. Tumor necrosis factor alpha gene regulation in activated t cells involves atf-2/jun and nfatp. *Mol Cell Biol*, 16(2):459–67, 1996. Tsai, E Y Jain, J Pesavento, P A Rao, A Goldfeld, A E CA-58735/CA/NCI NIH HHS/United States CA42471/CA/NCI NIH HHS/United States GM46227/GM/NIGMS NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states Molecular and cellular biology Mol Cell Biol. 1996 Feb;16(2):459-67.

[204] E. Y. Tsai, J. Yie, D. Thanos, and A. E. Goldfeld. Cell-type-specific regulation of the human tumor necrosis factor alpha gene in b cells and t cells by nfatp and atf-2/jun. *Mol Cell Biol*, 16(10):5232–44, 1996. Tsai, E Y Yie, J Thanos, D Goldfeld, A E CA42471/CA/NCI NIH HHS/United States Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, P.H.S. United states Molecular and cellular biology Mol Cell Biol. 1996 Oct;16(10):5232-44.

[205] J. H. Esensten, A. V. Tsytsykova, C. Lopez-Rodriguez, F. A. Ligeiro, A. Rao, and A. E. Goldfeld. Nfat5 binds to the tnf promoter distinctly from nfatp, c, 3 and 4, and activates tnf transcription during hypertonic stress alone. *Nucleic Acids Res*, 33(12):3845–54, 2005. Esensten, Jonathan H Tsytsykova, Alla V Lopez-Rodriguez, Cristina Ligeiro, Filipa A Rao, Anjana Goldfeld, Anne E CA42471/CA/NCI NIH HHS/ GM056492/GM/NIGMS NIH HHS/ England Nucleic Acids Res. 2005 Jul 12;33(12):3845-54. Print 2005.

[206] J. V. Falvo, C. H. Lin, A. V. Tsytsykova, P. K. Hwang, D. Thanos, A. E. Goldfeld, and T. Maniatis. A dimer-specific function of the transcription factor nfatp. *Proc Natl Acad Sci U S A*, 105(50):19637–42, 2008. Falvo, James V Lin, Charles H Tsytsykova, Alla V Hwang, Peter K Thanos, Dimitris Goldfeld, Anne E Maniatis, Tom AI-20642/AI/NIAID NIH HHS/ GM-54605/GM/NIGMS NIH HHS/ GM-56492/GM/NIGMS NIH HHS/ P50 CA89520/CA/NCI NIH HHS/ Proc Natl Acad

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Sci U S A. 2008 Dec 16;105(50):19637-42. doi: 10.1073/pnas.0810648105. Epub 2008 Dec 5.

[207] A. V. Tsytsykova, J. V. Falvo, M. Schmidt-Supprian, G. Courtois, D. Thanos, and A. E. Goldfeld. Post-induction, stimulus-specific regulation of tumor necrosis factor mrna expression. *J Biol Chem*, 282(16):11629–38, 2007. Tsytsykova, Alla V Falvo, James V Schmidt-Supprian, Marc Courtois, Gilles Thanos, Dimitris Goldfeld, Anne E GM076685/GM/NIGMS NIH HHS/ J Biol Chem. 2007 Apr 20;282(16):11629-38. Epub 2007 Feb 15.

[208] L. H. Kasper, T. Fukuyama, M. A. Biesen, F. Boussouar, C. Tong, A. de Pauw, P. J. Murray, J. M. van Deursen, and P. K. Brindle. Conditional knockout mice reveal distinct functions for the global transcriptional coactivators cbp and p300 in t-cell development. *Mol Cell Biol*, 26(3):789–809, 2006. Kasper, Lawryn H Fukuyama, Tomofusa Biesen, Michelle A Boussouar, Faycal Tong, Caili de Pauw, Antoine Murray, Peter J van Deursen, Jan M A Brindle, Paul K P30 CA021765/CA/NCI NIH HHS/ R01 CA076385/CA/NCI NIH HHS/ Mol Cell Biol. 2006 Feb;26(3):789-809.

[209] A. Visel, J. Bristow, and L. A. Pennacchio. Enhancer identification through comparative genomics. *Semin Cell Dev Biol*, 18(1):140–52, 2007. Visel, Axel Bristow, James Pennacchio, Len A HG003988/HG/NHGRI NIH HHS/ HL066681/HL/NHLBI NIH HHS/ R01 HG003988/HG/NHGRI NIH HHS/ U01 HL066681-05/HL/NHLBI NIH HHS/ England Semin Cell Dev Biol. 2007 Feb;18(1):140-52. Epub 2007 Jan 5.

[210] J. Y. Leung, F. E. McKenzie, A. M. Uglialoro, P. O. Flores-Villanueva, B. C. Sorkin, E. J. Yunis, D. L. Hartl, and A. E. Goldfeld. Identification of phylogenetic footprints in primate tumor necrosis factor-alpha promoters. *Proc Natl Acad Sci U S A*, 97(12):6614–8, 2000. Leung, J Y McKenzie, F E Uglialoro, A M Flores-Villanueva, P O Sorkin, B C Yunis, E J Hartl, D L Goldfeld, A E GM56492/GM/NIGMS NIH HHS/ HG-02150/HG/NHGRI NIH HHS/ HL-59838/HL/NHLBI NIH HHS/ Z99 TW999999/TW/FIC NIH HHS/ Proc Natl Acad Sci U S A. 2000 Jun 6;97(12):6614-8.

[211] G. Trinchieri. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu Rev Immunol*, 13:251–76, 1995. Trinchieri, G Annu Rev Immunol. 1995;13:251-76.

[212] M. P. Hayes, S. L. Freeman, and R. P. Donnelly. Ifn-gamma priming of monocytes enhances lps-induced tnf production by augmenting both transcription and mrna stability. *Cytokine*, 7(5):427–35, 1995. Hayes, M P Freeman, S L Donnelly, R P United states Cytokine Cytokine. 1995 Jul;7(5):427-35.

[213] Y. Qiao, E. G. Giannopoulou, C. H. Chan, S. H. Park, S. Gong, J. Chen, X. Hu, O. Elemento, and L. B. Ivashkiv. Synergistic activation of inflammatory cytokine genes by interferon-gamma-induced chromatin remodeling and toll-like receptor signaling. *Immunity*, 2013. Qiao, Yu Giannopoulou, Eugenia G Chan,

Chun Hin Park, Sung-Ho Gong, Shiaoching Chen, Janice Hu, Xiaoyu Elemento, Olivier Ivashkiv, Lionel B Immunity. 2013 Sep 4. pii: S1074-7613(13)00337-3. doi: 10.1016/j.immuni.2013.08.009.

[214] S. Garrett, K. Dietzmann-Maurer, L. Song, and K. E. Sullivan. Polarization of primary human monocytes by ifn-gamma induces chromatin changes and recruits rna pol ii to the tnf-alpha promoter. *J Immunol*, 180(8):5257–66, 2008. Garrett, Stacey Dietzmann-Maurer, Kelly Song, Li Sullivan, Kathleen E R01 AI051323/AI/NIAID NIH HHS/ R01 AI44127/AI/NIAID NIH HHS/ Baltimore, Md. : 1950 J Immunol. 2008 Apr 15;180(8):5257-66.

[215] R. Pine, T. Decker, D. S. Kessler, D. E. Levy, and Jr. Darnell, J. E. Purification and cloning of interferon-stimulated gene factor 2 (isgf2): Isgf2 (irf-1) can bind to the promoters of both beta interferon- and interferon-stimulated genes but is not a primary transcriptional activator of either. *Mol Cell Biol*, 10(6):2448–57, 1990. Pine, R Decker, T Kessler, D S Levy, D E Darnell, J E Jr AI07233-15/AI/NIAID NIH HHS/ GM09820/GM/NIGMS NIH HHS/ Mol Cell Biol. 1990 Jun;10(6):2448-57.

[216] H. Harada, M. Kitagawa, N. Tanaka, H. Yamamoto, K. Harada, M. Ishihara, and T. Taniguchi. Anti-oncogenic and oncogenic potentials of interferon regulatory factors-1 and -2. *Science*, 259(5097):971–4, 1993. Harada, H Kitagawa, M Tanaka, N Yamamoto, H Harada, K Ishihara, M Taniguchi, T New York, N.Y. Science. 1993 Feb 12;259(5097):971-4.

[217] T. Fujita, L. F. Reis, N. Watanabe, Y. Kimura, T. Taniguchi, and J. Vilcek. Induction of the transcription factor irf-1 and interferon-beta mrnas by cytokines and activators of second-messenger pathways. *Proc Natl Acad Sci U S A*, 86(24):9936–40, 1989. Fujita, T Reis, L F Watanabe, N Kimura, Y Taniguchi, T Vilcek, J AI12948/AI/NIAID NIH HHS/ CA47301/CA/NCI NIH HHS/ Proc Natl Acad Sci U S A. 1989 Dec;86(24):9936-40.

[218] M. P. Creyghton, A. W. Cheng, G. G. Welstead, T. Kooistra, B. W. Carey, E. J. Steine, J. Hanna, M. A. Lodato, G. M. Frampton, P. A. Sharp, L. A. Boyer, R. A. Young, and R. Jaenisch. Histone h3k27ac separates active from poised enhancers and predicts developmental state. *Proc Natl Acad Sci U S A*, 107(50):21931–6, 2010. Creyghton, Menno P Cheng, Albert W Welstead, G Grant Kooistra, Tristan Carey, Bryce W Steine, Eveline J Hanna, Jacob Lodato, Michael A Frampton, Garrett M Sharp, Phillip A Boyer, Laurie A Young, Richard A Jaenisch, Rudolf 5-R37-CA084198/CA/NCI NIH HHS/ 5-RO1-CA87869/CA/NCI NIH HHS/ 5-RO1-HDO45022/PHS HHS/ HG002668/HG/NHGRI NIH HHS/ P01-CA42063/CA/NCI NIH HHS/ P30-CA14051/CA/NCI NIH HHS/ R01 CA084198-12/CA/NCI NIH HHS/ R37 HD045022-09/HD/NICHD NIH HHS/ Proc Natl Acad Sci U S A. 2010 Dec 14;107(50):21931-6. doi: 10.1073/pnas.1016071107. Epub 2010 Nov 24.

[219] A. Rada-Iglesias, R. Bajpai, T. Swigut, S. A. Brugmann, R. A. Flynn, and J. Wysocka. A unique chromatin signature uncovers early developmental enhancers

in humans. *Nature*, 470(7333):279–83, 2011. Rada-Iglesias, Alvaro Bajpai, Ruchi Swigut, Tomek Brugmann, Samantha A Flynn, Ryan A Wysocka, Joanna England Nature. 2011 Feb 10;470(7333):279-83. doi: 10.1038/nature09692. Epub 2010 Dec 15.

[220] G. E. Zentner, P. J. Tesar, and P. C. Scacheri. Epigenetic signatures distinguish multiple classes of enhancers with distinct cellular functions. *Genome Res*, 21(8):1273–83, 2011. Zentner, Gabriel E Tesar, Paul J Scacheri, Peter C 5R01HG004722/HG/NHGRI NIH HHS/ 5T32GM008614/GM/NIGMS NIH HHS/ 5T32GM008615/GM/NIGMS NIH HHS/ R01HD056369/HD/NICHD NIH HHS/ R33 MH087877/MH/NIMH NIH HHS/ Genome Res. 2011 Aug;21(8):1273-83. doi: 10.1101/gr.122382.111. Epub 2011 Jun 1.

[221] G. van Haaften, G. L. Dalgliesh, H. Davies, L. Chen, G. Bignell, C. Greenman, S. Edkins, C. Hardy, S. O'Meara, J. Teague, A. Butler, J. Hinton, C. Latimer, J. Andrews, S. Barthorpe, D. Beare, G. Buck, P. J. Campbell, J. Cole, S. Forbes, M. Jia, D. Jones, C. Y. Kok, C. Leroy, M. L. Lin, D. J. McBride, M. Maddison, S. Maquire, K. McLay, A. Menzies, T. Mironenko, L. Mulderrig, L. Mudie, E. Pleasance, R. Shepherd, R. Smith, L. Stebbings, P. Stephens, G. Tang, P. S. Tarpey, R. Turner, K. Turrell, J. Varian, S. West, S. Widaa, P. Wray, V. P. Collins, K. Ichimura, S. Law, J. Wong, S. T. Yuen, S. Y. Leung, G. Tonon, R. A. DePinho, Y. T. Tai, K. C. Anderson, R. J. Kahnoski, A. Massie, S. K. Khoo, B. T. Teh, M. R. Stratton, and P. A. Futreal. Somatic mutations of the histone h3k27 demethylase gene utx in human cancer. *Nat Genet*, 41(5):521–3, 2009. van Haaften, Gijs Dalgliesh, Gillian L Davies, Helen Chen, Lina Bignell, Graham Greenman, Chris Edkins, Sarah Hardy, Claire O'Meara, Sarah Teague, Jon Butler, Adam Hinton, Jonathan Latimer, Calli Andrews, Jenny Barthorpe, Syd Beare, Dave Buck, Gemma Campbell, Peter J Cole, Jennifer Forbes, Simon Jia, Mingming Jones, David Kok, Chai Yin Leroy, Catherine Lin, Meng-Lay McBride, David J Maddison, Mark Maquire, Simon McLay, Kirsten Menzies, Andrew Mironenko, Tatiana Mulderrig, Lee Mudie, Laura Pleasance, Erin Shepherd, Rebecca Smith, Raffaella Stebbings, Lucy Stephens, Philip Tang, Gurpreet Tarpey, Patrick S Turner, Rachel Turrell, Kelly Varian, Jennifer West, Sofie Widaa, Sara Wray, Paul Collins, V Peter Ichimura, Koichi Law, Simon Wong, John Yuen, Siu Tsan Leung, Suet Yi Tonon, Giovanni DePinho, Ronald A Tai, Yu-Tzu Anderson, Kenneth C Kahnoski, Richard J Massie, Aaron Khoo, Sok Kean Teh, Bin Tean Stratton, Michael R Futreal, P Andrew 077012/Wellcome Trust/United Kingdom Wellcome Trust/United Kingdom Nat Genet. 2009 May;41(5):521-3. doi: 10.1038/ng.349. Epub 2009 Mar 29.

[222] G. Marsili, A. L. Remoli, M. Sgarbanti, and A. Battistini. Role of acetylases and deacetylase inhibitors in irf-1-mediated hiv-1 long terminal repeat transcription. *Ann N Y Acad Sci*, 1030:636–43, 2004. Marsili, Giulia Remoli, Anna Lisa Sgarbanti, Marco Battistini, Angela Ann N Y Acad Sci. 2004 Dec;1030:636-43.

[223] E. Calo and J. Wysocka. Modification of enhancer chromatin: what, how, and

why? *Mol Cell*, 49(5):825–37, 2013. Calo, Eliezer Wysocka, Joanna GM 095555-01/GM/NIGMS NIH HHS/ R01 GM095555/GM/NIGMS NIH HHS/ Mol Cell. 2013 Mar 7;49(5):825-37. doi: 10.1016/j.molcel.2013.01.038.

[224] F. Tie, R. Banerjee, P. A. Conrad, P. C. Scacheri, and P. J. Harte. Histone demethylase utx and chromatin remodeler brm bind directly to cbp and modulate acetylation of histone h3 lysine 27. *Mol Cell Biol*, 32(12):2323–34, 2012. Tie, Feng Banerjee, Rakhee Conrad, Patricia A Scacheri, Peter C Harte, Peter J R01GM084947/GM/NIGMS NIH HHS/ R01GM39255/GM/NIGMS NIH HHS/ R01HD056369/HD/NICHD NIH HHS/ R01HG004722/HG/NHGRI NIH HHS/ RR-017980-01/RR/NCRR NIH HHS/ RR-021228-01/RR/NCRR NIH HHS/ RR-024536-01/RR/NCRR NIH HHS/ Mol Cell Biol. 2012 Jun;32(12):2323-34. doi: 10.1128/MCB.06392-11. Epub 2012 Apr 9.

[225] G. Natoli and J. C. Andrau. Noncoding transcription at enhancers: general principles and functional models. *Annu Rev Genet*, 46:1–19, 2012. Natoli, Gioacchino Andrau, Jean-Christophe Annu Rev Genet. 2012;46:1-19. doi: 10.1146/annurev-genet-110711-155459. Epub 2012 Aug 16.

[226] D. C. Angus and T. van der Poll. Severe sepsis and septic shock. *N Engl J Med*, 369(9):840–51, 2013. Angus, Derek C van der Poll, Tom N Engl J Med. 2013 Aug 29;369(9):840-51. doi: 10.1056/NEJMra1208623.

[227] J. M. Cavaillon and D. Annane. Compartmentalization of the inflammatory response in sepsis and sirs. *J Endotoxin Res*, 12(3):151–70, 2006. Cavaillon, Jean-Marc Annane, Djillali England J Endotoxin Res. 2006;12(3):151-70.

[228] C. W. Dawson, A. M. Ledgerwood, J. C. Rosenberg, and C. E. Lucas. Anergy and altered lymphocyte function in the injured patient. *Am Surg*, 48(8):397–401, 1982. Dawson, C W Ledgerwood, A M Rosenberg, J C Lucas, C E Am Surg. 1982 Aug;48(8):397-401.

[229] M. K. Angele and E. Faist. Clinical review: immunodepression in the surgical patient and increased susceptibility to infection. *Crit Care*, 6(4):298–305, 2002. Angele, Martin K Faist, Eugen England London, England Crit Care. 2002 Aug;6(4):298-305. Epub 2002 May 24.

[230] H. D. Volk, P. Reinke, and W. D. Docke. Clinical aspects: from systemic inflammation to 'immunoparalysis'. *Chem Immunol*, 74:162–77, 2000. Volk, H D Reinke, P Docke, W D SWITZERLAND Chem Immunol. 2000;74:162-77.

[231] M. Adib-Conquy and J. M. Cavaillon. Compensatory anti-inflammatory response syndrome. *Thromb Haemost*, 101(1):36–47, 2009. Adib-Conquy, Minou Cavaillon, Jean-Marc Germany Thromb Haemost. 2009 Jan;101(1):36-47.

[232] C. Munoz, J. Carlet, C. Fitting, B. Misset, J. P. Bleriot, and J. M. Cavaillon. Dysregulation of in vitro cytokine production by monocytes during sepsis. *J Clin Invest*, 88(5):1747–54, 1991. Munoz, C Carlet, J Fitting, C Misset, B Bleriot, J P Cavaillon, J M J Clin Invest. 1991 Nov;88(5):1747-54.

[233] C. Munoz, B. Misset, C. Fitting, J. P. Bleriot, J. Carlet, and J. M. Cavaillon. Dissociation between plasma and monocyte-associated cytokines during sepsis. *Eur J Immunol*, 21(9):2177–84, 1991. Munoz, C Misset, B Fitting, C Bleriot, J P Carlet, J Cavaillon, J M GERMANY Eur J Immunol. 1991 Sep;21(9):2177-84.

[234] A. Draisma, P. Pickkers, M. P. Bouw, and J. G. van der Hoeven. Development of endotoxin tolerance in humans in vivo. *Crit Care Med*, 37(4):1261–7, 2009. Draisma, Annelies Pickkers, Peter Bouw, Martijn P W J M van der Hoeven, Johannes G Crit Care Med. 2009 Apr;37(4):1261-7. doi: 10.1097/CCM.0b013e31819c3c67.

[235] G. Monneret, F. Venet, A. Pachot, and A. Lepape. Monitoring immune dysfunctions in the septic patient: a new skin for the old ceremony. *Mol Med*, 14(1-2):64–78, 2008. Monneret, Guillaume Venet, Fabienne Pachot, Alexandre Lepape, Alain Cambridge, Mass. Mol Med. 2008 Jan-Feb;14(1-2):64-78.

[236] S. L. Foster, D. C. Hargreaves, and R. Medzhitov. Gene-specific control of inflammation by tlr-induced chromatin modifications. *Nature*, 447(7147):972–8, 2007. Foster, Simmie L Hargreaves, Diana C Medzhitov, Ruslan Research Support, N.I.H., Extramural Research Support, Non-U.S. Gov't Research Support, U.S. Gov't, Non-P.H.S. England Nature Nature. 2007 Jun 21;447(7147):972-8. Epub 2007 May 30.

[237] S. L. Foster and R. Medzhitov. Gene-specific control of the tlr-induced inflammatory response. *Clin Immunol*, 130(1):7–15, 2009. Foster, Simmie L Medzhitov, Ruslan Howard Hughes Medical Institute/United States Research Support, Non-U.S. Gov't Review United States Clinical immunology (Orlando, Fla.) Clin Immunol. 2009 Jan;130(1):7-15. Epub 2008 Oct 28.

[238] W. F. Carson, K. A. Cavassani, Y. Dou, and S. L. Kunkel. Epigenetic regulation of immune cell functions during post-septic immunosuppression. *Epigenetics*, 6(3):273–83, 2011. Carson, William F Cavassani, Karen A Dou, Yali Kunkel, Steven L HL089216/HL/NHLBI NIH HHS/ HL31237/HL/NHLBI NIH HHS/ R01 HL031237-27/HL/NHLBI NIH HHS/ Epigenetics. 2011 Mar;6(3):273-83. Epub 2011 Mar 1.

[239] J. Chen and L. B. Ivashkiv. Ifn-gamma abrogates endotoxin tolerance by facilitating toll-like receptor-induced chromatin remodeling. *Proc Natl Acad Sci U S A*, 107(45):19438–43, 2010. Chen, Janice Ivashkiv, Lionel B R01 AI046712-09/AI/NIAID NIH HHS/ R01 AI046712-10/AI/NIAID NIH HHS/ R01 AI046712-11/AI/NIAID NIH HHS/ R01 AI046712-12/AI/NIAID NIH HHS/ R01 AI046712-13/AI/NIAID NIH HHS/ R01 AR050401/AR/NIAMS NIH HHS/ R01 AR050401-05/AR/NIAMS NIH HHS/ R01 AR050401-06A2/AR/NIAMS NIH HHS/ R01

AR050401-07/AR/NIAMS NIH HHS/ Proc Natl Acad Sci U S A. 2010 Nov 9;107(45):19438-43. doi: 10.1073/pnas.1007816107. Epub 2010 Oct 25.

[240] M. El Gazzar, B. K. Yoza, X. Chen, J. Hu, G. A. Hawkins, and C. E. McCall. G9a and hp1 couple histone and dna methylation to tnfalpha transcription silencing during endotoxin tolerance. *J Biol Chem*, 283(47):32198–208, 2008. El Gazzar, Mohamed Yoza, Barbara K Chen, Xiaoping Hu, Jean Hawkins, Gregory A McCall, Charles E R01AI-065791/AI/NIAID NIH HHS/ R01AI-09169/AI/NIAID NIH HHS/ J Biol Chem. 2008 Nov 21;283(47):32198-208. doi: 10.1074/jbc.M803446200. Epub 2008 Sep 22.

[241] M. El Gazzar, B. K. Yoza, X. Chen, B. A. Garcia, N. L. Young, and C. E. McCall. Chromatin-specific remodeling by hmgb1 and linker histone h1 silences proinflammatory genes during endotoxin tolerance. *Mol Cell Biol*, 29(7):1959–71, 2009. El Gazzar, Mohamed Yoza, Barbara K Chen, Xiaoping Garcia, Benjamin A Young, Nicolas L McCall, Charles E MO-1RR 007122/RR/NCRR NIH HHS/ R01AI-065791/AI/NIAID NIH HHS/ R01AI-09169/AI/NIAID NIH HHS/ Mol Cell Biol. 2009 Apr;29(7):1959-71. doi: 10.1128/MCB.01862-08. Epub 2009 Jan 21.

[242] M. M. Worm, A. Tsytsykova, and R. S. Geha. Cd40 ligation and il-4 use different mechanisms of transcriptional activation of the human lymphotoxin alpha promoter in b cells. *Eur J Immunol*, 28(3):901–6, 1998. Worm, M M Tsytsykova, A Geha, R S AI3154/AI/NIAID NIH HHS/ GERMANY Eur J Immunol. 1998 Mar;28(3):901-6.