Title Histone acetylation and inflammatory mediators in inflammatory bowel disease

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HISTONE ACETYLATION AND INFLAMMATORY MEDIATORS IN INFLAMMATORY BOWEL DISEASE

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Abstract

During cell activation the tightly compacted DNA is made available to DNA-binding proteins allowing the induction of gene transcription. In the resting cell, DNA is packaged into chromatin whose fundamental subunit is the nucleosome, composed of an octamer of four core histones (H) 3, 4, 2A and 2B. During the induction of gene transcription, modification of histones, by acetylation, methylation etc., results in unwinding of the DNA, permitting access of large DNA-binding proteins, such as RNA polymerase II, and subsequent induction of gene transcription.

This investigation initially examined the effects of pro-inflammatory stimuli LPS and TNF-α on the production of IL-8 in a macrophage cell line (U937 cells) and in two T-cell lines (Jurkat and HUT-78 cells) as a marker of NF-κB-directed inflammatory gene expression. The ability of dexamethasone (Dex) and triamcinolone acetonide (TA) (synthetic glucocorticoid agonists) to suppress expression of the inflammatory cytokine IL-8 and to regulate histone acetylation was also investigated in these cells. LPS and TNF-α caused an increase in IL-8 expression, which was further enhanced by the histone deacetylases inhibitor trichostatin A (TSA), suggesting a role for histone acetylation in IL-8 production in these cells. Dex and TA, repressed LPS- and TNF-α -induced IL-8 expression in all three cell lines. This effect of both Dex and TA was attenuated by TSA in all cell lines studied, where the effect of TSA was greater in TA stimulated cells.

Stimulation of all cell lines with LPS and TNF-α induced acetylation of H4 lysine residues (K5, 8, 12 and 16), the highest elevation of which was for K8 and K12. Also demonstrate is a K5 and K16 specificity of acetylation by glucocorticoids, apparent in all cell lines studied. Dex and, to a greater extent, TA suppressed LPS- and TNFα-induced K8 and K12 acetylation. TSA attenuated the inhibitory effect of the glucocorticoids for all three cell lines. An increase in HDAC activity with GCs was observed and ChIP assay showed these events occur on the native IL-8 promoter via histone acetylation.

Further studies investigated whether there were any links between histone acetylation and the regulation of apoptosis. It was showed that TSA induced apoptosis in cells previously stimulated with the inducer of oxidative stress hydrogen peroxide (H_2O_2). Studies into the activation of caspase 3 in LPS- and TNF-α stimulated cells revealed that the combinatory effect of Dex or TA with TSA significantly enhanced expression of the marker in all three cell lines. In resting cells, Dex, and TA, in the presence of TSA downregulated caspase 3 expression. These findings support the notion that glucocorticoid actions on apoptosis is mediated, at least in part, through an action on histone acetylation.

Finally, histone acetylation was investigated *in vivo* in two rat models of inflammation and in human subjects with inflammatory bowel disease (IBD). The results showed an increase in histone H4 acetylation lysine specificity of acetylation on K8 and K12 in inflamed tissue and Peyer's patches in animal models and in IBD patients. Whereas H3 acetylation was not elevated to the same extent in tissue and was restricted to the mantle zone of Peyer's patches. In general, the present studies on histone acetylation and inflammation (in animal models and IBD patients) underlined the possibility of a general mechanism linking activation of the transcription factor NFκB with histone acetylation. The ultimate objective of this work is to aid in the understanding of the mechanisms of how deregulation of chromosome structure leads to progression of the disease state. This knowledge may aid in the development of new therapeutic approaches or improved glucocorticoids.

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Finally, last but certainly not least, I wish to thank George for always seeing the bright side of life and for reminding me about it, for his constant support and encouragement, but most of all for being the fourth giant in my life.

Again, thank you all for making possible this, my thesis.

If I have seen further, it is by sitting
in the shoulders of giants.

Sir Isaac Newton
1643-1727

Στους γονείς μου,

Κυριακή & Γιώργο

List of Publications

This is a list of publications resulting from the work described in this thesis.

1. Tsaprouni L.G., Ito K., Punchard N.A. and Adcock I.M. (2002). Triamcinolone acetonide and dexamethasome suppress TNF-α induced histone H4 acetylation on lysine residues 8 and 12 in mononuclear cells. *N.Y academy of science,* **973**: 481-483.

2. Kagoshima M., Wilcke T., Ito K., Tsaprouni L., Barnes P.J., Punchard N.A. and Adcock I.M. (2001) Glucocorticoid-mediated transrepression is regulated by histone acetylation and DNA methylation. *Eur. J. Pharmacol.,* **429(1-3):** 327-34.

3. Tsaprouni L., Adcock I.M., Evans S. and Punchard N.A. (2001) Histone Acetylation and Inflammation of the Bowel. *Inflammation Research*, **50(sup. 3):** S 168.

4. Tsaprouni L., Ito K., Adcock I.M., Evans S. and Punchard N.A. (2001) Increased levels of Histone Acetylation in the inflamed bowel. Poster Presentation in the *British Society of Immunology, Autumn Meeting 2001.*

5. Tsaprouni L., Ito K., Adcock I.M. and Punchard N.A. (2000) Stimulation of U937 Cells with LPS induces Histone Acetylation. *Immunology*, **101(sup. 1):** 250.

6. Tsaprouni L., Ito K., Adcock I.M., Paul C.and Punchard N. A.(2000) Comparison of Cytokine Production and apoptosis in U937 cells by stimulation with LPS, H_2O_2 and TNF-α. Poster Presentation in the *British Society for Cell Biology Autumn Meeting 2000.*

7. Tsaprouni L.G., Ito k., Adcock I.M. and Punchard N.A. Effect of steroids on macrophages and T-cells in inflammatory induced histone acetylation, *in preparation.*

8. Tsaprouni L.G., Ito k., Adcock I.M. and Punchard N.A. Acetylation of H4 lysine residues in inflammatory stimuli induced monocytes and lymphocytes, *submitted to Biochem. J.*

9. Tsaprouni L.G., Ito K., Adcock I.M. and Punchard N.A. Histone H4 lysine acetylation is upregulated during apoptosis in lymphocytes, *submitted to J. Biol. Chem.*

Contents

Chapter 2

Materials and Methods

Chapter 3

Role of histone acetylation in IL-8 expression

Chapter 4
Histone acetylation in an *in vitro* model of inflammation

Chapter 5

Role of histone 4 acetylation in the regulation of apoptosis

Chapter 6

Histone acetylation in *in vivo* inflammation induced animal models and human IBD Peyer's patches

Chapter 7
Conclusions and Further Work

References

Appendices

List of Tables

List of Figures

Abbreviations

α	Alpha
γ	Gamma
AP-1	activator protein-1
APS	ammonium persulfate
As_2O_3	Arsenic
BrDU	Bromodeoxyuridine
Br-dUTP	bromodeoxyuridine triphosphates
CBP	CREB-binding protein
c-Myc	myelocytomatosis viral oncogene homologue
CO_2	carbon dioxide
Dex	Dexamethasone
DMEM	Dulbeccos modified Eagles media
DNA	deoxyribonucleic acid
DSS	dextran sodium sulphate
DTT	Trans-1,2-dithiane-4,5-diol
E2F	E-box transcription factor 2
ECACC	European collection of cell cultures
EDTA	ethylenediamine tetra-acetic acid
ELISA	Enzyme linked immuno-sorbent assay
ERK	Extracellular-regulated Kinase
FCS	foetal calf serum
GM-CSF	granulocytes macrophage colony stimulating factor
GR	Glucocorticoid receptor
h	Hour
H1	histone 1
H2A	histone 2A
H2B	histone 2B
H_2O	Water
H_2O_2	Hydrogen peroxide
H_2SO_4	sulphuric acid
H3	histone 3
H4	histone 4
HAT	histone acetyltrasnsferase
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HDAC	histone deacetylases
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethane)sulfonic acid
IFN-γ	interferon-γ
IFN	Interferon
IL-1	interleukin 1
IL-8	interleukin-8
JNK	JUN-N-terminal kinase
KCl	potassium chloride
LPS	Lipopolysaccharide
Mad	Max dimeriser
MAPK	mitogen-activated protein kinase
Max	Myc activation factor X
MK-2	MAPK-activated protein kinase-2
MMTV	mouse mammary tumour virus
MPS	mononuclear phagocyte system
mRNA	messenger ribonucleic acid
MTD	Metronidazole
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NaCl	sodium chloride
NF-κB	nuclear factor kappa B

NH_3	amino terminus
NF-1	nuclear factor-1
NP-40	nonident P-40
p53	protein encoded by the p53 tumour suppresser gene
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween 20
PHA	phytohemagglutinin
PKA	protein kinase A
PKB	protein kinase B
PMA	phorbol 12-myristate 13-acetate
PMSF	phenyl methanesulfonyl fluoride
pt	paired T-test
RA	retinoic acid
RB	Retinoblastoma
RES	reticulo-endothelial system
RIP 160	retinoid interaction protein 160
RNA	ribonucleic acid
RPMI 1640	Rockwell Park Memorial Institute media 1640
RT-PCR	reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SFM	serum free media
SRE	steroid hormone response elements
STAT	signal transducer and activator of transcription
TA	Triamcinolone Acetonide
TEMED	1,2-Bis(dimethylamino)ethane
TGF-β	transforming growth factor-beta
TNBS	trinitrobenzene sulfonic acid
TNF-α	tumour necrosis factor alpha
TRAF	TNF receptor-associated factor
TRIS	(hydroxymethyl) aminomethane
TSA	trichostatin A
TUNEL	terminal deoxynuceotidyl transferase mediated dUTP nick end labeling
ut	unpaired T-test
v/v	volume for volume
w/v	weight for volume

Declaration

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Luton. It has not been submitted for any degree or examination in any other University.

Loukia G. Tsaprouni

19th December 2002

Chapter 1

Introduction

1.1 Inflammation

1.1.1 General aspects of inflammation

Inflammation is a complex response to localized injury, other trauma or infection, which involves various cells of the immune system and numerous inflammatory mediators that are released by cells of innate or acquired immunity during the inflammatory response. Such mediators are released by blood platelets, and leukocytes, including neutrophils, monocytes/macrophages, eosinophils, basophils and lymphocytes. These mediators trigger or enhance specific aspects of immunity. The hallmark signs of an inflammatory response, first described 2000 years ago, are swelling *(tumor)*, redness *(rubor)*, heat *(calor)* and pain *(dolor)* with the more recent additional sign of loss of function (Male *et al.,* 1996).

An inflammatory response can be acute or chronic. An acute inflammatory response involves both localized and systematic effects on the organism. The localized effect begins when tissue and endothelial damage, induce formation of mediators (enzymes) that lead to vasodilation and increased vascular permeability. Neutrophils and then monocytes migrate into the site (Male *et al.,* 1996). Two cytokines, IFN-γ and TNF-α, are central to the chronic inflammatory response, which often involves granuloma formation and tissue damage. Activation of tissue macrophages and degranulation of mast cells lead to release of numerous inflammatory mediators, some of which induce systematic acute phase effects. The systematic response induces fever, leucocytosis and production of corticosteroids. A chronic inflammatory response may accompany allergies, microbial infections, transplants, burns and autoimmune diseases (Kuby, 1997).

1.1.2 The phases of inflammation

The main purpose of inflammation is to bring fluid, proteins and cells from the blood into the damaged tissues. It should be remembered that the tissues are normally bathed in a watery fluid (extracellular lymph) that lacks most of the proteins and cells that are present in blood, since the majority of proteins are too large to cross the blood vessel endothelium. Thus there have to be mechanisms that allow cells and proteins to gain access to extravascular sites where and when they are needed if damage and infection has occurred (Male *et al.,* 1996).

The main features of the inflammatory response are, vasodilation, i.e. widening of the blood vessels to increase the blood flow to the infected area; increased vascular permeability, which

allows diffusible components to enter the site; cellular infiltration by chemotaxis, or the directed movement of inflammatory cells through the walls of blood vessels into the site of injury; changes in biosynthetic, metabolic, and catabolic profiles of many organs; and activation of cells of the immune system as well as of complex enzymatic systems of blood plasma. The degree to which these occur is normally proportional to the severity of the injury and the extent of infection (Male *et al.,* 1996).

Inflammation can be divided into several phases. The earliest, gross event of an inflammatory response is temporary vasoconstriction, i.e. narrowing of blood vessels caused by contraction of smooth muscle in the vessel walls, which can be seen as blanching (whitening) of the skin. This is followed by several phases that occur over minutes, hours and days later, outlined below (Sherman & Ward, 1998 and references therein)

The acute vascular response follows within seconds of the tissue injury and lasts for several minutes. This results from vasodilation and increased capillary permeability due to alterations in the vascular endothelium, which leads to increased blood flow (*hyperaemia*) that causes redness (*erythema*) and the entry of fluid into the tissues (*oedema*). This phase of the inflammatory response can be demonstrated by scratching the skin with a finger-nail. The "wheal and flare reaction" that occurs is composed of (a) initial blanching of the skin due to vasoconstriction, (b) the subsequent rapid appearance of a thin red line when the capillaries dilate; (c) a flush in the immediate area, generally within a minute, as the arterioles dilate; and (d) a wheal, or swollen area that appears within a few minutes as fluid leaks from the capillaries. It usually terminates within 30-60 minutes (Sherman & Ward, 1998).

If there has been sufficient damage to the tissues, or if infection has occurred, the acute cellular response takes place over the next few hours. The hallmark of this phase is the appearance of granulocytes, particularly neutrophils, in the tissues. These cells first attach themselves to the endothelial cells within the blood vessels (*margination*) and then cross into the surrounding tissue (*diapedesis*) (Sherman & Ward, 1998). During this phase erythrocytes may also leak into the tissues and a haemorrhage can occur (e.g. a blood blister). If the vessel is damaged, fibrinogen and fibronectin are deposited at the site of injury, platelets aggregate and become activated, and the red cells stack together in what are called "rouleau" to help stop bleeding and aid clot formation. The dead and dying cells contribute to pus formation (Sherman & Ward, 1998).

If the damage is sufficiently severe, a chronic cellular response may follow over the next few days. A characteristic of this phase of inflammation is the appearance of a mononuclear cell infiltrate composed of macrophages and lymphocytes. The macrophages are involved in microbial killing, in clearing up cellular and tissue debris, and they also seem to be very important in remodelling the tissues (Sherman & Ward, 1998).

Over the next few weeks, resolution may occur, meaning that the normal tissue architecture is restored. Blood clots are removed by fibrinolysis, and if it is not possible to return the tissue to its original form, *scarring* results from in-filling with fibroblasts, collagen, and new endothelial cells. Generally, by this time, any infection will have been overcome. However, if it has not been possible to destroy the infectious agents or to remove all of the products that have accumulated at the site completely, they are walled off from the surrounding tissue in *granulomatous tissue*. A granuloma is formed when macrophages and lymphocytes accumulate around material that has not been eliminated, together with epitheloid cells and giant cells (perhaps derived from macrophages) that appear later, to form a ball of cells (Sherman & Ward, 1998 and references therein).

In addition, a large number of more distant effects occur during inflammation. These include: the production of acute phase proteins, including complement components, by the liver; fever, caused by pyrogens acting on the hypothalamus in the brain; and systemic immunity, resulting in part from lymphocyte activation in peripheral lymphoid tissues (Sherman & Ward, 1998).

1.1.3 Interleukin-8/chemokines:

IL-8 and other low molecular weight chemokines (*e.g.* platelet factor 4, macrophage inflammatory protein (MIP)-1alpha and β, MIP-2, monocyte chemoattractant protein-1 (MCP-1/JE), belong to a chemotactic cytokine family and are responsible for the chemotactic migration and activation of neutrophils and other cell types (such as monocytes, lymphocytes, basophils, and eosinophils) at sites of inflammation (Miller & Krangel, 1992; M.Y. Stoeckle & Barker, 1990). The two subsets of the chemokine family, "CXC" (or alpha), "C-C" (or β) are divided based on presence or absence of an amino acid between the first two of four conserved cysteines. A recent third subset, "C", has only two cysteines and to date only one member, IL-16, has been identified (Strieter *et al.,* 1996). Chemokines have been implicated in inflammatory conditions from acute neutrophil-mediated conditions such as acute respiratory distress syndrome to allergic asthma, arthritis, psoriasis, and chronic inflammatory disorders. To date, at least 27 chemokines have been described.

The product of many cell types, including mononuclear phagocytes, antigen-activated T cells, endothelial and epithelial cells, and even neutrophils, IL-8 was previously known as neutrophil chemotactic factor (NCF) and neutrophil activating protein (NAP-1) (Baggiolini & Clark-Lewis, 1992) (Fig. 1-1). It is the most thoroughly studied chemokine and therefore serves as a prototype for discussing the biologic properties of this rapidly growing family of inflammatory mediators. It consists of a 6-8 kDa protein whose cDNA was cloned by three different laboratories between 1987 and 1989. The corresponding gene has been mapped to chromosome 4 in humans (Van Damme, 1994). Its main inflammatory impact lies in its

chemotactic effects on neutrophils and its ability to stimulate granulocyte activity. In addition, IL-8, IL-1, and TNF-α are involved in neutrophil recruitment by upregulating cell-surface adhesion molecule expression (such as endothelial leukocyte adhesion molecule, ELAM-1; and intracellular adhesion molecule, ICAM-1), thereby enhancing neutrophil adherence to endothelial cell and facilitating their diapedesis through vessel walls. Thus, IL-8 mediates the recruitment and activation of neutrophils in inflamed tissue (Feghali & Wright, 1997). IL-8 can be detected in synovial fluid from patients with various inflammatory rheumatic diseases (Seitz *et al.*, 1992), and mucosal levels of IL-8 are elevated in patients with active ulcerative colitis (Mahida *et al.*, 1992).

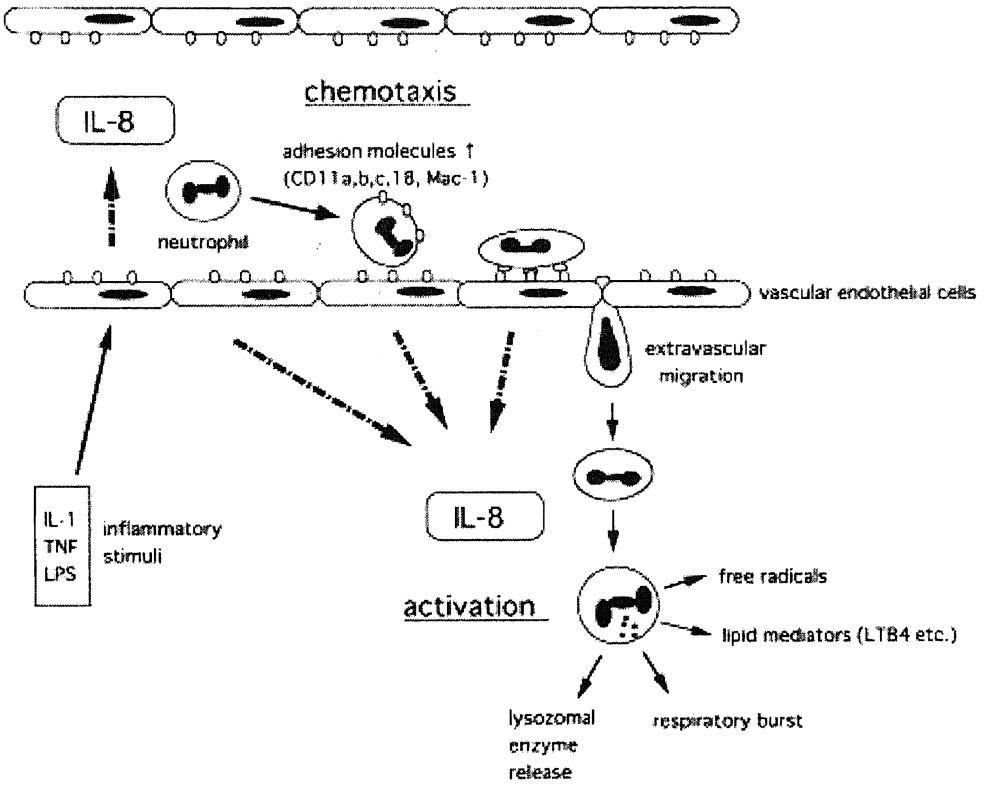

Figure 1-1 Schematic representation of the induction and activation of interleukin 8 (IL-8).

1.2 Inflammatory Gene Expression

Altered gene expression contributes to the aetiology of inflammatory diseases by modulation of the concentration of disease-related proteins. The expression of inflammatory genes is controlled through the concerted actions of specific transcription factors. Signal transduction, networks positively or negatively to regulate the activity of these transcription factors. Key components of these networks are protein kinases, which phosphorylate substrates on tyrosine, threonine or serine residues. During the disease process, pro-inflammatory signalling at the cell surface leads to a cascade of kinase activation, which ultimately culminates in modulation of the activity of transcription factors (Alton *et al.,* 2002). A schematic model of inflammatory gene expression and its regulation is shown in figure 1-2.

Post-transcriptional regulation of gene expression is generally mediated by the 5' or 3' untranslated regions (UTRs) of the mature mRNA, which flank the protein coding sequence. It is assumed that regulation is mediated by proteins that specifically interact with these regions. Much recent attention has focused on adenosine/uridine-rich elements (AREs), particularly repeats of the sequence AUUUA, which are present in the 3' UTRs of many transiently expressed cytokine and growth factor mRNAs, including tumour necrosis factor-alpha (TNF-α), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin 6 (IL-6) and IL-8, interferon gamma (IFN-γ) and granulocyte macrophage colony stimulating factor (GM-CSF) (Caput *et al.,* 1986).

TNF-α or endotoxin, induce the activation of two major transcription factors, nuclear factor-kappa B (NF-κB) and activating protein-1 (AP-1), which in turn induce genes involved in chronic and acute inflammatory responses. The activity of both of them is regulated by phosphorylation and subsequent interaction with the coactivator protein CREB-binding protein (CBP). Thus limiting amounts of CBP may play an important role in the development of critical illness (Matt, 2002).

Pro-inflammatory stimuli activate transcription factors such as AP-1, signal transducer and activator of transcription (STAT)s, CCAAT enhancer binding protein (C/EBP) and NF-κB. These transcription factors act co-ordinately to modulate gene expression via binding to specific response elements in the 5'-UTR. The activation of RNA polymerase II by these factors appears to be mediated by a number of common transducing molecules such as CBP, steroid receptor coactivator 1 (SRC-1) and retinoid interaction protein 160 (RIP 160) (Barnes & Adcock, 1998). The major role of glucocorticoids, which will be discussed later on, acting via a specific receptor (GR), appears to be by inhibition of transcription factors either by direct inhibition of DNA binding or by competition with these transcription factors for the common transducing proteins thus causing modulation of transcription (Almawi & Melemedjian, 2002)

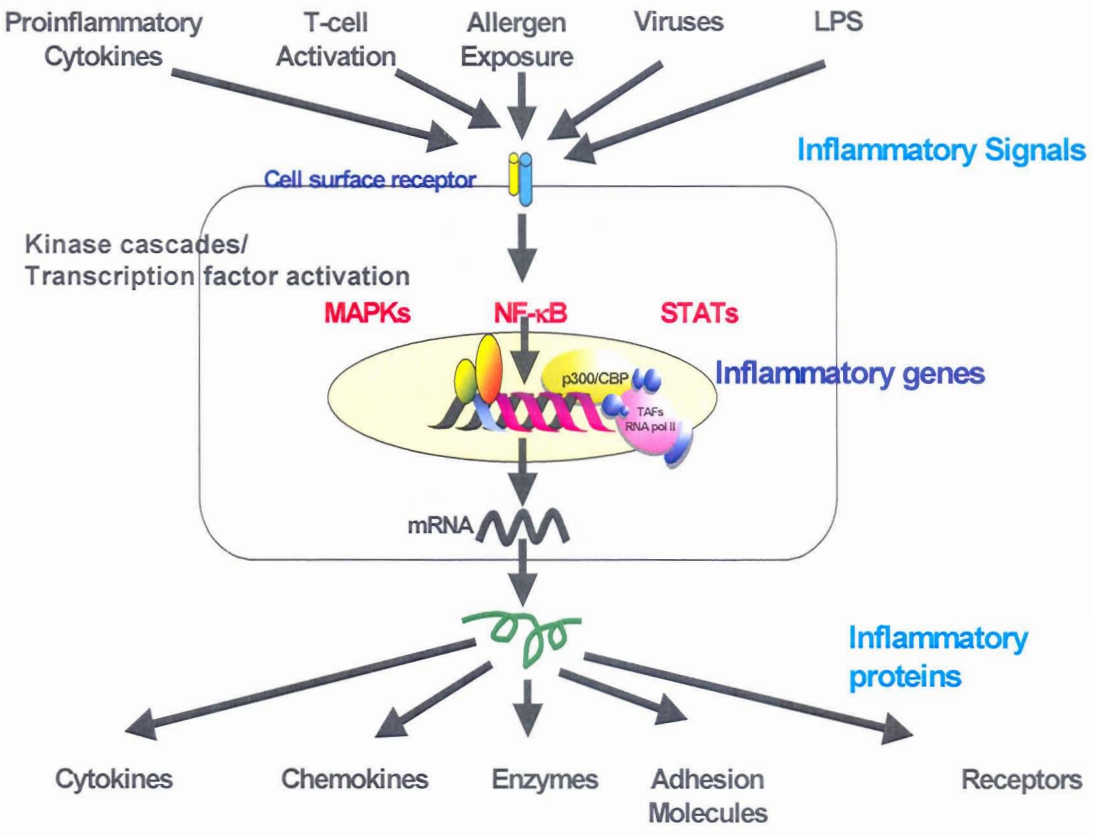

Figure 1-2 Schematic representation of signalling and gene regulatory mechanisms that regulate inflammatory gene synthesis. Extracellular signals including cytokines, allergens, viruses and lipolysaccharide (LPS) can stimulate cells via specific cell surface receptors. This leads to stimulation of intracellular signalling cascades (such as mitogen activated protein kinases, MAPKs) resulting in activation of transcription factors within the nucleus. Within the nucleus activated transcription factors such as nuclear factor kappaB (NF-κB) and signal transducers and activators of transcription (STATs) can interact with specific DNA sequences within the promoter regions of responsive genes leading to recruitment of co-activator proteins such as p300/CBP. Following chromatin modification (e.g. histone acetylation) and remodelling, basal transcription factors (TAFs) and RNA polymerase II (RNA pol II) are recruited and induction of mRNA for inflammatory genes is initiated. This results in the production of numerous pro-inflammatory proteins including cytokines, chemoklines, adhesion molecules cytokine and chemikine receptors and important inflammatory enzymes such as cycloxygenase (COX-2). Stimulation of T-cells by cross-linking of the T-cell receptor leads to induction of inflammatory mediators in a similar manner.

Pro-inflammatory cytokines (IL-1β, IL-8, IL-17, TNF-α) or microbial products (lipopolysaccharide; LPS) trigger receptor aggregation and subsequent TNF receptor-associated factor (TRAF) oligomerization, a crucial step in activation of the IκB kinase, extracellular-regulated kinase (ERK), JUN-N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signalling pathways (Dunn *et al.,* 2002). These kinases phosphorylate either IκB and NF-κB, or the transactivation domains of AP-1 proteins. This results in activation of both, nuclear NF-κB and AP-1. At least one activated MAP kinase pathway is required in addition to NF-κB to promote strong transcription. Signals from these pathways converge at gene promoters by promoting chromatin remodelling via histone acetylation and phosphorylation (Rahman, 2002). This is followed by the formation of multi protein complexes, so-called enhanceosomes. Within an enhanceosome NF-κB and AP-1 interact with DNA, other transcription factors, co-activators such as CBP/p300 and the RNA polymerase II holoenzyme. Variations within the enhanceosome composition result in activation of specific genes. The newly synthesized mRNA is stabilized by the p38- mitogen activated protein kinase-2 (MK-2) pathway (Shi & Gaestelts, 2002) (Fig. 1-2).

1.3 Histone Modifications

In the resting cell, DNA is tightly compacted into chromatin, a highly organized and dynamic protein-DNA complex, to prevent transcription factor accessibility. During activation of the cell the compact inaccessible DNA is made available to DNA-binding proteins, thus allowing the induction of gene transcription (Luger *et al.,* 1997) (Fig. 1-3).

The discovery of nucleosomes as a fundamental structural element for packaging DNA into ordered arrays was a historic breakthrough that initiated much of the contemporary research aimed at defining the structure and function of chromatin (Kornberg & Thomas, 1974). The nucleosome is composed of an octamer of 4 core histones, an H3/H4 tetramer and two H2A/H2B dimers surrounded by 146bp of DNA and a single molecular of a linker protein H1 (Beato, 1996). Histones are unusual proteins containing many basic amino acids (thought to be required for ionic interactions with the acidic phosphate-pentose backbone of DNA), each containing a structured histone fold domain and a basic amino (N-) terminal tail domain (Arents *et al.,* 1991). The core histone folding motif is conserved across the archaeal/eukaryotic boundary, and the overall amino acid sequences are highly similar across the entire eukaryotic range indicating the functional significance of such a structural features (Fig 1-4). This high degree of structural conservation is also reflected on the functional level: yeast and human core nucleosomes bind exactly the same amount of double-stranded DNA (146 base pairs)(Weinzierl, 1999).

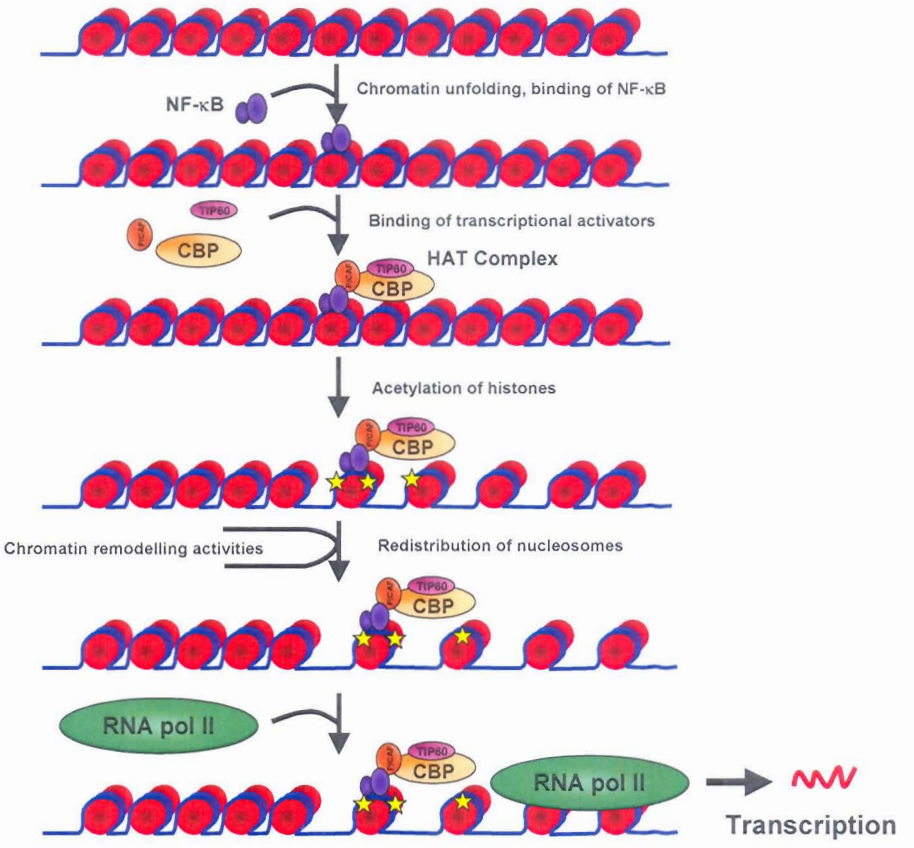

Figure 1-3 Schematic representation of the generation of transriptionally competent chromatin. Histone acetyltransferases together with nuclear binding factors and RNA polymerase allow the initiation of transcription.

The organization of the nucleosomes into the higher order "solenoid" or 30 nm filament, is the responsibility of linker histone 1 (Graziano *et al.*, 1994). Differential post-translational modification of H1 at various stages of the cell cycle may also contribute to a certain extent to the condensation/decondensation of chromosomes by regulating the DNA-binding ability of H1 (Roth & Allis, 1992).

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HMF-2 M E L P I A P I G R I I K D A - G A E R V S D D - - - - - A R I T L A K I L E E
H2A   L Q F P V G R V H R L L R K J N Y A E R V G A G - - - - - A P V Y L A A V L E Y
H2B   K E S Y S I Y I Y K V L K Q V H P D T G I S S K - - - - - A M G I M N S F V N D
H3    L L I R K L P F Q R L V R E I - A Q D F K T D L R F Q S S A V M A L Q E A S E A
H4    Q G I T K P A I R R L A R R G - G V K R I S G L - - - - - I Y E E T R G V L K V

HMF-2 M G R D I A S E A I K L A R H A G R K T I K A E D I E L A V R R F K K
H2A   L T A E I L E L A G N A A R D N K K T R I I P R H L Q L A I R N D E E
H2B   I F E R I A G E A S R L A H Y N K R S T I T S R E I Q T A V R L L L P
H3    Y L V G L F E D T N L C A I H A K R V T I M P K D I Q L A R R I R G
H4    F L E N V I R D A V T Y T E H A K R K T V T A M D V V Y A L K R Q G R
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Figure 1-4 Primary Amino Acid Sequence conservation of archaeal histone and the four eukaryotic core histones. The sequence alignment of an archaeal histone (HMF-2) with the central domains of the four eukaryotic histones H2A, H2B, H3 and H4 illustrates the conservation of key amino acid residues, especially towards the C-terminal end.

Apart from the histone fold-containing central domains, which allow the formation of stable nucleosome particle, all four histones contain N- and C-terminal tails that are mostly freely exposed, unstructured and located on the periphery of the nucleosome particle (Arents *et al.*, 1991). Genetic studies carried out in yeast show that different genes have different requirements for the histone N-termini. The presence of an intact H2A N-terminus is essential for the correct transcription of the *SUC2* gene (Hirschhorn *et al.*, 1995), and intact H3/H4 N-termini are required for the regulated expression of several other genes (including GAL1, GAL7 and GAL10) (Grunstein *et al.*, 1995) and nucleosome assembly (Ling *et al.*, 1996). The gene-specific phenotypes that mutations in particular histone N-termini impart on yeast cells thus provide a strong hint that histones affect the expression of genes in different manners. Many, if not all, of these effects are exerted by differential post-translational modifications of specific N-terminal amino acid residues by acetylation, phosphorylation, methylation and ubiquitination (Ling *et al.*, 1996; Wade *et al.*, 1997).

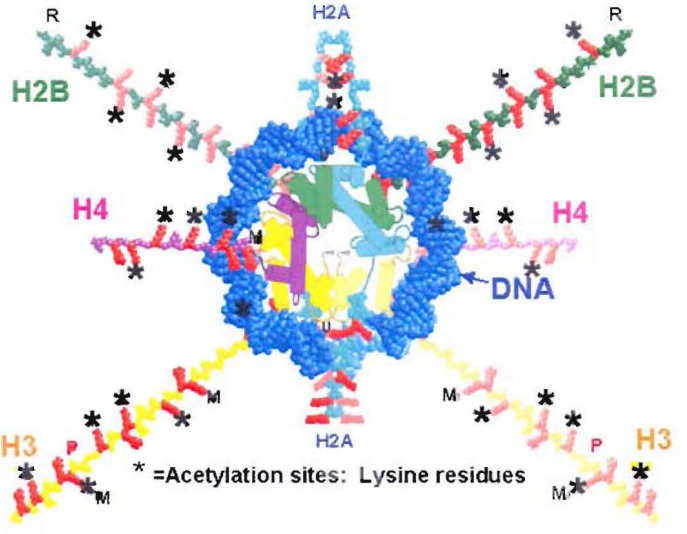


Figure 1-5 Model of the nucleosome. Histones form a tight core around which the DNA is tightly bound. The histone tails lie on the outside of the nucleosome where they are accessible to enzymes that carry out histone modifications. Here the lysine residue acetylation sites of all four core histones are shown (Steinbach, 1997).

The histone fold domains are involved in histone-histone interactions and in wrapping DNA. The core histone tails are the sites of post-translational modifications. Histone modifications are diverse: they include acetylation, methylation, phosphorylation, ADP ribosylation and ubiquitination (Berger, 2002). The tail domains lie on the outside of the nucleosome where they are accessible both to the enzymes that carry out the modification and to specific trans-acting factors that recognize the tail domains (Berger, 2002) (Fig. 1-5). The structural consequences of histone modification for the nucleosome have been determined in greatest detail for acetylation of the core histones. Specific lysine residues in the N-terminal tails of the core histone can be post-translationally modified by acetylation of the ε-amino group. H2A can be acetylated at lysine site 5, H2B at lysines 5, 12, 15 and 20, H3 can be acetylated at

lysines 4, 9, 14, 18, 23 and 27 and finally H4 can be acetylated at lysines 5, 8, 12 and 16. The dynamic equilibrium of core histone acetylation is established and maintained by histone acetyltransferase (HAT) that catalyze acetylation and histone deacetylase that remove it (HDAC) (Gray & The, 2001).

At least three critical roles may be assigned to HDACs and HATs: nucleosome assembly, transcriptional control and cell cycle checkpoint modulation, all being necessary mechanisms for a cell to choose a fate of cell growth or differentiation. Inhibition of HDACs leads to histone hyperacetylation, proliferation blockade and differentiation. These roles are accomplished by reversible acetylation of histones and formation of molecular complexes. Cycling cells thus need to dynamically modify their chromatin structure to coordinate the complex interplay of competing molecular complexes, in a process that is mediated by HDACs and HATs (Gray & The, 2001). Disruption of chromosome remodeling has been linked to a number of diseases, such as autoimmune diseases (List, 2002) and cancer (Batova *et al.,* 2002) and therefore elucidation of the differential responsiveness and specificities of HATs and HDACs may shed as light into potential therapeutic targets for intervention in autoimmunity and cancer. It is not clear yet whether histone modification plays a specific role in the activation of lymphocytes or other cell types during immune responses. It is assumed that these proteins allow the cells to respond during inflammatory conditions.

1.4 Glucocorticoid regulated inflammatory gene expression

1.4.1 Regulation of inflammatory gene expression

Glucocorticoids have been used for decades to control the progression of serious inflammatory diseases, such as inflammatory bowel diseases and asthma, by suppressing immune reactions. Until recently the mode of action of glucocorticoids was unknown, although it was clear that they suppressed transcription of many of the genes that are required for immune response functions.

Glucocorticoids penetrate the cell membrane and then bind to a steroid-binding site on the C-terminal of a cytoplasmic glucocorticoid receptor (GR) (Muller & Renkawitz, 1991). GR is a multi-domain 94 kDa member of a superfamily of steroid/thyroid/retinoic acid receptors. Binding of steroid triggers dissociation of heat shock proteins (hsp90) that bind to GR and mask the two nuclear translocation domains (Fig. 1-6). The GR alters gene expression in two ways. The first way is dependent on the GR binding directly to DNA and acting (positively or negatively) as a transcription factor. Active steroid-receptor complex translocates as a homodimer into the nucleus via the exposed translocation motifs and then binds to DNA at glucocorticoid response elements (GREs) (Gronemeyer, 1992) influencing transcription and inhibiting inflammatory or potentiating endogenous anti-inflammatory mechanisms, such as

IL-1ra and lipocortin-1 (Barnes, 1998). The second is dependent on GR binding to, and interfering with other transcription factors and this may be an important determinant of steroid actions and a key mechanism by which they exert such a broad range of anti-inflammatory activities (Barnes & Woolcock, 1998).

Both mechanisms could underlie suppression of inflammation. The liganded GR binds and inhibits the inflammatory transcription factors AP-1 and NF-κB (Stancovski & Baltimore, 1997). It also directly induces anti-inflammatory genes such as that encoding IκBα. Recent work has shown that glucocorticoids inhibit signalling in the MAPK pathways that mediate the expression of inflammatory genes. This inhibition is dependent on *de novo* gene expression (Lassa & Clarke, 2002). It is important to establish the significance of these different mechanisms for the various physiological effects of glucocorticoids, because it may be possible to produce steroid-related drugs that selectively target the inflammatory process (Saklatvala, 2002).

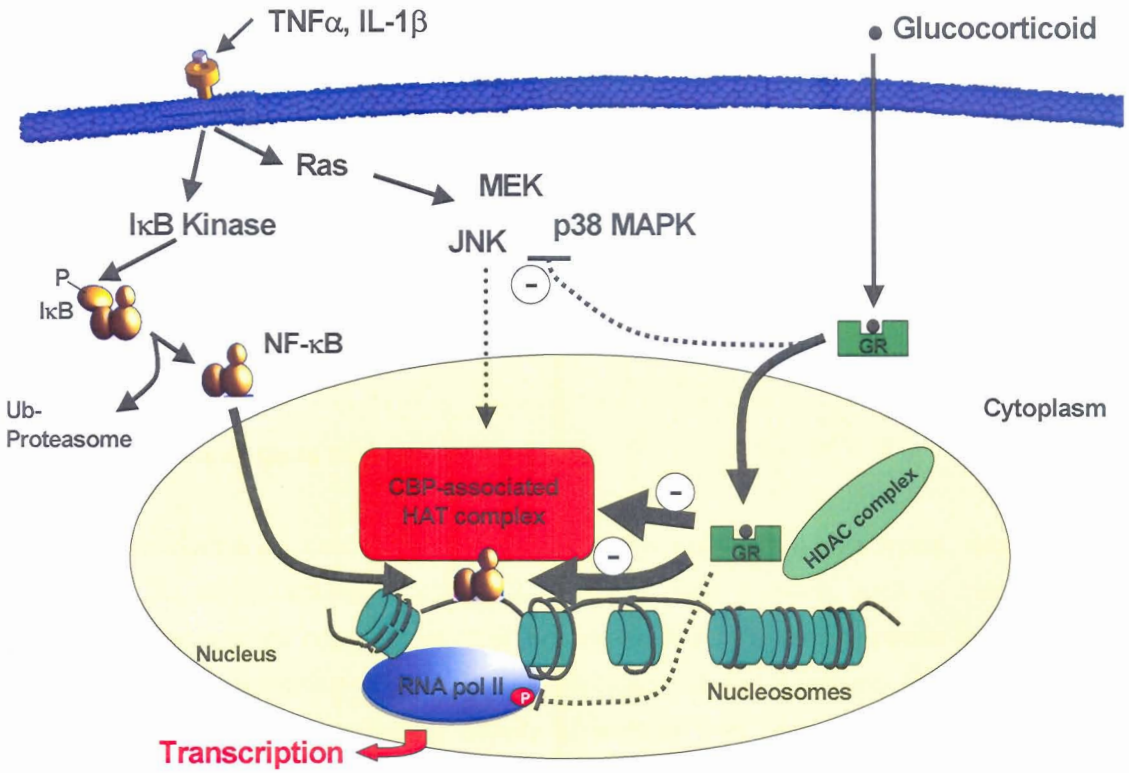


Figure 1-6 Schematic representation of gene expression/repression and its modulation by glucocorticoids. NF-κB is activated by proinflammatory stimuli such as TNF-α and IL-1β acting via IKK mediated IκBα phosphorylation and subsequent degradation. Activated NF-κB translocates to the nucleus where it recruits transcriptional coactivators containing HAT activity. This helps to unwind DNA from the surrounding nucleosomes and therefore making it available for the initiation of gene transcription. The activity of the transcriptional complex may also be enhanced by MAPKs activated by TNF-α and IL-1β. Glucocorticoids can freely diffuse through the plasma membrane where they bind to, and activate, the glucocorticoid receptor. This in turn translocates to the nucleus where it can inhibit the HAT activity stimulated by NF-κB either directly or by recruiting an HDAC complex. In addition, GR may also inhibit transcription by reducing phosphorylation of RNA polymerase II or by attenuating the activity of MAPKs.

1.4.2 How are genes inhibited by hormone-GR complexes?

How steroid repression of gene transcription may be achieved is not well understood. Although the existence of negative steroid response elements (nGRE) and their functional suppression of gene transcription has been established, for example in IL-1β gene (Zhang *et al.,* 1997), they are very similar to hormone response elements at which activation occurs, and not enough have yet been studied to enable one to state whether a different consensus sequence is involved.

An alternative possibility is that the binding site may be identical to that for activated genes, but its location is such that the bound receptor blocks, rather than enhances, the binding of other transcriptional activators. Support for this has come from studies of the genes encoding a α subunit of the glycoprotein hormones, which is repressed by glucocorticoids. It was found that the binding sites for the glucocorticoid receptor and a transcriptional activator of the gene (CREB) were overlapping. The critical feature may therefore be the location of the binding site for the receptor relative to those of other transcription factors (De Bosscher *et al.,* 2000).

Other effects may also be invoked, such as distortion of the DNA helix that may hinder binding of other factors distal to GRE (Ptashne, 1986). However, few of the inflammatory genes repressed by steroids have been shown to possess nGRE motifs (or indeed GREs) in their promoter region. Glucocorticoids therefore, must be exerting their effects through less direct mechanisms, which would suggest less specific mechanisms of protein-DNA interactions.

1.4.3 Mechanisms of gene activation

Since steroid hormones can activate different genes in different cell types, despite the presence of the same receptor protein, it is clear that other proteins, such as transcription factors or transcriptional coactivators, that are expressed in a tissue-specific manner are required for gene activation (Fig. 1-7). Appreciation of the profound effects that one transcription factor can have on the activity of another may prove of key importance in understanding how genes are regulated, both in homeostasis and in disease states. Allusion has already been made to the effects of heterodimerization (e.g. Fos/Jun combinations) and isoform variation within a transcription factor on gene transcription (Zhou *et al.,* 1999). The potential interaction of transcription factors triggered through different signaling pathways and networks makes the effects of such cross-talk even more complex.

Interaction of the activated receptors with other transcription factors can be directly demonstrated using DNA constructs in which a hormone response element and a binding site for another factor are coupled to the promoter for a reporter gene (Saklatvala, 2002). The

construct is transfected into recipient cells and the activity of the reporter gene is measured. This has shown that binding sites for a number of transcription factors, such as nuclear factor-1 (NF-1) and Sp1, can act synergistically with a hormone response element in promoting hormone-dependent gene expression, although the best response of all is obtained using two hormone response elements (Dufau *et al.*, 1995).

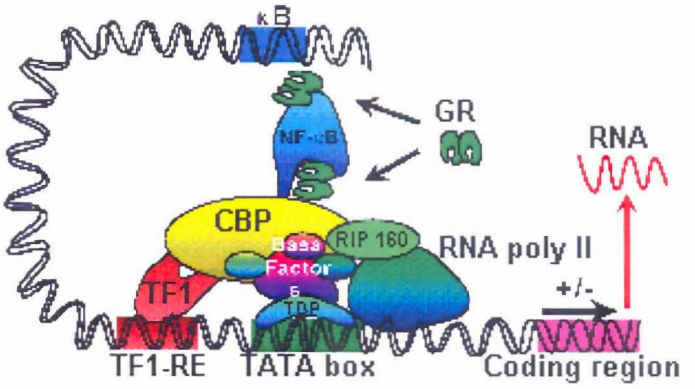


Figure 1-7 Schematic diagram of activation of inflammatory gene transcription and its modulation by glucocorticoids. Reproduced with permission from Dr Ian Adcock.

Experiments in which the spacing between the binding sites is varied have shown that this parameter is important, but that there is no single critical spacing. An alternative model is that the binding of the activated receptor may alter the chromatin structure around the gene, and thus indirectly allow binding of other transcription factor(s). Mild treatment with certain nucleases of chemicals, which degrade DNA, results in cleavage only in the linker regions (which are ~50 base pairs long), with the DNA in the nucleosome particles (~150 base pairs) being protected by association with the histones (Fajkus *et al.*, 1992).

Due to the great length and complexity of mammalian DNA, it is difficult to study the chromatin structure around individual genes in native chromosomes. However, experiments have been carried out using circular vectors (based on papillomavirus DNA), which acquire nucleosomes and replicate as "minichromosomes", independently of the host chromosomes, in mammalian cells (Venditti *et al.*, 1998). Constructs were made in which these vectors contained an inserted reporter gene, fused to a region of mouse mammary tumour virus (MMTV) containing two glucocorticoid response elements. In the absence of glucocorticoid, nuclease or chemical cleavage of minichromosomes occurred at specific locations, ~200 base pairs apart, on the 5' side of the transcription initiation site (at –60, -250, -450, -650, etc.). This shows that the nucleosomes were "phased", rather than being bound at random locations, with respect to the initiation site. Experiments using various different constructs showed that this phasing was produced by the inserted MMTV DNA, rather than being a function of the vector. Intriguingly, in the presence of glucocorticoid, the region between –60

and –250 was no longer protected against chemical cleavage: this is exactly the region containing the hormone response elements. Thus, the binding of the activated receptor appears to have displaced a nucleosome, or at least to have altered the nucleosome structure in this region. It has recently been demonstrated that this process is very rapid lasting only for ~6 seconds (McNally *et al.,* 2000). Further experiments showed that this also exposed a region from the initiation site to –60, which bound two other transcription factors, one of which was nuclear factor-1 (NF-1, Fryer & Archer, 2001; Deroo & Archer, 2001).

Although both of these factors appear to be present in extracts of unstimulated cells, they do not bind to the chromatin unless the cells are first treated with glucocorticoid. Therefore, binding of the activated hormone receptor may cause a local change in chromatin structure, which enables the binding of other proteins essential for initiation of transcription. At least some of the latter may be tissue-specific, explaining the different responses to the same hormone in different tissues (Collingwood *et al.,* 1999).

One of the best documented transcription factor interactions is the one between nuclear factor (NF-AT) and activator protein 1 (AP-1). NF-AT, once phosphorylated, has transient DNA binding and basal transcription capability. However, attachment of AP-1 stabilizes the transcription complex and this is likely to increase promotor activation (Hanson *et al.,* 1993). Co-operative activity of NF-AT and AP-1 is observed at several promoter sites including IL-2 (Prieschl *et al.,* 1995) and GM-CSF (Cockerill *et al.,* 1995). This process requires the interaction of several signaling pathways *in cellulo.*

Transcription factor interaction may prove to be the major mechanism through which glucocorticoids exert their peiotropic effects. Two early observations of steroid activity confirm this concept. Firstly, sub-transcriptional activation doses of steroid hormone can have a significant inhibitory effect on AP-1 activity, with GR and AP-1 co-precipitating (Jonat *et al.,* 1990). Secondly, GR monomers repress AP-1 activity (Heck *et al.,* 1994). Other observations have supported transcription factor interaction as a likely *modus operandi* (Schule *et al.,* 1990). In asthmatic inflammation GR has been shown to inhibit AP-1 DNA binding in both human lung and peripheral blood mononuclear cells (Adcock *et al.,* 1995). Immunosuppressant activity through GR-AP-1 interaction may also be achieved via trans-repression of NF-AT activity. The facts that steroids are known to suppress AP-1 binding to DNA and that AP-1 is a component of NF-AT transcription complex, lead to the conclusion that NF-AT is a likely target for GR-mediated trans-repression (Paliogianni *et al.,* 1993). Such an interaction in T-lymphocytes could profoundly suppress the inflammatory process.

Cross talk between GR and NF-κB has also been implicated in the anti-inflammatory effects of steroids. The NF-κB/Rel dimer is a major transcription factor associated with inflammatory cytokine production (Barnes & Adcock, 1993). As discussed previously, activity of NF-κB is

regulated through association of the inhibitor IκBα, which sequesters NF-κB in the cytoplasm and must be released for transcriptional activation. It is known that steroid-GR binding to the IκBα promotor induces expression of the inhibitor, explaining some of the repressing actions of steroids on NF-κB driven gene regulation. However, it has also been demonstrated that increased synthesis of IκBα is neither requisite nor sufficient for hormone-mediated suppression of NF-κB (Heck *et al.,* 1997). Therefore, through NF-κB interaction, other transcription factors may be expected to influence the inflammatory process. A similar mutual repression to that observed between GR and AP-1 is demonstrated between GR and NF-κB (Scheinman *et al.,* 1995), with the corollary that NF-κB/Rel can in turn repress GR/GRE trans activation (Ray & Prefontaine, 1994).

The above suggest a theoretical GR intervention in the many inflammatory systems militated by NF-κB. In support to this, the steroid-GR complex is able to repress NF-κB promotion of ICAM-1 expression through direct physical interaction with the Rel-A p65 family protein (Caldenhoven *et al.,* 1995). GR-DNA binding was not required for this repression, since GR with a P-box mutation (making it unable to recognize GRE) still repressed NF-κB. However, there is a possibility that GR repression of NF-κB is also due to GR competing with NF-κB for limiting amounts of transcriptional co-activator CBP (Sheppard *et al.,* 1998). The Rel(p65) component is thought to be the target for many transcription factor interactions with the NF-κB dimer since p50 does not appear to be targeted (Ray & Prefontaine, 1994).

1.5 Macrophages and T-cells

1.5.1 Macrophages and monocytes

Originally, monocytes and macrophages were classified as cells of the reticulo-endothelial system (RES) (Aschoff, 1924). Van Furth et al. (1972) proposed the mononuclear phagocyte system (MPS), and monocytes and macrophages became basic cell types of this system. Their development takes in the bone marrow and passes through the following steps: stem cell - committed stem cell - monoblast - promonocyte - monocyte (bone marrow) - monocyte (peripheral blood) - macrophage (tissues). Monocyte differentiation in the bone marrow proceeds rapidly (1.5 to 3 days). During differentiation, granules are formed in monocyte cytoplasm and these can be divided as in neutrophils into at least two types. However, they are fewer and smaller than their neutrophil counterparts (azurophil and specific granules). Their enzyme content is similar (Male *et al.,* 1996).

The blood monocytes are young cells that already possess migratory, chemotactic, pinocytic and phagocytic activities, as well as receptors for IgG Fc-domains (FcγR) and iC3b complement. Under migration into tissues, monocytes undergo further differentiation (taking at least one day) to become multifunctional tissue macrophages. Monocytes are generally,

therefore, considered to be immature macrophages. However, it can be argued that monocytes represent the circulating macrophage population and should be considered fully functional for their location, changing phenotype in response to factors encountered in specific tissue after migration (Kuby, 1992).

Macrophages can be divided into normal and inflammatory macrophages. Normal macrophages include macrophages in connective tissue (histiocytes), liver (Kupffer's cells), lung (alveolar macrophages), lymph nodes (free and fixed macrophages), spleen (free and fixed macrophages), bone marrow (fixed macrophages), serous fluids (pleural and peritoneal macrophages), skin (histiocytes, Langerhans's cell) and in other tissues. The macrophage population in a particular tissue may be maintained by three mechanisms: influx of monocytes from the circulating blood, local proliferation and biological turnover. Under normal steady-state conditions, the renewal of tissue macrophages occurs through local proliferation of progenitor cells and not via monocyte influx. Originally, it was thought that tissue macrophages were long-living cells. More recently, however, it has been shown that depending on the type of tissue, their viability ranges between 6 and 16 days (Roitt *et al.,* 1995).

Inflammatory macrophages are present in various exudates. They may be characterized by various specific markers, e.g. peroxidase activity, and since they are derived exclusively from monocytes they share similar properties. The term exudate macrophages designates the developmental stage and not the functional state (Male *et al.,* 1996).

Macrophages are generally a population of ubiquitously distributed mononuclear phagocytes responsible for numerous homeostatic, immunological, and inflammatory processes. Their wide tissue distribution makes these cells well suited to provide an immediate defence against foreign elements prior to leukocyte immigration. Because macrophages participate in both specific immunity via antigen presentation and IL-1 production and nonspecific immunity against bacterial, viral, fungal, and neoplastic pathogens, it is not surprising that macrophages display a range of functional and morphological phenotypes (Male *et al.,* 1996).

1.5.2 T-Cells

Lymphocytes recognize antigens presented by macrophages, proliferate after contact with antigens, attack antigen-labelled cells, and manufacture antibodies. These cells belong to many different functional groups, which interact in a complex way, resembling a large military organization (Kuby, 1992).

T-cells are lymphocytes, which develop and differentiate in the thymus before seeding the secondary lymphoid tissues. Resting mature T-lymphocytes recognize antigen and MHC

molecules when they are triggered via their antigen- specific T-cell receptor (TCR) molecule. TCR, distinct from but related to immunoglobulin, consists of an antigen binding domain formed by two different polymophic chains, which is associated with CD3, a complex of polypeptides involved in signaling cellular activation. The antigen binding domain may consist of an $\alpha\beta$ or $\gamma\delta$ heterodimer, but the great majority of T-cells contain the $\alpha\beta$ heterodimer. In humans the markers cluster of differentiation 2 (CD2) and CD5 are also present on all T-cells. In some species, including humans, activated T-cells also carry endogenously synthesized MHC class II molecules, although these are absent from resting T-cells. Activated T-cells may also be induced to express CD25, which forms part of the high affinity IL-2 receptor and is important in clonal expansion. Other markers differentiate T cell subsets (Kuby, 1992).

There are two main subpopulations of T cells, which can be distinguished according to their expression of CD4 or CD8. These molecules act as receptors for class II and class I MHC molecules, respectively, and contribute towards both T cell immune recognition and cellular activation. Most $CD4^+$ T cells recognize antigens associated with MHC class II molecules and these cells act predominantly as helper T cells (Th). $CD8^+$ T cells recognize antigens associated with MHC class I molecules and are primarily responsible for cytotoxic destruction of virally infected cells. Th1 cells interact preferentially with mononuclear phagocytes, while Th2 cells tend to promote B cell division and differentiation. The balance of activity between those two subsets is related in part to how antigen is presented to the cells and it ultimately determines the type of immune response, which develops (Kuby, 1992).

The surface phenotypes of T cell population change during development. In humans, virgin T cells express CD45RA, while activated cells express CD45R0 and higher levels of adhesion molecules such as the β_1 integrins (CD29). The relationship of activated cells to resting memory T cells is not completely understood. The enhanced secondary response to antigens is only partly due to the increased numbers of activated cells available. Primed T cells also respond to antigens more efficiently than virgin T cells but this does not appear to be due to affinity maturation of the T cell receptor.

Under physiological conditions, T-cell production is a fine balance between proliferation and apoptosis, both of which require on-going gene induction regulated, at least in part, by histone acetylation and deacetylation as stated previously. In contrast, pre-activated T-cells can undergo antigen-induced cell death (AICD) in response to the same signals. Stimulation of activated T-cells upregulates the expression of the Fas-ligand and the interaction of Fas-ligand with the corresponding Fas-receptor (cell surface receptor) triggers an apoptosis program that culminates in cellular suicide usually associated with the fragmentation of DNA into oligonucleosomal bands (Kabelitz and Janssen, 1997).

Molecular evidence indicates that proteases relating to interleukin-1β converting enzyme play an essential role in the execution of cell death. Apoptosis of mature T-lymphocytes can be efficiently triggered by monoclonal antibodies against the CD3/TCR complex, or by superantigens such as bacterial enterotoxins. Although it is more difficult to induce apoptosis by conventional peptide antigens, it is now clear that antigen-induced AICD is a powerful means of eliminating antigen-reactive T-cells. Therefore, AICD contributes to the regulation (i.e., termination) of cellular immune responses. In addition, AICD might play a role in the establishment of peripheral immune tolerance (Kabelitz and Janssen, 1997 and references therein).

1.6 Apoptosis and Inflammation

1.6.1 Cell death and Apoptosis

For many years, cellular injury was seen in black and white terms. A cell subjected to minor damage recovered, whereas a cell subjected to a more severe injury died by uncontrolled necrotic cell death, in which cells and their organelles swell and rupture. Leakage of cellular components, characteristics of necrosis, elicits an inflammatory response, which can further damage tissues. Kerr and colleagues in 1972 (Kerr *et al.,* 1972) described a morphologically distinct form of cell death, termed apoptosis. The term is derived from the Greek word meaning "dropping off", as with the leaves of a tree in the autumn. Apoptotic cell death is characterized by the controlled removal of a cell, without leakage of intracellular contents, in the absence of an inflammatory response. The distinction between apoptotic and necrotic cell death that occurs during development, in response to tissue damage, and in association with uncontrolled cellular proliferation has revolutionized the study of cell death. This is particularly since the manipulation of apoptosis during development and/or disease states, promises major therapeutic implications permitting the removal or preservation of specific cells in diseased tissues (Bamford *et al.,* 2000).

Apoptotic cell death is characterized by cell shrinkage, loss of cell-cell contacts, nuclear chromatin condensation, plasma membrane budding, and removal of cell debris by macrophages and surrounding cells in the absence of an inflammatory response (Wylie *et al.,* 1980) (Fig. 1-8). The requirement for energy in apoptotic cell death, in the form of ATP, is absolute. Cells that are damaged and unable to maintain adequate ATP levels will, by default, undergo necrotic cell death (Leist *et al.,* 1997; Lelli *et al.,* 1998). Necrotic cell death is characterized by cellular swelling, plasma membrane blebbing/disruption, swelling of the mitochondria (that show amorphous densities), chromatin clumping, release of intracellular contents into the interstitium and removal of cell debri by inflammatory cells (Savill & Fadok, 2000).

The study of apoptosis in cells in culture has added a further level of complexity to the definition of cell death. Whereas apoptotic cells *in vivo* are phagocytosed by surrounding cells, in tissue culture models this does not occur and apoptotic cells often undergo anoikis (loss of cell-cell contacts associated with cell surrounding), followed by "secondary necrosis". Therefore, "necrotic" cell death is the common end point of both apoptotic cell death and uncontrolled cell death *in vitro*. A new term, "oncosis", was introduced to represent uncontrolled (previously termed necrotic) cell death. Necrosis is now regarded as the cellular degradation that is the common end point of both apoptotic and oncotic cell death (Table 1-1) (Majno and Joris, 1995).

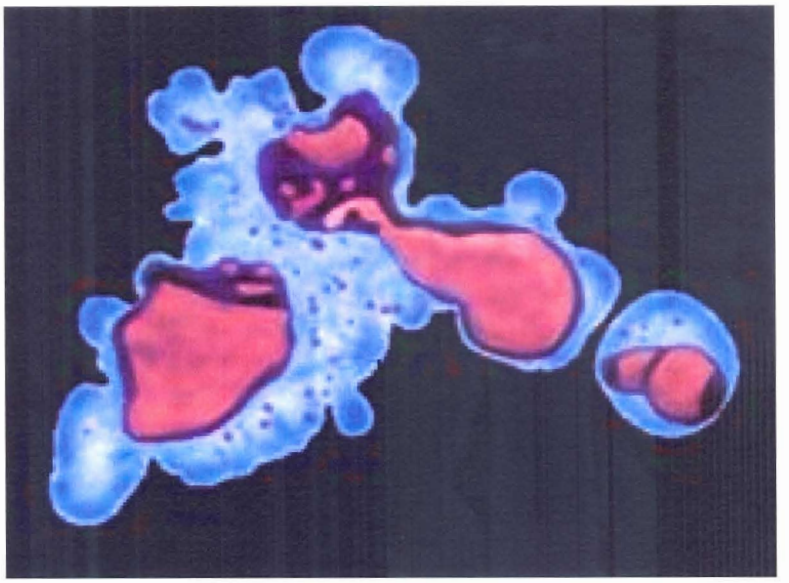


Figure 1-8 Final stages of apoptotic cell death. The cell nucleus is ruptured but enclosed into cell membrane pockets. The disintegrated cell will be phagocytosed by macrophages eliminating any remnants that might cause inflammatory responses. Figure adapted from www.cellsalive.com

In multicellular organisms, apoptosis provides a means of removing cells without stimulating an inflammatory response. This is important during development where tissues are sculpted or shaped (Jacobson *et al.*, 1997; Vaux and Korsmeyer, 1999) and in the adult where cells can be removed without damaging the surrounding tissue (Granville *et al.*, 1998). It is also increasingly apparent that apoptosis and cell growth/proliferation are inter-linked and interdependent processes. Uncontrolled cell growth (cancer) is detrimental to the organism as a whole; therefore strictly regulated controls of cell growth have evolved in metazoans. If uncontrolled cell growth/proliferation occurs, apoptotic pathways are activated effecting the removal of the offending cell(s). Failure to activate apoptotic pathways may result in cancerous growth and ultimately, death of the organism.

Apoptosis	Oncosis
Single/clusters of cell affected	Areas of tissue destroyed
Small, contracted cells	Cellular oedema
Mitochondrial swelling	Mitochondrial rupture (amorphous densities)
Chromatin condensation	Coarse chromatin
Intact plasma membrane	Plasma membrane rupture
Phagocytosis of apoptotic bodies	Inflammatory response

Table 1-1 Comparison of the gross morphological features of apoptotic and oncotic cell death

1.6.2 Regulation of apoptosis by intracellular signaling pathways

Regulation of ubiquitously expressed pro-apoptotic and anti-apoptotic factors is afforded by subcellular localization, oligomerisation and/or cleavage. Key elements of the apoptotic process are also regulated by protein phosphorylation, linking the control of apoptosis with the complex intracellular signaling pathways, which are activated within a cell by intrinsic factors and cellular stresses (Gallaher *et al.,* 2001). The best-characterized group of protein kinases implicated in the control of apoptosis is the Ser/Thr protein kinases, although tyrosine kinases may also be involved (Yuan *et al.,* 1999). Ser/Thr protein kinases involved in the control of apoptosis include the MAPKs, protein kinase B (PKB) and protein kinase A (PKA) (Cross *et al.,* 2000). Other Ser/Thr protein kinases [e.g. protein kinase C (PKC) and p21-activated protein kinase-1 (PAK)] (Swannie & Kaye, 2002) are also likely to be involved in the regulation of apoptosis, but their role is less well established.

1.7 Caspases – the effectors of apoptosis

1.7.1 The caspases

Most of our initial understanding of the molecular mechanisms controlling apoptosis came from developmental studies of the nematode *Caenorhabditis elegans (C.elegans)*. A group of cell death (CED) genes were identified that was central to the process of apoptotic cell death, which occurred during the development of C. elegans (Yuan, 1996). Following the discovery of the CED genes and their corresponding proteins, mammalian homologues were identified.

CED-3 homologues were identified as a group of proteases (Martin and Green, 1995), now referred to as caspases (for cysteine aspases) (Alnemri *et al.,* 1996). Caspases cleave substrates after specific aspartic acid residues and are central to the execution of apoptosis. However, there may be some instances where caspase-independent apoptosis occurs (Xiang *et al.,* 1996; Susin *et al.,* 1999).

Prior to the use of the generic term caspase, the enzymes had a bewildering array of unrelated names. Standardized nomenclature has now clarified the situation (Table 1-2). Caspases are pro-apoptotic and exist as pro-enzymes that are activated by cleavage at specific peptide sequences. To date, at least 14 isoforms have been identified and some caspases may be alternatively spliced (Cohen, 1998; Thornberry and Lazebnik, 1998). Following activation, these enzymes have proteolytic activity and cleave substrates at specific cleavage sites. Caspase-6, caspase-8, caspase-9 preferentially act at V/L-E-T/H-D, whereas caspase-3 and caspase-7 show high selectivity for the peptide motif DEVD. Caspases activated early in the apoptotic process (initiators; e.g. capsase-8 and caspase-9) act on downstream caspases (effectors; e.g. caspase-3) in a caspase cascade that affords amplification. Ultimately, the caspase cascade results in the cleavage of target intracellular molecules resulting in: recruitment and activation of other caspases and proteins involved in the apoptotic process (Cardone *et al.,* 1997; Sakahira *et al.,* 1998), inactivation of survival/repair proteins (Widmann *et al.,* 1998) and the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (Szabo and Dawson, 1998) and finally, cleavage of proteins involved in cytoskeletal regulation, including gelosin (Kothakota *et al.,* 1997) and focal adhesion kinase (FAK, Wen *et al.,* 1997). The role of caspases therefore, is to reorganize the cell cytoskeleton, shut down DNA replication/repair processes and induce cells to display "eat me" signals that mark them for phagocytosis.

1.7.2 Activation of the caspase cascade

Caspase precursors are ubiquitously expressed in living cells (including terminally differentiated cells) and can be quickly activated, initiating apoptotic cell death. Caspase activity is, under tight regulation involving a combination of regulatory proteases, cofactors, feedbacks and thresholds that converge to control the activity of individual caspases. Activation of the caspase cascade occurs when initiator caspases at the plasma membrane (caspase-8) or at the mitochondria (caspase-9) bind specific cofactors. The means of caspase activation by cofactors is unclear, but the formation of caspase dimers/oligomers, which have intermolecular autocatalytic activity, may be important (Martin *et al.,* 1998).

Caspases	Old nomenclature
Caspase-1	Interleukin 1β converting enzyme (ICE)
Caspase-2	ICH-1, Nedd2
Caspase-3	Apopain, CPP32, Yama
Caspase-4	ICH-2, ICErel-II, TX
Caspase-5	ICErel-III, TY
Caspase-6	Mch2
Caspase-7	ICE-Lap3, Mch3, CMH-1
Caspase-8	FLICE, Mch5, Mach
Caspase-9	ICE-Lap6, MCH6
Caspase-10	Mch4

Table 1-2 New and old nomenclature of CED-3 homologues

1.7.3 Inhibition of caspase activity

Caspase activity can be inhibited by decoy proteins, inhibitor proteins and by post-translational modifications of caspase residues. The interaction between cytokine receptor death domains and their respective death domain associated proteins can be inhibited by FADD-like interleukin 1β-converting enzyme-inhibitory proteins (FLIPs). FLIPs have a high degree of sequence homology with caspase-8, but lack a catalytic site and therefore function as decoy proteins (Irmler *et al.,* 1997). Direct inhibition of caspase activity is also possible through the activity of mammalian homologues of viral inhibitor of apoptosis proteins (IAPs) and related molecules. The X-linked IAP is a potent and selective inhibitor of caspase-3 and caspase-7 activity (Deveraux *et al.,* 1998), and a protein with a similar mode of action, the apoptosis repressor with caspase recruitment domain (ARC) protein, directly inhibits caspase-8 (Koseki *et al.,* 1998). Modification of residues within caspases is a further means of controlling caspase activity. Phosphorylation of serine-196 of human pro-caspase-9 prevents its activation (Cardone *et al.,* 1998) and S-nitrosylation of the active site cysteine residues of pro-caspase prevents their proteolytic activity (Mannick *et al.,* 1999). The control and implications of caspase inhibitors remain to be determined, but may be of particular importance in tissues where they are highly expressed.

1.8 B-cell lymphoma leukemia 2 (Bcl-2) family proteins

1.8.1 Regulators of mitochondrial apoptotic processes

CED-3 (the *C. elegans* caspase homologue) promotes apoptosis, whereas CED-9 protects cells from apoptosis (Wu *et al.,* 1997). CED-9 was found to have sequence homology with the

human Bcl-2 proto-oncogene, and subsequently an ever-increasing family of related Bcl-2 family proteins has been identified. So far, over 20 members of the Bcl-2 family have been identified, each possessing up to four conserved Bcl-2 homology domains (BH1, BH2, BH3 and BH4), which correspond to α-helical domains (Adams and Cory, 1998; Gross *et al.,* 1999). Bcl-2 family members can be either pro-apoptotic or anti-apoptotic. The anti-apoptotic members Bcl-2 and Bcl-X_L display sequence conservation in all four domains and localize to mitochondrial, endoplasmic reticulum and nuclear membranes (Hockenberry *et al.,* 1990; Boise *et al.,* 1996).

Deletion and mutagenesis studies suggest that the BH3 domain serves as a critical death domain. This is supported by an emerging subset of "BH3-domain-only" members (e.g. Bad, Bim and Bid) that are all pro-apoptotic and are primarily localized to the cytosol in healthy cells (Lutz, 2000). Bcl-2 family proteins influence the apoptotic process upstream of irreversible cell damage and primarily act to control the mitochondrial response to apoptotic signals (Green and Reed, 1998). Activation of mitochondrial apoptotic processes is regarded as the "point of no return" in apoptosis and therefore Bcl-2 family proteins play a pivotal role in deciding the fate of a cell.

Bcl-2 family proteins act to register cellular damage and growth/cytoprotective stimuli, integrate these signals and determine whether or not to initiate mitochondrial apoptotic processes. Once initiated, mitochondrial involvement in apoptosis is characterized by the translocation of cytochrome *c* and apaf-1 and other pro-apoptotic factors to the cytoplasm, disruption of the electron transport chain, generation of reactive oxygen species and, ultimately, deterioration of the mitochondrial membrane potential ($\Delta\Psi_m$) (Gross *et al.,* 1999). The role of the mitochondrial permeability transition pore (MPTP), a non-specific pore comprising of the voltage-dependent anion channel (VDAC; outer mitochondrial membrane) in apoptosis is unclear. Some studies suggest it is a pre-requisite to mitochondrial apoptotic processes and others that it is not involved at all (Green and Reed, 1998 and references therein).

In addition to their role in the control of apoptosis, Bcl-2 family proteins may modulate cell cycle progression in some cell types (e.g. thymocytes). Bcl-2 promotes exit into quiescence and retards re-entry into the cell cycle (Brady *et al.,* 1996; Lind *et al.,* 1999; Vairo *et al.,* 2000). Bcl-2 can also modulate cyclin dependent kinase activity (Gil-Gomez *et al.,* 1998). By way of contrast, the pre-apoptotic proteins, Bax and Bad, promote entry to the cell cycle (Mok *et al.,* 1999). The role of Bcl-2 family proteins in cell cycle control remains poorly characterized and understood.

1.8.2 Regulation of Bcl-2 family protein function

The mechanisms of regulation of mitochondrial function by Bcl-2 family proteins has been the subject of much debate, Anti-apoptotic Bcl-2 family members prevent activation of mitochondrial apoptotic processes, whereas pro-apoptotic family members promote apoptotic mitochondrial processes. However, the means by which Bcl-2 family proteins exert these effects at the mitochondria is far from clear. One theory suggests that some Bcl-2 family proteins can oligomerise to form ion channels, which regulate mitochondrial function. This is supported by evidence that the anti-apoptotic proteins Bcl-2 and Bcl-X_L contain seven α-helices, which show high structural homology with pore-forming domains of bacterial toxins (Muchmore *et al.*, 1996). Indeed, both Bcl-2 and Bcl-X_L form homodimers, which act as ion channels in synthetic membranes (Minn *et al.*, 1997; Schendel *et al.*, 1997). Anti-apoptotic Bcl-2 family proteins can directly regulate mitochondrial homeostasis (Vander Heiden *et al.*, 1997) and the MPTP (Zamzani *et al.*, 1996; Shimuzu *et al.*, 1998), preventing cytochrome *c* release from the mitochondria, possibly by preserving $\Delta\psi m$ (Kluck *et al.*, 1997; Bossy-Wetzel *et al.*, 1998). Pro-apoptotic Bcl-2 family proteins may dimerise with Bcl-2/Bcl-X_L through domains of sequence homology (Yang *et al.*, 1995), disrupting the protective ion channel function of Bcl-2/Bcl-X_L, thus promoting apoptosis. The regulation of Bcl-2 family proteins are therefore of paramount importance to the fate of the cell.

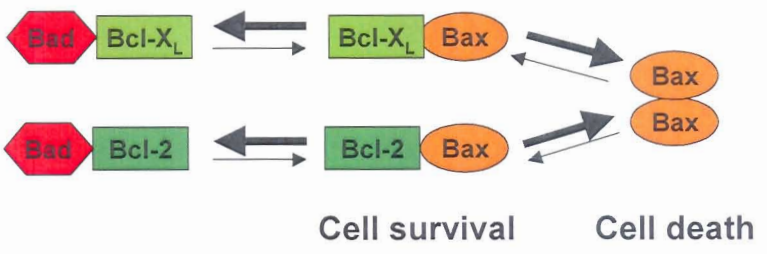


Figure 1-8b Bad is a negative regulator of apoptosis. Bad displaces Bax from Bcl-X_L-Bax and Bcl-2-Bax heterodimers allowing more Bax-Bax homodimer formation which permits more cell death.

1.9 Inflammatory Bowel Disease (IBD)

1.9.1 Clinical and biological aspects of IBD

Inflammatory bowel disease is now recognized as one of the most serious non-malignant disorders involving the gastrointestinal tract, and is of an unknown etiology. The origin of inflammatory bowel disease appears to be multifactorial; environmental and microbiological factors initiate and perpetuate an immune response in the intestine of genetically susceptible individuals. It can be divided in two major forms, Ulcerative colitis (UC) and Crohn's disease (CD) (Jones, 1989) (Table 1-3). In UC the diffused inflammation extends proximally from the rectum and may extend to the entire colon, where the lining of the intestine (including the rectum) becomes inflamed and ulcerated. UC can occur at any age (between 20-40 most commonly effected). 5000 people develop UC per year. The pathology comprises of hyperemia, oedema, mucous gland depletion and in severe cases, mucosal ulceration. The

inflammatory cell infiltrate is confined to the mucosa and when acute often crypt abscesses are observed. Clinically the patients suffer cramping abdominal pains, weight loss, loss of appetite, urgency and frequently loose stool containing blood and mucus. The disease is characterized by frequent periods of remission (Rayhorn & Rayhorn, 2002 and references therein).

Ulcerative Colitis	Crohn's Disease
Static Incidence	Incidence increasing
Colon affected only	Small bowel can also be affected
Marked tendency to relapse and remit	Similar but less marked tendency
No abdominal mass, signs of malabsorption, fistula formation or gross perianal disease	Clinical features of abdominal mass, signs of malabsorption, fistula formation, gross perianal disease
Changes affect rectum and proximal colon in continuity	Deep fissuring and ampoid ulceration, "skip" lesions and small bowel involvement
Mucosal inflammation, crypt abscesses, goblet cell depletion	Transmural inflammation, fissuring, granulomas, goblet cell preservation

Table 1-3 Differences between Ulcerative Colitis and Crohn's Disease

Crohn's disease is a transmural inflammatory process, characterized by the presence of granulomas, mucosal ulceration and the formation of fistulas and sinuses mainly affecting the terminal ileum of the small intestine, parts of the colon and the rectum. The symptoms involve anal bleeding, abdominal pain, weight loss, diarrhea with mucous and/or pus, fever, nausea, lethargy and loss of appetite (Rayhorn & Rayhorn, 2002). UC and CD share very similar extra-intestinal manifestations including mouth ulcers, involvement of the skin, joints, liver and uveal tracts (McCay *et al.,* 1997). Moreover, when the colon alone is involved a clinical distinction between UC and CD may be difficult (MacDonald *et al.,* 2000).

Immune phenomena linked to the etiopathogenesis of inflammatory bowel disease have also been extensively investigated, and findings have underscored the differences between the responses of normal and affected individuals. For example, it is well known that the distribution of specific cytokine-producing lymphocytes differs between normal persons and patients with CD or UC (Ardizzone & Porro, 2002). However, much less is known about how microbial agents affect the disease process. Speculations that viruses may be involved in the pathogenesis of inflammatory bowel disease have been advanced for some time because of the clinical association of respiratory virus infections with subsequent disease flares (Blumberg, 1990). With inflammatory bowel disease, the mucosal immune cell population increases dramatically, and the infiltrate is predominantly comprised of mononuclear leukocytes (Lakatos, 2000) (Fig. 1-9). Furthermore, the muscularis mucosae cell layer thickens to nearly 300 times its normal depth secondary to smooth muscle cell hyperplasia and extracellular matrix deposition. These features suggest that interactions between

recruited leukocytes and mesenchymal smooth muscle cells are important in the development and propagation of inflammatory bowel disease.

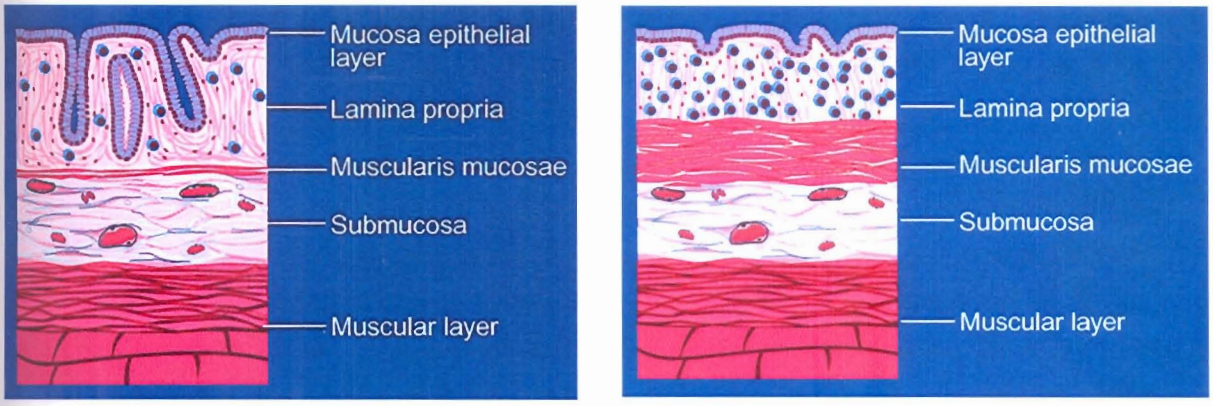


Figure 1-9 Morphology of normal versus IBD mucosa. IBD induces changes in all layers of the gut wall from the epithelial mucosal layer to the submucosa.

1.9.2 Peyer's patches

Peyer's patches are named after Johann Konrad Peyer, who more than 300 years ago described aggregation of lymphoid tissue in the wall of the small intestine (Mac Donald, & Spencer, 1990) (Fig. 1-10). Peyer's patches anatomically are termed as "folliculi lymphatici aggregati". They form the bulk of the organized lymphoid tissue in the gut-associated lymphoid tissue and are mainly localized opposite the mesentery. At least two lymphoid follicles are required to form a Peyer's patch (Mac Donald & Spencer, 1990).

In humans, Peyer's patches are developed well before birth and about 50 can be identified 6 months of gestation on (McNicol *et al.*, 1985). In adolescence, more than 240 patches can be identified and by the age of 90, still about 50 of them can be localized which means that there is a steady decrease occurring with age (MacDonald & Carter, 1982).
Peyer's patches can be found in the ileum, the duodenum and the jejunum. The patches contain B cell follicles each of which comprises a germinal center and mantle zone. The germinal center contains dividing B cells together with follicular dendritic cells and macrophages. A mantle of small lymphocytes with the phenotype $CD22^+$, $CD5^-$, IgM^+, IgD^+ surrounds the germinal center, which in turn, is surrounded by B cells with irregular nuclear morphology similar to centrocytes (small cleaved cells in the germinal center). These centrocyte-like cells are phenotypically $CD22^+$, $CD5^-$, IgM^+/IgM^-, IgD^+ (Spencer *et al.*, 1986). The Peyer's patch T-cell zone containing the high endothelial venules is situated on the serosal aspect of the follicle. The T-cell ratio is approximately 3:1 helper/inducer: suppressor/cytotoxic (MacDonald & Monteleone, 2001). However, the T cells within the germinal center are exclusively of helper/inducer phenotype. In the dome region of the Peyer's patches, a few plasma cells are also present, while the macrophages are

concentrated in the T cell zone, the germinal center and beneath the epithelium (MacDonald & Carter, 1982).

Peyer's patches are not only aggregations of lymphoid follicles. They consist of definite compartments with a number of basic structural elements and specific compositions of lymphocyte subsets and accessory cells. Along with other gut-associated lymphoid tissue (GALT) sites, they can be divided into four compartments. A Peyer's patch is a site of continuous cell proliferation and cell death in the gut. The lymphoid follicles are localized below the muscularis mucosae. Proliferating B-lymphocytes form germinal centers that also contain macrophages that phagocytose remnants of lymphocyte nuclei (Spencer *et al.,* 1987).

Cell death occurs in sheep ileal Peyer's patches and is believed to be important for B cell selection. A small corona of lymphocytes surrounds the follicles. In many of the lymphocyte cell surface, IgM and IgD antibodies are expressed. The inter-follicular area is characterized by high endothelial venules (HEV) that are surrounded by densely packed lymphocytes, most of which are T cells. These HEV serve as a pathway for the lymphocytes to enter Peyer's patches. This compartment is also called the traffic area of Peyer's patches. Lymphocytes are also found on top of the follicle toward the gut lumen, in the dome area. Specialized M cells in the follicle-associated epithelium of Peyer's patches represent an intimate interface between luminal antigens and gut-associated lymphoid tissue (Farstad *et al.,* 1994). M cells form pockets that contain clusters of leukocytes probably involved in the first encounter with antigens from the gut lumen (Braegger *et al.,* 1992).

In untreated human tissue, Peyer's patches, are macroscopically invisible and they are distributed in small clusters throughout the gastrointestinal tract, being most concentrated in the terminal ileum. The Peyer's patches of mice and rats however, are found in approximately 10 clusters along the length of the ileum. Their size varies in humans, while in rodents they consist of only a few follicles. It is believed now that the size and the location of each Peyer's patch are genetically determined. It is also believed that the transposition of gut segments or the removal of a large portion of Peyer's patches does not influence the number of the remaining patches (McDonald *et al.,* 1987 and references therein).

Lymphoid sinuses are situated around the follicles as shown by scanning electron microscopy of sheep ileal Peyer's patches. These are connected to septal vessels, lymph vessels and to the deep mucosal network that contains valves. In general, the function of Peyer's patches as antigenic sampling sites involve the complex interplay of a variety of mechanisms that aim to recognize luminal antigens, induce an immunological response to decrease the incidence of antigen translocation across the mucosal epithelium (Finke and Kraehenbuhl, 2001 and references therein).

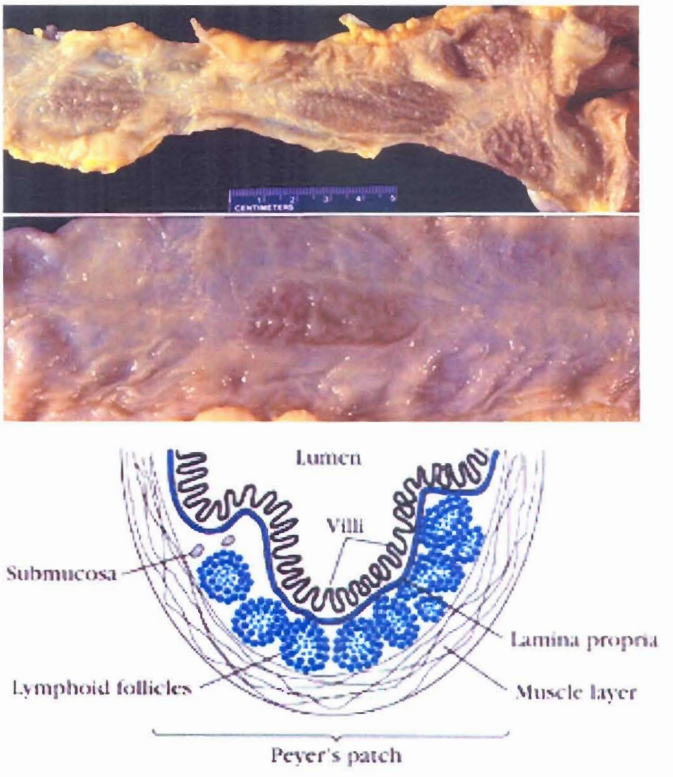


Figure 1-10 Photograph of Peyer's patches and schematic representation of their structure. The diagram is cross sectional of the mucous membrane lining the intestine showing nodule of lymphoid follicles constituting a Peyer's patch in the submucosa. The intestinal lamina propria contains loose clusters of lymphoid cells and diffuse follicles.

1.9.3 The role of NOD proteins and NF-κB in IBD

The role of genetic factors in the pathogenesis of IBD has always been suggested since disease susceptibility has been associated with genetic markers for particular subsets of IBD patients. In the last few years studies using genome-wide screening have provided the first link between NOD2 mutations and the clinical characterization of Crohn's disease (Cuthbert et al., 2002). NOD proteins are cytosolic receptors for bacterial signals and studies have demonstrated that mutant forms or single nuclear polymorphisms of NOD2 are highly associated with Crohn's disease in that defects of the protein cause or contribute to the development of the disease. NOD2 is expressed in monocytes and activates nuclear factor kB (NF-κB).

NOD1 and NOD2 contain nucleotide-binding oligomerisation domains (NOD) and are members of a family of homologous intracellular proteins that are composed of an N-terminal caspase recruitment domain (CARD), a central nucleotide binding domain (NBD) and a C-terminal regulatory domain which is composed of a leucine-rich repeat sequence (Inohara &

Gunez, 2001). Apaf-1 and other proteins that regulate apoptosis through the CARD domains are included in this family. NOD1 and NOD2 act in a similar fashion in that they both self associate, oligomerise and then bind to Rip2 through CARD-CARD interactions which then interacts with IKKγ, leading to the activation of NF-κB in a caspase independent manner (Inohara *et al.,* 1999). Although neither NOD1 Or NOD2 induce apoptosis on their own, both promote caspase-9 induced apoptosis although the exact mechanism by which they do so in currently unknown. Whilst NOD1 is expressed in adult heart, placenta, lung, liver, kidney, spleen, thymus and ovaries (Inohara *et al.,* 1999), NOD2 is only expressed in monocytes (Ogura *et al.,* 2001).

NOD2 is highly homologous to a class of plant disease resistance gene products and its leucine rich repeat region is homologous to that of Toll-like receptors, important for the recognition of microbial products (Hugot J.P. *et al.,* 2001) Unlike Toll-like receptors however, NOD2 does not possess a Toll-IL-1 receptor domain and as previously stated is cytosolic. The association between mutant forms of NOD2 and CD must therefore relate to one (or both) of the similarities with other inflammatory cell signalling pathways mentioned above.

Study of the location and degree of activation of NF-κB within the bowel wall is relevant to the understanding of the mechanisms of inflammation in Crohn's disease and Ulcerative colitis (Ellis et al., 1998). It has recently been shown that corticosteroids exert their diverse anti-inflammatory effects by inhibition of activation of NF-κB (Adcock, 2001) and this occurs post NF-κB nuclear translocation. This may account for the lack of corticosteroids effect seen by Ellis and co-workers on NF-κB nuclear localization.

The perpetuated activation of NF-κB in patients with active IBD suggests the regulation of NF-κB activity as a very attractive target for therapeutic intervention. Such strategies include antioxidants, proteasome inhibitors, inhibition of NF-κB by adenoviral IκBα expression vectors and antisense DNA targeting of the transcription factor (Neurath et al., 1998). These approaches together with the latest association of NF-κB with the discovery of the NOD proteins will hopefully permit the design of new treatment strategies for chronic intestinal inflammation.

1.10 A *Priori Rationale*

Inflammation and inflammatory responses have been studied by many groups, but generally in certain cell models, mainly focusing on the cytokines produced by these cells following inflammatory stimuli. In this thesis, the aim is to investigate down-stream events activated by cytokines. It is currently unclear how histone acetylation and cell death (apoptosis/necrosis) are regulated during progression of inflammation or during the immunologic response of the animal to inflammation.

Current research in asthma showed increased histone acetylation, in epithelial cells (A549 lung epithelial cells) and macrophages (U937 cell), stimulated with inflammatory mediators, suggesting that ongoing gene transcription occurs during the early inflammatory responses of the cells. The reports in asthma, another inflammatory disease, provided some clues into the possible involvement of histone acetylation in the pathogenesis of IBD. However, it is not clear which of the four histones are playing the most significant role in inflammatory gene transcription. Since the pathogenesis and progress of IBD is not well understood, this thesis aims to investigate whether specific histones are being acetylated during the course of the disease. Prior to *in vivo*, *in vitro* studies are performed to assess whether acetylation of histones depends on certain inflammatory mediators and if so, whether all histones are acetylated in a similar fashion. The ultimate goal is to identify whether specific patterns of histone acetylation can be identified in IBD following *in vitro* studies in macrophages and T-lymphocytes.

Studies are required to reveal the functional outcomes resulting from the acetylation of the genes being transcribed during histone modifications. Furthermore, it should be evaluated whether distinct inflammatory markers, such as IL-8, are produced as a result of histone acetylation of specific histone lysine residues and whether the latter are acetylated during inflammatory cell responses. Glucocorticoids, acting through glucocorticoid receptors, are thought to inhibit histone acetylation. Being one of the most effective treatments for IBD, the aim is to provide further evidence on how glucocorticoids work and whether their action is associated with histone acetylation in the cell lines studied and in IBD. It should also be assessed whether cell death, as part of the functional response to inflammation, is encouraged (or discouraged) and whether it correlates with post transcriptional modifications of histones.

As both macrophages and lymphocytes play a pivotal role in the immune responses during inflammation, investigation of the above questions is performed in the monocytic cell line U937 and in the T-cell lines Jurkat and HUT-78. Both cell types are known as two of the major inflammatory cells involved in the pathogenesis of IBD. The role of the macrophages is to eliminate apoptotic and necrotic cells and the role of the T-cells is to orchestrate the immune response.

Finally, this project investigates whether the *in vitro* findings regarding histone acetylation and inflammatory gene expression in the monocytic and T-cell lines correlate with *in vivo* processes occurring in IBD. Specific interest is given to the acetylation of histones in Peyer's patches since, although, these organs have been known to play a regulatory role in the activation of IBD, their detailed mechanism of action has not yet been identified. In summary, this project attempts to investigate the role of histone acetylation in the process of inflammation, in the activation of cells and in disease.

Chapter 2

Materials and Methods

2.1 Cell culture

All cell lines used for this work were acquired as live cultures from the European Collection of Cell Cultures (ECACC, Salisbury, UK). After receipt, all work carried out on them was completed in sterile conditions inside a class II hood using sterile, pyrogen free reagents, purchased (unless stated otherwise) from Sigma (Poole, UK), and sterile disposable plastic wear purchased from Helena Bioscience (Helena Bioscience, Sunderland, UK) (unless stated otherwise). The cells were incubated in a cell culture cabinet (BOC, Crawley, UK) at 37°C in a 5% CO_2 humidified atmosphere. Cells were culture in disposable 25 cm^2 or 50 cm^2 cell culture flasks, in 7 or 14 ml of culture medium, unless stated otherwise.

The cell lines used were U937 cells, HUT-78 cells and Jurkat E6.1 cells. All cell lines used were grown in suspension. U937 cells are a human Caucasian histiocytic lymphoma cell line. HUT-78 cells are a human T cell lymphoma cell line. Finally, Jurkat E6.1 cells are a human leukaemic T cell lymphoblast cell line (for further information on all cell lines used see appendix).

2.1.1 U937 cell culture

U937 cells were grown as per instructions from ECACC, in Roswell Park Memorial Institute (RPMI) 1640 containing 10% (v/v) Foetal Calf Serum (FCS) (Helena Bioscience, Sunderland, UK) and 2 mM L-glutamine until approximately 90% confluence had been attained. Upon reaching confluence, cells were passaged by centrifuging the cells for 5 min at 700 rpm at 37°C, disposing of the culture medium and washing the cells, followed by centrifugation under the same conditions, twice with Hank's Balanced Salt solution (HBSS). Finally, the cells were resuspended in fresh culture medium to a 3x the volume they were cultured in (unless otherwise stated: 3x7=21 ml). The diluted suspension was divided into fresh 25 cm^2 flasks at 7 ml per flask and incubated as described above. The cell medium was replaced once every two days during incubation. Cells would take approximately 5 days to reach confluence after each passage.

All U937 cells used for experiments were maintained within 10 passages of one another. For stimulation, cells were grown to the appropriate degree of confluence (depending on the experiment) in 25 cm^2 flasks, the medium was removed, the cells were washed twice with HBSS, as described above, and the cells were further incubated with FCS (serum) free medium (containing 0.25% FCS) for 48-72 hrs to achieve synchronisation. Following serum starvation, the medium was removed and replaced with fresh serum free medium containing the appropriate stimulant. The cells were incubated for an appropriate time period depending on the experiment.

2.1.2 HUT-78 cell culture

HUT-78 cells were grown (following the ECACC instructions) in the same manner as described for the U937 cells. HUT-78 cells usually required 7 days to reach confluence. Stimulation procedures were the same as described for the U937 cells.

2.1.3 Jurkat E6.1 cells

Jurkat cells were grown (following the ECACC instructions) in the same way as U937 cells. Jurkat cells usually required 7 days to reach confluence. Stimulation procedures were the same as described for the U937 cells.

2.1.4 Cell treatments

Following serum starvation all three cells lines were treated similarly.
The cell density used for each experiment was 10^6 cells/ml. For Western blotting (see section 2.6) and Chromatin IP (see section 2.8) experiments, 10^7 cells were used. Cells were stimulated as follows:
LPS and TNF-α were used at concentrations of 10ng/ml for 1 hour in all three cell lines.
TSA was used at concentrations of 1ng/ml in U937 cells and 10ng/ml in HUT-78 and Jurkat cells respectively and was added to the medium 30 min prior to addition of LPS or TNF-α.
Dexamethasone and Triamcinolone Acetonide were used at concentration of 10^{-8}M and 10^{-10}M respectively in all three cell lines. The steroids were added simultaneously with the addition of TSA and were incubated in the medium for 30 min prior to addition of the inflammatory stimuli (LPS and TNF-α).
For ELISA purposes (see section 2.7) the cells were incubated to stimuli for 17-24 hrs.

2.2 Collection of animal tissue

The 2,4,-trinitrobenzene sulfonic acid (TNBS) model of intestinal inflammation, based on that of Morris *et al.*, (1989), was used. All handling of live animals was completed off site, by Tim Bourne (at Celltech Chiroscience, Slough), with the appropriate licences and ethical permission already having been granted. Eighteen male Sprague-Dawley rats (Charles River, UK) with a median weight of 337.5 g were fed with basic rat chow. The animals were divided into two groups each consisting of nine animals. The first group was treated intrarectally with 30 mg of TNBS in 30% w/v ethanol, using a syringe and tube, on day zero. At the same time and using the same technique, the second, Sham operated (control), was treated with 30% ethanol alone. The animals were sacrificed on day seven and tissue was resected from two separate areas of the large intestine- two centimetres distal to the caecum (proximal colon) and three centimetres proximal to the anus (distal colon). Within the TNBS treated group these two areas constituted

the inflamed (distal) and non-inflamed (proximal) regions of the colon. All samples were snap frozen in liquid nitrogen immediately after excision. Tissue was subsequently maintained in a frozen state at –80°C until use.

2.3 Collection of human tissue

Collection of human intestinal tissue was based on a technique previously described (Ellis *et al.*, 1998). Human tissue was obtained during routine surgery at St. Thomas' hospital, or routine endoscopy procedures at St. Thomas' hospital or the Middlesex hospital. In Crohn's disease patients, biopsies were collected from either macroscopically inflamed or non-inflamed regions of the large and small intestine. In control patients, biopsies were collected at least 4 cm from macroscopic disease. Upon collection, tissue was immediately snap frozen in liquid nitrogen and then stored until use in liquid nitrogen or at –80°C. Patient information, supplied by the surgeons, included sex, age, disease type and location as well as treatment regime. None of the patients were smokers.

2.4 Preparation of tissue sections

For microscope analysis, the biopsies were fixed in 4% (w/v) paraformaldehyde/PBS for 3 h at 4°C, cryoprotected in sterile 4%(w/v) sucrose/PBS at 4°C overnight, mounted in OCT mountant (BDH, Atherstone, UK) on labeled cork discs and frozen in liquid nitrogen-cooled isopentane. Tissue samples were stored at -70°C. The sections were cut in a cryostate in the appropriate depth, generally 5µm thick, and were positioned in electrostatically charged slides. The slides were allowed to dry completely in air. Finally, the slides were covered in foil (shiny side touching the sections) and stored at -20°C.

2.5 The BioRad™ Bradford method for protein quantitation

The BioRad™ Bradford assay is a rapid and accurate method for the estimation of protein concentrations (between 1-10 µg), and is an adaptation of that described by Bradford (Bradford, 1976). The Bradford assay relies on the binding of the dye Coomassie blue G250 to protein. The anionic form of the dye, which binds to protein, has maximum absorbance at a wavelength of 595 nm. Thus, the amount of dye bound to protein can be quantified by measuring the absorbance of the dye/protein solution at 595 nm. The advantage of the BioRad assay compared to the Bradford assay is that the BioRad assay is compatible with a wider range of detergents and is therefore compatible with the lysis buffers used herein.

The protein assay detergent was prepared by diluting the BioRad dye 1/5 with deionised water (diH_2O), as per instructions. For all estimates of protein content, a standard curve (0-10 µg protein, 2 µg increments) was used, in duplicate using a 96 well microtitre plate. The protein

standard was a 1 mg/ml solution of BSA in dH_2O (stored at 20°C). Protein extracts were added to separate wells and 200µl of diluted dye was added to all wells using a multichannel pipette. Plates were incubated for five min in darkness at room temperature before being read as 595 nm using an ELISA plate spectrophotometer (Rosys Anthos HIII; Anthos Labtech Instruments, Salzburg, Austria). The protein concentration of the samples was then read off the standard curve.

2.6 Western Blotting

Western blotting allows the semi-quantitive measurement of a specific protein within a given sample (Sambrook & Gething, 1989). The sample proteins are separated by gel electrophoresis and transferred to a more stable matrix, in this case a nitrocellulose membrane, which is incubated with an antibody raised against the antigen of interest. The first incubation is followed by a second, using an antibody raised against the first antibody and which is conjugated to an enzyme. This enzyme is then allowed to react with a chemi-luminescent substrate, which causes the evolution of blue light wherever the first and the second antibodies have bound, forming distinctive bands. These bands of blue light can be captured on film and after X-ray development of the film; the presence of the target proteins can be quantified by computer analysis of the image density of the bands captured on film. The protocol used here was based on that of Sambrook (Sambrook *et al.*, 1989).

2.6.1 Cell harvesting

The stimulated cells (methods described is sections 2.1.2, 2.1.3 and 2.1.4) were washed (centrifuged at 12.000g for 5 min) in ice cold phosphate buffered saline pH 7.4 (PBS). The supernatant was removed and the pelleted cells underwent nuclear/cytosolic fractionation or histone isolation depending on the proteins that required measurement.

2.6.2 Nuclear/cytosolic fractionation

The nuclear/cytosolic fractionation method was similar to those previously described (Latchman, 1992).

The cell pellet was resuspended (10µl / $5x10^6$ cells, 70µl / $25cm^2$ flask) in a solution that consisted of 10mM N-(2-hydroxyethyl)piperazine-N'-(2-ethane)sulfonic acid (HEPES) pH 7.9, 1.5mM $MgCl_2$, 10mM KCl (Merck, Poole, UK), 0.5mM trans-1,2-dithiane-4,5-diol (DTT) and 0.25% v/v nonident P-40 (NP-40). The solution was allowed to incubate for 20 min on ice during which time lysis of the plasma membrane occurred. To retrieve the cytosolic fraction the samples were centrifuged for 15 sec at 12.000g to pellet the nuclei. The supernatant (cytosolic fraction) was removed and stored subsequently at -20°C.

The remaining nuclei pellet was resuspended in 15 µl of a solution consisting of 20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 0.42M NaCl, 0.5mM DTT, 25% glycerol and 0.5mM phenyl methanesulfonyl fluoride (PMSF) in diH_2O. The cell suspension was allowed to incubate for 20 min on ice and was then centrifuged at 12.000g for 10 min in a microcentrifuge (Sanyo, MSE II, CN Biosciences, Nottingham, UK) to pellet the cellular debris. The supernatant (nuclear fraction) was removed to a fresh eppendorf. The fresh nuclear fraction was diluted in 75µl of a third solution consisting of 20mM HEPES pH 7.9, 50mM KCl, 0.5mM Dtt, 0.2mM ethylenediamine tetraacetic acid (EDTA) and 0.5mM PMSF. Prior to storing the nuclear and cytosolic fractions at -20°C, a BioRad™ protein assay (see 2.4) was performed to determine sample protein concentrations.

2.6.3 Histone isolation

Note: All procedures were carried out on ice

The cells were cultured in 6 well plates until they reached a subconfluent state (40%-60% confluent). The medium was changed to serum (FCS) free 24-48 hours prior treatment. The cells were stimulated with the appropriate concentration of the stimulant for a required established time. Cell suspensions were washed twice with cold HBSS. 500µl of cold lysis buffer consisting of 10 mM Tris-HCl pH 6.5, 50mM sodium bisulfate, 10mM $MgCl_2$, 8.6% sucrose, 1% Triton X-100, one protease cocktail tablet (Complete Mini; Boehringer Manheim, Lewes, UK) and 100 ng/ml TSA, were added in each well. The cells were harvested into eppendorf tubes and allowed to stand on ice for 10 min. The cells were washed three times with 200µl of lysis buffer (centrifuged at 12000 rpm, for 5 minutes, at 4°C). Cell suspensions were washed once with nuclear washing buffer and 150µl of an acid mixture consisting of 0.2M HCl and 0.4 M H_2SO_4 were added to the pellet and sonicated for 3 seconds (making sure that the cells were kept on ice while sonicating). The solution was allowed to stand on 4°C for at least 2 hours. The cell suspension was centrifuged at 12000 rpm, for 10 minutes, at 4°C. 120µl of the supernatant were aliquoted in 1ml of chilled acetone. Allowing the samples to stand in the freezer overnight precipitated histones. The samples were centrifuged at 12000 rpm, for 10 minutes, at 4°C. The pellets were washed twice with chilled acetone and the pellets were allowed to air dry. The dry pellets were diluted in deionised water (usually 20-30µl depending on the concentration of histones isolated). Finally, a Bradford (Biorad) assay was performed to determine the final protein concentration.

2.6.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) was used to separate the sample proteins. An SDS-PAGE gel is a vertical gel that contains two distinct layers of gel; a resolving gel at the bottom and a stacking gel at the top. The gels were poured between

two glasses (10x10 cm^2; Novex, Hamburg, Germany) separated by one mm thick spacers which had previously been thoroughly cleaned with 70% IMS. The resolving gel consisted of 3.55ml diH_2O, 2.5ml Acrylamide/Bisacrylamide (40% 19:1; Anachem), 3.75ml of 1.5M Tris buffer pH8, 100µl SDS (10% w/v), 100µl ammonium persulfate (APS) (10% w/v) and 10µl 1,2-Bis(dimethylamino)ethane (TEMED). This was poured into the bottom of the plate assembly. Water saturated isobutanol (isobutanol and 10% v/v diH_2O) was added on top of the resolving gel whilst it set, creating an airtight seal (O_2 is inhibitory to gel polymerization) whilst at the same time smoothing the surface of the gel.

Once the resolving gel was set, the isobutanol was poured and the gel was washed with diH_2O to completely remove any isobutanol. The stacking gel that consisted of 3.64ml diH_2O, 0.63ml Acrylamide/Bisacrylamide (40% 19:1), 0.63ml of 1M Tris buffer pH 6.8, 50µl SDS (10% w/v), 50µl APS (10% w/v) and 5µl TEMED, was poured on top of the resolving gel. A ten-tooth gel comb was quickly placed on top of each gel thereby creating the sample wells. Once the gel had set the gel combs were removed and the gel (still supported by the glass plates) was placed into an electrophoresis tank (Novex), filled with running buffer (25mM Tris base, 250mM Glycine and 0.1% w/v SDS in diH_2O). The tank was filled so that the level of the buffer completely covered the gel(s).

With the gels prepared, four times required concentration (4x) loading buffer was added to all samples in the correct volume to yield the correct working concentration and the samples were boiled for five minutes. The 4X loading buffer consisted of 1M Tris-HCl (pH 6.8), 8% w/v SDS, 40% v/v Glycerol, 0.04% w/v Bromophenol Blue and 4% v/v 2 Mercaptoethanol. Subsequently, the samples were loaded into each well, with one well being set aside for the addition of 5µl of molecular weight markers (Rainbow Markers; Amersham, Little Chalfont, UK). The gels were run at 40mA for approximately 90min until all the samples had migrated to the base of the gel. With this complete the proteins in the gel were transferred to a nitrocellulose membrane by electroblotting.

2.6.5 Electroblotting

Electroblotting buffer was prepared consisting of 700ml diH_2O, 100ml of 10x-transfer buffer (200mM Tris-base and 1.92M Glycine in diH_2O), and 200ml Methanol (AnalaR grade; Merck, Harlow, UK). Gel sized (10cm^2) blotting paper and nitrocellulose membrane (ECL Hybond; Amersham, Cardiff, UK) were cut and soaked in transfer buffer for 5min. The SDS-PAGE gel was removed from the supporting glass plates and ECL membrane was placed on top of the gel. The gel and the membrane were sandwiched between blotting paper and foam pads placed either side. This construction was placed inside the electroblotting module (Novex, Hamburg, Germany) orientated so that the gel was on the anode side and the membrane on the cathode. After filling

the module with electroblotting buffer, a 200mA current was passed through the module for 45min.

2.6.6 Immunoblotting

With electroblotting complete the membrane was recovered and protein transfer was verified by staining with Ponceau S solution (Sigma) for approximately 5min. Ponceau S stains proteins in red allowing verification of even protein loading and transfer. Subsequently, the membrane was blocked in PBS containing 0.05% v/v Tween-20 (PBS-T) and 5% w/v non-fat powdered milk, for 1h at room temperature (incubation could be extended to overnight at 4°C).

Once blocked, the membrane was washed twice for 5min in PBS-T and finally placed in the primary (1°) antibody solution. The primary antibodies (Serotec, Oxford, UK) that had been raised to specific peptide were; polyclonal rabbit anti-histone H4 (acetylated) (1/600 dilution in blocking buffer), rabbit anti-histone H4 (Ac5) (1/600 dilution), rabbit anti-histone H4 (Ac8) (1/600 dilution), rabbit anti-histone H4 (Ac12) (1/600 dilution), rabbit anti-histone H4 (Ac16) (1/600 dilution), rabbit anti-histone H3 (1/600 dilution), polyclonal goat Annexin V (Santa Cruz Biotechnology, Loughborough, UK) (1/400 dilution), rabbit polyclonal Caspase-3 (Santa Cruz) (1/400 dilution) and rabbit polyclonal Bcl-2 (Santa Cruz) (1/400 dilution). The primary antibody incubation was carried out at room temperature with agitation for 90min.

Subsequently the membranes underwent by two 5min washes in PBS-T followed by incubation with the secondary (2°) antibody at 1/4000 dilution for 60min. The secondary antibody was goat polyclonal anti-rabbit, horseradish peroxidase (HRP) conjugate (Dako, Cambridge, UK), for all the primary antibodies except the goat anti-Annexin V antibody. For this a mouse anti-goat was used followed by a wash sequence and a further incubation with the rabbit anti-goat-HRP conjugate. With the final antibody incubation complete, the membranes were subjected to three 5min washes in PBS-T. The membranes were blotted dry and covered with ECL substrate for 1min in darkness followed immediately by the exposure of the membrane to a blue light sensitive film (X-ograph). The film was developed by incubation in GBX developer for 5min followed by a 30 second wash in tap water, then 8min incubation in GBX fixative with a final wash in tap water. All manipulations of the developed film were carried out in a photographic dark room. The films were analysed using digital optical densitometry software (GelPro, MediaCybernetics, Silver Spring, USA) on a computer linked to a flatbed scanner configured to scan using transmitted light.

2.7 Interleukin-8 ELISA

To determine cell culture medium concentrations of interleukin-8 (IL-8) an enzyme linked immuno-sorbent assay (ELISA) kit was used (Pharmingen, Oxford, UK). The kit was used

according to manufacturers instructions. Briefly, all reagents were brought to room temperature before use. The ELISA standards were reconstituted in 1ml of diH_2O and allowed to equilibrate for 15min. Aliquots of these standards were stored at -70°C. To prepare a standard curve, serial dilutions of the standards that ranged from 200-31 pg/ml were made in assay diluent, consisting of PBS (pH 7) and FCS. Assay diluent alone served as the control.

The ELISAs were carried out in 96 well microtitre plates that were coated with the IL-8 capture antibody. The antibody was diluted (according to kit specific ratios) in coating buffer consisting of 0.1M Carbonate buffer (8.4gr $NaHCO_3$ and Na_2CO_3 made up into 1ltr of diH_2O) pH 9.5. 100µl of this solution were added per well to the microtitre plate, the plate sealed and incubated overnight at 4°C. After coating, the plates were washed three times with 300µl wash buffer (PBS-T) per well per wash. Plates were blocked by the addition of 200µl assay diluent per well and incubated at room temperature for one hour. After two washes, as described above, 100µl of standard or sample were added to the appropriate wells and the plate sealed and incubated at room temperature for a further two hours. Subsequently, the plates were washed five times and 100µl per well of detector antibody diluted as per kit instructions was added. Again, the plate was sealed, incubated at room temperature for one hour and then washed seven times. Finally, 100µl per well of TMB substrate solution (Pharmingen, Oxford, UK) were added and the plates were incubated for 30min at room temperature in darkness. The absorbance of each well was then read at 450 nm (with subtraction of 570nm from 450nm) and the concentration of the samples determined by plotting the unknowns from the standard curve.

2.8 Chromatin Immunoprecipitation assay

The protocol used was based on a method described by Ito *et al.* (2000).

Cells were treated with LPS (10ng/ml) and TNF-α (10ng/ml) in the presence of dexamethasone (10^{-8}M) and triamcinolone acetonide (10^{-10}M) as described above. After a 4-h incubation, protein-DNA complexes were fixed by formaldehyde (1%, final concentration) and treated as previously described (Ito *et al.*, 2000). Cells were resuspended in 200µl of SDS lysis buffer (50 mM Tris, pH 8.1; 1% SDS; 5mM EDTA; complete proteinase inhibitor cocktail) and sonicated (three 10s pulses) on ice. Sonicated samples were centrifuged to spin down cell debris, and the soluble chromatin was immunoprecipitated using Pan-AcH4 antibody (20µl) (Serotec) and salmon sperm DNA agarose A slurry (25µl) (Upstate Biotechnology, Buckingham, United Kingdom). Protein-bound immunoprecipitated DNA was washed with LiCl wash buffer and Tris-EDTA (TE), and immune complexes were eluted by adding elution buffer (1% SDS, 0.1M $NaHCO_3$). The elution was treated successively for 4h at 65°C in 200mM NaCl-1% SDS to reverse cross-links and then incubated for 1h at 45°C with 70µg of proteinase K per ml, DNA extracted with phenol-chloroform, precipitated with ethanol/0.3 M NaHCOOH/20µg of glycogen, and resuspended in 50µl of TE. Semi-quantitative PCR was performed with 10µl of DNA sample for 30 cycles. Primer

pairs to detect NF-κB binding site on the IL-8 promoter region were as follows: forward, 5-AACAGTGGCTGAACCAGAG-3; and reverse, 5-AGGAGGGCTTCAATAGAGG -3. PCR products were resolved by using 3% agarose gel and visualized with ethidium bromide.

2.9 Immunocytochemistry/Immunohistochemistry

The Immunocytochemical and immunohistochemical techniques used varied according to the target proteins in question.

2.9.1 Preparation of cytospins

Cytospins were prepared from cell suspensions at 0.5 x 10^6 cells/slide coated with 0.1% poly-L-lysine (BDH, Atherstone, UK). The purpose of slide treatment was mainly to minimize non-specific attachment of radio-labeled probes to slides and maximize section of cell retention on the slides through the various treatments involved in immunocytochemical procedures.

2.9.2 Protocol for rabbit polyclonal antibodies

Note: Protocol used for Immunocytochemistry and immunohistochemistry using anti-histone antibodies

The slides were fixed for 10min in chilled acetone and allowed to air dry for a further 10mins. They were then incubated for 1hr in Quench Endogenous Peroxidase (3% H_2O_2 in PBS containing 0.02% Sodium Azide). Subsequently, they were washed 3x5mins in PBS and were pre-blocked with 5% normal swine serum (NSS, depending on the secondary antibody) for 20mins. The slides were incubated with the primary antibody diluted in PBS. The time of the incubation as well as the dilutions depended on the recommendation of the manufacturer. They were then washed twice for 5mins in PBS and the secondary antibody was added (the time of the incubation as well as the dilutions depended on the recommendation of the manufacturer). Slides were again washed 2x5mins in PBS. Avidin-HRP was added in a dilution 1/500 in PBS for 45mins before two final washes in PBS for 5mins. DAB (one tablet diluted in 5ml of PBS) was added and allowed to incubate until colour had developed. The slides were washed for 5mins in distilled water and were counterstained in 20% Harris haematoxylin for 10sec. Finally, they were air-dried and mounted in DPX.

2.9.3 Protocol for glucocorticoid receptors and goat polyclonal antibodies

Note: Protocol most appropriate for Bcl-2 and Annexin-V antibodies

The slides were allowed to stand for 10mins in 2% formaldehyde in PBS followed by 10mins in 0.5% NP-40 in PBS. Slides were subsequently incubated in 100mM glycine in PBS for 5mins. Glycine solution was discarded and the slides were immersed for 10mins in PBG (0.5% BSA, 0.1% gelatin in PBS). The primary antibody (4°C) was diluted in PBG and added to the slides. Time and dilution depended on recommendation by the manufacturer. The slides were washed 4x5mins in PBG. The slides were allowed to stand on secondary antibody (room temperature) diluted in PBG. Time and dilution depended on recommendation by the manufacturer. The slides were washed 4x5mins in PBG. Slides were incubated for 1hr in FITC-Streptavidin in a dilution 1/100 in PBG. The slides were washed 4x5mins in PBG. The slides were washed for 10mins in PBS. The slides were counterstained in 20% Harris haematoxylin. Finally, slides were air dried and mounted in Citifluor (DPX was used if immunofluorescence was not required).

2.10 HDAC fluorescent activity assay

An HDAC fluorescent activity assay/ Drug discovery kit was used to measure histone deacetylase (HDAC) activity in cell nuclear extracts (Biomol, Exeter, UK).

In a 96 well plate, assay buffer (25mM Tris/Cl, pH 8.0, 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$) and diluted TSA were added to appropriate wells. Diluted (supplied) HeLa extracts and U937, HUT-78 and Jurkat cell extracts were added to the rest of the wells. Diluted Fluor de Lys™ extract (supplied) was allowed to equilibrate in the microtiter plate to assay temperature (25°C). The HDAC reaction was initiated by adding diluted substrate (25µl) to each well and mixing thoroughly. The HDAC reaction was allowed to proceed for 1h and then stoped by the addition of Fluor de Lys™ developer (50µl). The plate was incubated at room temperature for 15 min. The samples were read in a microtiter-plate reading fluorimeter capable of excitation at a wavelength in the range 350-380nm and detection of emitted light in the range 440-4660nm.

2.11 Detection of cell viability and apoptosis

2.11.1 MTT cell viability assay

The 3-[4,5-Dimethylthiazol-2yl]-2,5-dephenyletetrazolium (MTT) viability assay, based on that of Denizot & Lang (1986) and Carmichael *et al.,* (1987) was used for the purpose of assessing U937, HUT-78 and Jurkat cell viability in 96 well plates. The cells grown into 96 well plates and stimulated (section 2.1.2). At the end of the stimulation period the culture medium was removed and replaced with 25µl of MTT solution consisting of 5mg/ml MTT in phenol free RPMI-1640 medium. After a two hour incubation period, the MTT solution was removed leaving behind the insoluble MTT product. This was re-dissolved in a SDS-isopropanol mix, consisting of 1% w/v SDS and 5% v/v distilled water in absolute propyl alcohol. 100µl of this solution were added per well and mixed by repeated aspiration through a pipette. The absorbance of the solution was

measured at 570nm with background subtraction at 620nm using an ELISA plate spectrophotometer (Rosys Anthos HIII, Salzburg, Austria).

2.11.2 Hoechst 33342 staining for apoptosis detection

The cells were harvested and to 1ml of cell suspension (10^6cells), 100µl of 10µg/ml Hoechst 33342 solution were added. The cell suspension was incubated for 5min at room temperature. 100µl of the dead cell discriminator propidium iodide (50µg/ml) were added. The cells were viewed under a fluorescent microscope and cells stained with the dye were counted using a cytometer.

2.11.3 DNA laddering

A DNA laddering kit was used to assay cells for apoptosis by detecting internucleosomal DNA fragmentation and displaying DNA laddering (R&D Systems, Abingdon, UK).

2.11.3.1 DNA isolation

Cells were cultured and stimulated as described previously (section 2.1). $1x10^7$ cells were collected by centrifugation at 2000g for 5min at 4°C. The cells were resuspended in 100µl of sample buffer (provided by kit). The cell suspension was incubated at 18-24°C for 10min. The samples were lysed and the DNA stabilized by addition of 100µl of a supplied lysis solution. The samples were transferred to a 2ml microcentrifuge tube and 700µl of extraction solution were added. 400µl of the second extraction buffer was added and the samples were vortexed for 10sec. The samples were microcentrifuged at 12,000g for 5min. The upper (aqueous) layer, which was formed into the tube, was transferred to a new microcentrifuge tube. 0.1 volume of sodium acetate was added to the aqueous DNA solution. To the total volume in the microcentrifuge tube, an equal volume of 2-propanol was added. The samples were microcentrifuged at 12,000g for 10min. The supernatant was carefully removed and discarded. 1ml of 70% ethanol was added to the DNA pellet. The DNA solution was microcentrifuged at 12,000g for 5min. the supernatant was discarded and the DNA pellet was allowed to air-dry. The DNA was resuspended in 100µl of DNase-free water. DNA was quantified using a spectrophotometer (5µl of the DNA were diluted in 995µl of water and the optical density was read at a wavelength of 260nm.

2.11.3.2 Ethidium Bromide labeling and detection

1µl of DNA was diluted in 9µl DNase-free water and 2µl of gel loading buffer (provided by kit) were added. The samples were loaded into a 1.5% TreviGel 500 gel (provided by kit) in TAE buffer (242g Tris-base, 57.1ml glacial acetic acid, 100ml o.5M EDTA, pH 8.0 in 1lt of dH_2O). The

samples were run at 100V for 2h. The gel was then stained for 15min in 0.5µl/ml ethidium bromide prepared in DNase-free water. Ethidium bromide stained DNA was visualized using a UV transIluminator and photographed using Kodak 22A yellow filter.

2.12 Statistical Analysis

Results are expressed as mean±SEM with n being the number of independent observations. Statistical significance of differences between means were evaluated by computer program (Graphpad Prism, version 3), using one-way ANOVA, followed by a Tukey post-hoc analysis, or a Student's t-test where appropriate. $p<0.05$ was considered significant.

Chapter 3

Role of histone acetylation in IL-8 expression

3.1 Introduction

In order to fit into the nucleus, DNA has to be condensed about 10,000-fold. For this reason, the DNA is first wound around a histone octamer core consisting of histones (H) 2A, 2B, 3 and 4. Assembling higher-order structures with the help of histone 1 and other non-histone proteins achieve further compaction. Under resting conditions, DNA is tightly compacted into the nucleosome complex excluding the binding of RNA polymerase II (Pol II), the key enzyme that results in transcription of the gene and the formation of messenger RNA, and other large protein complexes. The tightly wound DNA forms a *"closed chromatin structure"*, also excluding binding of transcription factors, so that the gene is silent or repressed (Grunstein, 1997; Wolffe, 1999). In stimulated cells, transcription factors such as NF-κB, AP-1 and STATS, are activated within the nucleus and bind to co-activator molecules, such as CREB-binding protein (CBP), p300, GCN5 and PCAF (Torchia *et al.,* 1997; Kamei *et al.,* 1996). These co-activator molecules are part of multiprotein complexes that are recruited by DNA-bound transcription factors to the transcription start site and act as molecular switches. When transcription factors bind to these co-activator molecules, the intrinsic histone acetyltransferase (HAT) activity on these proteins is activated resulting in acetylation on core histone proteins (Ogryzko *et al.,* 1996). When the histone basic lysine residues, situated in the N-terminal tails, are acetylated by HAT this neutralises the charge on the histone tail and allows DNA to unwind. This opens up the chromatin structure, allowing RNA Pol II and other factors to bind, thus switching on gene transcription.

Therefore, high levels of histone acetylation are associated with transcriptionally active chromatin (Turner, 1993). Actively transcribed chromatin regions have been associated with hyperacetylation and histone acetyltransferase (HAT) recruitment. Histone deacetylase (HDAC)-mediated deacetylation on histones is thought to promote the return to a repressive, higher order chromatin structure. This balance between acetylation and deacetylation is an important factor in regulating gene expression and is thus linked to the control of cell fate (Timmermann *et al.,* 2001). As a consequence, hyperacetylation of normally silenced regions or deacetylation of normally actively transcribed regions can lead to various disorders, including developmental and proliferative diseases (Marks *et al.,* 2001).

Glucocorticoids, which induce changes in cell proliferation and differentiation, exert their effects through specific intracellular steroid hormone receptors. It is well established that glucocorticoids down regulate the transcription of pro-inflammatory genes (Ray & Prefontaine, 1994; Mukaida *et al.,* 1994). Those genes are themselves activated by the NF-κB transcriptional regulator, but the

mechanism by which glucocorticoids preclude activation has been a matter of debate (Ito *et al.*, 2000; Nissen & Yamamoto, 2000). The anti-inflammatory effects of glucocorticoids are due to the glucocorticoid receptor acting as a hormone-dependent transcription factor to modify chromatin structure. On ligand binding, these receptors are activated and translocate to the nucleus where they interact directly with glucocorticoid response elements (GREs) or associate with other transcription factors through protein:protein:DNA complexes to induce local DNA unwinding and subsequent gene induction. Glucocorticoid receptors interact with CREB-binding protein (CBP) and several other transcriptional co-factors including SRC-1 to enhance gene expression. Increased transcription is associated with uncoiling of DNA, which in turn is associated with acetylation on histones by the enzymic action of the CBP and other HATs. Glucocorticoids may also modify chromatin structure indirectly by recruiting HDACs to the promoter site of inflammatory genes resulting in histone deacetylation, tighter coiling of DNA and reduced access of the large basal transcription complex and RNA pol II and thereby suppressing inflammatory gene expression (Barnes, 1998).

In this chapter, the effect of whole cell histone acetylation status in inflammatory gene expression is investigated. To assess whether histone acetylation influences transcription and chromatin modifications it was determined whether TSA, an antifungal antibiotic with differentiating properties in a mammalian cell culture and a potent and specific inhibitor of HDAC activity, influenced basal, inflammatory stimuli-mediated and glucocorticoid-mediated release of pro-inflammatory cytokines in a monocytic cell line (U937) as well as in two T-cell lines (Jurkat and HUT-78).

3.2 Results

3.2.1 Evidence of a role of histone acetylation in IL-8 production in macrophages and lymphocytes

IL-8 production in three cell lines was the focus of this study because of its physiological importance during inflammation. The 5' flanking region of the IL-8 gene contains potential binding sites for several nuclear factors such as NF-κB, activation factor-1 (AP-1) and a glucocorticoid responsive element. The cell lines studied were: U937, HUT-78 and Jurkat. Both LPS (10 ng/ml) and TNF-α (10 ng/ml) stimulated IL-8 production in each cell line after 18h (Fig. 3-1). No induction of IL-8 release was seen before 4h and a maximum was reached after 24h. In U937 cells LPS (1430 ± 140 versus 210 ± 30 pg/ml; $p<0.01$) and TNF-α (1220 ± 100 versus 140 ± 30 pg/ml; $p<0.01$) both induced IL-8 expression. A similar effect was seen in HUT-78 cells, where LPS (542 ± 41 versus 120 ± 12 pg/ml, $p<0.05$) and TNF-α (683 ± 35 versus 200 ± 22 pg/ml, $p<0.05$) stimulated IL-8 production but to a lower extent compared to that seen in U937 cells. Finally, both LPS and TNF-α also stimulated IL-8 production in Jurkat cells (453 ± 23 versus 123 ± 15 pg/ml, $p<0.05$) and (690 ± 38 versus 193 ± 15 pg/ml, $p<0.05$) respectively.

To determine whether histone acetylation played a role in LPS- or TNF-α- induced IL-8 release, cells were further stimulated with increasing concentrations of Trichostatin A (TSA), a potent histone deacetylase inhibitor (Fig. 3-2). For U937 cell the optimal concentration of TSA stimulation was 1 ng/ml causing the highest release of the cytokine. In both T-cell lines, TSA had the most potent effect at 10 ng/ml (Fig. 3-2). In all three cell lines investigated higher concentrations of TSA suppressed cytokine release.

Figure 3-3 shows the additional effect of TSA on IL-8 production stimulated by LPS or TNF-α. TSA enhanced LPS-induced IL-8 production in all three cell lines. In U937 cells IL-8 production increased by 22% (1740 ± 120 versus 1530 ± 140 pg/ml, $p<0.1$). Smaller increases were seen in HUT-78 cells (630 ± 42 versus 542 ± 41 pg/ml LPS alone, $p<0.05$) and in Jurkat cells (628 ± 35 versus 453 ± 23 pg/ml LPS alone, $p<0.05$).

TSA also enhanced the release of IL- 8 in cells stimulated with TNF-α (10 ng/ml) (Fig. 3-4). Here the increase in IL-8 production observed was: 1453 ± 121 versus 1220 ± 100 pg/ml ($p<0.05$) for U937 cells; 872 ± 44 versus 683 ± 23 pg/ml ($p<0.05$) for HUT-78 cells and 800 ± 52 versus 690 ± 28 pg/ml ($p<0.05$) for Jurkat cells.

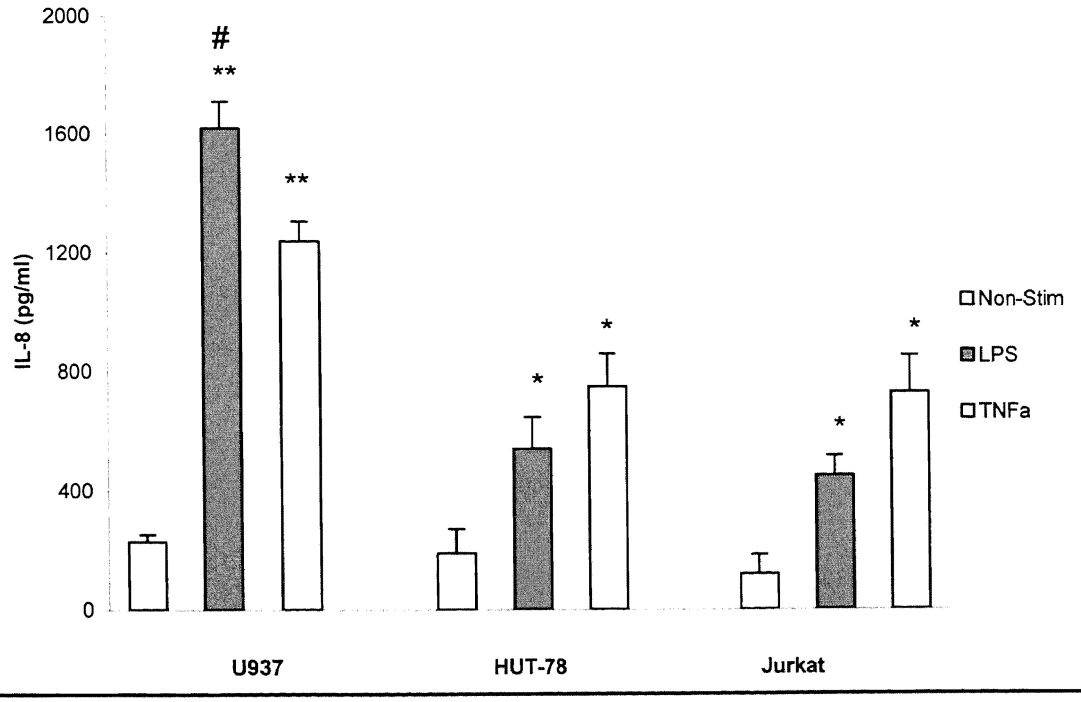


Figure 3-1 Effect of lipopolysaccharide (LPS) (10 ng/ml) and tumour necrosis factor-α (TNF-α) (10 ng/ml) in interleukin 8 (IL-8) release in U937, Jurkat and HUT-78 cells. Both inflammatory stimuli induced IL-8 production in all three cell lines. In U937 the release of IL-8 induced by both inflammatory stimuli is significantly higher than that of the T-cell lines (*$p<0.01$, **$p<0.05$) (#$p<0.05$ U937 vs HUT-78 and Jurkat cells). (n=4).

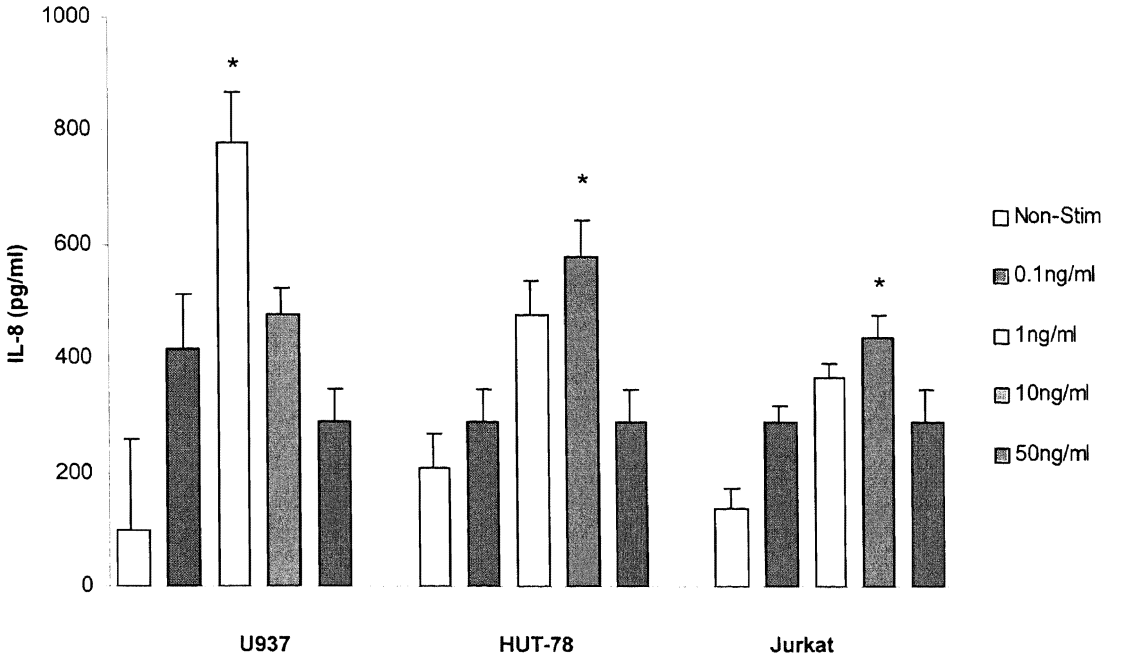


Figure 3-2 Concentration response curve of Trichostatin A (TSA) in U937, HUT-78 and Jurkat cells. The concentration of TSA was increased from 0.1 ng/ml to 50 ng/ml. In macrophages, a significant increase in interleukin 8 (IL-8) production is observed after stimulation of 1 ng/ml of TSA. In both T-cell lines maximum IL-8 release was observed with a 10 ng/ml concentration of TSA. (*$p<0.05$). ($n=4$).

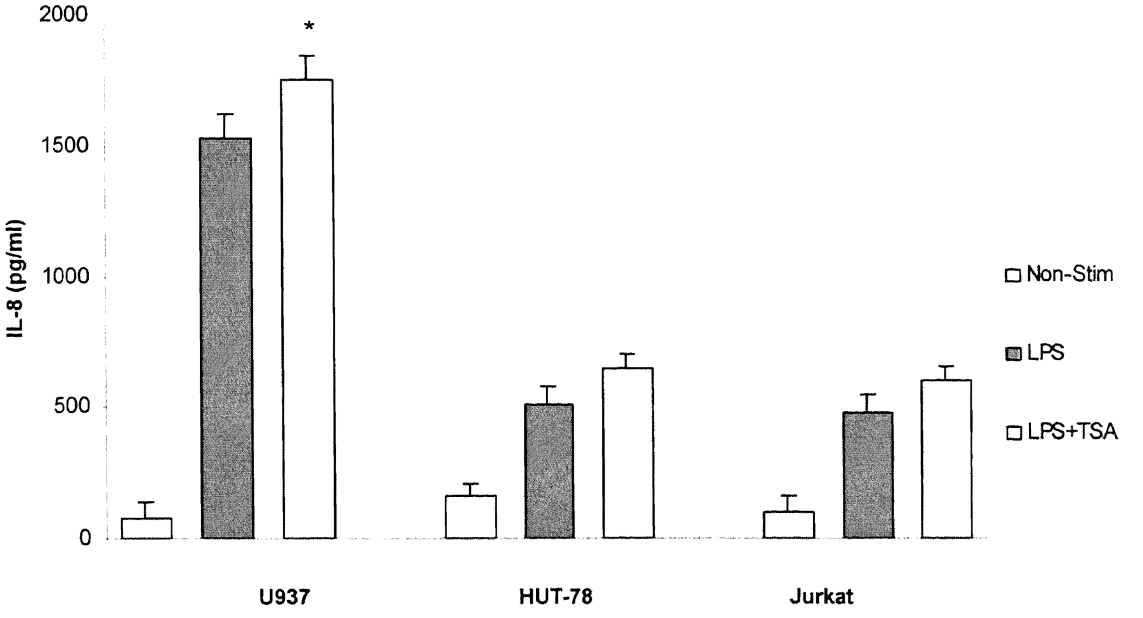


Figure 3-3 Effect of Trichostatin A (TSA) on the production of interleukin 8 (IL-8). Lipopolysaccharide (LPS) (10 ng/ml), enhanced production of IL-8 in all three cell lines with the most significant increase observed at the macrophage cell line. In all three cell lines the production of IL-8 was significantly increased following addition of TSA at concentrations determined in figure 3-2 (1 ng/ml in U937 cells and 10 ng/ml in HUT-78 and Jurkat cells). (*$p<0.1$). ($n=4$).

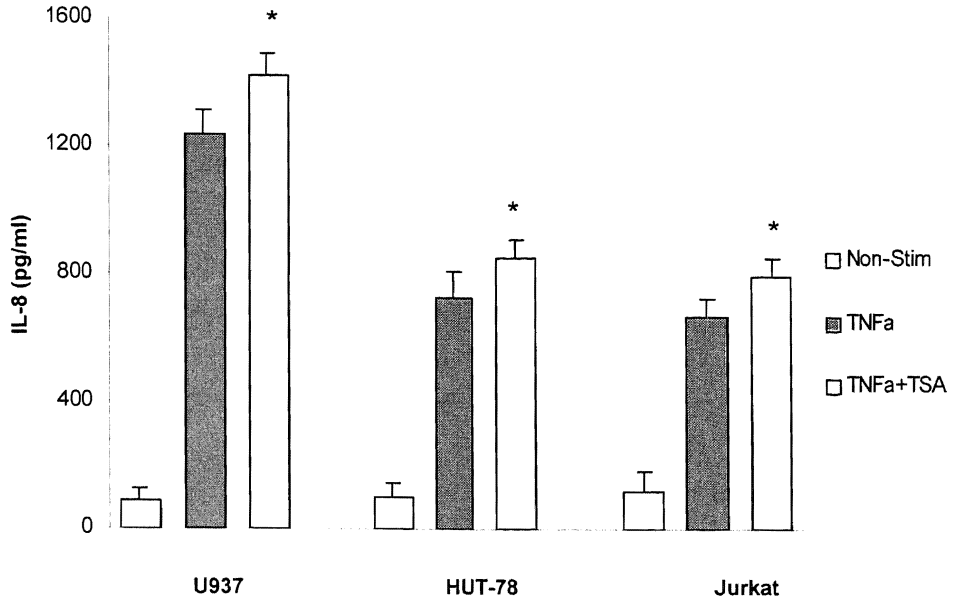


Figure 3-4 Effect of Trichostatin A (TSA) on the production of interleukin 8 (IL-8). Tumour necrosis factor α (TNF-α) (10 ng/ml) induced elevated production of IL-8 in all three cell lines with the most significant increase observed in the U937 cells. In all three cell lines the production of IL-8 was significantly increased following further addition of TSA at concentrations determined in figure 3-2 (1 ng/ml in U937 cells and 10 ng/ml in HUT-78 and Jurkat cells). (*$p<0.05$). (n=4).

3.2.2 Role of histone acetylation in dexamethasone and triamcinolone acetonide-mediated actions

Next, the effect of glucocorticoid agonists, dexamethasone (Dex) (10^{-8} M) and triamcinolone acetonide (TA) (10^{-10} M), on LPS and TNF-α -stimulated release in all three cell lines was investigated. Cells were pre-incubated with either Dex or TA for 30min before incubation with either LPS or TNF-α at pre-determined concentrations (10 ng/ml for both LPS and TNF-α). In figure 3-5 the effect of Dex on LPS stimulated cells is demonstrated. Dex (10^{-8} M) down-regulated IL-8 production (Fig. 3-5) in U937 cells by 28% (1150 ± 105 versus 1600 ± 140 pg/ml, $p<0.05$), in HUT-78 cells by 43% (308 ± 29 versus 570 ± 35 pg/ml, $p<0.05$), and in Jurkat cells by 33% (323 ± 30 versus 518 ± 22 pg/ml, $p<0.05$).

TSA enhanced IL-8 release and also altered the suppressive effect of Dex on LPS-induced IL-8 release. The percentage inhibition by Dex was reduced by 50% in U937 cells (1548 ± 125 versus 1150 ± 105 pg/ml, $p<0.05$) and 51% HUT-78 cells (506 ± 23 versus 308 ± 29 pg/ml,$p<0.05$). In both cases the production of IL-8 remained at almost pre-steroid treatment levels. In Jurkat cells TSA, only altered dexamethasone suppression by 9% (390 ± 31 versus 323 ± 30 pg/ml LPS plus Dex).

Similar results were obtained when the cells were treated with the more potent synthetic glucocorticoid, TA (10^{-10} M) (Fig. 3-6). TA suppressed LPS-induced IL-8 production significantly in all cell lines. The percentage inhibition by TA was reduced by 66% reduction in U937 cells, (510 ± 58 versus 1430 ± 140 pg/ml, $p < 0.01$), 55% in HUT-78 cells (261 ± 14 versus 542 ± 41 pg/ml, $p < 0.01$) and 43% in Jurkat cells (310 ± 19 versus 453 ± 23 pg/ml, $p< 0.05$).

TSA similarly to the findings obtained in figure 3-5, enhanced IL-8 release and also altered the suppressive effect of TA on LPS-induced IL-8 release. The suppression percentage was 65% in U937 cells (1347± 100 versus 510 ± 58 pg/ml LPS plus TA, $p<0.05$), 72% in HUT-78 cells (552 ± 42 versus 261 ± 14 pg/ml LPS plus TA, $p<0.05$) and 76% in Jurkat cells by (538 ± 23 versus 310 ± 19 pg/ml LPS plus TA, $p<0.05$).

Dex (10^{-8} M) also suppressed TNF-α-induced IL-8 production. By 28% in U937 cells (872 ± 36 versus 1220 ± 100 pg/ml TNF-α alone), by 20% in HUT-78 cells (620 ± 23 versus 683 ± 35 pg/ml TNF-α alone) and by 16% in Jurkat cells (613 ± 28 versus 690 ± 38 pg/ml TNF-α alone) (Fig. 3-7).

TSA altered the suppressive effect of Dex on TNF-α-induced IL-8 release by 27% in U937 cells (1140 ± 90 versus 872 ± 36 pg/ml TNF-α plus Dex), by 55% in HUT-78 cells (770 ± 35 versus 620 ± 23 pg/ml TNF-α plus Dex) and by 12.5% in Jurkat cells (690 ± 25 versus 613 ± 28 pg/ml TNF-α plus Dex) (Fig. 3-7).

TA suppressed TNF-α-induced IL-8 release more potently than dexamethasone. The percentage that TA suppressed TNF-α-induced IL-8 release was 40% in U937 cells (780 ± 43 versus 1220 ± 100 pg/ml TNF-α alone, $p<0.01$), 42% in HUT-78 cells (462 ± 28 versus 683 ± 35 pg/ml TNF-α alone, $p<0.01$) and 46% in Jurkat cells (410 ± 20 versus 690 ± 38 pg/ml TNF-α alone, $p<0.01$) (Fig. 3-8).

TSA altered the suppressive effect of TA on TNF-α induced IL-8 release by 72.5% in U937 cells (1223 ± 110 versus 780 ± 43 pg/ml TNF-α plus TA, $p<0.05$), by 83% in HUT-78 cells (790 ± 31 versus 462 ± 28 pg/ml TNF-α plus TA, $p<0.05$) and by 86% in Jurkat cells (748 ± 21 versus 410 ± 20 pg/ml TNF-α plus TA, $p<0.05$). In all three cell lines TSA restored IL-8 levels to those observed following TNF-α stimulation alone. Table 3-1 shows a summary of the results.

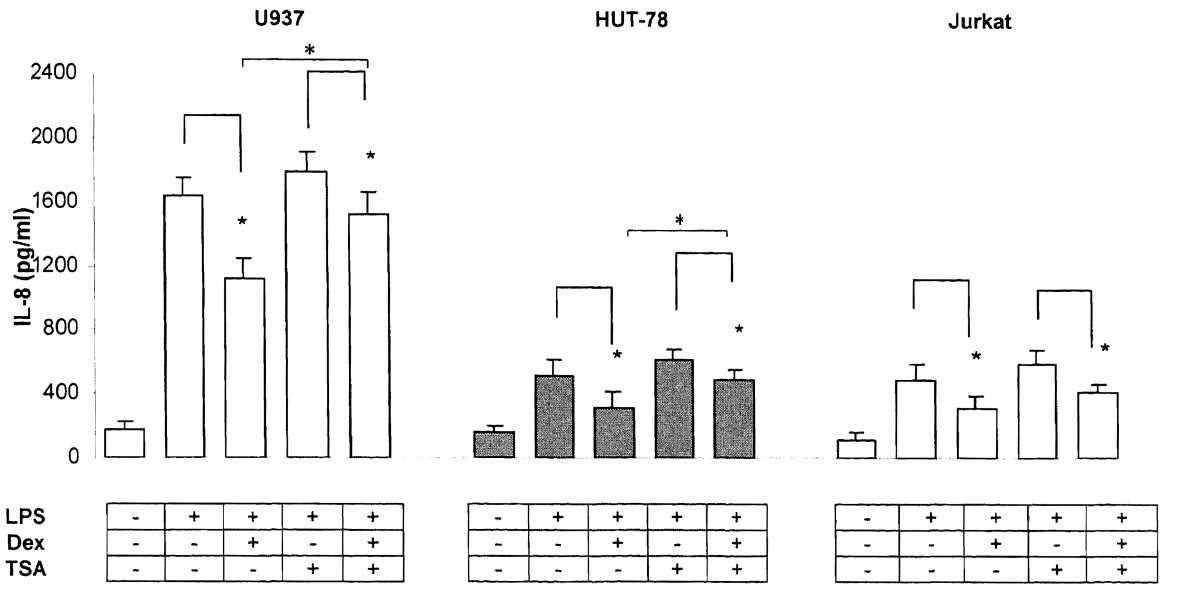


Figure 3-5 Effect of dexamethasone (Dex; 10^{-8} M) and Trichostatin A (TSA) (1 ng/ml in U937 cells and 10 ng/ml in HUT-78 and Jurkat cells) on interleukin 8 (IL-8) release in lipopolysaccharide (LPS) treated cells. LPS (10 ng/ml) induced a high production of IL-8 in all three cell lines with the most significant increase observed in the U937 cells. Addition of Dex inhibited IL-8 release in all three cell lines with significant results observed for U937 and HUT-78 cells. Further addition of TSA resulted in the upregulation of the IL-8 levels in all three cell lines. (*p<0.05). (n=6)

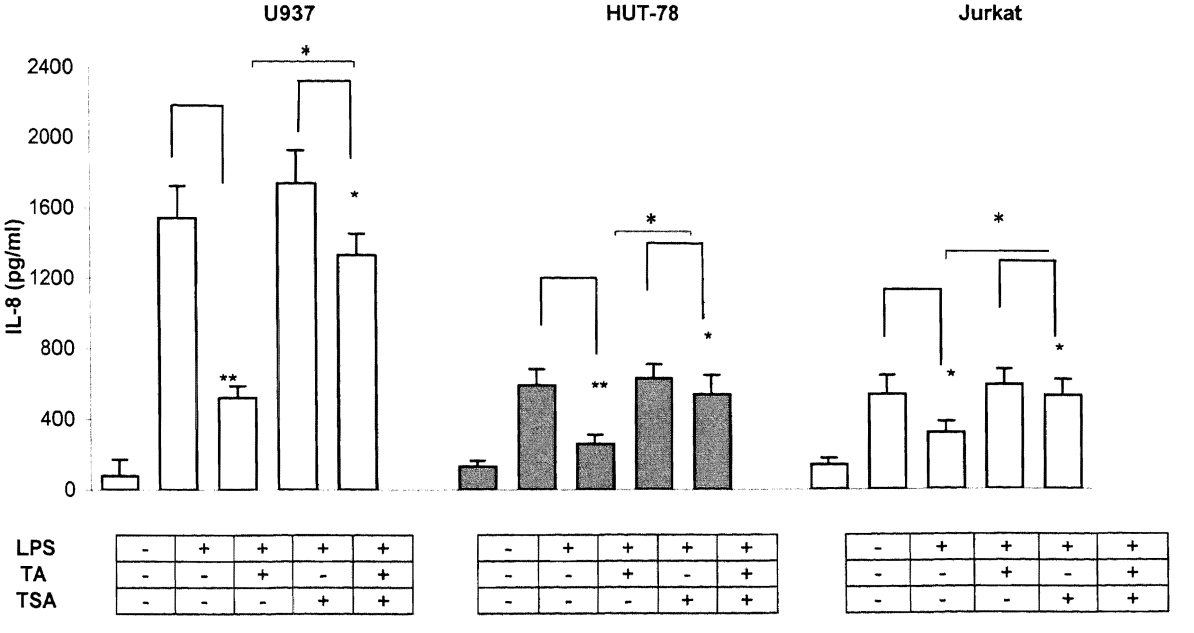


Figure 3-6 Effect of triamcinolone acetonide (TA) and Trichostatin A (TSA) on the production of lipopolysaccharide (LPS)-induced interleukin 8 (IL-8) in U937, HUT-78 and Jurkat cells. LPS (10 ng/ml) induced a high production of IL-8 in all three cell lines, an effect that was significantly blocked by further addition of TA (10^{-10} M). The levels of IL-8 for all cell lines were decreased to 40% for the macrophage cell line and almost 50% for the T-cell lines. In all three cell lines the production of IL-8 was significantly increased following further addition of TSA at concentrations determined in figure 3-2. (*p<0.05, **p<0.01). (n=6).

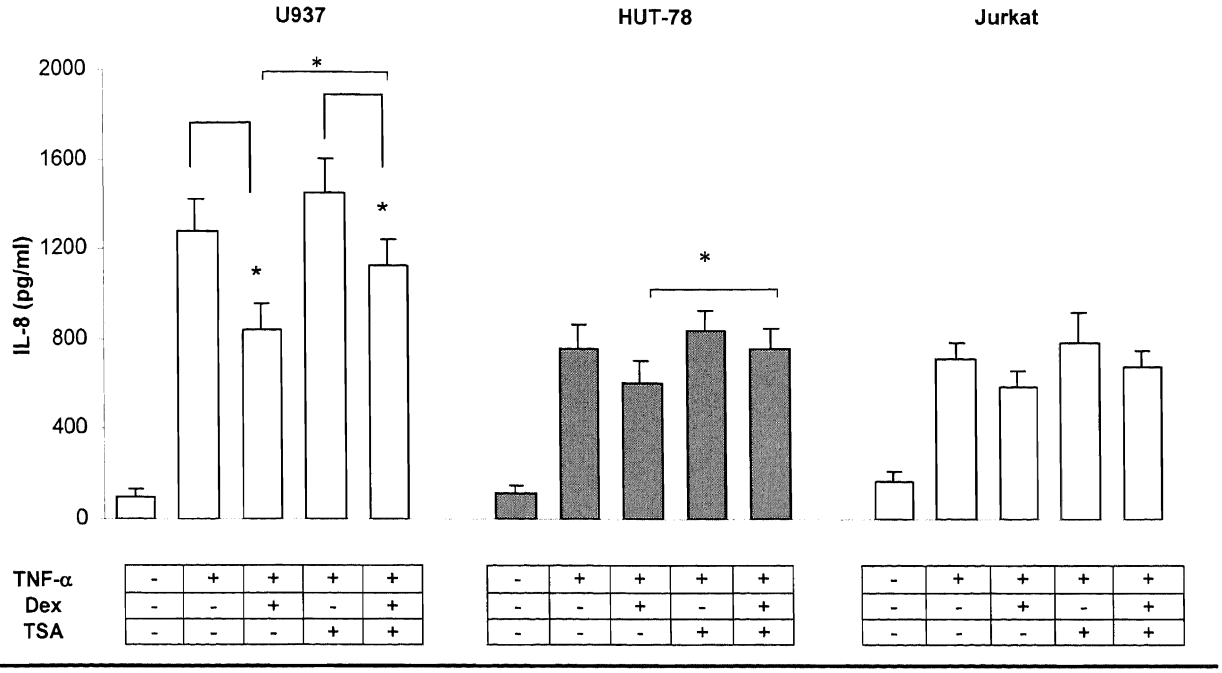


Figure 3-7 Effect of dexamethasone (Dex; 10^{-8}M) and Trichostatin A (TSA; 10 ng/ml) on tumour necrosis factor α (TNF-α)-induced interleukin 8 (IL-8) release in U937, HUT-78 and Jurkat cells. TNF-α (10 ng/ml) induced IL-8 in all three cell lines. Dex decreased IL-8 levels, in U937 and HUT-78 cells. Addition of TSA resulted in upregulation of the production of IL-8 in all cell lines. (* $p<0.05$). (n=6).

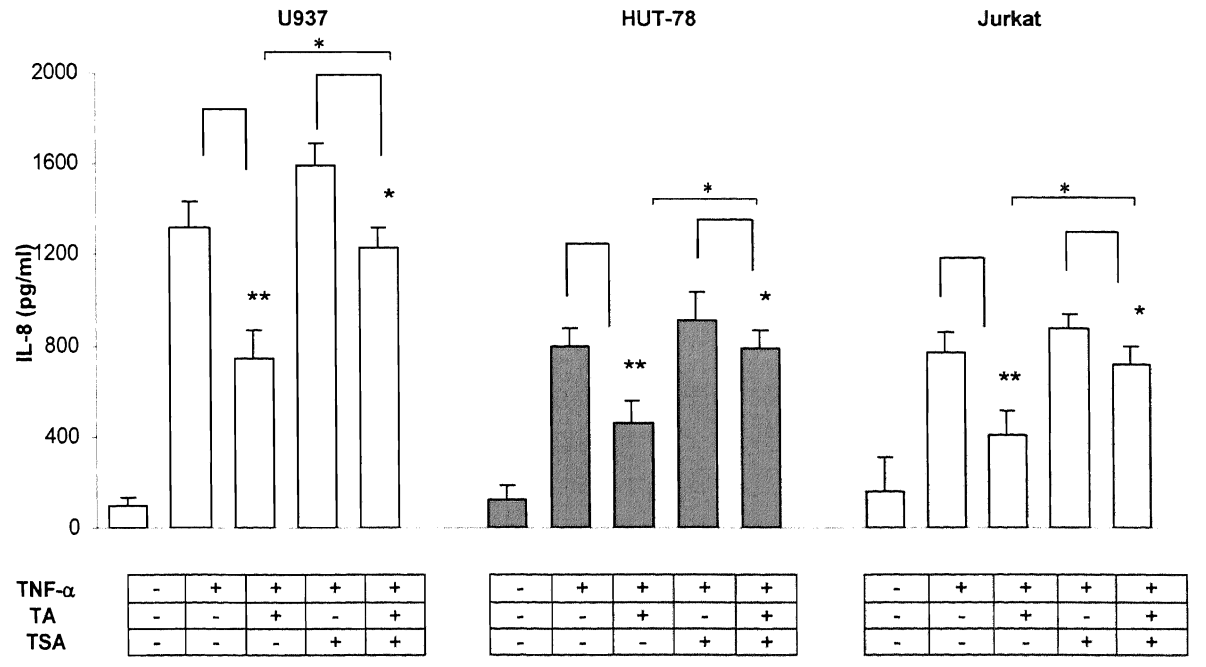


Figure 3-8 Effect of triamcinolone acetonide (TA; 10^{-10} M) and Trichostatin A (TSA; 10 ng/ml) on the production of interleukin (IL-8) in three cell lines stimulated with tumour necrosis factor α (TNF-α). TA downregulated the production of IL-8 significantly in all cell lines. Further treatment of the cells with TSA blocked the effect of TA, with the greatest effect seen in the U937 cells where the levels of IL-8 almost reached TNF-α stimulated levels. (*$p<0.05$, **$p<0.01$, comparisons made as in figure 3-7). (n=6).

Induction of IL-8	U937	HUT-78	Jurkat
LPS+Dex	28 ± 3%	43 ± 5%	33 ± 3%
LPS+Dex+TSA	14 ± 4%	21 ± 9%	30 ± 15%
Blocking effect (%) of TSA versus Dex	**50 ± 7% (p<0.05)**	**51 ± 4% (p<0.05)**	**9 ± 1%**
LPS+TA	66 ± 9%	55 ± 6%	43 ± 3%
LPS+TA+TSA	23 ± 3%	15 ± 4%	10 ± 2%
Blocking effect (%) of TSA versus TA	**65 ± 7% (p<0.05)**	**72 ± 6 (p<0.05)**	**76 ± 4% (p<0.05)**
TNF-α+Dex	28 ± 4%	20 ± 3%	16 ± 2%
TNF-α+Dex+TSA	21.5 ± 5%	9 ± 2%	14 ± 4%
Blocking effect (%) of TSA versus Dex	**27 ± 4 % (p<0.05)**	**55 ± 7% (p<0.05)**	**12.5 ± 3%**
TNF-α+TA	40 ± 4%	42 ± 3%	46 ± 4%
TNF-α+TA+TSA	11± 3%	7 ± 2%	6.5 ± 2%
Blocking effect (%) of TSA versus TA	**72.5 ± 8% (p<0.05)**	**83 ± 5% (p<0.05)**	**86 ± 6% (p<0.05)**

Table 3-1. Percentage representation of the blocking effect of Trichostatin A (TSA) of glucocorticoid actions in repressing interleukin 8 (IL-8)-stimulated cytokine release in U937, HUT-78 and Jurkat cells. In all cell lines the effect of TSA was more potent in downregulating triamcinolone acetonide (TA)-stimulated IL-8 release. The two T-cell lines (HUT-78 and Jurkat) responded differently in dexamethasone (Dex) stimulation (Dex was more potent in downregulating IL-8 production in the HUT-cells). TA was a more potent glucocorticoid in all three cell lines compared to the effect of Dex.

3.2.3 Transcriptional regulation is associated with TNF-α and LPS-induction of IL-8

This far, it has been shown that LPS and TNF-α –induction of the pro-inflammatory cytokine, IL-8 involves histone modifications. Further studies were required to investigate if these modifications occurred on the IL-8 promoter and to determine whether GR blocks histone acetylation at the IL-8 promoter. The cells were stimulated as previously with LPS (10 ng/ml) or TNF-α (10 ng/ml) in the presence or absence of TA (10^{-10} M), as TA was the most potent glucocorticoid shown from previous results.

Using chromatin immunoprecipitation (ChIP) assays, immunoprecipitation (IP) with an antibody directed against pan-acetylated histone 4 resulted in the enrichment for the DNA segments encompassing the κB site in the IL-8 promoter following both LPS and TNF-α treatments. (Fig. 3-9; lane 3). This was apparent in all three cell lines stimulated with both LPS and TNF-α. These data demonstrate that increased histone acetylation occurs at the κB site of the IL-8 promoter *in vitro* and is associated with increased IL-8 expression. The presence of the glucocorticoid (TA), caused a reduction in the enrichment of histone 4 –associated IL-8 promoter fragments an effect which was seen in U937, HUT-78 and Jurkat cells. This effect correlated with TA repression of IL-8 release shown in previous results.

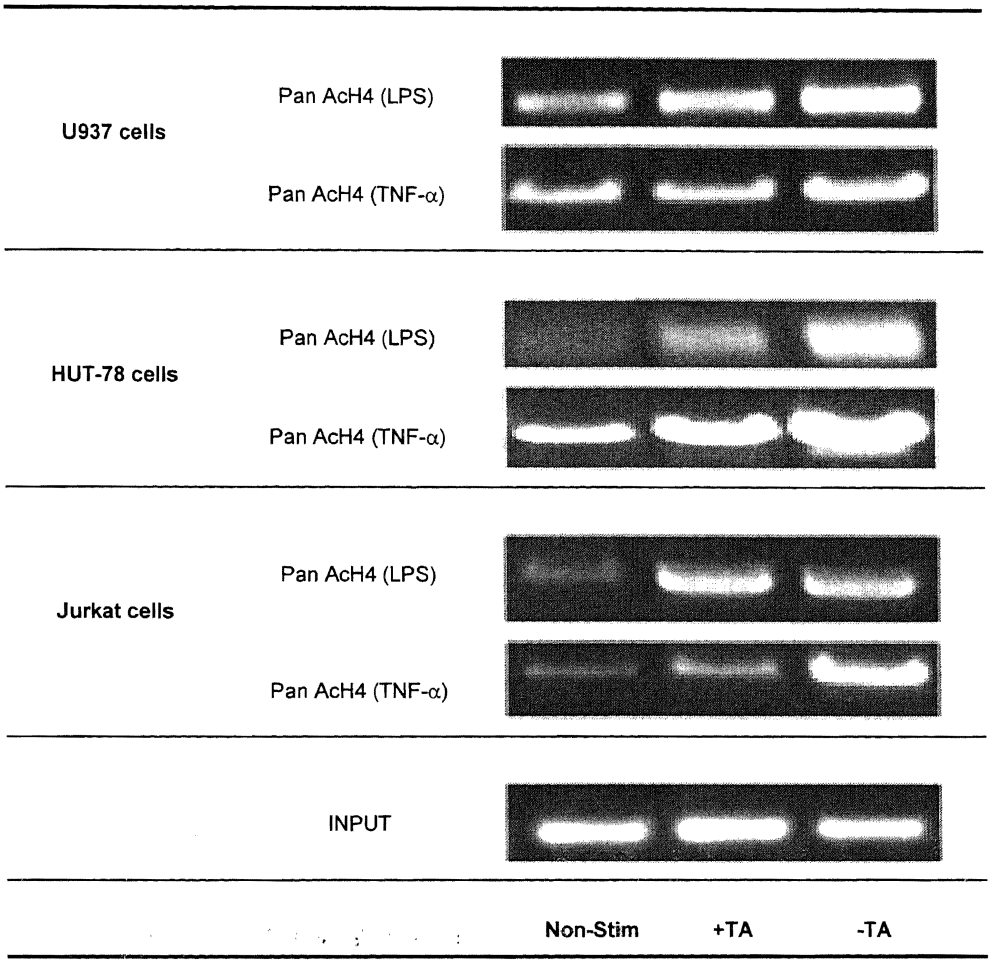


Figure 3-9 Association of histone acetylation with the interleukin 8 (IL-8) gene promoter. Cells were incubated with either lipopolysaccharide (LPS; 10 ng/ml) or tumour necrosis factor α (TNF-α; 10 ng/ml) in the presence (+) and absence (-) of triamcinolone acetonide (TA; 10^{-10} M). Both LPS and TNF-α induced histone acetylation at the IL-8 promoter in all three cell lines. The presence of TA caused the reduction of the density of the IL-8 promoter fragments in all three cell lines. The results are representative of three independent experiments.

3.3 Discussion

In this chapter the effects of inflammatory stimuli, LPS and TNF-α, in the production of the pro-inflammatory cytokine IL-8 in a macrophage cell line (U937) and in two T-cell lines (HUT-78 and Jurkat) were investigated. LPS and TNF-α caused an increase in IL-8 expression, which was inhibited by the synthetic glucocorticoids, dexamethasone (Dex) and triamcinolone acetonide (TA), in all three cell lines. TSA produced a concentration-dependent increase in IL-8 release, which reached a peak at 1 ng/ml for the macrophage cell line and at 10ng/ml for the two T-cell lines. LPS (10ng/ml) and TNF-α (10ng/ml) –stimulated release of IL-8 was enhanced by TSA, suggesting a role for histone acetylation in IL-8 production in these cells. Similar results have been reported for HeLa cells where it has been shown that inhibition of HDAC activity causes increased expression of the IL-8 gene (Ashburner *et al.*, 2001). The same report interestingly shows that only the level of expression, not the kinetics of IL-8 expression,

was altered by TSA treatment. This implies that HDACs are associated directly with factors that induce IL-8 production and that the effect of TSA is not secondary to induction of other transcription factors. These results suggest that treatment of the cells with TSA blocks the activity of HDAC proteins, resulting in hyperacetylation on histones and subsequently a higher level of gene expression. Similar data have also been reported by Ghosh *et al.,* (2002) where HDAC1 associates with p65 or p50 in resting cells.

Dex and TA repressed LPS and TNF-α -induced IL-8 expression in all three cell lines. TA was significantly more potent than Dex in all cell lines studied. In addition, the effect of Dex was significantly less in Jurkat cells compared to HUT-78 cells. In U937 cells, Dex caused a 28% reduction in both LPS- and TNF-α-induced IL-8 release compared to a 66% reduction in LPS – induced and 40% reduction in TNF-α induced IL-8 production by TA. In both T-cell lines the difference in the effects of the two glucocorticoids was even greater. In HUT-78 cells Dex caused a 43% (LPS-induced) and a 20% (TNF-α induced) reduction of IL-8 levels compared to a 55% (LPS-induced) and a 42% (TNF-α induced) downregulation caused by TA. Similarly, in Jurkat cells Dex caused a 33% (LPS-induced) and a 16% (TNF-α induced) downregulation whilst TA caused a 43% (LPS-induced) and a 46% (TNF-α induced) decrease of IL-8 levels. It is shown that the glucocorticoid action in repressing LPS and TNF-α –stimulated cytokine release is mediated by HDACs. TSA (1 ng/ml and 10 ng/ml) caused a decline in the ability of the glucocorticoids to inhibit both LPS and TNF-α -stimulated IL-8 release (Table 3-1).

In U937 cells the presence of TSA blocked Dex –induced IL-8 downregulation (by 50% in LPS-stimulated cells and by 27% in TNF-α stimulated cells). Similarly in HUT-78 cells TSA blocked the Dex actions by 51% (in LPS stimulated cells) and 55% (in TNF-α stimulated cells). In Jurkat cells the blocking effect of TSA in Dex actions was significantly lower compared to the other cell lines (9% in LPS stimulated cells and 12.5% in TNF-α stimulated cells). TSA blocked the suppressive action of both Dex and TA on LPS- and TNF-α-induced IL-8 production. Similar investigation in all three cell lines on the effect of TA showed that TSA blocked significantly the effect of the glucocorticoid regardless of cell type and inflammatory stimuli. This variation in the response of the two T-cell lines to Dex (and the slight difference in their response to TA) might reflect altered GR expression present in the two cell lines or a difference in the regulation of IL-8 in the two cell lines. Further studies would therefore be required, in ideally primary cells.

Nissen and Yamamoto (2000) reported, but did not show, that GR repression of NF-κB-mediated IL-8 expression was resistant to the effects of a single concentration of TSA in the epithelial lung cell line A549 (Nissen & Yamamoto, 2000). Enhanced IL-8 transcription was associated with increased phosphorylation of serine residues 2 and 5 of the carboxyl terminal domain (CTD) of RNA pol II. CTD phosphorylation allows RNA pol II-mediated mRNA

transcriptional initiation, elongation and subsequent re-initiation of RNA pol II to the IL-8 start site. Nissen and Yamamoto report that GR receptor inhibited serine 2 phosphorylation of the CTD. Contrary to these findings here it is shown that in U937, Jurkat and HUT-78 cells, TSA upregulated the production of LPS and TNF-α -induced IL-8 both in the presence and absence of glucocorticoids. Similar results have been reported in the A549 lung epithelial cells for IL-1β-stimulated GM-CSF release (Ito *et al.,* 2000). Altered TSA responsiveness may reflect differential HDAC expression in cell lines and recruitment to distinct promoters in the same cell lines and this might explain the reported discrepancies. These contradictory findings also enhance the theory that transcriptional regulators such as GR are likely to exploit a diversity of mechanisms across different cellular and promoter contexts.

Previous data have suggested a role for CBP in the nuclear integration of NF-κB and glucocorticoid actions (Wolffe, 1997). Acetylation on histones produces a loose, less-regulated, chromatin structure that allows transcription factors to bind to nearby promoter sequences, thus, activating gene transcription. Although CBP overexpression can modify NFκB and glucocorticoid actions, squelching of limited cellular amounts of CBP is not now thought to be important (de Boscher *et al.,* 2000). The use of the histone de-acetylase inhibitor shows that the actions of glucocorticoids may result from a functional reversal of the ability of CBP to regulate histone acetylation (Sheppard *et al.,* 1998) rather than a direct inhibition of CBP activity *per se*.

Having showed that LPS and TNF-α -induction of IL-8 involves histone modifications it is also demonstrated that these events occurred on the native IL-8 promoter using chromatin immunoprecipitation. In addition, TA suppressed the amount of acetylated histone 4 associated with the IL-8 promoter following cell stimulation. No obvious differences were detected between cell lines. The PCR products encompassed the NF-κB response element within the IL-8 promoter and it is tempting to speculate that these events are mediated via NF-κB activation. This needs confirmation using specific inhibitors of NF-κB.

A previous report has shown a histone 4 lysine residue dependent transcriptional activation, where IP with an antibody against acetylated K8 and K12 resulted in the enrichment for the DNA segments encompassing the NF-κB site in the GM-CSF promoter following IL-1β treatment (Ito *et al.,* 2000). It is also important to take into account that the effect of TSA has been shown to be non-specific. The HDACs involved in the corepressor complex, which interacts with the CBP-associated coactivator complex, are not clear. It has been shown that HDAC3, HDAC2 and HDAC1 participate in activating complexes but it is possible that other HDACs may be activated. Therefore, the use of specific HDAC inhibitors could help to clarify which types of HDAC are involved in the transcriptional regulation of cytokine release and whether their effect is cytokine or GR specific.

Glucocorticoids exert their anti-inflammatory effects largely by interfering with the ability of cytokine-activated pro-inflammatory transcription factors to induce inflammatory gene transcription. This interaction may affect transactivation by the pro-inflammatory transcription factor, association with histone acetylation, DNA methylation, and subsequently, activation of RNA polymerase II. The exact contribution of each mechanism may vary between cell types and depend upon the cell stimulus. Attempting to determine the involvement of histone acetylation in the inflammatory gene expression and the mechanism through which the glucocorticoid receptor and chromatin activation interact, it was firstly required to determine whether a histone deacetylase inhibitor (TSA) would influence the inflammatory stimuli and the glucocorticoid actions *in vitro*. The blockade of TSA in the effect of glucocorticoids, Dex and TA, for macrophages as well as T-lymphocytes, indicated a role of histone deacetylases in inflammatory gene expression in these cells. Further study in the role of histones and their acetylation on the native IL-8 promoter would help the understanding of the mechanism of which gene activation and repression occur in these cells under inflammatory conditions (Chapter 4). The central role of histone acetylation in mediating these actions makes these potentially important targets for future anti-inflammatory drug interventions.

Chapter 4

Histone acetylation in an *in vitro* model of inflammation

4.1 Introduction

On the tails of histones, extensive post-translational modifications, such as acetylation, phosphorylation and methylation occur. The best studied of these, is the acetylation of core histone tails (Hebbes *et al.,* 1988). The correlation between acetylation and increased transcription has been known for years and acetylated histones have been shown to preferentially associate with transcriptionally active chromatin (Sealy *et al.*, 1978). The discovery of GCN5 (Brownwell *et al.*, 1996), the first nuclear acetylase, and HDAC1 (Taunton *et al.*, 1996), the first deacetylase, verified that acetylation of histones is an important controlling step in transcription and has also aided the discovery of novel nuclear targets for acetylation. Some of these targets are well-known and extensively characterized transcription factors. There are now several reported families of acetylases exemplified by PCAF/GCN5, p300/CBP, TAF250, SRC1 and MOZ (Kouzarides, 1999). However, despite the many attempts to understand the role of acetylation in chromatin assembly, transcription factor accessibility and nucleosome remodeling, the fact remains that the precise mechanism by which acetylation of histones augments transcription remains elusive. The truth is likely to be complicated, since acetylation of histones most probably effects a combination of events, which allows for higher processivity of RNA polymerase II (Kouzarides, 2000).

Acetylation occurs at conserved lysine residues on the amino terminal tails of core histones, such as lysines 5, 8, 12 and 16 on histone 4 and lysines 9 and 14 on histone 3 (Davie, 1998). Acetylases modify very few lysines within a given protein, an indication of specificity. Alignment of sequences surrounding modified lysines and mutagenesis of Rch1 (human importin-α) suggest that the amino sequence GK may be part of a recognition motif. The crystal structure of GCN5, a gene regulatory protein, with a histone identifies GKXXP as a possible recognition motif and site selection for deacetylases (Rojas *et al.*, 1999). The consequence of acetylation depends on where within the protein acetylation takes place. In the case of four site-specific DNA-binding transcription factors, p53, E2F1, EKLF and GATA1, the acetylation site falls directly adjacent to the DNA-binding domain and acetylation results in stimulation of DNA binding (Boyes *et al.*, 1998; Zhang and Beaker, 1998; Martinez-Balbas, 2000). In contrast, the lysines acetylated within the HMGI(Y) transcription factor fall within the DNA-binding domain and result in disruption of DNA binding.

As previously mentioned (Chapter 3), glucocorticoids are the most effective therapy for the treatment of inflammatory diseases such as inflammatory bowel disease and asthma. Functionally glucocorticoids act partly by inducing some anti-inflammatory genes (IL-1

receptor agonist) (Levine *et al.*, 1996), but mainly by repression of inflammatory genes, such as cytokines, inflammatory enzymes and receptors. Within the nucleus, the activated glucocorticoid receptor (GR) either induces gene transcription by binding to specific DNA elements in the promoter-enhancer regions of responsive genes, or reduces gene transcription by transrepression (Truss and Beato, 1993). GR reduces gene transcription by interaction with pro-inflammatory transcription factors such as AP-1 and NF-κB (Barnes and Adcock, 1998)(Fig. 4-1). Both transcription factors and GR mutually repress each other's ability to activate transcription and require CBP for maximal activity. It has also been reported that sodium butyrate, a histone deacetylase inhibitor, interferes with GR-activated transcription (Plesko *et al.*, 1983). These findings together with findings, as mentioned in Chapter 3, suggest that alterations in chromatin structure must play a role in modulating glucocorticoid actions.

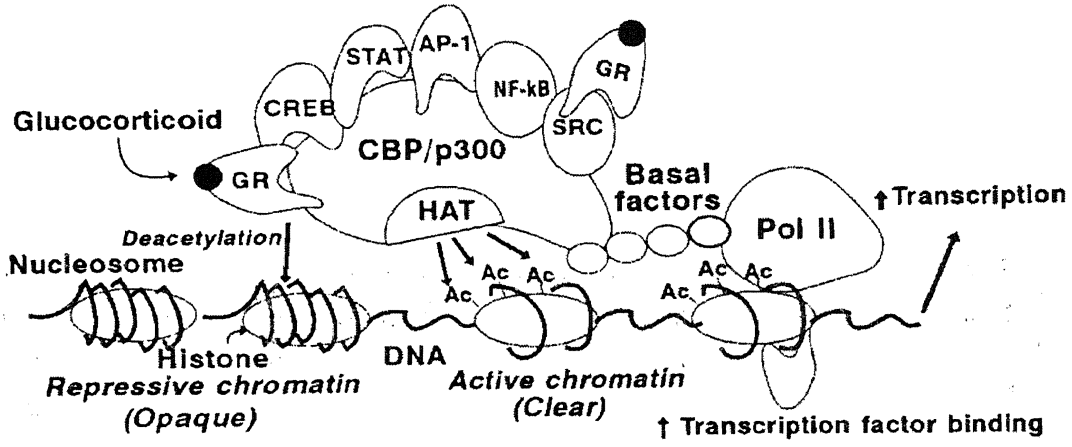


Figure 4-1 Schematic representation of glucocorticoids on chromatin structure. Chromatin can become alternatively transparent (clear) or opaque to facilitate or restrict the access of transcription factors and RNA polymerase to DNA. Transcription factors (i.e. NF-κB) bind to co-activator molecules (i.e. CBP), resulting in acetylation of the histone residues. This leads to unwinding of the DNA and allows increased binding of transcription factors resulting in increased gene transcription (figure adapted from Barnes, 1998).

Many of the above mentioned studies relied on the overexpression of components of these pathways, which could lead to misinterpretation of the results. Therefore, the examination of the role of histone acetylation and associated factors in the regulation of the glucocorticoid functions was performed in non-transfected cells. The ability of glucocorticoids dexamethasone and triamcinolone acetonide to regulate histone acetylation and deacetylation in three cell lines (U937, HUT-78 and Jurkat cells), was investigated. Changes in the phosphorylation status of histone 1 were also monitored as this would reveal that this linker histone dissociated from the chromatin complex allowing other core histones to be post-translationally modified. In this chapter it is demonstrated that both glucocorticoids show a different pattern of histone 4 acetylation from that seen with inflammatory stimuli LPS and TNF-α and in low doses (as determined in Chapter 3) repress LPS and TNF-α histone acetylation.

4.2 Results

4.2.1. Histone acetylation in three cell lines stimulated with LPS and TNF-α

In all three cell lines acetylation levels of the four core histones were investigated under the effect of inflammatory stimuli. This was to determine the acetylation pattern and therefore the role of each core histone in inflammatory responses of the cells. Acetylation of histones 2A and 2B failed to show any significant changes upon stimulation of the cells with LPS and TNF-α. Figure 4-2 illustrates a set of western blot analysis bands obtained from these experiments after 1h stimulation. Results obtained from analysis of U937 cells are shown as representative of all the experiments performed on all three cell lines (n=6).

Figure 4-3 illustrates changes in acetylation status of histones 2A, 2B, 3 and 4 in LPS (10 ng/ml) and TNF-a (10 ng/ml) induced U937, Jurkat and HUT-78 cells after 1h stimulation.

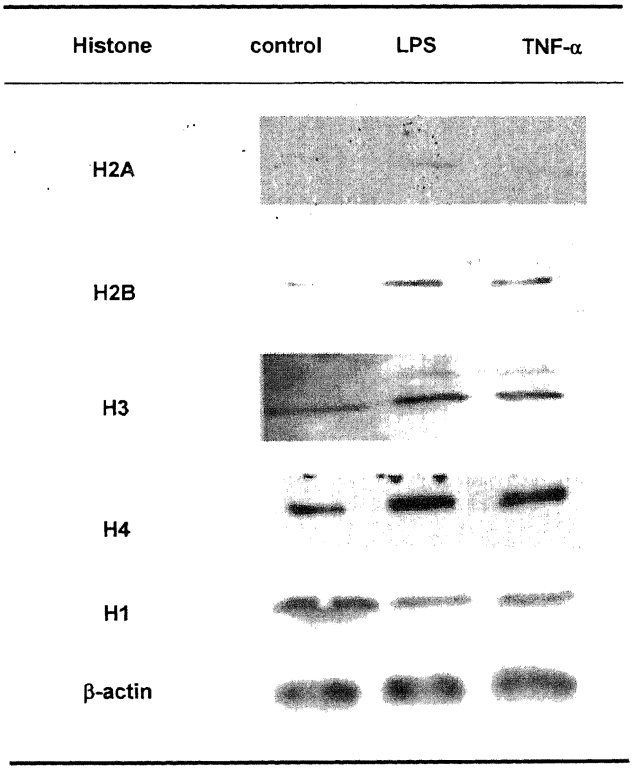


Figure 4-2 Western Blot analysis of lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) stimulated histone acetylation (and phosphorylation of H1) in a monocytic cell line (U937 cells). Lanes: (1) control, (2) LPS (10 ng/ml) stimulated, (3) TNF-α (10 ng/ml) stimulated. Results are shown for 1h stimulations. Similar results were obtained from experiments performed for T-cell lines Jurkat and HUT-78. β-actin was measured to ensure equal protein loading. The results are representative of six independent experiments.

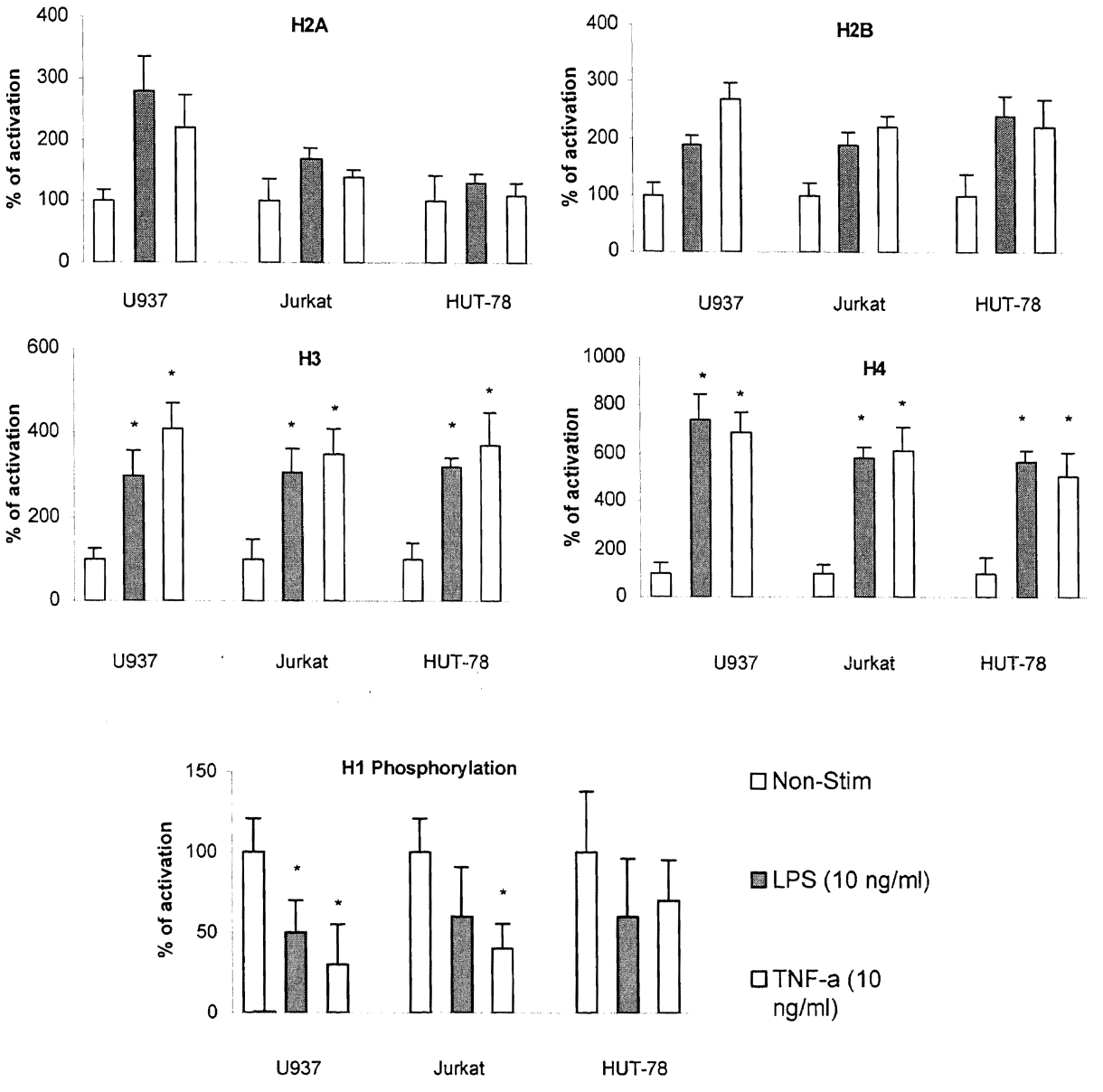


Figure 4-3 Acetylation of core histones and histone 1 phosphorylation in U937, Jurkat and HUT-78 cell lines as determined by Western blotting. The cells were stimulated with lipopolysaccharide (LPS; 10 ng/ml) or tumour necrosis factor α (TNF-α; 10 ng/ml) [concentrations determined previously (see Chapter 3)] for 1h. Histones 2A and 2B showed a small increase in acetylation following stimulation. Histones 3 and 4 however showed the highest up regulation with a significant elevation of acetylation levels for histone 4. Phosphorylation levels of histone 1 decreased indicating the weakening of the basic tails of the histones with DNA and therefore the transcriptional activation occurring in the cells. (*$p<0.05$) (n=6).

Treatment with both LPS and TNF-α for 1h affected all three cell lines studied in a similar manner. Changes in the levels of acetylation however were both cell and histone specific being greatest in the U937 cells (Fig. 4-3). Whereas H2A and H2B acetylation showed no significant increase in U937 cells following LPS and TNF-α treatment, acetylation of the other two core histones (H3 and H4) showed significant elevation. Specifically, acetylation of histone 4 was significantly up-regulated following stimulation by both LPS (740 ± 110 versus 100 ± 43 non-stimulated cells, $p<0.05$) and TNF-α (690 ± 81 versus 100 ± 43 non-stimulated cells, $p<0.05$). Histone 3 levels were also elevated but to a lesser extent for LPS (300 ± 58 versus 100 ± 26 non-stimulated cells, $p<0.05$) and TNF-a (410 ± 62 versus 100 ± 26 non-stimulated cells, $p<0.05$). Phosphorylation of linker histone 1 was also investigated. Down regulation of phosphorylation of H1 is interpreted as dissociation of this linker protein enabling activation of other histone proteins (Fig. 4-3). Both LPS and TNF-α reduced histone 1 phosphorylation in U937 cells whereas this effect was only significant following TNF-α stimulation in Jurkat cells. HUT-78 cells showed no difference in histone 1 phosphorylation after either stimulation.

4.2.2 Histone 4 lysine residue acetylation in three cell lines

Following the results shown in figure 4-2 and 4-3 further studies were required to investigate whether H4 acetylation was localised to specific lysine residues. Experiments were performed in LPS (10 ng/ml) and TNF-α (10 ng/ml) stimulated macrophage and T-cell lines in which acetylation of lysine residues 5, 8, 12 and 16 was investigated with the use of specific antibodies by both Western blotting and immunocytochemistry. Figure 4-4 shows band changes in the acetylation status of specific lysine residues for histone 4 when cells were treated with LPS and TNF-α for 1h. Due to similarities of the bands obtained in all cell lines, results obtained by U937 cells are shown as representative of all three cell lines. A graphical representation of the acetylation status of specific histone 4 lysine residues in all cells stimulated with LPS and TNF-α is shown in figure 4-5. All lysine residues show an elevation in acetylation in all three cell lines. Interestingly and in accordance to previous findings the highest levels of acetylation are observed for lysine residues 8 and 12 in U937 and T-cell lines, where acetylation of these lysines was significantly increased ($p<0.05$). Both inflammatory stimuli had a similar effect on acetylation levels in all three cell lines studied.

Western blot data at 1h was confirmed using confocal microscopy (Fig. 4-6). The micrographs show localised histone 4 lysine residue acetylation and increased acetylation of lysines 8 and 12 in cells stimulated with the pro-inflammatory stimuli LPS and TNF-α. Treatment with TSA alone showed an upregulation of acetylation of all lysine residues. Similar results were obtained from both T-cell lines stimulated in the same manner. The photomicrographs are representative of three individual experiments performed.

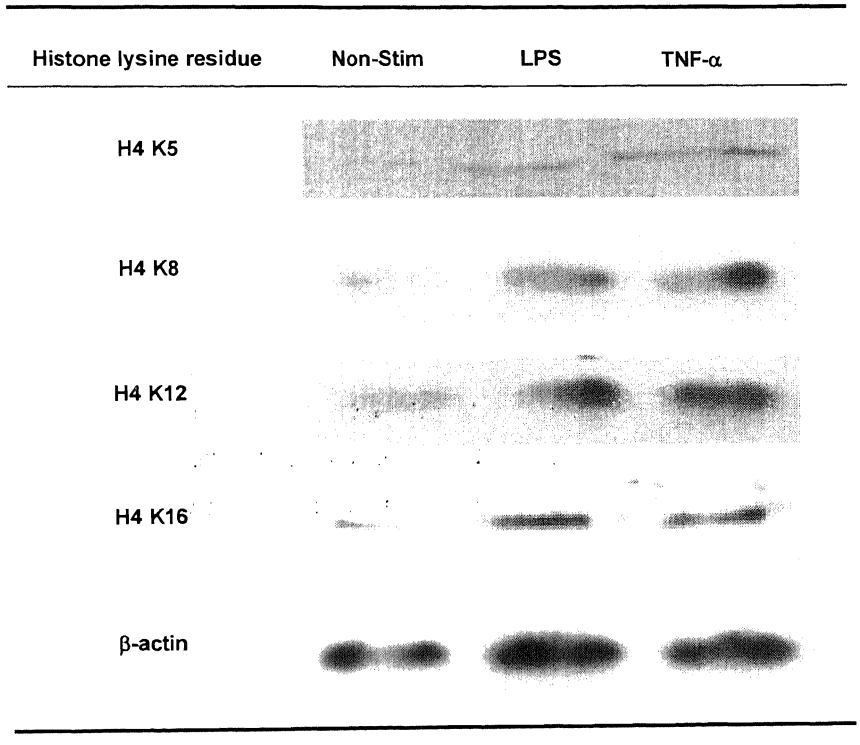


Figure 4-4 Western Blot analysis of histone 4 (H4) acetylation of specific lysine residues in a monocytic cell line (U937) stimulated for 1h with lipopolysaccharide (LPS) and tumour necrosis factor α (TNF-α). Lanes: (1) control, (2) LPS (10 ng/ml) stimulated, (3) TNF-α (10 ng/ml) stimulated. Similar results were obtained from experiments performed for T-cell lines Jurkat and HUT-78. β-actin was detected to ensure that equal amounts of protein were loaded onto the gels. The results are representative of six independent experiments.

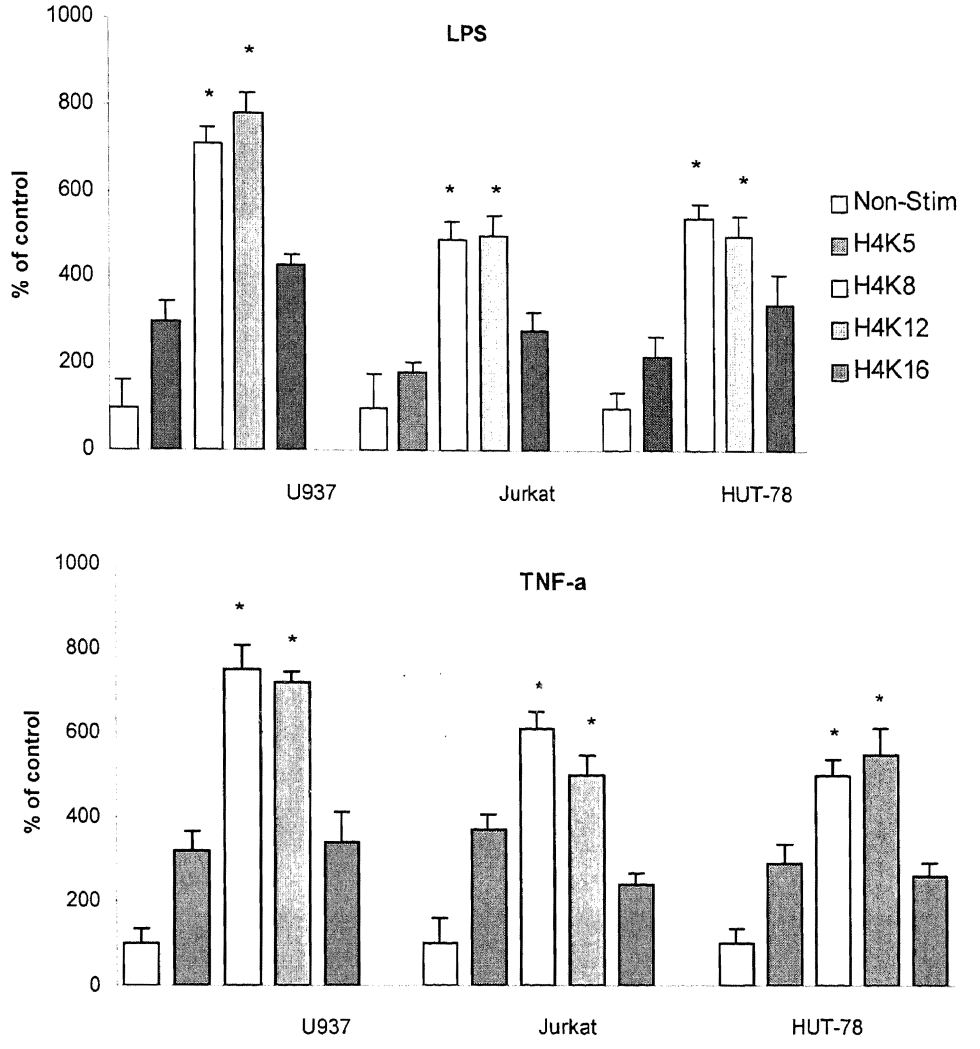


Figure 4-5 Western blotting analysis of lysine residue acetylation on histone 4 (H4) in U937, Jurkat and HUT-78 cells after 1h. Treatment of the cells with lipopolysaccharide (LPS; 10 ng/ml) and tumour necrosis factor α (TNF-α; 10 ng/ml) induced activation of acetylation on all four lysine residues. Acetylation on lysine residues 8 and 12 is elevated predominantly. Statistical analysis of the results revealed that in all three cell lines acetylation on lysines 8 and 12 was significantly upregulated in comparison to the remaining lysine residues. Results were similar in all cell lines investigated. (*$p<0.05$) (n=6).

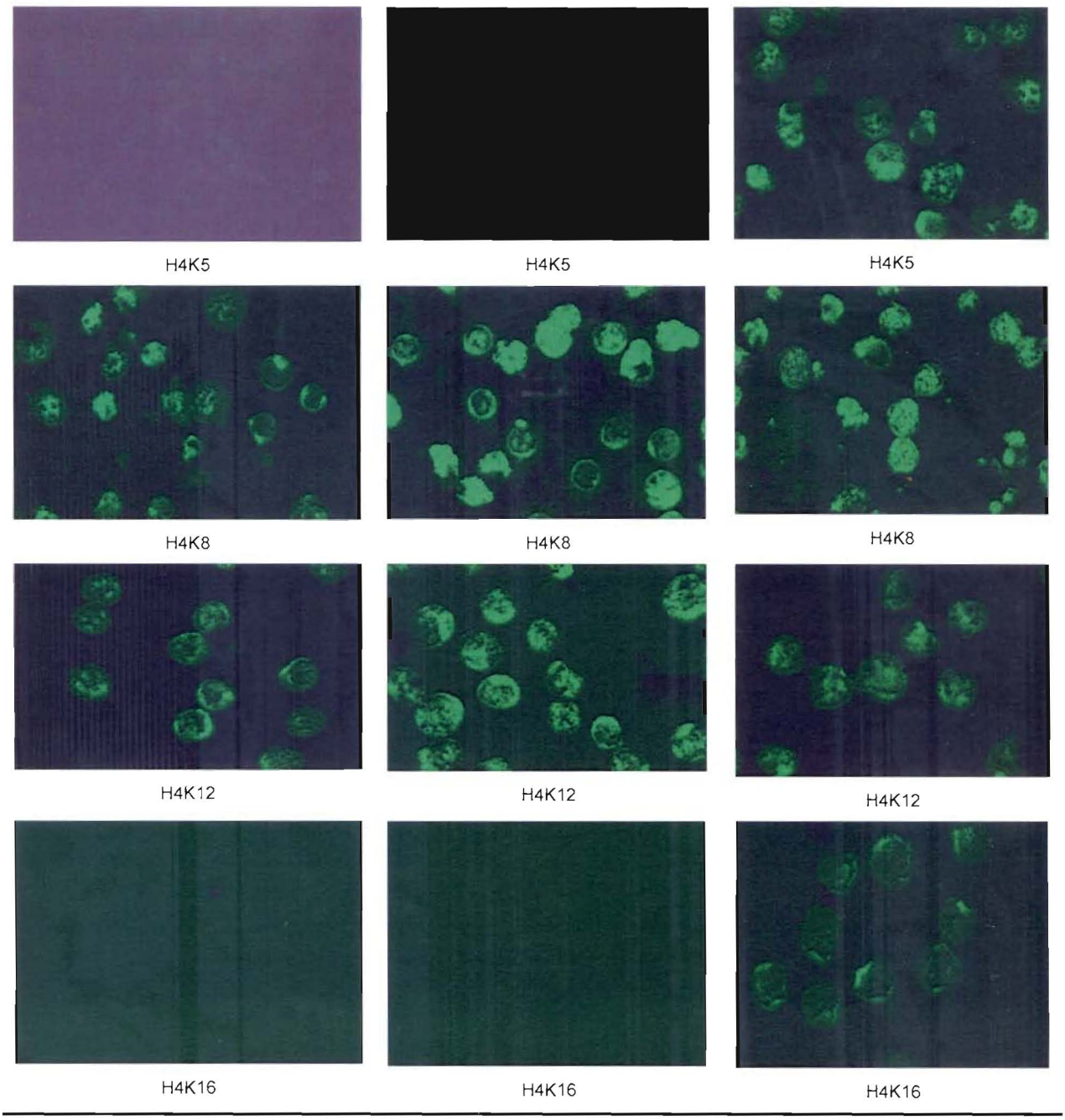


Figure 4-6 Immunocytochemical staining for specific histone 4 (H4) acetylated lysine residues of Jurkat cells stimulated with lipopolysaccharide (LPS), tumour necrosis factor α (TNF-α) and Trichostatin A (TSA). LPS and TNF-α acetylate specific and distinct lysine residues. TSA acetylates all four histone 4 lysine residues. Cells were incubated with LPS (10 ng/ml), TNF-α (10 ng/ml) and TSA (100 ng/ml) for 6 h before staining for acetylated forms of histone 4 lysine residues 5, 8, 12 and 16. For reference, micrographs of non-stimulated cells in all instances appeared similar to the LPS stimulated K5 and K16 micrographs. Counterstaining with DAPI confirmed that the staining was nuclear and not cytoplasmic (data not shown for clarity). Results are representative of three independent experiments.

4.2.3 Effect of TSA in lysine residue acetylation in three cell lines

In chapter 3 it was established that the histone deacetylase inhibitor, Trichostatin A (TSA), enhanced the LPS and TNF-α induced IL-8 production in monocytes and T-cells suggesting a role for chromatin acetylation in IL-8 production. Here, whether the effect of TSA is associated with acetylation on specific lysine residues within histone 4 is investigated. Cells were stimulated as previously with LPS and TNF-α with an additional stimulation of TSA. Western blotting experiments were performed and in figure 4-7 representative bands of the results obtained from western blot analysis of U937 cells stimulated with LPS and TNF-α as well as the additional effect of TSA are shown. Similar results were obtained for Jurkat and HUT-78 cells. The data from these experiments are illustrated in figure 4-8 in representative histograms. TSA as expected upregulated the acetylation levels of all lysine residues in all cell lines. In the T-cell lines the effect was similar to the effect observed in the macrophage cell line but less potent. Whereas further addition of TSA in the U937 cells resulted in an almost two-fold increase in acetylation, the acetylation levels for the T-cells were not elevated to the same extent.

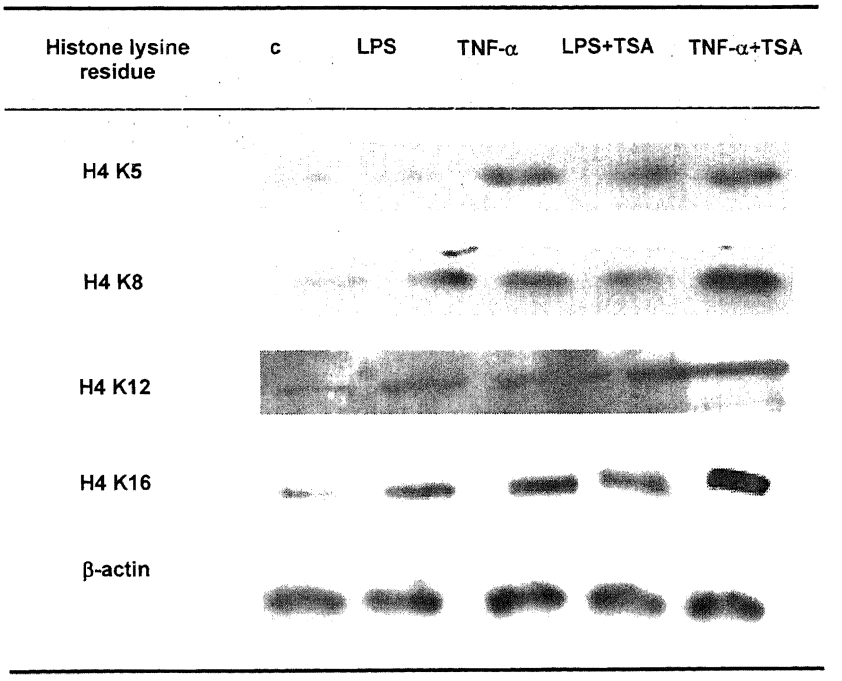


Figure 4-7 Western Blot analysis showing the effect of Trichostatin A (TSA) on histone 4 (H4) lysine (K) acetylation in a monocytic cell line (U937 cells) stimulated by lipopolysaccharide (LPS)- and tumour necrosis factor α (TNF-α). Lanes: (1) control, (2) LPS (10 ng/ml) (1hr incubation), (3) TNF-α (10 ng/ml) (1hr incubation), (4) LPS+TSA (LPS: 1 ng/ml, TSA: 10ng/ml) (1hr incubation), (5) TNF-α+TSA (TNF-α: 1 ng/ml, TSA: 10ng/ml) (1hr incubation). Increased acetylation on all lysine residues is evident with the addition of TSA. Similar results were obtained from experiments performed for T-cell lines Jurkat and HUT-78. β-actin was measured to ensure equal protein loading. The results are representative of six independent experiments. TSA (10ng/ml) alone enhanced acetylation of all lysine residues (data not shown).

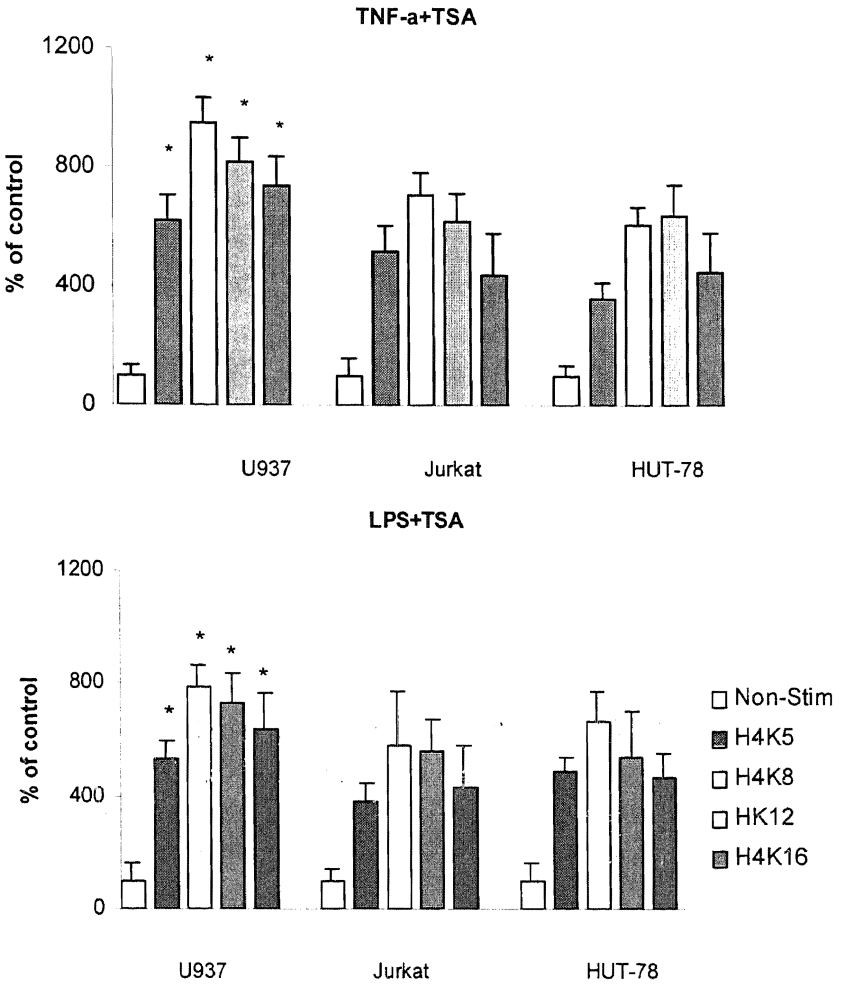


Figure 4-8 Changes in lysine residue acetylation of histone 4 (H4) in U937, Jurkat and HUT-78 cells following treatment with Trichostatin A (TSA). Co-stimulation of cells with TSA and lipopolysaccharide (LPS) or tumour necrosis factor α (TNF-α) for 1h resulted in an increase of acetylation levels of all H4 lysine residues as seen on figure 4-5. Results were similar in all cell lines investigated. (*$p<0.05$) (n=6).

4.2.4 Effect of steroids in histone acetylation

Next, the effect of the glucocorticoids, dexamethasone (Dex; 10^{-8} M) and triamcinolone acetonide (TA; 10^{-10} M) alone, and on LPS- and TNF-α -stimulated mediator release, on histone acetylation, was investigated. In addition, the pattern of histone acetylation following glucocorticoid stimulation of cells and glucocorticoid suppression of LPS or TNF-α –induced acetylation, was determined.

First, the effect of Dex and TA alone, in all three cell lines was studied. The cells were stimulated with relatively high concentrations of steroids, as these have been shown to transactivate the expression of responsive genes (Truss & Beato, 1993). There was no significant effect of TA and Dex on acetylation of histones 2A, 2B and 3 (data not shown). Both glucocorticoids, as illustrated in figure 4-10 (figure 4-9 illustrates representative bands from experiments), targeted acetylation on H4 K5 and K16 (with weaker signals apparent at K8 and K12) (values are shown in table 4-1). TA induced higher levels of histone acetylation compared to those seen with Dex.

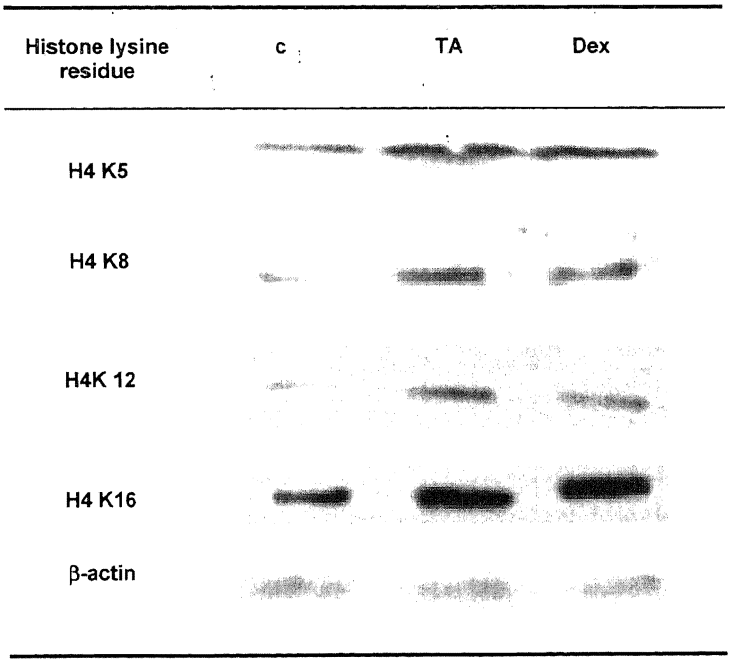


Figure 4-9 Western Blot analysis of Triamcinolone Acetonide (TA) and Dexamethasone (Dex) actions in a monocytic cell line (U937 cells). Lanes: (1) control, (2) TA (10^{-10} M), (3) Dex (10^{-8} M). Increased acetylation in histone 4 (H4) lysine (K) residues 5 and 16 is evident in the presence of both glucocorticoids. Similar results were obtained from experiments performed for T-cell lines Jurkat and HUT-78. β-actin was measured to ensure equal protein loading. The results are representative of six independent experiments performed after 1h stimulation.

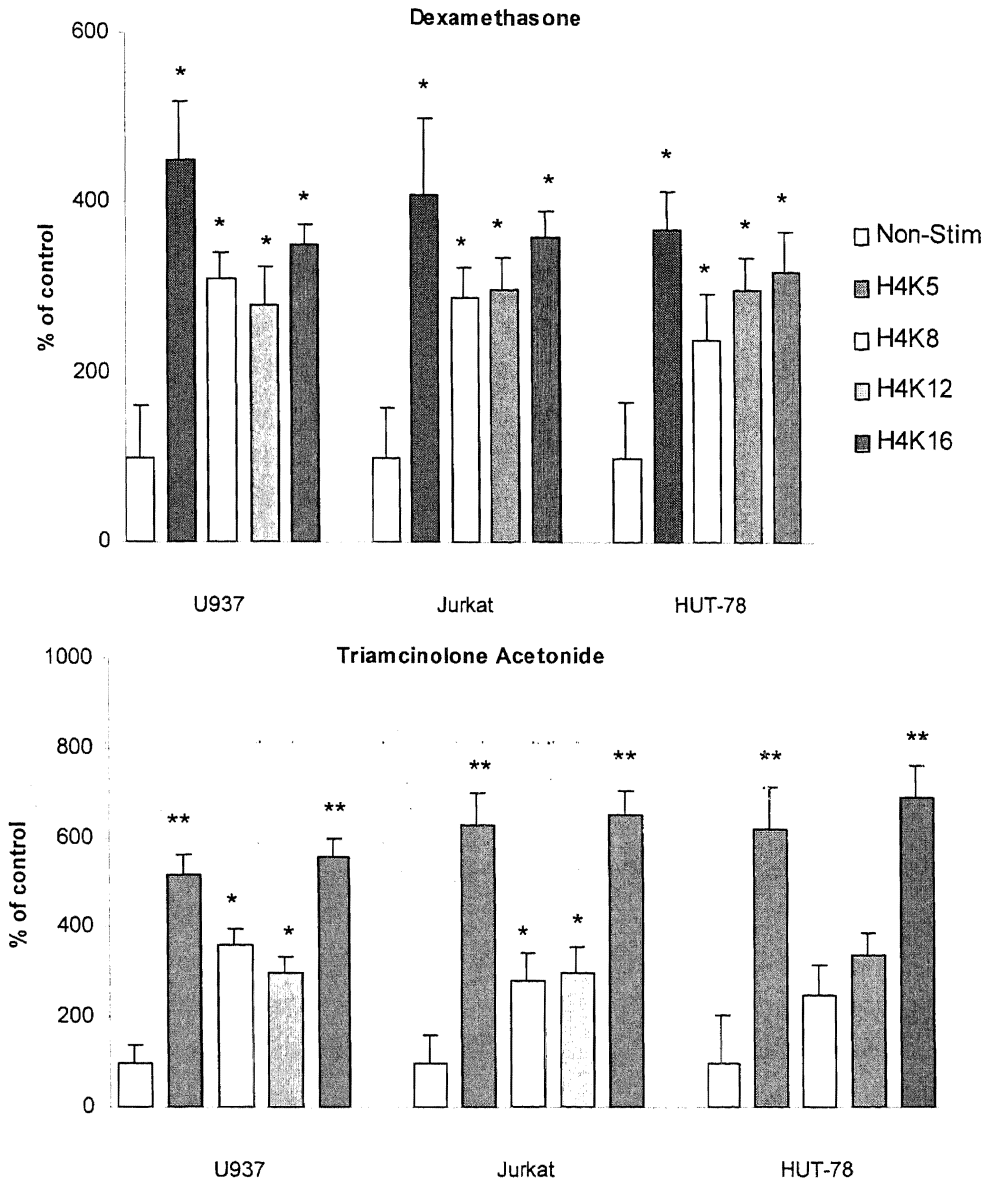


Figure 4-10 Western Blot analysis of the effect of Dexamethasone (Dex) and Triamcinolone Acetonide in histone 4 (H4) lysine (K) residue acetylation in U937, Jurkat and HUT-78 cells. Treatment of the cells with Dex (10^{-8} M) and TA (10^{-10} M) significantly induced activation of acetylation on residues K5 and K16. Acetylation on residues K8 and K12 was also upregulated but to a lesser degree. The results shown were obtained by Western Blotting and are expressed as a percentage of the control after 1h (*$p<0.05$, **$p<0.01$) (n=6).

		Increase in histone 4 lysine acetylation			
Cell line	**Stimulation**	**H4K5**	**H4K8**	**H4K12**	**H4K16**
U937	Dex	503 ± 70 ($p<0.05$)	316 ± 30	323 ± 45	414 ± 45 ($p<0.05$)
	TA	526 ± 40 ($p<0.01$)	333 ± 37	330 ± 36	570 ± 36 ($p<0.01$)
Jurkat	Dex	480 ± 90 ($p<0.05$)	310 ± 34	330 ± 36	383 ± 32 ($p<0.05$)
	TA	563 ± 70 ($p<0.01$)	283 ± 65	343 ± 58	600 ± 55 ($p<0.01$)
HUT-78	Dex	416 ± 45 ($p<0.05$)	293 ± 55	330 ± 36	393 ± 47 ($p<0.05$)
	TA	593 ± 92 ($p<0.01$)	410 ± 68	356 ± 47	613 ± 75 ($p<0.01$)

Table 4-1 Increases in acetylation of histone 4 (H4) lysine residues in cells stimulated with Dexamethasone (Dex) and Triamcinolone Acetonide (TA). In all three cells lines (U937, Jurkat and HUT-78) acetylation of all lysine residues increased following glucocorticoid stimulation (Dex, 10^{-8} M; TA, 10^{-10} M). Acetylation of lysine (K) residues 5 and 16 was noted as significantly higher than that of K8 and K12 in U937, Jurkat and HUT-78 cells. The results were obtained via Western blotting and presented as mean ± SEM increase in band density at 1h.

Photomicrographs in figure 4-11 illustrate H4 lysine activation in U937 cells stimulated with TA. A strong nuclear signal of cells stained with K5 and K16 antibodies is shown. Activation of cells stained with K8 and K12 antibodies is also present but to a much weaker degree. Due to the similarity of photomicrographs obtained for Jurkat and HUT-78 cells and also for cells stimulated with Dex, only one set of results is shown. The figure is representative of results from three individual experiments.

It was further examined whether Dex and TA could suppress K8 and K12 acetylation induced by LPS and TNF-α. Figure 4-12 shows the effect of glucocorticoids in LPS- and TNF-α- induced histone lysine acetylation expressed as a percentage of the control. A significant reduction of acetylation levels is observed for residues K8 and K12 in all cell lines.

In LPS-stimulated U937 cells, Dex suppressed K8 acetylation by 52% (396 ± 68% versus 826 ± 41%, $p<0.01$) and K12 acetylation by 47% (406 ± 61% versus 773 ± 104%, $p<0.01$). TA suppressed K8 acetylation by 61% (323 ± 55% versus 826 ± 41%, $p<0.01$) and K12 acetylation by 52% (370 ± 60 versus 773 ± 104%, $p<0.01$).

In TNF-α stimulated U937 cells, Dex suppressed K8 acetylation by 52% (380 ± 65% versus 796 ± 57%, $p<0.01$) and K12 acetylation by 42% (400 ± 62% versus 693 ± 35%, $p<0.01$). TA had a more profound effect and suppressed K8 acetylation by 56% (350 ± 26% versus 796 ± 57%, $p<0.01$) and K12 acetylation by 50% (340 ± 30% versus 693 ± 35%, $p<0.01$).

In LPS-stimulated Jurkat cells, Dex suppressed K8 acetylation by 46% (300 ± 66% versus 560 ± 72%, $p<0.01$) and K12 acetylation by 18% (470 ± 40% versus 580 ± 53%, $p<0.05$). TA was more potent as it suppressed K8 acetylation by 51% (270 ± 49%, $p<0.01$) and K12 acetylation by 47% (296 ± 55%, $p<0.01$).

In TNF-α -stimulated Jurkat cells, Dex suppressed K8 acetylation by 48% (310 ± 35% versus 603 ± 40% TNF-α alone, $p<0.01$) and K12 acetylation by 32% (330 ± 36% versus 483 ± 40% TNF-α alone, $p<0.01$). TA, similarly, suppressed K8 acetylation by 51% (293 ± 46%, $p<0.01$) and K12 acetylation by 37% (303 ± 70%, $p<0.01$).

Finally, the effect that the two glucocorticoids had on LPS and TNF-α -stimulated HUT-78 cells was investigated. Dex suppressed K8 acetylation by 43% (313 ± 32% versus 553 ± 60% LPS alone, $p<0.01$) and K12 acetylation by 30% (403 ± 72% versus 573 ± 50% LPS alone, $p<0.01$). TA also suppressed K8 by 47.5% (290 ± 79%, $p<0.01$) and K12 by 48% (300 ± 55%, $p<0.01$) acetylated levels.

In TNF-α-stimulated HUT-78 cells, Dex suppressed K8 acetylation by 47% (273 ± 86% versus 516 ± 38% TNF-α alone, $p<0.01$) and K12 acetylation by 50% (250 ± 81% versus 500 ± 62% TNF-α alone, $p<0.01$). Only in this cell type was the TA effect less potent on both K8 (41% suppression, 306 ± 70%, $p<0.01$) and K12 (36% suppression, 320 ± 46%, $p<0.01$) residues.

Interestingly, there was a mutual repression of K5 and K12 acetylation induced by dexamethasone and TA by both LPS and TNF-α (compare Figures 14a and 14b with results in Table 4.0).

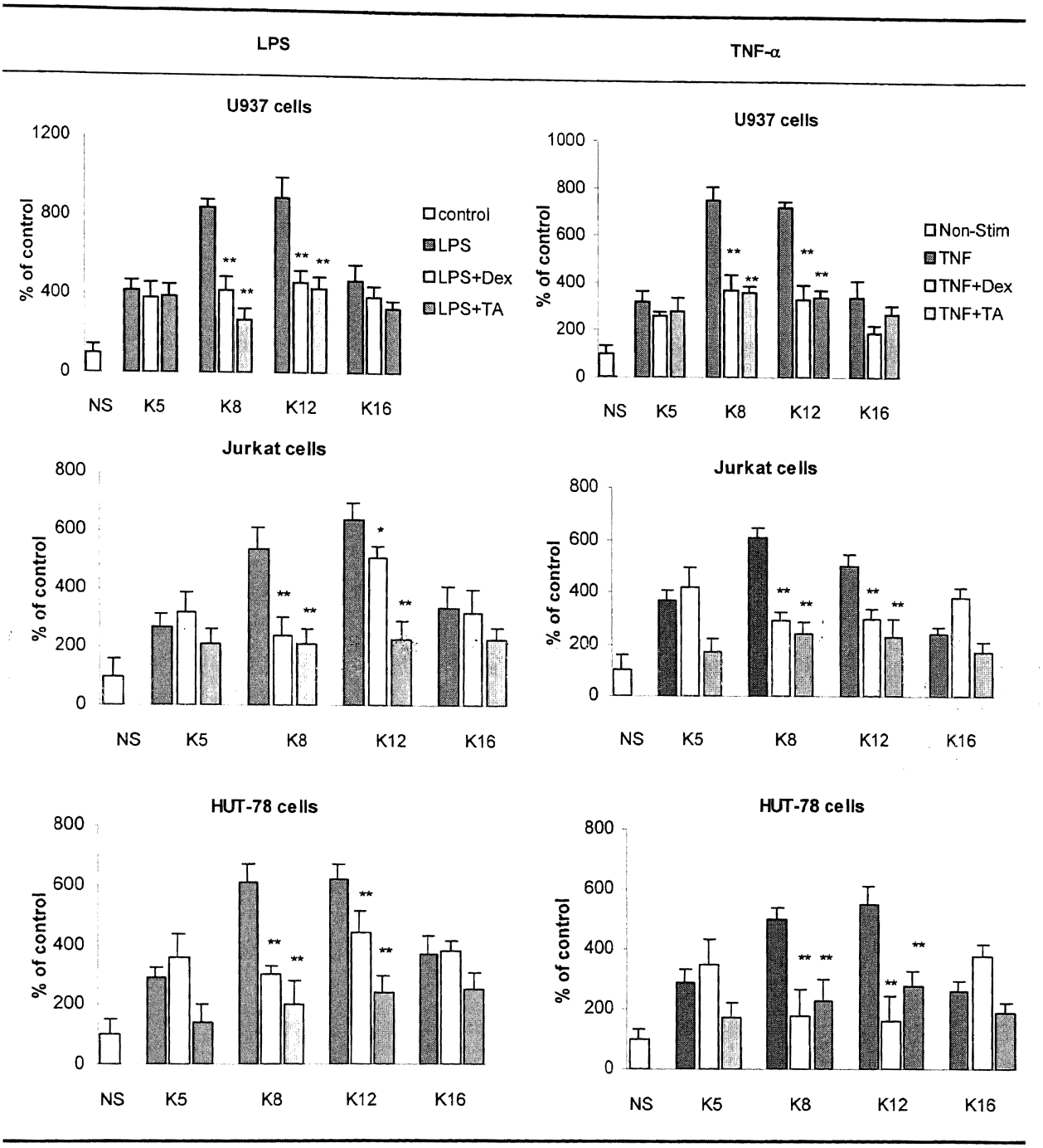


Figure 4-12 Effect of Dexamethasone (Dex) and Triamcinolone Acetonide (TA) lipopolysaccharide (LPS)- and tumour necrosis factor α (TNF-α) -induced histone 4 (H4) lysine (K) acetylation in U937, Jurkat and HUT-78 cells. Treatment of the cells with LPS or TNF-α (both at 10 ng/ml) significantly induced activation of acetylation on lysine residues 8 and 12. Addition of Dex (10^{-8} M) or TA (10^{-10} M) reduced the levels of acetylation of all lysine residues with a more potent decrease on K8 and K12. The results shown were obtained by Western Blotting and expressed as a percentage of the control at 1h (*p<0.05, **p<0.01) (n=6).

4.2.5 Effect of TSA in histone 4 inflammatory stimuli –induced acetylation in the presence of glucocorticoids

TSA, as shown previously, elevated the acetylation levels of all histone 4 lysine residues in cells stimulated with LPS and TNF-α (Figure 4-8). The effect of TSA in the presence of Dex and TA was further investigated. U937, Jurkat and HUT-78 cells were stimulated as previously (pg. 80). Dex or TA in the presence of TSA (1 ng/ml for U937 cells and 10 ng/ml for Jurkat and HUT-78 cells), were added 30 min prior to stimulation with the inflammatory stimuli.

Western blotting revealed that in U937 cells, TSA partially overcame the effect of the steroids. In LPS-stimulated U937 cells, TSA attenuated Dex suppression on K8 acetylation (470 ± 30% versus 353 ± 29% LPS alone, $p<0.05$) and K12 acetylation (517 ± 30 versus 380 ± 45 LPS alone, $p<0.05$). A lesser effect was noted on K5 acetylation (410 ± 65% versus 320 ± 34% LPS alone, $p<0.05$) and K16 acetylation (243 versus 216 ± 32 LPS alone, $p<0.05$).

TSA attenuated TA suppression on K8 acetylation (390 ± 75% versus 340 ± 26% LPS alone, $p<0.05$) and K12 acetylation (483 ± 65 versus 326 ± 38 LPS alone, $p<0.05$). TSA had a lesser effect on K5 acetylation (313 ± 293% versus 293 ± 30% LPS alone, $p<0.05$) and K16 acetylation (306 versus 226 ± 25 LPS alone, $p<0.05$) (Fig. 4-14.a).

In LPS-stimulated Jurkat cells, TSA also attenuated Dex suppression but to a lesser extent. TSA attenuated Dex suppression on K8 acetylation (360 ± 70% versus 310 ± 40% LPS alone, $p<0.05$) and K12 acetylation (520 ± 94% versus 496 ± 38% LPS alone). TSA had a lesser effect on K5 acetylation (510 ± 105% versus 430 ± 62% LPS alone) and K16 acetylation (360 ± 66% versus 280 ± 57%).

TSA attenuated TA suppression on K8 acetylation (380 ± 46% versus 200 ± 61% LPS alone, $p<0.05$) and K12 acetylation (490 ± 66% versus 290 ± 53% LPS alone, $p<0.05$). TSA had a lesser effect on K5 acetylation (420 ± 44% versus 266 ± 50% LPS alone, $p<0.05$) and K16 acetylation (480 ± 56% versus 290 ± 49% LPS alone, $p<0.05$) (Fig. 4-14.a).

In LPS-stimulated HUT-78 cells, TSA also attenuated Dex suppression on K8 acetylation (480 ± 53% versus 340 ± 52% LPS alone, $p<0.05$) and K12 acetylation (510 ± 45% versus 405 ± 66% LPS alone, $p<0.05$). TSA had a lesser effect on K5 acetylation (470 ± 50% versus 320 ± 76% LPS alone, $p<0.05$) and K16 acetylation (440 ± 46% versus 310 ± 74%, $p<0.05$).

TSA attenuated TA suppression on K8 acetylation (270 ± 42% versus 190 ± 31% LPS alone) and K12 acetylation (350 ± 61% versus 220 ± 34% LPS alone, $p<0.05$). TSA had a lesser effect on K5 acetylation (250 ± 35% versus 170 ± 47% LPS alone) and K16 acetylation (420 ± 65% versus 320 ± 55% LPS alone) (Fig. 4-14.a).

In TNF-α-stimulated U937 cells, TSA had a more variable effect. It blocked considerably the Dex effect (decreased acetylated lysine levels) but the effect of TA was not significantly altered on any of the lysine residues when cells were further stimulated with TSA. In Jurkat cells however, the inhibitor had a similar effect as the one observed in cells induced with LPS and under the influence of both corticosteroids.

Likewise, in TNF-α-stimulated U937 cells TSA attenuated the Dex effect on K8 acetylation (112 ± 35%, $p<0.05$) and K12 acetylation (70 ± 25%, $p<0.05$). TSA also attenuated the effect of Dex on K5 acetylation (110 ± 37%, $p<0.05$) and K16 acetylation (80 ± 28%, $p<0.05$) (Fig. 4-14.b).

In TNF-α-stimulated U937 cells TSA attenuated the TA effect on K8 acetylation (170 ± 43%) and K12 acetylation (60 ± 32%). TSA also attenuated the effect of TA on K5 acetylation (160 ± 50%) and K16 acetylation (20 ± 12%) (Fig. 4-14.b).

In the T-cell lines, the TSA blocking effect was similar, in that it increased acetylation levels of all lysine residues proportionally to the previous effect of each steroid. In TNF-α-stimulated Jurkat cells, TSA attenuated Dex suppression on K8 acetylation (160 ± 65%, $p<0.05$) and K12 acetylation (170 ± 64%, $p<0.05$). TSA also attenuated Dex suppression on K5 acetylation (160 ± 56%, $p<0.05$) and K16 acetylation (120 ± 32%, $p<0.05$).

In TNF-α-stimulated Jurkat cells, TSA attenuated TA suppression on K5 acetylation (100 ± 46%, $p<0.05$), K8 acetylation (130 ± 62%, $p<0.05$), K12 acetylation (40 ± 27%) and K16 acetylation (120 ± 32%, $p<0.05$).

Finally, in TNF-α-stimulated HUT-78 cells TSA attenuated the Dex effect on K8 acetylation (120 ± 46%, $p<0.05$) and K12 acetylation (160 ± 68%, $p<0.05$). TSA also attenuated the effect of Dex on K5 acetylation (70 ± 55%) and K16 acetylation (180 ± 57%, $p<0.05$) (Fig. 4-14.b).

In TNF-α-stimulated HUT-78 cells TSA attenuated the TA effect on K8 acetylation (120 ± 46%, $p<0.05$) and K12 acetylation (190 ± 58%, $p<0.05$). TSA also attenuated the effect of TA on K5 acetylation (90 ± 55%, $p<0.05$) and K16 acetylation (200 ± 45%, $p<0.05$) (Fig. 4-14.b).

Representative bands of the results obtained are shown in figure 4-13 for U937 cells, since they were the only cell line not to be affected by TSA under the effect of triamcinolone acetonide.

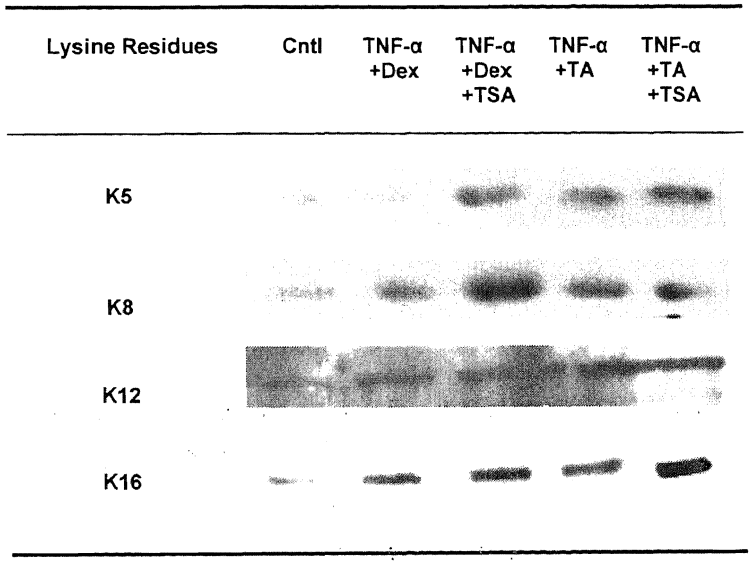


Figure 4-13 Western Blot analysis of Trichostatin A (TSA) actions on tumour necrosis factor α (TNF-α) - stimulated histone 4 (H4) lysine (K) acetylation in the presence of Dexamethasone (Dex) (10^{-8} M) and Triamcinolone Acetonide (TA) (10^{-10} M) in U937 cells. Lanes: control, TNF-α + Dex, TNF-α + Dex + TSA, TNF-α + TA and TNF-α + TA + TSA. Increased acetylation of all lysine residues is evident with the addition of the inhibitor. Similar results were obtained from experiments performed for the Jurkat and HUT-78 T-cell lines. The results are representative of four independent experiments performed at 1h post stimulation.

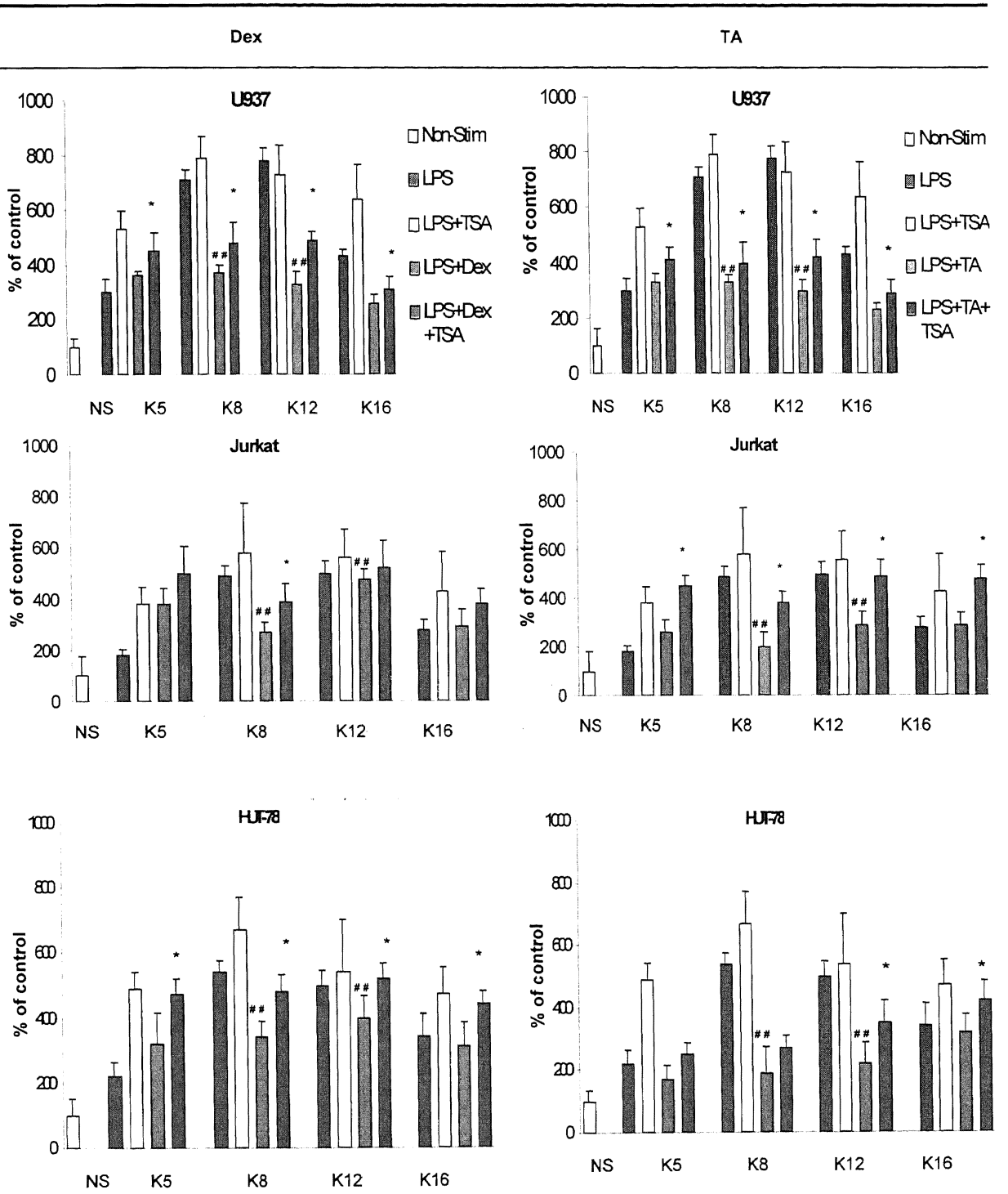


Figure 4-14.a Effect of Trichostatin A (TSA) in U937, Jurkat and HUT-78 cells stimulated with lipopolysaccharide (LPS) and glucocorticoids. Cells were stimulated with either LPS (10 ng/ml) in the presence of Dexamethasone (Dex; 10^{-8} M) or Triamcinolone Acetonide (TA; 10^{-10}M) and histone acetylation levels were monitored after further addition of TSA (1 ng/ml for U937 cells and 10 ng/ml for Jurkat and HUT-78 cells) by Western blotting. The histograms show percentage changes in acetylation levels of all histone 4 (H4) lysine (K) residues. Increased levels of acetylation are observed in all lysine residues in all three cell lines with TSA. (*$p<0.05$) (n=4). Dex and TA suppressed LPS-stimulated K8 and K12 histone acetylation in all 3 cell lines (##$p<0.01$).

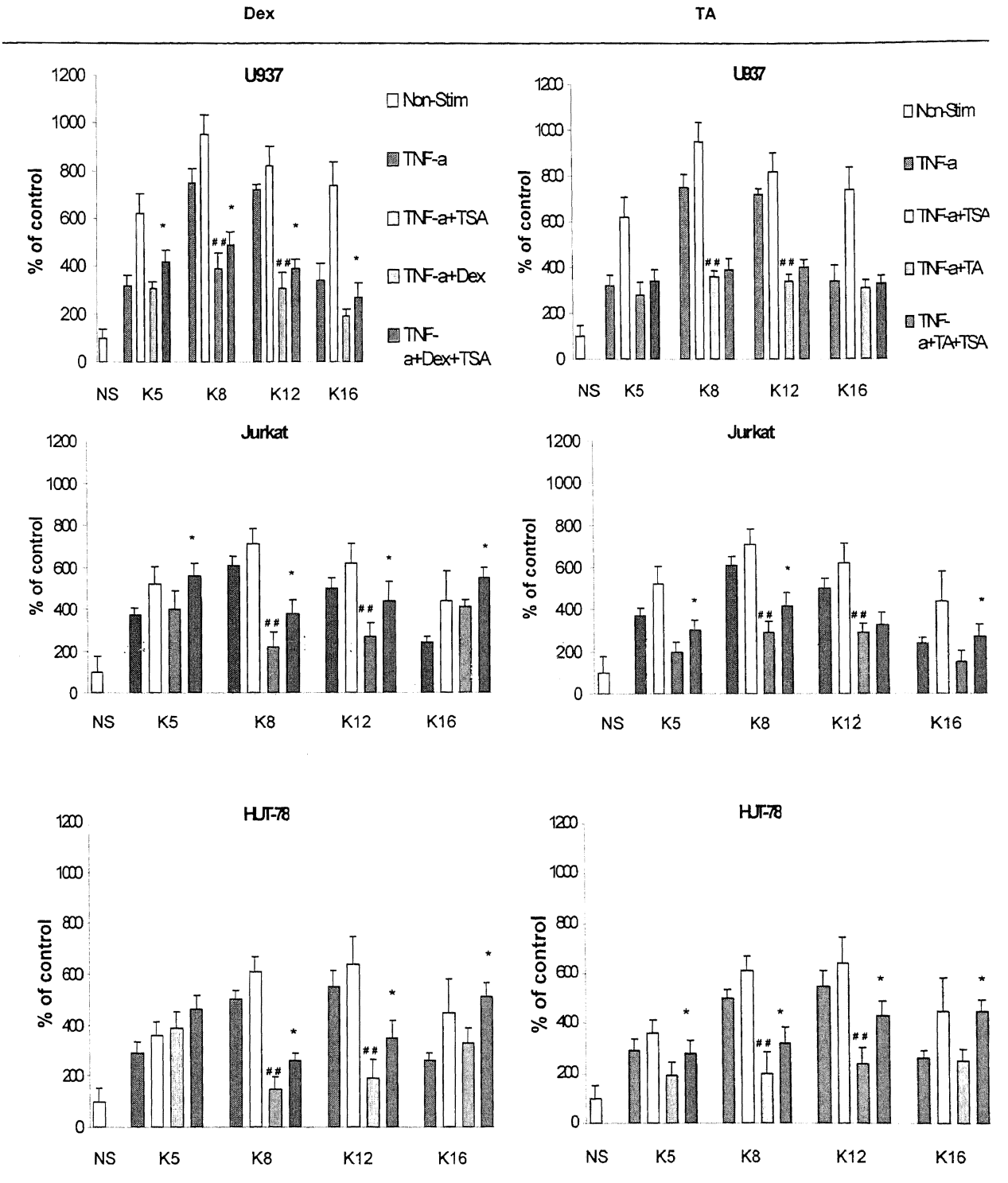


Figure 4-14.b Effect of Trichostatin A (TSA) in U937, Jurkat and HUT-78 cells stimulated with tumour necrosis factor α (TNF-α) and glucocorticoids. Cells were stimulated with either TNF-α (10 ng/ml) in the presence of Dexamethasone (Dex; 10^{-8} M) or Triamcinolone Acetonide (TA; 10^{-10} M) and histone acetylation levels were monitored after further addition of TSA (1 ng/ml for U937 cells and 10 ng/ml for Jurkat and HUT-78 cells) by Western blotting. The histograms show changes in acetylation levels of all histone 4 (H4) lysine (K) residues. Increased levels of acetylation are observed on all lysine residues in all three cell lines with TSA, with the exception of TNF-α-stimulated U937 cells where TSA did not affect histone acetylation. (*$p<0.05$) (n=4). Dex and TA suppressed TNF-α-stimulated K8 and K12 histone acetylation in all 3 cell lines (##$p<0.01$).

4.2.6 Histone deacetylase enzyme activity assay

Since HDACs are enzymes it was important to investigate the effect of LPS and TNF-α on HDAC activity in the presence and absence of glucocorticoids. U937, Jurkat and HUT-78 cells were stimulated with Dex (10^{-8} M) or TA (10^{-10} M) for 30 min followed by stimulation with either LPS or TNF-α for 1hr and nuclear extracts isolated. Figure 4-15 illustrates changes in HDAC activity in all three cell lines. HDAC enzymic activity, as measured by a fluorescent activity assay, did not significantly increase following stimulation of the cells with LPS or TNF-α compared to the non-stimulated cells. Similar results were seen in all three cell lines. In U937 cells, addition of Dex and TA resulted in a significant increase in HDAC activity. Addition of Dex caused an upregulation in cells stimulated with LPS (435 ± 83% versus 200 ± 45% LPS alone) and TNF-α (475 ± 50% versus 180 ± 25% TNF-α alone). In addition, TA resulted in greater upregulation following both LPS- (570 ± 80% versus 200 ± 45% LPS alone) and TNF-α-stimulation (680 ± 145% versus 180 ± 25% TNF-α alone).

In the T-cell lines HDAC activity was also increased when cells were stimulated with either Dex or TA in the presence of LPS or TNF-α but to lower levels than seen in U937 cells. In Jurkat cells the upregulation reached a maximum of 435 ± 83% versus 210 ± 25%, increase (in cells stimulated with TNF-α and TA). In HUT-78 cells, TA was more potent than Dex. Addition of TA to both LPS- (250 ± 43% versus 160 ± 25% LPS alone) and TNF-α- (480 ± 43% versus 170 ± 26% TNF-α alone) stimulated cells resulted in the highest upregulation of HDAC activity. Addition of TSA inhibited HDAC activity in all cell lines as expected to baseline levels. The data represent the means ± the SEM of three independent experiments (*$p<0.05$, **$p< 0.01$).

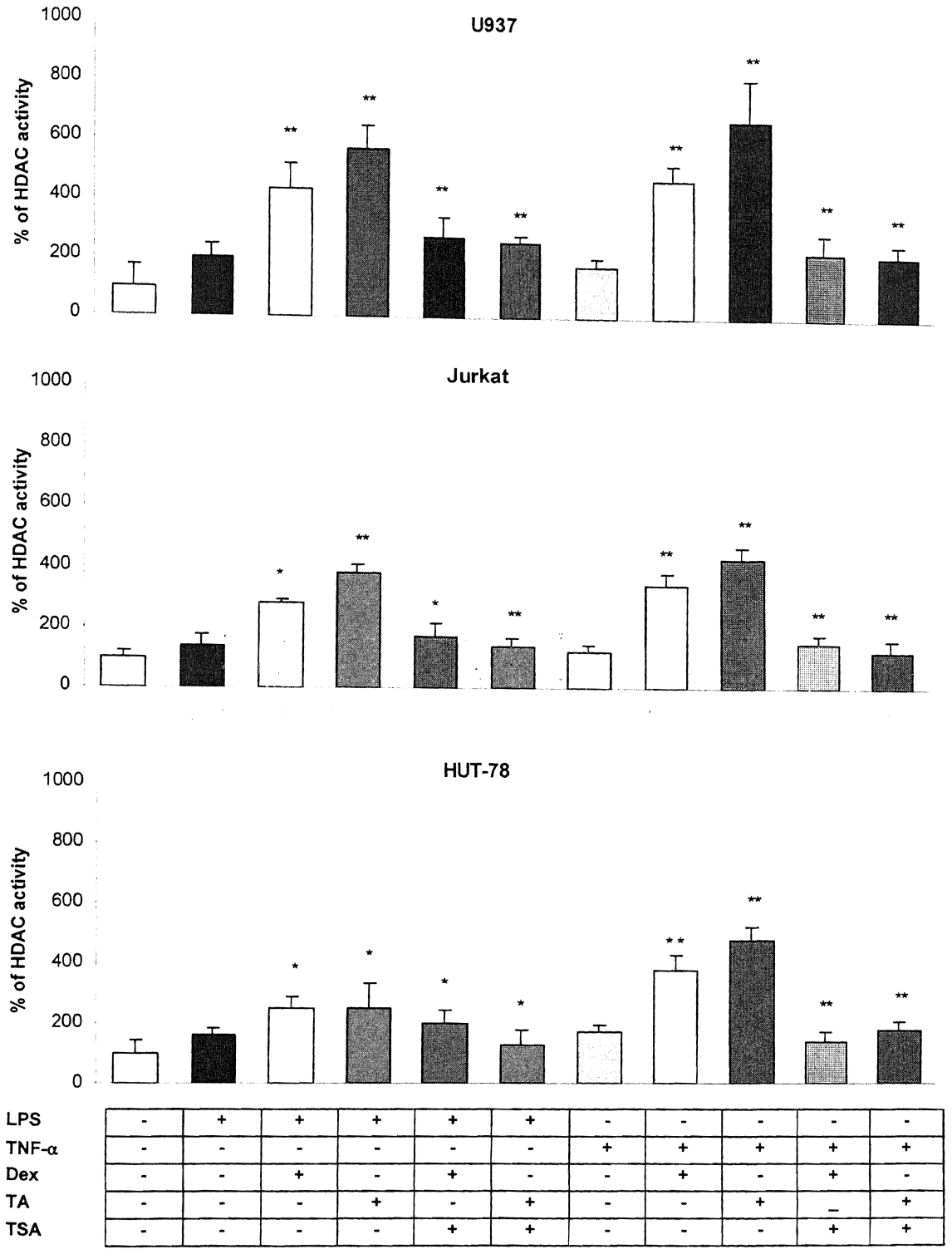


Figure 4-15 Effect of lipopolysaccharide (LPS), tumour necrosis factor α (TNF-α) and glucocorticoids on HDAC activity. HDAC activity was measured by a fluorescent activity assay. Dexamethasone (Dex; 10^{-8} M) and Triamcinolone Acetonide (TA; 10^{-10}M) enhanced HDAC activity in the presence of LPS or TNF-α. This was in contrast to the lack of effect seen with LPS and TNF-α. The findings were consistent in monocytes and T-cells. TSA downregulated HDAC activity to basal levels in all cell lines. The results are representative of three independent experiments and are presented as the mean ± SEM. (*p<0.05, **p< 0.01) (n=4).

4.3 Discussion

The work described in this chapter set out to characterise histone acetylation, as the most extensively studied histone modification, which occurs in response to inflammatory-stimuli in monocytes and T-cells. This was undertaken because of the important role that both macrophages and lymphocytes play in the regulation of inflammation.

In the U937 cells, as well as in the T-cell lines Jurkat and HUT-78, both LPS and TNF-α induced histone acetylation. Acetylation of histones 2A, 2B and 3 increased by a small factor but acetylation of histone 4 was predominant (seven-fold increase in the monocytic cell line and six-fold increase in the T-cell lines). Histone 4 acetylation has been previously shown to be upregulated in a number of cell lines. H4 acetylation was induced in the lung epithelial cell line A549 by IL-1β (Ito *et al.,* 2000) and in MCF-7 cells, where estradiol was used for induction of the progesterone receptor (Ruh *et al.,* 1999). This core histone dependent acetylation, where acetylation levels on H4 were higher than H3 and much higher than H2A and H2B (H2A≅H2B), suggests a role of histone 4 in inflammatory transcriptional regulation, while involvement of other core histones does not seem to be of major importance. These *in vivo* results also differ with findings in yeast, where acetylation on H3 is predominant compared with other core histones (Van Driel & Otte, 1997).

The core histones have been associated however, with other cell functions such as the cell cycle and cell proliferation. Acetylation of specific lysine residues in histone 3, for example, is associated with processes apart form transcription (Turner & O'Neil, 1995). During DNA replication, new histones are rapidly synthesized and assembled onto the replicated DNA. H3 (and H4) is brought to replicating chromatin in a pre-acetylated state that becomes erased after replication is completed and the newly assembled chromatin matures (Turner & O'Neil, 1995). Histone 2A is phosphorylated during mitosis (Paulson & Taylor, 1982) while ADP ribosylation of histone 2B introduces a negatively charged branched molecule into chromatin that resembles a single stranded nucleic acid and therefore such a structure disrupts higher order chromatin structure and potentially displaces the more weakly bound histones from nucleosomes.

The finding that replication-coupling assembly factor (RCAF), a chromatin assembly complex in *Drosophila*, contains H4 specifically acetylated at lysines 5 and 12 suggests that these acetylation sites play an important role in chromatin assembly (Tyler *et al.,* 1999). Following the results demonstrating that H4 was highly upregulated by inflammatory stimuli in monocytes and T-cells, acetylation of the specific lysine residues in these cells was further investigated. Lysines 8 and 12 were significantly upregulated in all three cell lines suggesting that a common pathway occurred in the inflammatory gene expression process, whilst LPS and TNF-α did not show any significant differences in the upregulated levels of histone

acetylation in any of the cell lines in question. Ito et al. (2000) reported an upregulation of the same lysine residues in A549 cells. The same paper showed that p65 mediated activation of the GM-CSM promoter *in vitro* is concomitant with the acetylation of H4 K8 and K12 residues. The agreement of the present results with Ito et al. (2000), in conjunction with findings presented in Chapter 3, also suggests that p65 mediated activation of the IL-8 promoter *in vitro* could be concomitant with the acetylation of H4 K8 and K12 residues.

Trichostatin A has been shown to release transcriptional repression in some model systems. Studying the effect of TSA in the activation of histone 4 lysines showed that the actions of TSA were not lysine residue specific. It was however shown that TSA significantly upregulated acetylation of all histone 4 lysine residues in both monocytes and T-cells.

The effect of glucocorticoids in lysine specific histone 4 acetylation was further investigated. Western blotting and immunocytochemistry showed lysine 5 and 16 specificity for glucocorticoids alone in all three cell lines. Prior to this investigation a concentration response curve of the effect of glucocorticoids Dex and TA on all three cell lines was performed and the results showed the maximal effect at 10^{-8}M for Dex and 10^{-10}M for TA. The effect of the glucocorticoids on pro-inflammatory stimuli LPS and TNF-α induced cells was again lysine residue specific, but the pattern revealed was different. Here, both dexamethasone and triamcinolone acetonide decreased acetylation of the lysine residues (K8 and K12) that were upregulated by the effect of the inflammatory stimuli used indicating a lysine specificity of the GR receptor. This, corresponds to reports from Ito *et al.*, (2000) that dexamethasone targeted the same lysine residues in A549 cells pre-treated with IL-1β. Their findings in relation to histone acetylation and glucocorticoid actions, suggested that CBP-associated factors, but not CBP itself, is the most likely target for competition between GR and p65 or other transactivating proteins.

In the present chapter, it is also shown that TSA blocked the inhibitory effect of steroids on histone acetylation in all cell lines. It is noteworthy that in the U937 cells the effect of TSA in cells stimulated with TA was not as potent as expected compared to the T-cell lines. The data to this point suggested that TA was a more effective glucocorticoid than dexamethasone and therefore it would be interesting to study the effect of other steroids and more specific histone deacetylase inhibitors such as SAHA in the TA actions. Similar studies using sodium butyrate, another HDAC inhibitor, have proved inconclusive because of the high toxicity and low half-life that this inhibitor has been reported to have (Santini *et al.*, 2001). Trichostatin A repressed the steroid effect to almost baseline levels as shown by the HDAC assay in all cell lines. The greatest increase in HDAC activity in cells stimulated with TA compared to Dex, suggested TA as the most potent glucocorticoid in monocytes and T-cells stimulated with either LPS or TNF-α.

Two major possible models are available at the moment in explaining the cross-talk between GR complexes and histone acetylation. The first suggests that deacetylation of histones increases tightening of the DNA around histone residues and therefore, reduces access of transcription factors such as AP-1 and NF-κB to their binding sites and of RNA polymerase II to the activation complex resulting in repression of inflammatory genes (Ito *et al.,* 2000). The second model proposes that GR interferes with p65 association with the TATA box environment, therefore, inhibiting p65 actions (Vanden Berghe, 2000). These models are not necessarily exclusive since altered histone acetylation will block bromodomain-regulated recruitment of cofactors including other TFs and TAFs. Another possibility suggested by Nissen & Yamamoto (2000), is that GR inhibits NF-κB by interfering with serine-2 phosphorylation of the mRNA polymerase II carboxy-terminal domain.

Acetylation of histones has acquired clinical importance with the findings that histone deacetylase (HDAC) inhibitors administered with retinoic acid can cause differentiation in promyelocytic leukaemia cells that had previously acquired resistance to retinoic acid (Lin *et al.,* 1998). The molecular mechanism of the HDAC inhibition was uncovered by the finding that histones in TSA treated cells are acetylated to unusually high degrees (Yoshida *et al.,* 2001). Pulse-chase experiments revealed that histone hyperacetylation induced by TSA is not due to increased acetylation but to decreased deacetylation of histones (Yoshida *et al.,* 2001). In a recent study in K562 cells where Trichostatin A induced H4 hyperacetylation, it was shown that changes in cellular phosphatase activity inhibited H4 acetyltransferase activity (Galasinski *et al.,* 2002). Since HDACs are also phosphoproteins, it is possible to speculate that changes in HDAC activity may also be modulated at by kinase and phosphatase activities affected by steroids and inflammatory mediators. This raises the possibility that phosphatases released from the GR/hsp90 complex may directly modulate HDAC activity.

Although not shown here *in vitro*, it is possible that inflammation *in vivo* reduces HDAC activity. Cigarette smoke, another pro-inflammatory stimulus, is associated with a reduction in HDAC2 expression and activity in bronchial biopsies and alveolar macrophages (Ito *et al.,* 2001). Furthermore, there was an inverse correlation between HDAC activity and the ability of glucocorticoids to suppress TNF-α-induced IL-8 release. The present findings, although not HDAC specific, showed an increase in HDAC activity in cells by glucocorticoids in the presence of inflammatory stimuli. The increase was significant in all three cell lines suggesting a similar mechanism of cross talk between GR activation, HDAC activity and pro-inflammatory transcription factors resulting in the regulation of inflammatory gene expression. The repressive action of glucocorticoids may therefore, at least in part, result from recruitment of activation of HDACs to sites in the promoters of inflammatory genes regulated by TFs, including AP-1 and NF-κB (Adcock, 2001). Alternatively, activated GR could bind to one of

the several transcription corepressor molecules, such as RIP140, which associate with proteins that have a differing histone deacetylase activity (Adcock and Caramori, 2001).

In summary, in this chapter it is shown that both inflammatory stimuli and glucocorticoids induced histone acetylation, albeit with different patterns of histone 4 acetylation. Low concentrations of both glucocorticoids in combination with LPS or TNF-α, repressed H4 acetylation on lysines 8 and 12, while glucocorticoids alone induced acetylation of different lysine residues (K5 and K16). The HDAC activity assay also showed a more potent effect of TA in repressing LPS and TNF-α induced histone acetylation compared to Dex, which agree with the functional data. Most important though, was the suggestion that monocyte and lymphocyte gene expression follows a similar and distinct pattern of histone acetylation. This leads to the conclusion that a more general pharmacological manipulation of specific histone acetylation status is a potentially useful approach for the treatment of inflammatory diseases. Extensive knowledge of the exact mechanism by which activated GR recruits HDACs may reveal new targets for the development of drugs that may dissociate the anti-inflammatory actions of glucocorticoids from their side effects that are largely due to gene induction. Alternatively, it could assist in the development of drugs that prevent K8 and K12 acetylation. So far, available drugs are non-specific in targeting histone lysine residue acetylation. The finding that K12 was less sensitive to GC actions than K8, could lead in the development of new drugs that will be lysine residue specific and therefore target K8 acetylation rather than acetylation of both K8 and K12 residues.

A number of lines of evidence indicate that the enzymatic activity of acetylases is regulated by proliferation and differentiation signals (Ait-Si-Ali *et al.,* 1998). *In vitro* studies with TSA indicated that it produces an irreversible growth arrest (at least in the short term) in keratinocyte-derived squamous carcinoma cell (Yoshida *et al.,* 1995). TSA has also recently been reported as a potent inhibitor of proliferation with an antitumor efficacy without measurable toxicity in human breast cancer cell lines (Vigushin *et al.,* 2001). The evidence provided in this chapter, along with reports on the effects of TSA in cell death and proliferation (Dagmond *et al.,* 1998), suggest that the role of histone acetylation in the regulation of inflammation is not only due to its effects in inflammatory gene transcription but also to its regulation of cell cycle progress and apoptosis. In chapter 5, early and late markers of apoptosis are studied, under the same conditions used in this chapter, in order to evaluate the role of histone acetylation in programmed cell death.

Chapter 5

Role of histone 4 acetylation in the regulation of apoptosis

5.1 Introduction

One of the cardinal signs of inflammation is loss of function. This has long been associated with cell death and apoptosis. The current chapter investigates the role of histone acetylation and inflammatory gene expression in programmed cell death. As it has previously been described, nucleosomal histones play key regulatory functions, as their acetylation state can be modified by histone acetylatransferase (HAT) and histone deacetylase (HDACs) enzymes. In addition to this, the use of HDAC inhibitors has revealed, a complex role for HDACs in cell function, as these agents block proliferation (Bohmig *et al.,* 1995), cause G1 (Gilbert and Weigle, 1993) or G2 cell cycle arrest (Yoshida and Beppu, 1988) and lead to cell differentiation (Hoshikawa *et al.,* 1994) and apoptosis (Conway *et al.,* 1995; Chang & Yung, 1996). These findings together with the understanding that histone acetylation plays a major role in the control of inflammatory gene expression has opened the gate to new and exciting possibilities on the understanding of the regulation of gene transcription.

HDACs are known to associate with two important cell cycle regulators: Myc activation factor X (Max)/Max dimeriser (Mad) and retinoblastoma (RB) (Sears *et al.,* 1997). Mad/Max heterodimers are essential for the repression of E-box-containing growth stimulatory genes during cellular differentiation (Sears *et al.,* 1997). Transcriptional repression by Mad/Max requires the assembly of a multisubunit repressor complex that carries HDAC activity. Disruption of this repressor by overexpression of myelocytomatosis viral oncogene homologue (c-Myc) or sarcoma viral oncogene homologue (v-Ski) results in re-induction of cell cycle progression and transformation (Fig. 5-1). RB is critical for the regulation of S-phase entry in eukaryotic cells. It performs its function through association with the E-box transcription factor (E2F) and repression of E2F-dependent promoters. It has been demonstrated that the repressive function of RB is mediated by its interaction with a histone deacetylase (Brehm *et al.,* 1998; Luo *et al.,* 1998). Mutations or deletion of RB disaggregates this repressive complex and can lead to uncontrolled proliferation, irregulation in apoptosis rates and tumour formation. Since aberrant histone acetylation has been linked to malignant diseases in some cases, HDAC inhibitors, such as sodium butyrate and Trichostatin A (TSA), have potential as new drugs due to their ability to modulate transcription and to induce differentiation and apoptosis (Marks *et al.,* 2000).

Butyrates, such as sodium butyrate and phenylbutyrate have been shown to induce differentiation in non-acute promyelocytic leukemia cell lines such as U937 and HL-60 cells (Guidez *et al.,* 1998). DNA repair processes are rapidly downregulated during differentiation induction of leukemic cells and consequently intracellular levels of previously administered cytotoxic agents are more effective in producing leukemia cell death by apoptosis (Waxman, 2000). The effects of butyrate and TSA also include direct apoptosis induction.

The effective concentration of sodium butyrate that cause histone hyperacetylation is in the millimolar range, which may also have nonspecific effects on cellular function. However, other HDAC inhibitors such as TSA and trapoxin are effective in the nanomolar range in inducing hyperacetylation of histones (Luo and Dean, 1999). TSA appears to be a reversible inhibitor of histone deacetylase activity, whereas trapoxin irreversibly inhibits histone deacetylase. The short half life of TSA could explain its reversable action. Siavoshian *et al.,* compared the effects of butyrate and TSA in HT-29 colonic cells, and found that the two compounds showed different histone H4 hyperacetylation kinetics. The effect of butyrate on histone H4 hyperacetylation was maintained after 24 h, whereas the TSA effect was no longer detectable after 15 h (Siavoshian *et al.,* 2000). After 16 h of exposure to butyrate, most H4 histones are acetylated whereas around 60% of H4 is acetylated in the presence of TSA after 2 h and the amount of non-acetylated H4 returns to control levels by 16 h. This difference in kinetics may explain the variance of HDAC inhibitors in their effect in apoptosis regulation observed in a number of cell lines, it does however reinforce the conclusion that histone acetylation may play a significant role in the regulation of programmed cell death. Apoptosis-inducing concentrations of TSA and butyrate upregulated the expression of HDAC mRNAs in a differential manner and acted synergistically with phytohemagglutinin (PHA) to induce HDAC expression, suggesting the presence of independent HDAC regulatory mechanisms (Dangond & Gullans, 1998). In addition, TSA abrogated interferon gamma (IFN-γ) production at a time-dependent manner in Th1 T cells and blocked proliferation (Dangond & Gullans, 1998). IFN-γ plays an anti-apoptotic, protective role during Shigella flexneri-induced apoptosis (Hilbi *et al.,* 1997) and induced the expression of Bcl-$_x$L in human macrophages (Okada *et al.,* 1998). Furthermore, treatment with TSA and retinoic acid (RA) markedly enhanced neuronal differentiation in P19 embryonal carcinoma cells, although TSA alone did not induce differentiation but caused extensive apoptosis (Minucci *et al.,* 1997). Finally, micromolar amounts of arsenic (As_2O_3) lead to growth arrest and apoptosis induction following induction of hyperacetylation of histones in leukemia cells (Perkins *et al.,* 2000).

Glucocorticoids can significantly reduce the survival of certain inflammatory cells such as T-lymphocytes. Exposure of the cells to glucocorticoids can block the effect of cytokines (i.e GM-CSF) that are required for survival and therefore lead to programmed cell death (Owens *et al.,* 1991). In contrast, glucocorticoids decrease apoptosis and therefore increase cell survival in other cell types such as neutrophils (Meagher *et al.,* 1996). In thymocytes,

inhibition of HDACs, by butyrate and TSA, is unable to augment dexamethasone-induced apoptosis. Even more pronounced was the antagonistic relationship between dexamethasone and Trichostatin A, as TSA-induced apoptosis was not only blocked by the presence of dexamethasone but dexamethasone-induced apoptosis was also partially inhibited in the presence of TSA (Bernhard *et al.*, 1999). The fact that the antagonistic relationship with dexamethasone for apoptosis was also observed with butyrate, suggests that in thymocytes this phenomenon may be related to histone acetylation. The molecular mechanisms that account for the opposing effects of glucocorticoids on these two types of cell, are yet unclear and in need of investigation. The molecular mechanism of action of glucocorticoids in increasing T-cells apoptosis is still poorly understood and there are many potential sites of action, including effects of histone deacetylase inhibitors.

There is some controversy as to the precise mechanism of HDAC inhibitors on cell apoptosis (Kelly *et al.*, 2002). HDAC inhibitors are reported to induce apoptosis through induction of $p21^{CIP/WAF}$ (Richon *et al.*, 2000) but there is evidence that the concentrations of HDAC inhibitors used to induce apoptosis are far greater than those required to enhance histone acetylation (Vigushin and Coombes, 2002).

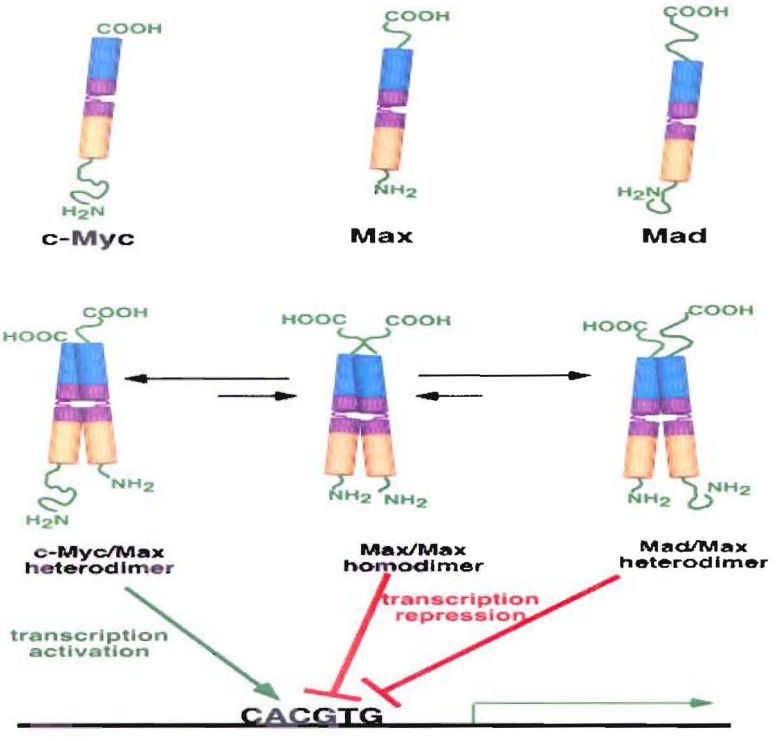


Figure 5-1 Model for transcriptional regulation by c-Myc, Max and Mad

While a number of groups have studied the effects of HDACs and HDAC inhibitors *in vivo* and *in vitro*, little work has been reported examining directly histone acetylation and apoptosis. The pro-inflammatory cytokine IL-8 and controlled cell suicide are associated with signaling and increased induction of inflammation (see chapter 2). The aim of this study was to compare the effects of LPS, a known stimulator of pro-inflammatory mediator production, H_2O_2, an oxidative stress inducer resulting in apoptosis or necrosis depending on the concentrations used and TNF-α, a pro-inflammatory cytokine, on the production of IL-8 and apoptosis in these cells. In this chapter the induction of histone acetylation in these conditions is studied. Also the effects of glucocorticoids dexamethasone and triamcinolone acetonide in apoptosis and the interrelationship between the chromatin remodeling machinery and steroid-induced programmed cell death in these cell lines are investigated.

5.2 Results

5.2.1 Inflammatory stimuli cell death in three cell lines

To detect cell death induced in U937, Jurkat and HUT-78 cells when stimulated with inflammatory stimuli and steroids an MTT assay was used. Figure 5-2 illustrates the changes in the viability of U937, Jurkat and U937 cells following treatment with LPS (10 ng/ml), TNF-α (10 ng/ml and 100 ng/ml) and hydrogen peroxide (H_2O_2; 100 μM). Increased or decreased viability by the MTT assay would be detected as changes in the absorbance (increased viability: increases in absorbance and decreased viability, cell death: decrease in absorbance). In U937 cells, LPS did not affect cell viability, during the 1 hr incubation period. H_2O_2 and TNF-α however, induced a significant reduction in cell viability ($p<0.05$). In both HUT-78 and Jurkat cells, LPS and H_2O_2 ($p<0.05$) also induced a reduction in cell viability. Cells were also treated with TSA and its effect on cell viability was monitored. The effect of TSA in all three cells was concentration dependent. TSA did not affect cell viability at 10 ng/ml but at 100 ng/ml it reduced cell viability to a similar extent to that observed with H_2O_2, in all three cell lines ($p<0.05$). Similar experiments were also performed for an additional 24 h incubation. These showed a contradictory increase in the viability of the cells (results not shown). This result may be interpreted as being due to the necrotic or apoptotic cells having already been eliminated from the cell suspension or surviving cells proliferating following removal of dead cells at 1hr.

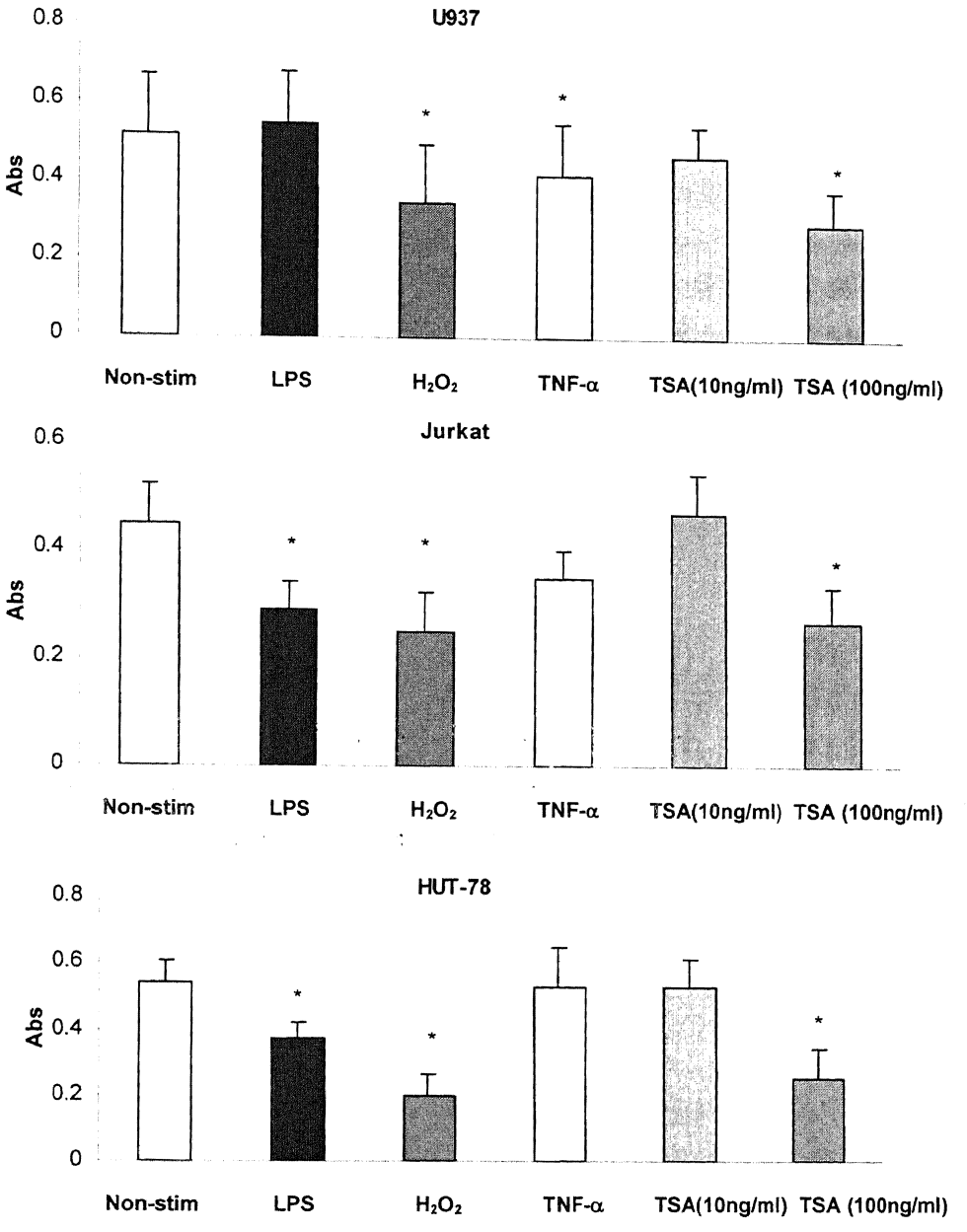


Figure 5-2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) viability assay in U937, Jurkat and HUT-78 cells. The cells were stimulated with pro-inflammatory stimuli lipopolysaccharide (LPS; 10 ng/ml) or tumour necrosis factor α (TNF-α; 10 ng/ml), the oxidant stress inducer hydrogen peroxide (H_2O_2 ; 100μM) and the HDAC inhibitor Trichostatin A (TSA) for 1hr and changes in the cell viability and therefore cell death were detected. In the U937 cells LPS did not change cell viability, while both TNF-α and H_2O_2 decreased cell viability shown by the reduced absorbance in the chart. In both T-cell lines the effects of LPS and H_2O_2 were similar in that they affected cell viability. Addition of TSA (10 ng/ml) did not alter cell viability, while addition of 100 ng/ml induced cell death in all three cell lines. Columns represent the mean ± SD (bar) of three independent experiments (*$p<0.05$ compared to non-stim).

5.2.2 Stimuli induced apoptosis in three cell lines

Following the results in section 5.2.1 it was examined whether the cell death detected was necrotic or apoptotic. Using a Hoechst 33342 stain, apoptotic bodies within cells were visualised under a fluorescence microscope and the ratio of apoptotic versus total cell numbers was measured. The cells were stimulated as stated previously, in order to detect signs of apoptosis however the incubation periods were increased to 6 hrs. Table 5-1 shows the percentage of cells undergoing apoptosis after each treatment. The data was generally inversely related to the results obtained from the MTT assay and showed an increase in apoptotic cell death in all cell lines following stimulation with LPS (except in the U937 cells where LPS did not induce apoptosis or changes in cell survival), TNF-α (except in the HUT-78 cells, were no significant induction of apoptosis or cell survival was detected), H_2O_2 and TSA (100 ng/ml). At a concentration of 10 ng/ml, TSA did not induce apoptosis in any cell line. These results verified that the reduction of cell viability detected by the MTT assay was related to programmed cell death.

Cell line	Stimulant	Apoptotic cell No (%)
U937	Non-Stim	9 ± 4 %
	LPS	27 ± 8%
	H_2O_2	45 ± 12%
	TNF-α	57 ± 15%
	TSA (10 ng/ml)	18 ± 7%
	TSA (100 ng/ml)	64 ± 13%
Jurkat	Non-Stim	13 ± 5%
	LPS	49 ± 13%
	H_2O_2	68 ± 17%
	TNF-α	63 ± 14%
	TSA (10 ng/ml)	20 ± 5%
	TSA (100 ng/ml)	73 ± 17%
HUT-78	Non-Stim	5 ± 2%
	LPS	51 ± 14%
	H_2O_2	68 ± 16%
	TNF-α	24 ± 9%
	TSA (10 ng/ml)	18 ± 5%
	TSA (100 ng/ml)	72 ± 16%

Table 5-1 Percentage of apoptotic cells following cell stimulation.
Results are expressed as mean ± SEM (n=6)

The hoechst stain is an efficient method of detecting apoptosis at its late stages where changes in cell morphology can be detected. However apoptotic cell signalling pathways are initiated as soon as 1 h following stimulation. It was therefore required to investigate whether apoptotic regulating proteins were activated during stimulation. The cells were stimulated as above for 1 hr and changes in the activation status of B cell lymphoma leukemia 2 (Bcl-2) and Annexin V were measured by Western blotting. Figure 5-3 shows representative bands obtained by Western blotting and figure 5-4 illustrates changes in the expression status of Bcl-2 in all three cell lines when stimulated as above.

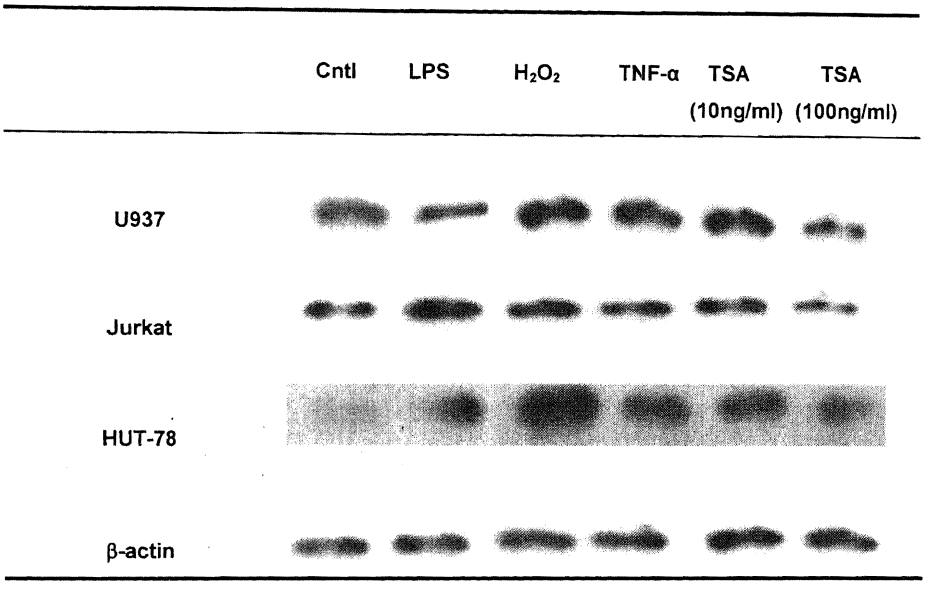


Figure 5-3 Representative bands of B-cell lymphoma leukemia 2 (Bcl-2) expression as obtained by Western blotting in three cell lines. Lanes represent: control, lipopolysaccharide (LPS; 10 ng/ml), hydrogen peroxide (H_2O_2,; 100μM), tumour necrosis factor α (TNF-α; 10 ng/ml), Trichostatin A (TSA; 10 ng/ml) and TSA (100 ng/ml) stimulations for 1hr. The results are representative of three independent experiments. β-actin controls for loading were for HUT-78 cells.

In U937 cells LPS significantly downregulated Bcl-2 levels (57 ± 12% versus 100 ± 26% control U937 cells, $p<0.05$). H_2O_2, TNF-α and TSA did not affect Bcl-2 expression.

In the T-cell lines LPS, TNF-α and H_2O_2 induced Bcl-2 expression to a similar extent. LPS, H_2O_2, and TNF-α induced an upregulation of the protein levels. TSA, as in U937 cells, did not affect Bcl-2 expression in either low or high concentrations.

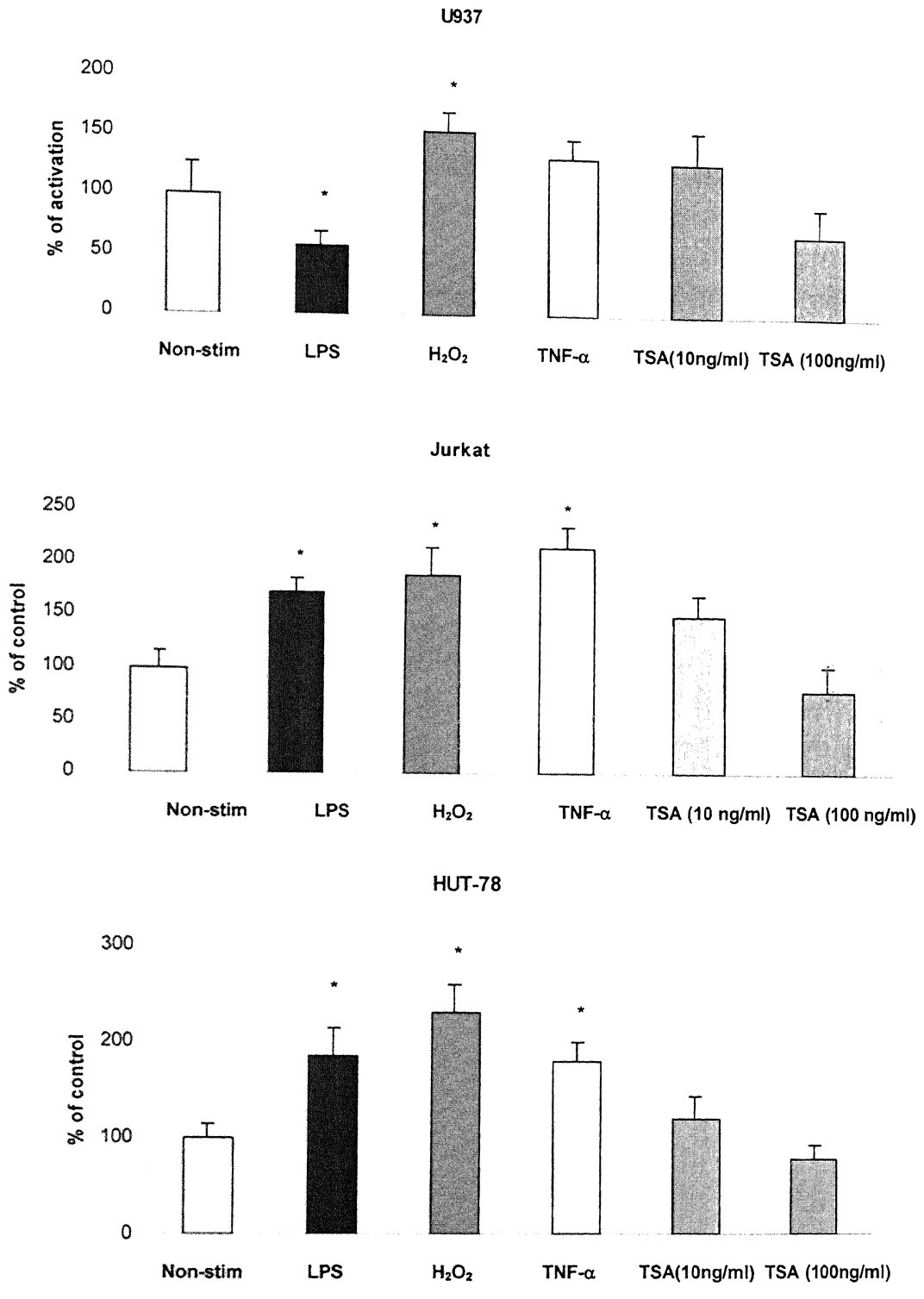


Figure 5-4 B-cell lymphoma leukemia 2 (Bcl-2) expression, in stimulated U937, Jurkat and HUT-78 cells. Cells were stimulated with lipopolysaccharide (LPS; 10 ng/ml), hydrogen peroxide (H_2O_2; 100μM), tumour necrosis factor α (TNF-α; 10 ng/ml) and Trichostatin A (TSA; 10 and 100 ng/ml) for 1hr and changes in the expression of the protein were detected by Western blotting. LPS downregulated protein levels in the U937 cells while it had the opposite effect in the T-cell lines. H_2O_2 and TNF-α induced activation of Bcl-2 in T-cells, whilst TSA was ineffective in all 3 cell types (*$p<0.05$ compared to non-stim).

Activation of annexin V is an effective method of identifying early stages of apoptosis by detecting the translocation of phosphatidylserine from the inner to the outer cell membrane. Suspensions of all three cell lines were stimulated as above and following a 1h incubation period whole cell extracts were isolated. The histograms in figure 5-5 illustrate expression of annexin V in U937, Jurkat and HUT-78 cells. The data were obtained by Western blotting and the results are representative of three independent experiments. Representative Western blots are shown in the Appendix.

In U937 cells, LPS did not induce annexin V expression. However, when cells were stimulated with H_2O_2 (261 ± 34% versus 123 ± 25%, $p<0.05$) annexin V expression was significantly upregulated. Similarly, stimulation of the cells with TNF-α, significantly induced annexin V expression (300 ± 45%, $p<0.05$). Finally, TSA (10 ng/ml) upregulated annexin V expression (258 ± 45%, $p<0.05$). In contrast TSA (100 ng/ml) had no effect on annexin V levels.

The data obtained in the T-cell lines for annexin V levels were very similar to those observed with the Bcl-2 protein with the exception of the effect of TNF-α. Stimulation of both Jurkat and HUT-78 cells with H_2O_2 induced upregulation of annexin V expression (in Jurkat: 334 ± 66% versus 115 ± 21%, $p<0.05$ and in HUT-78: 333 ± 76% versus 109 ± 19%, $p<0.05$). In contrast, only TNF-α significantly upregulated annexin V levels in Jurkat cells (346 ± 35%, $p<0.05$). LPS did not affect annexin V expression in either T-cell line. Finally, a similar effect of TSA to that seen observed in U937 cells was seen. Low concentrations (10ng/ml) of the inhibitor TSA significantly induced annexin V expression (in Jurkat cells: 260 ± 50%, $p<0.05$ and in HUT-78 cells: 350 ± 62%, $p<0.05$) whilst high concentrations (100ng/ml) resulted in no enhancement of annexin V expression.

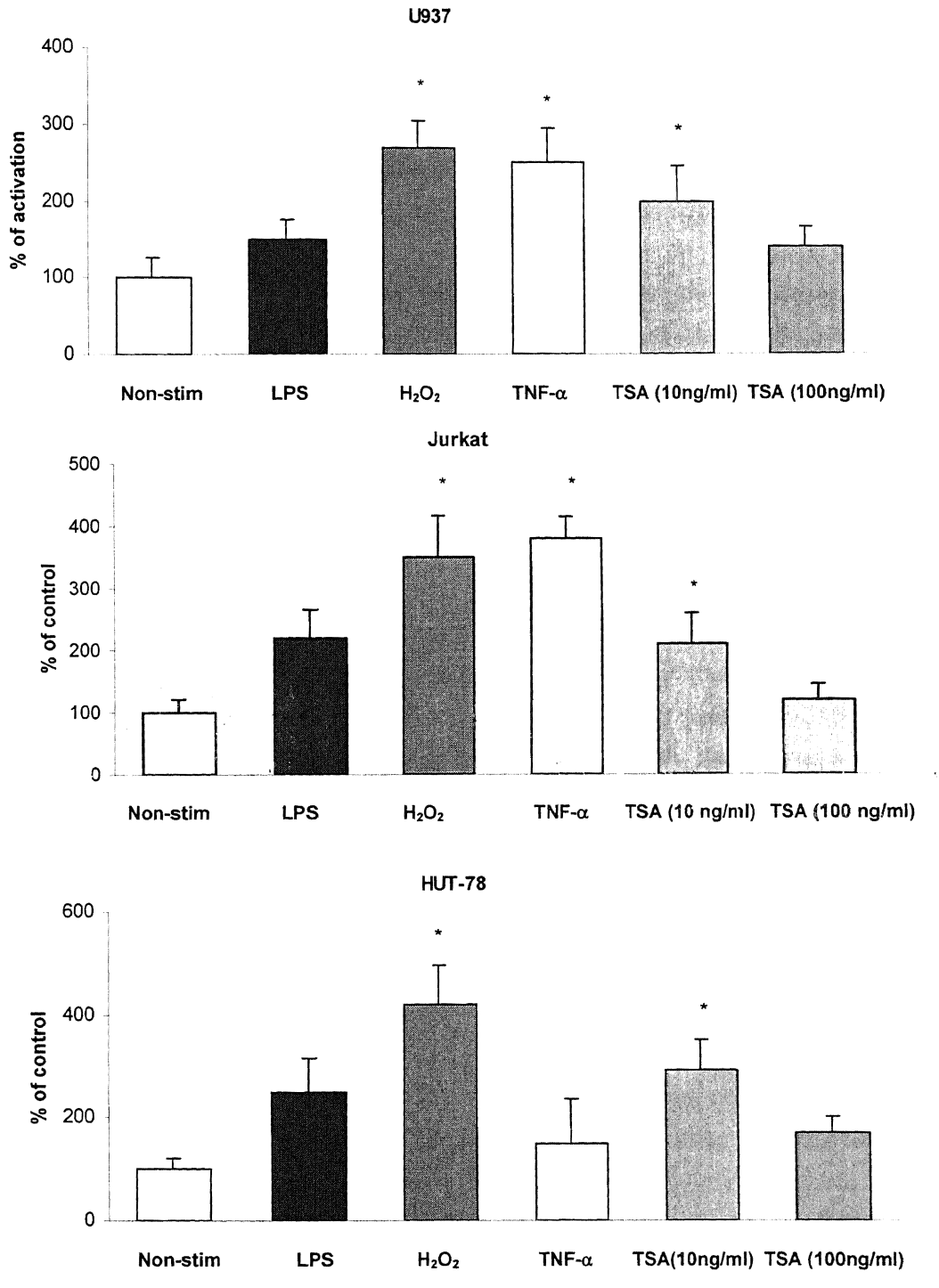


Figure 5-5 Annexin V expression, in stimulated U937, Jurkat and HUT-78 cells, indicating early stages of apoptosis. Cells were stimulated with lipopolysaccharide (LPS; 10 ng/ml), hydrogen peroxide (H_2O_2 ; 100 μM), tumour necrosis factor α (TNF-α; 10 ng/ml) and Trichostatin A (TSA; 10 and 100 ng/ml) for 1hr and upregulation of the protein was detected by Western blotting. LPS had no effect in any cell line. H_2O_2 induced Annexin V expression in all three cell lines, while the effect of TSA was concentration dependent. At 10 ng/ml TSA slightly upregulated Annexin V expression, while at 100 ng/ml no significant upregulation of Annexin V expression. TNF-α induced Annexin V expression in U937 and Jurkat cells. Columns represent the mean ± SD (bar) of three independent experiments (*$p<0.05$ compared to non-stim).

5.2.3 Glucocorticoid induced apoptosis in three cell lines

Following the investigation of the glucocorticoid effects on IL-8 production and histone acetylation described in chapters 3 and 4, the effects of dexamethasone and triamcinolone acetonide in the induction of apoptosis in the cells were also studied. Cells were stimulated as in chapters 3 and 4 and apoptosis was detected indirectly via Western blotting for Bcl-2 and by immunocytochemistry for annexin V.

In all three cell lines, addition of the steroids had no effect on expression of Bcl-2 (Fig. 5-6.a). In U937 cells, addition of Dex to TNF-α and H_2O_2 or TSA stimulated cells did not alter Bcl-2 levels. However, Dex significantly increased Bcl-2 levels in LPS stimulated cells (137 ± 32% versus 56 ± 12% LPS alone, $p<0.05$). TA had no effect on Bcl-2 expression (Fig. 5-6.b) except in LPS stimulated cells where addition of TA significantly upregulated Bcl-2 levels (168 ± 16% versus 56 ± 12% LPS alone, $p<0.05$). In the two T-cell lines investigated addition of either steroid did not affect Bcl-2 levels in LPS, TNF-α, H_2O_2 or TSA stimulated cells.

Figure 5-7 illustrates expression of annexin V in the three cell lines in the presence of steroids when stimulated as previously. Regardless of the upregulation of Bcl-2 levels shown above, indicating protection of the cells against apoptosis, it is shown that annexin V levels were upregulated in stimulated cells in the presence of both glucocorticoids. Glucocorticoids alone did not induce annexin V levels in the U937 cells or the T-cell lines. However, annexin V levels were upregulated significantly in stimulated cells.

Dex enhanced annexin V expression in LPS-stimulated U937 cells (315 ± 27% versus 177 ± 26% LPS alone, $p<0.05$) only. In contrast, Dex enhanced annexin V expression in all cell types stimulated by H_2O_2 (U937 cells: 448 ± 41% versus 261 ± 34% H_2O_2 alone, $p<0.05$; in Jurkat cells: 413 ± 55% versus 333 ± 67% H_2O_2 alone, $p<0.05$ and in HUT-78 cells: 490 ± 85% versus 312 ± 62% H_2O_2 alone, $p<0.05$). In TNF-α stimulated cells Dex enhanced annexin V expression in U937 (442 ± 45% versus 300 ± 41% TNF-α alone, $p<0.05$) and HUT-78 cells (364 ± 34% versus 183 ± 75% TNF-α alone, $p<0.05$) (Fig. 5-7.a) but not in Jurkat cells.

In contrast, TA enhanced annexin V expression in LPS-stimulated U937 cells (444 ± 44% versus 177 ± 26% LPS alone, $p<0.05$), Jurkat cells (440 ± 55% versus 230 ± 45% LPS alone, $p<0.05$) and HUT-78 cells (323 ± 40% versus 260 ± 57% LPS alone, $p<0.05$).

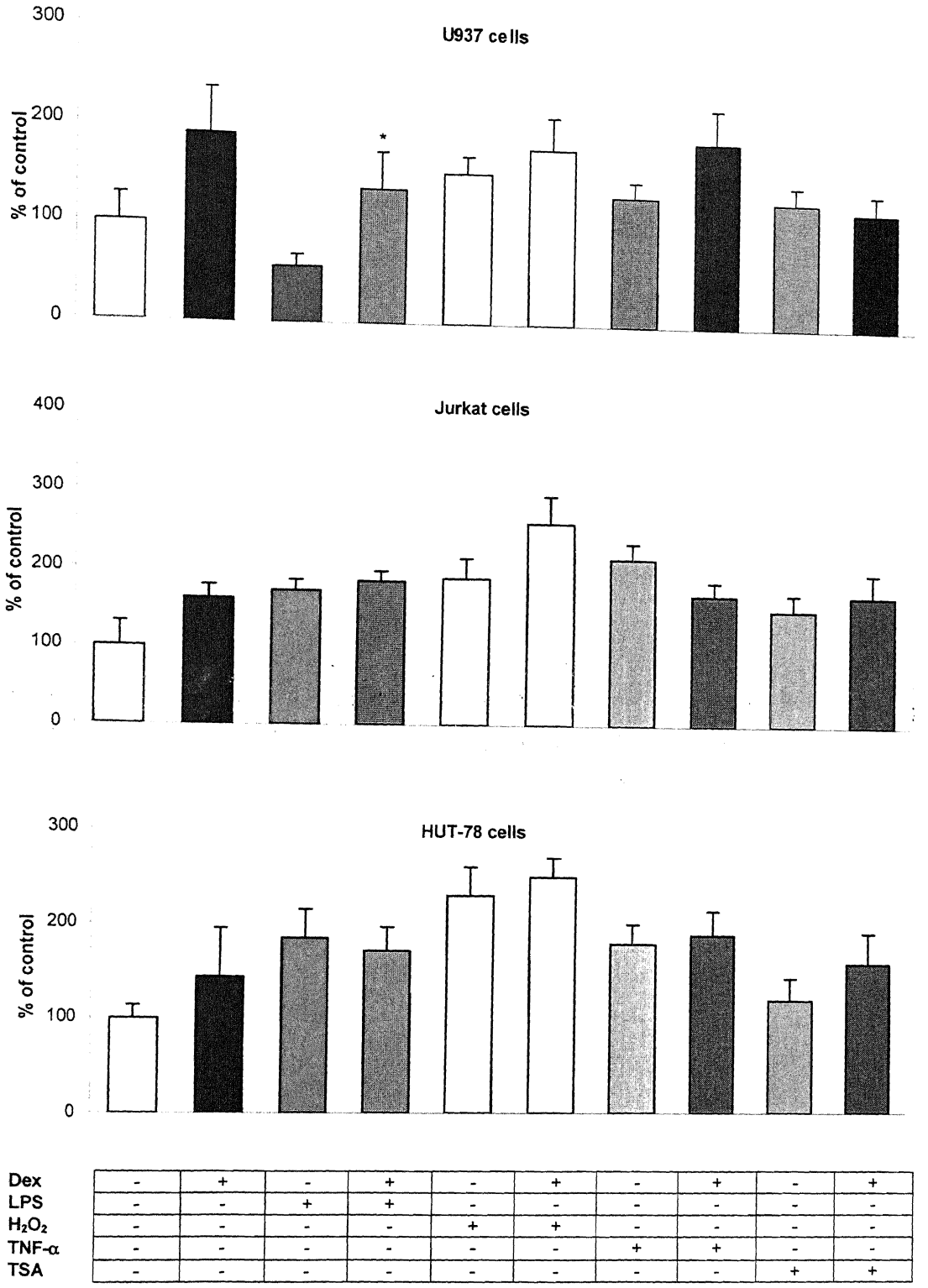


Figure 5-6.a Western Blot analysis of B-cell lymphoma leukemia 2 (Bcl-2) expression, in stimulated U937, Jurkat and HUT-78 cells. Cells were stimulated with lipopolysaccharide (LPS; 10ng/ml), hydrogen peroxide (H_2O_2; 100μM), tumour necrosis factor α (TNF-α; 10ng/ml) and Trichostatin A (TSA; 10 and 100ng/ml) in the presence of dexamethasone (Dex; 10^{-8} M). Cells were stimulated with Dex and TSA 30min prior to the addition of the inflammatory stimuli and then allowed to incubate for a further 1hr. Dex alone failed to induced Bcl-2 upregulation in all three cell types. Dex enhanced Bcl-2 expression in the presence of LPS in U937 cells. Columns represent the mean ± SD (bar) of three independent experiments. (*$p<0.05$ compared to LPS).

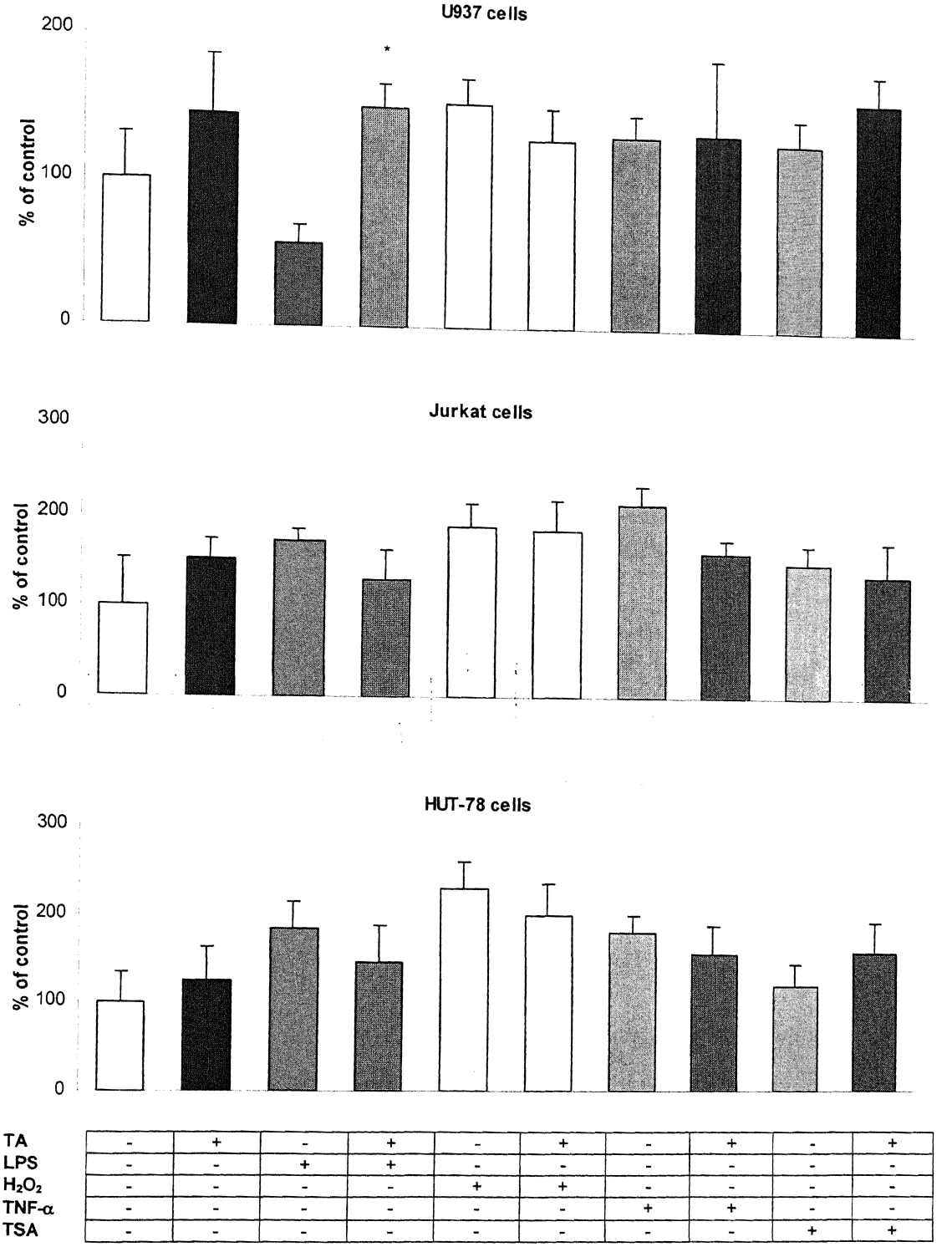


Figure 5-6.b Western Blot analysis of B-cell lymphoma leukemia 2 (Bcl-2) expression, in stimulated U937, Jurkat and HUT-78 cells. Cells were stimulated with lipopolysaccharide (LPS; 10ng/ml), hydrogen peroxide (H_2O_2; 100μM), tumour necrosis factor α (TNF-α; 10ng/ml) and Trichostatin A (TSA; 10 and 100ng/ml) in the presence of triamcinolone acetonide (TA; 10^{-10} M). Cells were stimulated with TA and TSA 30min prior to the addition of the inflammatory stimuli and then allowed to incubate for a further 1hr. TA alone had no effect on Bcl-2 levels in any cell type. In U937 cells, TA enhanced Bcl-2 expression in the presence of LPS. Columns represent the mean ± SD (bar) of three independent experiments. (*$p<0.05$ compared to LPS).

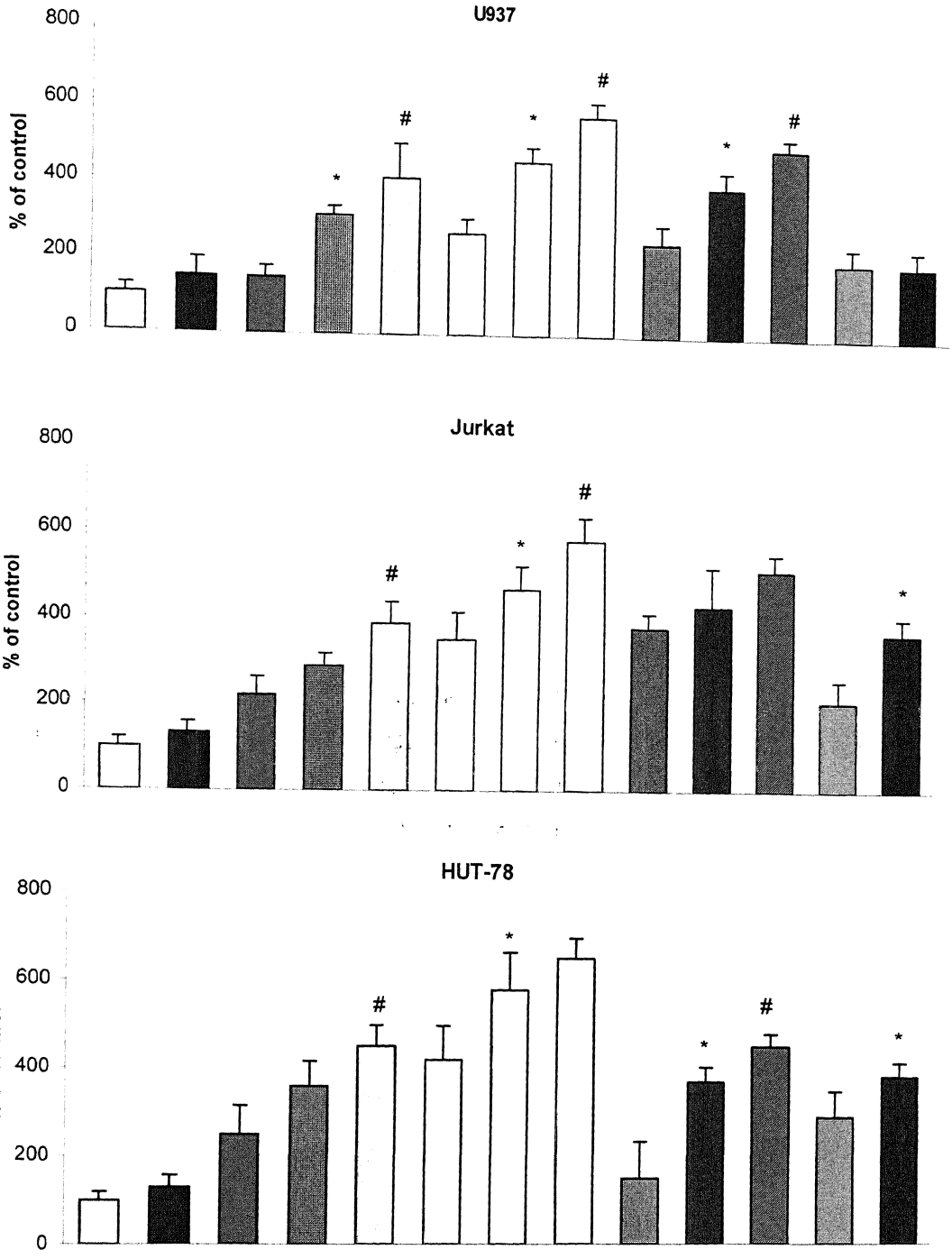
Similar effects were seen with H_2O_2-treated cells (U937 cells: 493 ± 42% versus 261 ± 34% H_2O_2 alone, $p<0.05$; Jurkat cells: 540 ± 40% versus 333 ± 67% H_2O_2 alone, $p<0.05$; and HUT-78 cells: 550 ± 48% versus 312 ± 62% H_2O_2 alone, $p<0.05$) and TNF-α treated cells (U937 cells: 533 ± 49% versus 300 ± 41% TNF-α alone, $p<0.05$; Jurkat cells: 572 ± 56% versus 300 ± 41% TNF-α alone, $p<0.05$; and HUT-78 cells: 443 ± 30% versus 183 ± 75% TNF-α alone, $p<0.05$ (Fig. 5-7.b).

Finally the effect of TSA on Dex and TA actions on annexin V expression was investigated. Previous results in this chapter showed no effect of TSA alone on annexin V expression. However, in both T-cell lines, the presence of glucocorticoids synergistically interacted with TSA to significantly enhance annexin V expression. Specifically, Dex enhanced Annexin V expression in the presence of TSA in Jurkat cells (405 ± 42% versus 242 ± 38% TSA alone, $p<0.05$) and in HUT-78 cells (405 ± 38% versus 308 ± 31% TSA alone, $p<0.05$)(Fig. 5-7.a). More potent effects were seen with TA in Jurkat cells (593 ± 58% versus 242 ± 38% TSA alone, $p<0.05$) and in HUT-78 cells (435 ± 41% versus 308 ± 31% TSA alone, $p<0.05$)(Fig. 5-7.b).

Addition of TSA to LPS+Dex (493 ± 91% versus 315 ± 27% LPS+Dex alone; $p<0.05$), H_2O_2+Dex (534 ± 39% versus 448 ± 41% H_2O_2+Dex alone, $p<0.05$) and TNF-α+Dex (469 ± 32% versus 442 ± 45% TNF-α+Dex alone, $p<0.05$) –stimulated U937 cells resulted in enhanced annexin V expression. Similar effects were seen in Jurkat (LPS+Dex: 382 ± 50% versus 298 ± 35% LPS+Dex alone, $p<0.05$ and H_2O_2+Dex: 530 ± 52% versus 413 ± 55% H_2O_2+Dex alone, $p<0.05$) and HUT-78 cells (LPS+Dex: 467 ± 48% versus 253 ± 39% LPS+Dex alone, $p<0.05$; H_2O_2+Dex: 652 ± 46% versus 490 ± 85% H_2O_2+Dex, $p<0.05$ and TNF-α+Dex: 482 ± 27% versus 364 ± 34% TNF-α+Dex alone, $p<0.05$) (Fig. 5-7.a).

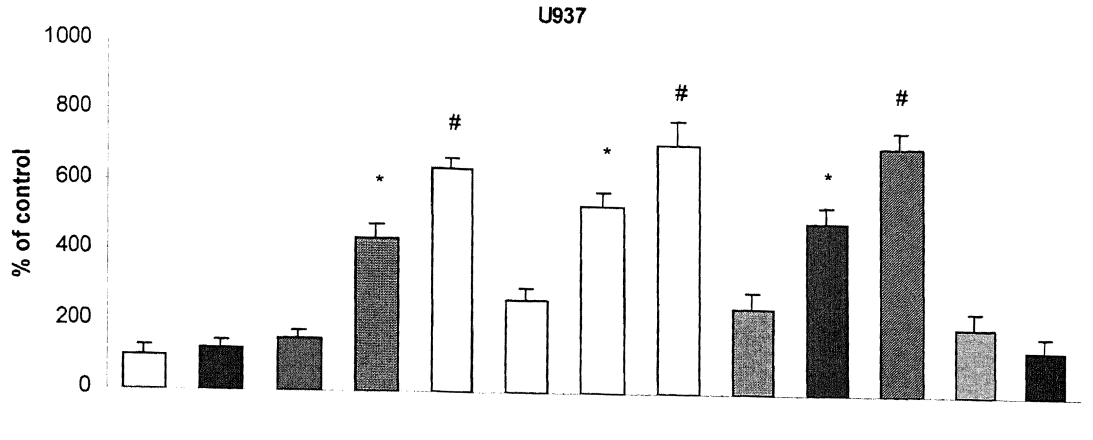
Addition of TSA to LPS+TA (U937: 617 ± 33% versus 444 ± 44% LPS+TA, $p<0.05$; Jurkat: 506 ± 76% versus 440 ± 55% LPS+TA, $p<0.05$ and HUT-78: 529 ± 30% versus 323 ± 40% LPS+TA, $p<0.05$), H_2O_2+TA (U937: 673 ± 69% versus 493 ± 42% H_2O_2+TA, $p<0.05$; Jurkat: 666 ± 59%, versus 540 ± 40% H_2O_2+TA, $p<0.05$ and HUT-78: 688 ± 50%, versus 550 ± 48% H_2O_2+TA, $p<0.05$) and TNF-α (U937: 672 ± 49% versus 533 ± 49% TNF-α+TA, $p<0.05$; Jurkat: 676 ± 59% versus 572 ± 56% TNF-α+TA, $p<0.05$ and HUT-78: 622 ± 51% versus 443 ± 30% TNF-α+TA, $p<0.05$) –stimulated cells resulted in enhanced annexin V expression in all three cell lines.

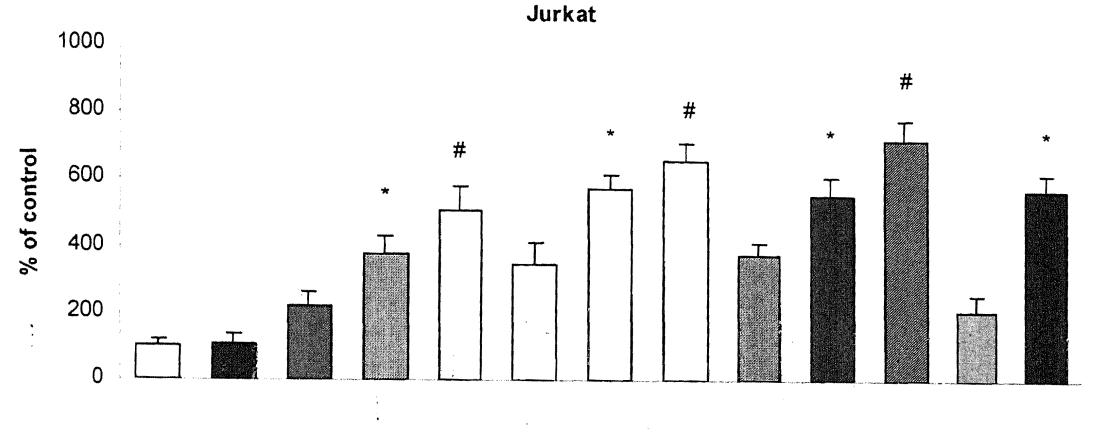
It is therefore demonstrated that glucocorticoids and TSA possibly act synergistically towards the induction of programmed cell death in both monocytes and lymphocytes depending on the inflammatory stimuli.

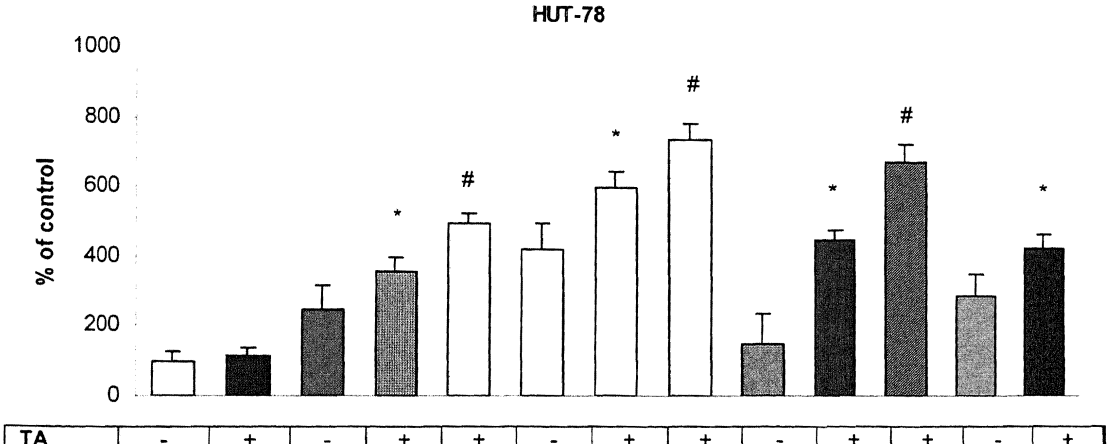


Dex	-	+	-	+	+	-	+	+	-	+	+	-	+
LPS	-	-	+	+	+	-	-	-	-	-	-	-	-
H_2O_2	-	-	-	-	-	+	+	+	-	-	-	-	-
TNF-α	-	-	-	-	-	-	-	-	+	+	+	-	-
TSA	-	-	-	-	+	-	-	+	-	-	+	+	+

Figure 5-7.a Western Blot analysis of Annexin V expression, in stimulated U937, Jurkat and HUT-78 cells, in the presence of dexamethasone (Dex; 10^{-8} M). Cells were stimulated with Dex and TSA 30min prior to the addition of the inflammatory stimuli and then allowed to incubate for a further 1hr. Dex did not upregulate annexin V levels alone. In all stimulated cells, Dex induced annexin V expression except following lipopolysaccharide (LPS)-stimulation in T-cells. Co-stimulation of the cells with Trichostatin A (TSA) and Dex also induced apoptosis in all three cell lines. Columns represent the mean ± SD (bar) of three independent experiments (*$p<0.05$ compared to inflam. stim; #$p<0.05$ compared to inflam. stim. plus Dex).







TA	-	+	-	+	+	-	+	+	-	+	+	-	+
LPS	-	-	+	+	+	-	-	-	-	-	-	-	-
H_2O_2	-	-	-	-	-	+	+	+	-	-	-	-	-
TNF-α	-	-	-	-	-	-	-	-	+	+	+	-	-
TSA	-	-	-	-	+	-	-	+	-	-	+	+	+

Figure 5-7.b Western blot analysis of annexin V expression, in stimulated U937, Jurkat and HUT-78 cells, in the presence of triamcinolone acetonide (TA; 10^{-10} M). Cells were stimulated with TA and TSA 30min prior to the addition of the inflammatory stimuli and then allowed to incubate for a further 1hr. TA did not upregulate annexin V levels alone. In stimulated cells (monocytes and T-cells) TA upregulated annexin V expression. Co-stimulation of the cells with Trichostatin A (TSA) and TA also induced annexin V expression in all three cell lines. Columns represent the mean ± SD (bar) of three independent experiments (*$p<0.05$ compared to inflam. stim; #$p<0.05$ compared to inflam. stim. plus Dex).

5.2.4 The role of histone acetylation in glucocorticoid induced apoptosis in three cell lines

Having proved that stimulation of cells induced programmed cell death, caspase activation was also investigated. To distinguish between necrosis and apoptosis a caspase 3 assay, and a caspase 3 monoclonal antibody for inmmunocytochemical staining of the cells were used. Cells were stimulated as previously in the presence of TSA and glucocorticoids and the activation of caspase 3 was recorded (Fig. 5-8). In all three cell lines Dex and TA alone did not significantly induce caspase 3 activation. Preliminary results also indicated that TSA did not induce caspase 3 activation in any of the three cell lines (results not shown). However the presence of Dex induced caspase 3 activation in all H_2O_2-stimulated cells lines ($p<0.05$). TA had a more potent effect in U937 and HUT-78 cells as it induced caspase 3 activation with all inflammatory stimuli (LPS, H_2O_2 and TNF-α), but it was only effective in H_2O_2-stimulated Jurkat cells.

In U937 cells costimulation with TSA and Dex only significantly activated caspase three in TNF-α-stimilated cells. Costimulation with TA and Dex however, resulted in increased caspase 3 levels in all inflammatory stimuli.

In Jurkat cells TSA only significantly upregulated TNF-α+Dex-stimulated cells whilst TA upregulated caspase 3 activation in all cells stimulated with TA plus inflammatory stimuli. Finally, in HUT-78 cells only the TA effect was enhanced by further addition of TSA in LPS, H_2O_2 and TNF-α stimulated cells.

The micrographs illustrated in figure 5-9, exhibit staining of caspase 3 in U937 cells. The results are representative of similar results obtained for the lymphocytic cell lines. The intensity of green fluorescence indicated caspase 3 expression. Neither Dex nor TA enhanced annexin V expression whereas caspase 3 expression was elevated in LPS, H_2O_2, and TNF-α -stimulated cells. Upon addition of TSA the fluorescence intensity is further increased. Consistent with the caspase 3 assay the micrographs showed that caspase 3 is not expressed in U937 cells stimulated with TA and TSA. In the Jurkat and the HUT-78 cells, however, caspase 3 was visualised (Fig. 5-10).

Finally apoptosis in these cell types under the same stimulatory conditions using DNA laddering was examined. In figure 5-11, DNA fragmentation in Jurkat cells is shown. As shown in the figure all treatments induced DNA fragmentation to a greater or lesser extent indicating late stages of apoptosis. The results confirmed those obtained for annexin V expression (Fig. 5-6) in Jurkat cells. Similar data was found in HUT-78 and U937 cells except that TSA plus steroid treatment in U937 cells did not result in DNA fragmentation.

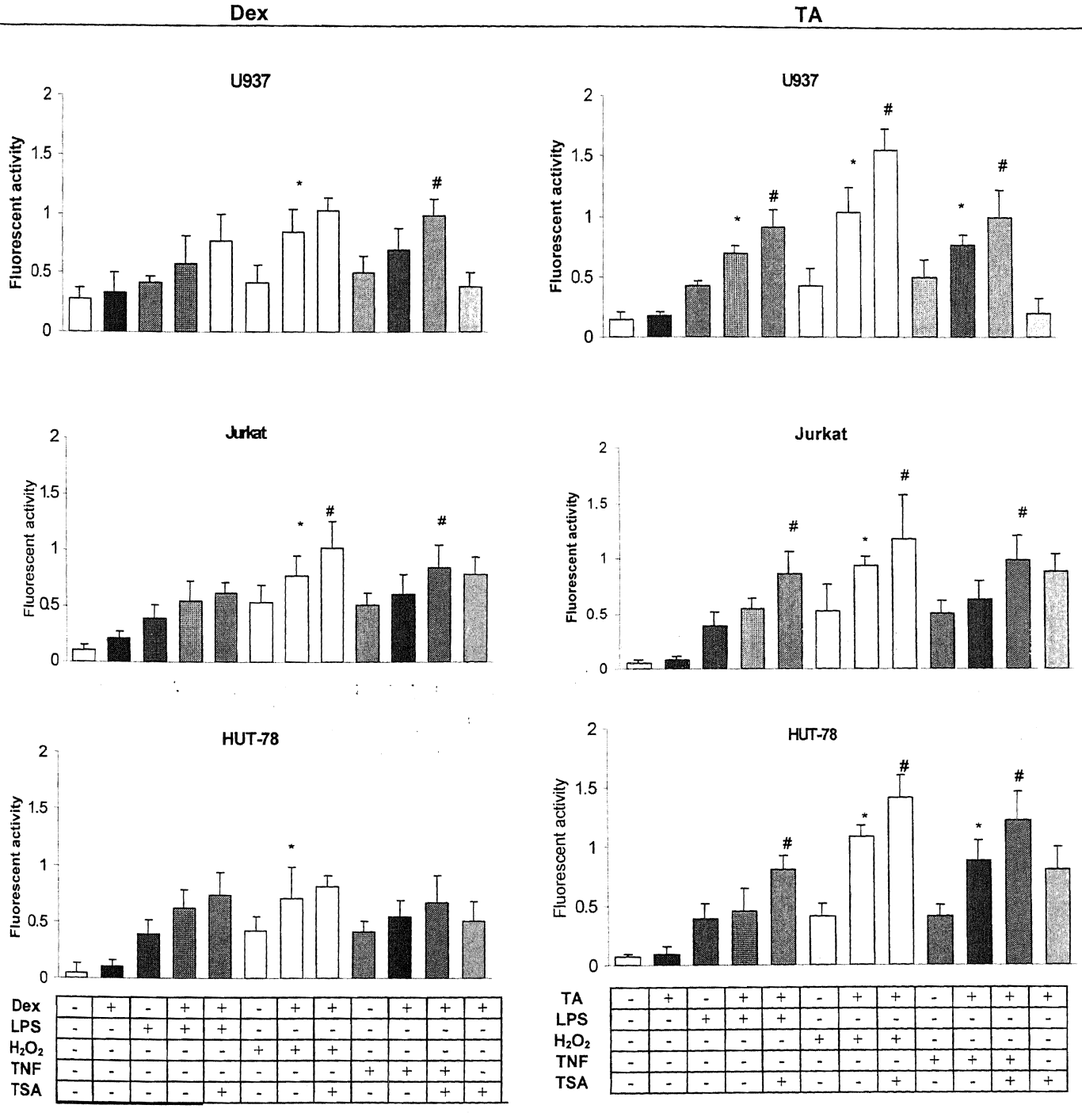


Figure 5-8 Caspase 3 activation assay, in stimulated U937, Jurkat and HUT-78 cells, in the presence of glucocorticoids. Cells were stimulated with glucocorticoids and TSA 30min prior to the addition of the inflammatory stimuli and then allowed to incubate for a further 1hr. Both steroids did not affect caspase 3 levels alone. In stimulated cells (monocytes and T-cells). In the presence of dexamethasone (Dex; 10^{-8} M) and triamcinolone acetonide (TA; 10^{-10} M) increases in caspase levels were inflammatory stimuli and cell type dependent, with triamcinolone being more potent in the upregulation of higher levels of the caspase. Co-stimulation of the cells with Trichostatin A (TSA) and TA resulted in an increase in the activation of caspase 3. Columns represent the mean ± SD (bar) of three independent experiments (*$p<0.05$ compared to inflam. stim; #$p<0.05$ compared to inflam. stim plus steroid).

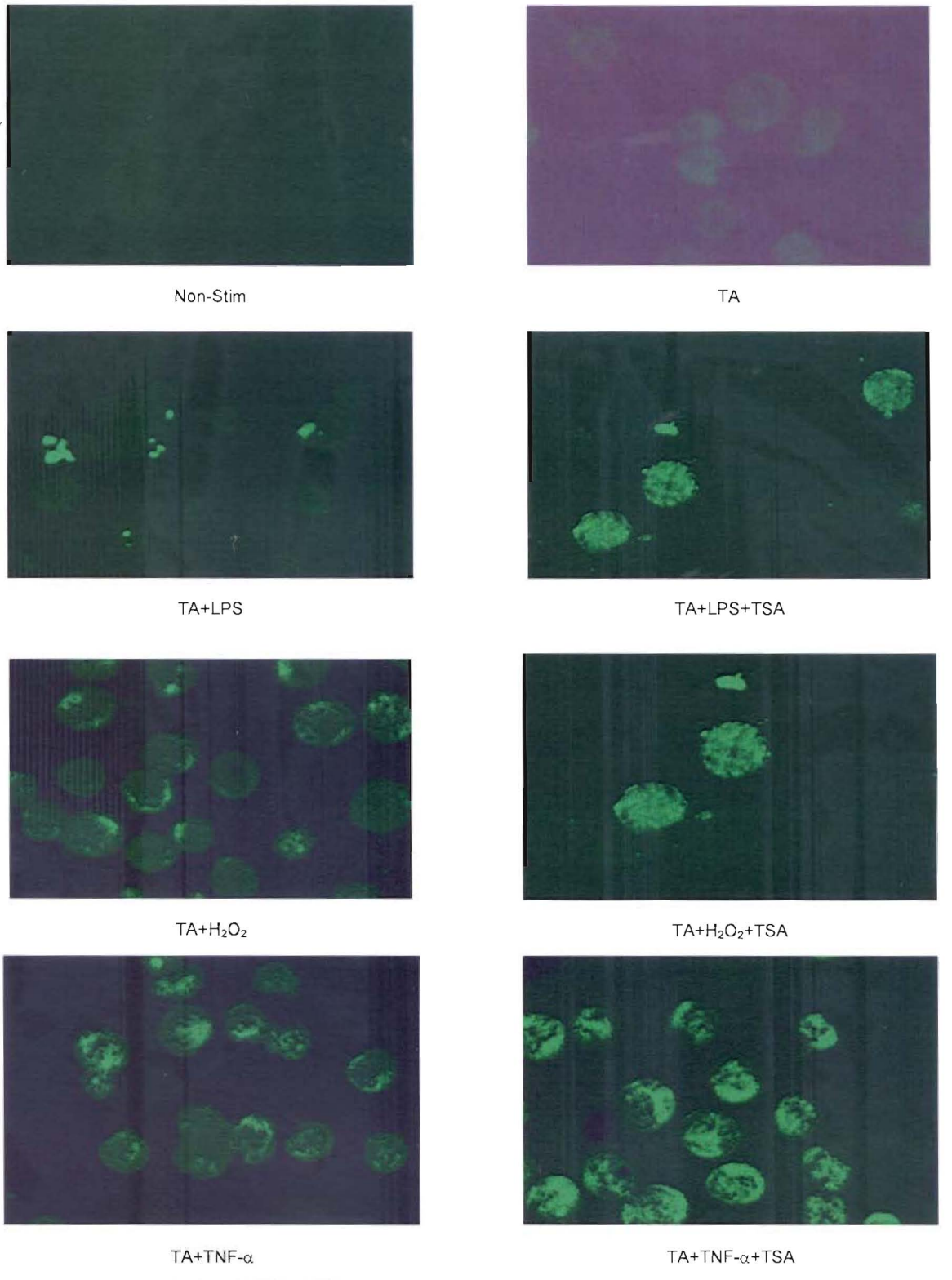
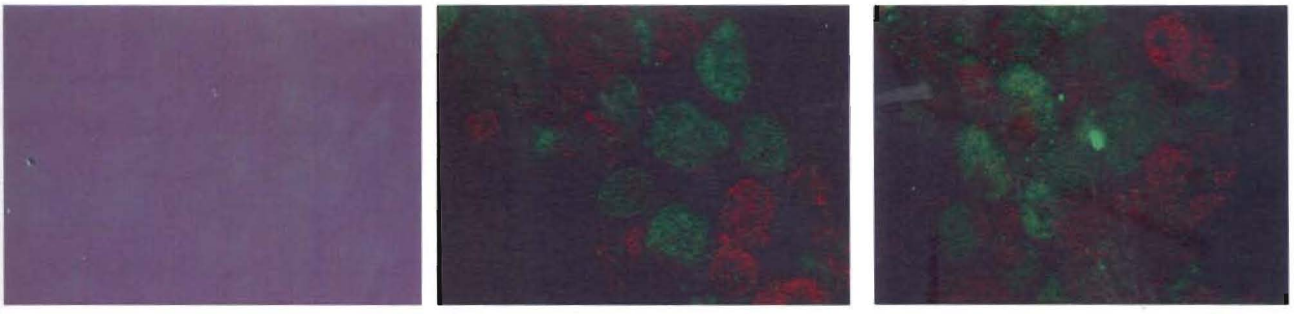


Figure 5-9 Immunocytochemical detection of caspase 3 in U937 cells. The cells were stimulated with lipopolysaccharide (LPS), hydrogen peroxide (H_2O_2) and tumour necrosis factor α (TNF-α) for 1hr and caspase 3 was detected. In experiments conducted iin the presence of triamcinolone acetonide (TA; 10^{-10}M), TA was added 30 minutes before cell activation. TA alone did not induce activation of the protein. In stimulated cells, however, TA induced apoptosis. Co-stimulation of the cells with Trichostatin A (TSA; 10ng/ml) and TA also induced apoptosis. The micrographs are representative of 3 independent experiments. Similar results were obtained for Jurkat and HUT-78 cells. TSA alone had no effect on Caspase 3 expression.



U937 Jurkat HUT-78

Figure 5-10 Immunocytochemical detection of caspase 3 in U937, Jurkat and HUT-78 cells. The cells were stimulated with Trichostatin A (TSA) and caspase 3 was detected in the presence of triamcinolone acetonide (TA; 10^{-10} M). Caspase 3 was not activated in the U937 cells but it was detected in both T-cell lines. Similar results were obtained in cell stimulated with dexamethasone (Dex) and TSA.

Lanes	Cntrl	Blank	Dex +H_2O_2	Dex +H_2O_2 +TSA	TA +H_2O_2	TA +H_2O_2 +TSA	Dex +TSA	TA +TSA

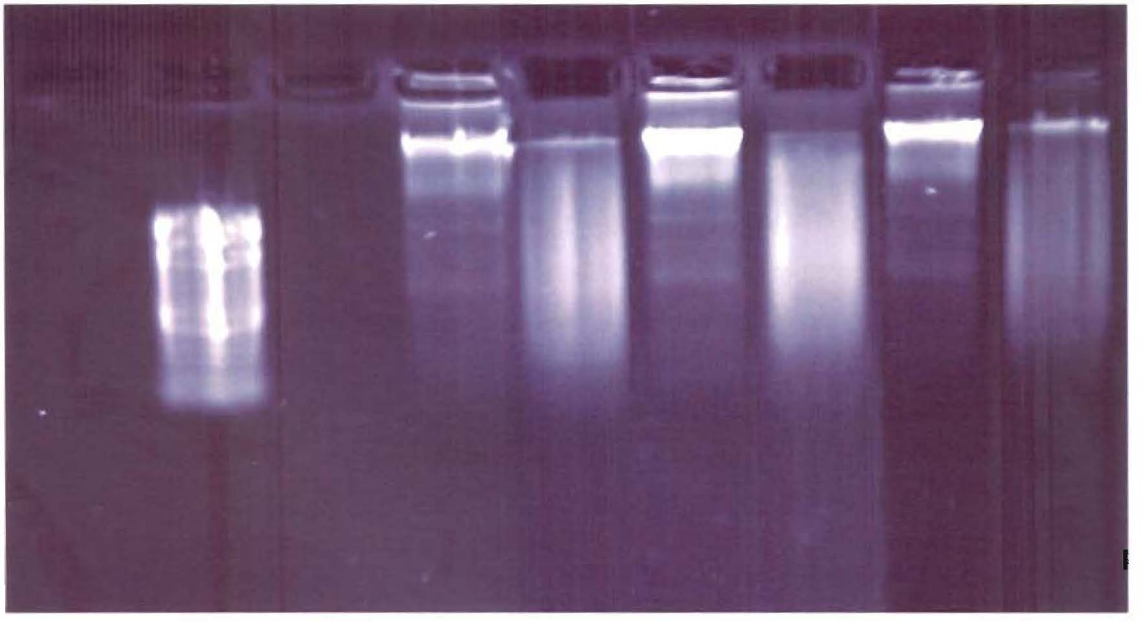


Figure 5-11 DNA fragmentation in Jurkat cells indicating late stages of apoptosis. The lanes represent different cell treatments. Lane: kit control, blank, dexamethasone (Dex)+hydrogen peroxide (H_2O_2), Dex+H_2O_2+Trichostatin A (TSA), triamcinolone acetonide (TA)+H_2O_2, TA+H_2O_2+TSA, Dex+TSA, TA+TSA. Similar results were obtained in U937 and HUT-78 cells. Treatment of the cells with glucocorticoids and TSA induced DNA fragmentation in the T-cells (evident in lanes 7 and 8). This was not seen in U937 cells treated with glucocorticoids and TSA (results not shown). Results are representative of three independent experiments.

5.3 Discussion

The mechanisms involved with induction of cell death by histone deacetylase inhibitors remain unclear. In lymphoid and colorectal cells, the histone deacetylase inhibitor butyrate acted synergistically with staurosporine to promote apoptosis by upregulating the expression of caspase-3 (Medina *et al.*, 1997). In the human leukaemia cell line CEM-C7H2, low concentrations of butyrate promoted cell death that was triggered by dexamethasone (Bernhard *et al.*, 1999). This synergism however, was not seen with TSA. Increasing the concentration of these inhibitors produced an antagonistic effect on Dex-induced apoptosis. In this chapter, supportive evidence of a role for histone acetylation in apoptosis are provided. Cells under the effect of inflammatory stimuli can either activate cell signalling pathways that lead to restoration of function or undergo programmed cell death. Having established from previous work that increased histone acetylation occurs in cells when induced with inflammatory stimuli, here, whether histone hyperacetylation leads to programmed cell death was investigated.

Following previous findings this investigation concentrated on the induction of apoptosis in the monocytic cell line U937. In the immune system, especially in lymphocytes, apoptosis plays an important role in maintaining the T cell repertoire and in deleting autocrine lymphocytes, thus regulating the immune response, however it has been reported that some T-cell subtypes are particularly resistant to various inducers of apoptosis. For this reason two lymphocytic cell lines (Jurkat and HUT-78 cells) were studied. All three cell lines were primarily stimulated as previously with LPS (10ng/ml), TNF-α (10ng/ml) and TSA (10 ng/ml and 100 ng/ml) over a period of 1hour. TSA at 100 ng/ml is associated with induction of apoptosis in a number of cell lines while at the lower concentration of 10 ng/ml the inhibitor is known to induce histone hyperacetylation. The cells were also stimulated with H_2O_2 (100μM) a known inducer of oxidative stress and consequently apoptosis in many cell lines.

Preliminary studies on the initiation of cell death following stimulation showed that in LPS stimulated U937 cells, cell death was not detectable whereas in both T-cell lines, cell death was induced. Stimulation of the cells with H_2O_2 and also TSA (100 ng/ml) induced cell death in all three cell lines. In U937, Jurkat and HUT-78 cells viability was not altered by the addition of TNF-α. Finally TSA (10 ng/ml) did not appear to induce cell death in any of the cell lines.

Studies into programmed cell death showed that in the U937 cells, cell death was due to apoptosis (over 50% apoptotic cells detected) when TNF-α and TSA (100 ng/ml) treated. Despite its toxicity, H_2O_2 did not induce apoptosis in U937 cells. This finding is in agreement with a report stating that H_2O_2 suppressed cell death in U937 cells by two different mechanisms depending on its concentration (Lee and Um, 1999) and in alveolar macrophages from smokers (Tomita, 2002). The T-cell lines were shown to be more

susceptible to apoptosis. In the Jurkat cells H_2O_2, TNF-α and TSA caused more than 60% apoptosis and LPS caused more than 40% of the cells to undergo apoptosis. Similar effects were seen in HUT-78 cells. TNF-α treatment alone however did not induce apoptosis in these cell lines. This is in contrast to a previous showing that in contrast to Jurkat cells, HUT-78 cells were resistant to the apoptotic effects of TNF (Giri *et al.*, 1998).

Our investigation on the expression of Bcl-2, an anti-apoptotic protein, suggested that different cells respond differently to the same apoptotic stimulus. In general upregulation of Bcl-2 is associated with an increase in the resistance of cells to apoptosis. In the U937 cells Bcl-2 expression was only significantly downregulated following treatment with LPS. It has been reported (Vanhaesebroeck *et al.*, 1993) that in U937 cells as well as in the human breast carcinoma cell line MCF7 and promyelocytic leukaemia cell line HL60, despite constitutive expression of the endogenous Bcl-2 gene, that cells underwent apoptosis following TNF-α stimulation. Bcl-2-overexpressing derivatives of these cell lines did not acquire reduced TNF sensitivity and still exhibited the characteristic pattern of internucleosomal DNA fragmentation of TNF-induced apoptosis. An absence of a correlation between Bcl-2 gene expression and cellular sensitivity to TNF-induced cell lysis was proposed. Moulding *et al.*, (2000) showed that in the U937 cells the induction of Mcl-1 is required to prevent apoptosis during differentiation, and the constitutive expression of Bcl-2 is unable to compensate for the loss of Mcl-1. Also, in nitric oxide stimulated U937 cells, Bcl-2 is downregulated as caspase activation occurs (Brockhaus & Brune, 1998).

Bcl-2 expression in T-cell lines showed a similar pattern of activation for both Jurkat and HUT-78 cells. Bcl-2 levels were increased with all stimulants with the exception of TSA (100 ng/ml). Trichostatin A has been demonstrated to increase the expression of the Bcl-2 related protein Bad, although the expression of Bcl-2, Bcl-xL, Bax, and Fas was not changed by the addition of TSA in human glioma cells (Sawa *et al.*, 2001). The same report suggested that HDAC inhibitors such as TSA and sodium butyrate induce apoptosis through an increase in Bad protein in human glioma cells *in vitro*.

Investigation of the activation of annexin V as a method for the detection of early stages of apoptosis showed that in U937 cells H_2O_2, TNF-α and TSA (10 ng/ml) significantly upregulated this protein. In Jurkat cells LPS, H_2O_2, TNF-α and TSA (10 ng/ml) also upregulated annexin V upregulation but in contrast, in HUT-78 cells TNF-α did not induce annexin V expression. TSA (10 ng/ml) caused the upregulation of annexin V despite the fact that high levels of apoptosis were not detectable at this concentration. 100 ng/ml of the inhibitor did not upregulate annexin V levels. This might be due to the fact that TSA at high concentrations causes rapid apoptosis and therefore at the later time points investigated annexin V may have been downregulated.

In a recent study, an increased constitutive expression of a Fas-associated death domain protein (FADD) was reported in ageing subjects at the protein and not at the mRNA level, suggesting a post translational modification of FADD in ageing humans (Green & Scott, 1994). In another paper it has been reported that in peripheral blood monocytes that apoptosis increased with increased levels of of histone 4 acetylation (Sourlingas *et al.,* 2001). Finally, the HDAC inhibitor HDAI, has been shown to augmented TNF family-related apoptosis in the human colonic adenocarcinoma cell lines, LS 180 cells and HT-29. The same report suggested that HDAI sensitises human colonic adenocarcinoma cell lines to TRAIL-mediated apoptosis (Inoue *et al.,* 2002). Our studies, in contrast, show that TSA and presumably changing histone acetylation status did not affect induction of apoptosis in monocytic and T-cell lines since only high concentrations of the inhibitor caused cells to undergo apoptosis. This, however, is attributed to the toxic effect of TSA rather than its deacetylation inhibitory abilities.

Steroid hormones are potent regulators of programmed cell death in many steroid-dependent cells and tissues such as the mammary gland, prostate, ovary and testis where they can affect or facilitate apoptotic process either by presence or absence. Here the effects of the glucocorticoids dexamethasone and triamcinolone acetonide on the induction of apoptosis in cells treated with inflammatory stimuli were studied. Glucocorticoids alone failed to show any significant changes in the activation of Bcl-2 in all three cell lines. The only significant effect of dexamethasone and triamcinolone acetonide on Bcl-2 expression was seen in LPS-stimulated U937 cells where Bcl-2 levels were elevated 2-fold. It has been previously demonstrated that Bcl-2 did not markedly affect glucocorticoid-mediated growth arrest, thereby separating the anti-proliferative from the apoptosis-inducing effect of glucocorticoids (Hartmann *et al.,* 1999). Moreover, Bcl-2 did not prevent the dramatic reduction in the levels of several mRNAs observed during glucocorticoid treatment, including the transgenic Bcl-2 mRNA. The same report therefore suggested that Bcl-2 can be placed upstream of effector caspase activation, but downstream of other glucocorticoid-regulated events, such as growth arrest and the potentially critical repression of steady state levels of multiple mRNA. In the CCRF-CEM acute-lymphatic-leukaemia model, mutations in the GR-gene coding region has been suggested to represent one cause of glucocorticoid apoptosis resistance (Hala *et al.,* 1996).

However, investigation of the activation of annexin V in stimulated cells in the presence of glucocorticoids revealed an upregulation of the protein in all cell lines with the exception of TNF-α-stimulated Jurkat cells where the presence of Dex in TSA stimulated cells did not induce upregulation of annexin V. This phenomenon was distinct from that of a recent report by Bernhard *et al.,* showing that in cultured rat thymocytes the apoptosis induced by dexamethasone alone did not increase following butyrate addition (Bernhard *et al.,* 1999). The authors also reported a non-additive interrelationship between Dex and TSA, as TSA-

induced apoptosis was not only blocked by the presence of Dex but Dex-induced apoptosis was also partially inhibited in the presence of TSA, suggesting that in thymocytes this phenomenon is related to histone acetylation. Furthermore, in CEM-C7H2 cells, at higher concentrations of butyrate or trichostatin A, there was a minor but reproducible antagonistic effect of dexamethasone on apoptosis induced by each of the two histone deacetylase inhibitors, suggesting that this antagonistic effect too, is related to histone hyperacetylation. In contrast to this, in the human T cell-derived leukaemia cell line CEM-C7H2, Dex did not block butyrate- or TSA-induced apoptosis; moreover, butyrate, over a range of concentrations, had a marked synergistic effect on Dex-induced apoptosis. This synergism, however, was not mimicked by TSA, indicating that the effect is not related to histone acetylation but rather due to a pleiotropic effect of butyrate. It could also be explained by differences in cell specificity or apoptotic stimulus.

Investigation of Caspase 3 activation of the cells under the same conditions revealed that the combinatory effect of steroids and TSA in stimulated cells enhanced this marker of apoptosis in all three cell lines. Here, the effect of triamcinolone acetonide was significantly more potent compared to that of dexamethasone. The findings in this chapter complement a study of the structural changes that occur in chromatin of apoptotic cells by Allera *et al.* (1997). The authors reported that histones became deacetylated in rat thymocytes that were induced into apoptosis by glucocorticoids. Triton-acid-urea gels revealed that with increasing cell death, the percentages of monoacetylated and deacetylated histone H4 decreased with a corresponding increase in the percentage of unmodified histone H4. It was proposed that either this histone deacetylation could function to suppress gene expression during apoptosis, or the bulk deacetylation could promote chromatin condensation by allowing greater DNA-histone interactions and conformational changes in the nucleosomal level. This loss of histone acetylation in apoptotic cells was also reported by Hendzel *et al.,* (1998) in several cell lines that either entered apoptosis simultaneously or were induced with programmed cell death.

IBD has been reported to be associated with changes in apoptosis of inflammatory cells as well as enhanced expression of inflammatory mediators (Sturm and Fiocchi, 2002). Several key proteins including p300, HDAC1 and HDAC2, have intrinsic acetyl- or deacetyl-transferase activity, which as previously discussed has been linked to transcriptional activation and repression. The present results suggest that TSA may induce or prevent apoptosis due to its histone deacetylase ability and not due to its toxicity. Therefore lower concentrations of TSA could reduce the inflammation seen in IBD without the toxic side effects of the inhibitor being manifested. This is also demonstrated in combination to the glucocorticoid effects in inflammatory stimuli induced cells. TSA acted synergistically with glucocorticoids to enhance apoptosis in stimulated U937, Jurkat and HUT-78 while preventing programmed cell death in the absence of these other stimuli. These findings support the notion that histone acetylation regulates apoptosis and this may be the target for the actions

of glucocorticoids. The variable results that Dex and TA had on macrophages and lymphocytes suggest that probably regulation of apoptosis via histone acetylation is cell type and inflammatory stimuli dependent. Finally, in agreement to findings in chapter 4 where triamcinolone acetonide actions were more potent compared to dexamethasone actions in the regulation of histone acetylation, triamcinolone acetonide appears to be effective in the regulation of apoptosis in both macrophage and T-cell lines.

Some of the cutting edge discoveries that have been made during the last few years are based on the growing realisation that several drugs in widespread use, such glucocorticoids, act on the transcriptional level, although often in an indirect manner. Instead of modulating directly the function of gene-specific transcription factors, such drugs often target various signalling pathways that ultimately control the activity of whole transcriptional networks in the nucleus. Histone deacetylase inhibitors have recently been used in cancer therapy trials and the results have proved to be very promising (Marks *et al.,* 2001). Having established the role of histone H4 acetylation and deacetylation in inflammatory gene expression and the regulation of apoptosis this investigation finally expanded in the role of histone acetylation in disease, specifically in inflammatory bowel diseases and Peyer's patches.

Chapter 6

Histone acetylation in *in vivo* inflammation induced animal models and human IBD Peyer's patches

6.1 Introduction

The cause of inflammatory bowel disease (IBD) remains unknown. It has been proposed that epithelial abnormalities are the central defect, and that they underlie the development of mucosal inflammation and its chronicity (Gibson, 1997). IBD can be effectively treated by enemas containing short chain fatty acids (SCFA) such as butyrate, propionate, and acetate (Perrin *et al.,* 1994) in combination with steroid treatment. The molecular mechanisms that lead to this response have not been well characterized. It is well known that intestinal inflammation leads to an alteration in patterns of epithelial differentiation with an increase in epithelial proliferation and an expansion of cell populations in an undifferentiated state. SCFAs such as butyrate are capable of inhibiting cell proliferation and inducing a differentiated phenotype *in vitro.*

Butyrate is the preferred energy source for colonocytes (Ahmad *et al,* 2000) and its therapeutic potential in colon cancer has been proposed. It has been suggested that colitis may be caused by impaired colonocyte oxidation of butyrate (Hague *et al.,* 1995). The most commonly reported mechanism by which butyrate modulates gene expression involves an alteration of chromatin structure subsequent to increased histone acetylation.

In the Caco-2 colon cancer cell line the effect of SCFAs and the process of cellular differentiation on the expression of the pro-inflammatory cytokine, interleukin 8 (IL-8) has been studied. SCFAs and Trichostatin A, structurally unrelated compounds which both induce histone hyperacetylation, both led to a concentration-dependent inhibition of IL-8 expression (Huang *et al.,* 1997). The same report suggested that a possible mechanism by which SCFAs may be effective in the treatment of ulcerative colitis may be through their ability to increase histone acetylation states and inhibit the production of pro-inflammatory substances by the intestinal epithelium. In the human intestinal epithelial cells HT-29, the HDAC inhibitor TSA, only partly mimicked the effects of butyrate, and although both compounds induced histone hyperacetylation, they did so with different kinetics of action (Siavoshian *et al.,* 2000). Although these findings might seem contradictory to the present study, the results reported may be cell and cytokine specific and relate to the high concentrations of drugs used. However, they do suggest that histone acetylation may be involved in the control of inflammatory gene expression in inflammation of the bowel.

The ideal animal model of inflammatory bowel disease, should be a naturally or inducible animal disease that is identical in every aspect to the human disease. This means that the disease is induced and maintained by the same primary and secondary factors (same aetiology and pathophysiology), has an equivalent clinical spectrum and is treatable with therapeutic agents (Kim & Berstad, 1992). Several animal models of experimental ulcerative colitis have been described (Table 6-1) and, of these, colitis induced in mice by oral dextran sulfate sodium (DSS) has been widely used because of many similarities to human ulcerative colitis involving a T_H2 type inflammation (Dieleman *et al*, 1997; Dohi *et al.*, 1999). Ulcerative colitis can be induced in hamsters (Ohkusa, 1985) and mice (Okayasu *et al.*, 1990) by giving DSS in their drinking water. On postmortem examination, multiple erosions and inflammatory changes including crypt abscesses can be found on the descending and sigmoid colon and the rectum. Mice develop chronic colitis, including dysplasia, shortening of the large intestine and prominent lymphoid follicles after five administration cycles, each cycle consisting of 7 days with 5% dextran sulfate sodium in the drinking water followed by 10 days consumption of distilled water. The observation that shortening of crypts occurs early after exposure to oral DSS and precedes development of significant inflammation has been used to suggest that the primary defect in DSS colitis is an abnormal colonic epithelium (Cooper *et al*, 1993). Okayasu *et al.* (1990), also reported swollen macrophages in the inflamed colonic wall of the DSS model, a finding that was consistent with other sulphate type-induced colitis (i.e. carrageenan and amylopectin sulphate induced colitis).

	Affected site	Pathology	Pathogenic mechanism of injury
	Chemically induced models		
Acetic Acid	Colon, ileum	Acute	Toxic
TNBS/Ethanol	Colon, ileum	Acute/Chronic	Toxic
Cyclosporin	Stomach, colon	Acute	Altered T cell function
	Microbial and polymer induced models		
Dextran sulphate	Colon, cecum	Acute	Unknown
	Genetically engineered models		
T-cell receptor mutant	Colon	Acute/Chronic	Absent T-cells, present B-cells
Disrupted interleukin-2 gene	Colon	Acute/Chronic	Absent IL-2
Disrupted interleukin-10 gene	Jejunum, ileum, colon	Acute/Chronic	Absent IL-10
	Spontaneous models		
C3H/HEJ mice	Colon	Acute/Chronic	Genetic, environmental

Table 6-1 Models of IBD (adapted from Kirsner, 1995)

The trinitrobenzene sulfonic acid (TNBS) model of colitis (Morris *et al.*, 1989), is a commonly used experimental model of colitis very similar to IBD and particularly Crohn's disease (Fig. 6-1) (Vilaseca *et al.*, 1990). In this model an acute inflammation of the classical delayed-type hypersensitivity with the T_H1 type profile is generated (Neurath *et al.*, 2000) resulting in a

transmural granulomatous colitis with characteristics very similar to Crohn's disease, such as transmural inflammation with granuloma, skip-segment ulceration and inflammation, cobblestone-like appearance of the mucosa, mast cell and lymphoid infiltrates, and crypt distortion (Parronchi *et al.,* 1997). Inflammation is induced through sensitization with TNBS, which is a chemical hapten that binds to tissue proteins and is capable of stimulating cell-mediated immunity (Kim *et al.,* 1992). However, TNBS induced colitis is not purely T cell driven, as colonocytes can also metabolize TNBS to form reactive oxygen species that are themselves pro-inflammatory (Grisham *et al.,* 1991).

The nuclear factor kappa B (NF-κB)-p65 subunit has been shown to be activated in response to a number of immune and inflammatory stimuli. NF-κB has been demonstrated to be essential in DSS induced colitis through the use of p65 antisense to prevent inflammation (Murano *et al.,* 2000) and through the use of gliotoxin a non-specific inhibitor of NF-κB, (Herfarth *et al.,* 2000; Fitzpatrick *et al.,* 2000). Preliminary results showed that NF-κB-p65 protein expression levels were increased by DSS in inflamed tissue, where also, the p65 subunit was suggested as being of greatest significance in the early stages of inflammation. In the TNBS model, active NF-κB-p65 has been localized to both lamina propria and epithelium of intestinal tissue (Segain *et al.,* 2000). Also, dexamethasome has been shown to suppress the TNBS induced NF-κB activity and inflammation of the intestine (Nakase *et al.,* 2001). Since dexamethasone suppresses TNBS induced NF-κB activity and is also shown to effect histone acetylation levels (see chapter 4), it could be hypothesized that histone acetylation could be involved in the regulation of inflammatory bowel disease.

Currently, whilst an essential role for histone acetylation has been demonstrated by the use of HDAC inhibitors as treatment for IBD in cell models and in patients, a more thorough examination of the chromatin remodeling machinery that is activated during intestinal inflammation, has yet to be undertaken. To date, no relevant work has been performed on the role of histone acetylation in Peyer's patches or in intestinal tissues from IBD animal models or patients. Thus, this chapter investigates the activation of histone 4 acetylation in Peyer's patches of two *in vivo* models of inflammation (Lewis and Sprague-Dawley) as well as in Peyer's patches of IBD patients.

It is clear that many different immune defects or alterations can lead to similar IBD phenotypes. Interaction between genetic factors and environment play a crucial role in the development of IBD and it has been shown that the onset and severity of colitis are dependent on the background strain of the animals, for this reason, for the *in vivo* model two mouse strains were used as DSS animal models. Acetylation on histones 4 and 3 was investigated in the Peyer's patches of these animal models as well as acetylation on the specific H4 lysine residues 5, 8, 12 and 16. Finally in this chapter the induction of histone 4 acetylation in the Peyer's patches of IBD patients studied.

6.2 Results

6.2.1 Localisation of histones 4 and 3 in animal models

The investigation into the activation of histone acetylation into Peyer's patches was initiated by a study into the activation of histones 3 and 4 in Peyer's patches of Lewis and Sprague-Dawley rats treated with 5% DSS in drinking water. Tissue of sacrificed animals following treatment showed severe inflammation. However, the Peyer's patches did not show any macroscopic signs of inflammation. This was due to the fact that the inflammation induced by DSS more closely resembles ulcerative colitis i.e. inflammation is observed on the descending and sigmoid colon and the rectum, but is not apparent along the wall of the small intestine where Peyer's patches are situated. Western blotting was once again employed to study histone 3 and 4 acetylation. In figure 6-1 representative bands obtained from Western blotting are illustrated where increased acetylation on histones is shown by the increase of density in the bands that represent of proteins isolated from Peyer's patches of DSS-treated animal. Protein levels of β-actin are also shown to indicate equal protein loading. The graphs in figure 6-2 show acetylation on histones 3 and 4 as shown by three independent experiments. In both Lewis and Sprague-Dawley non-treated animals, acetylation on both histones 4 and 3 was evident. However, when animals were treated with DSS histone 4 acetylation was significantly upregulated three fold in the Lewis rat Peyer's patches. Similar results were also found for the Sprague-Dawley rats. Histone 3 acetylation levels were also upregulated in DSS treated rats but to a lesser extent (2-fold increase) than with histone 4 acetylation.

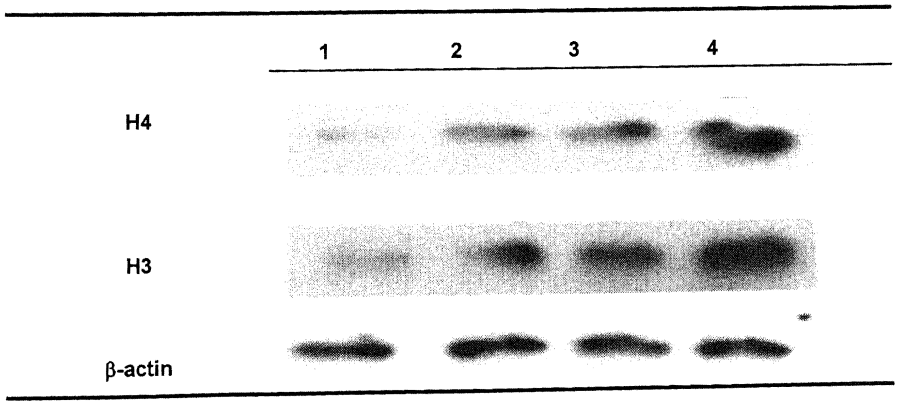


Figure 6-1 Representative bands of histone 4 (H4) and 3 (H3) acetylation as obtained by Western blotting in two dextran sulfate sodium (DSS) rat strains of inflammation (animals were treated with 5% DSS in drinking water). Lanes represent: (1) non-DSS treated Lewis rats (control), (2) DSS-treated Lewis rats, (3) non-DSS treated Sprague-Dawley rats (control) (4) DSS-treated Sprague-Dawley rats. β-actin levels were also measured to ensure equal protein loading in all samples. The results are representative of three independent experiments.

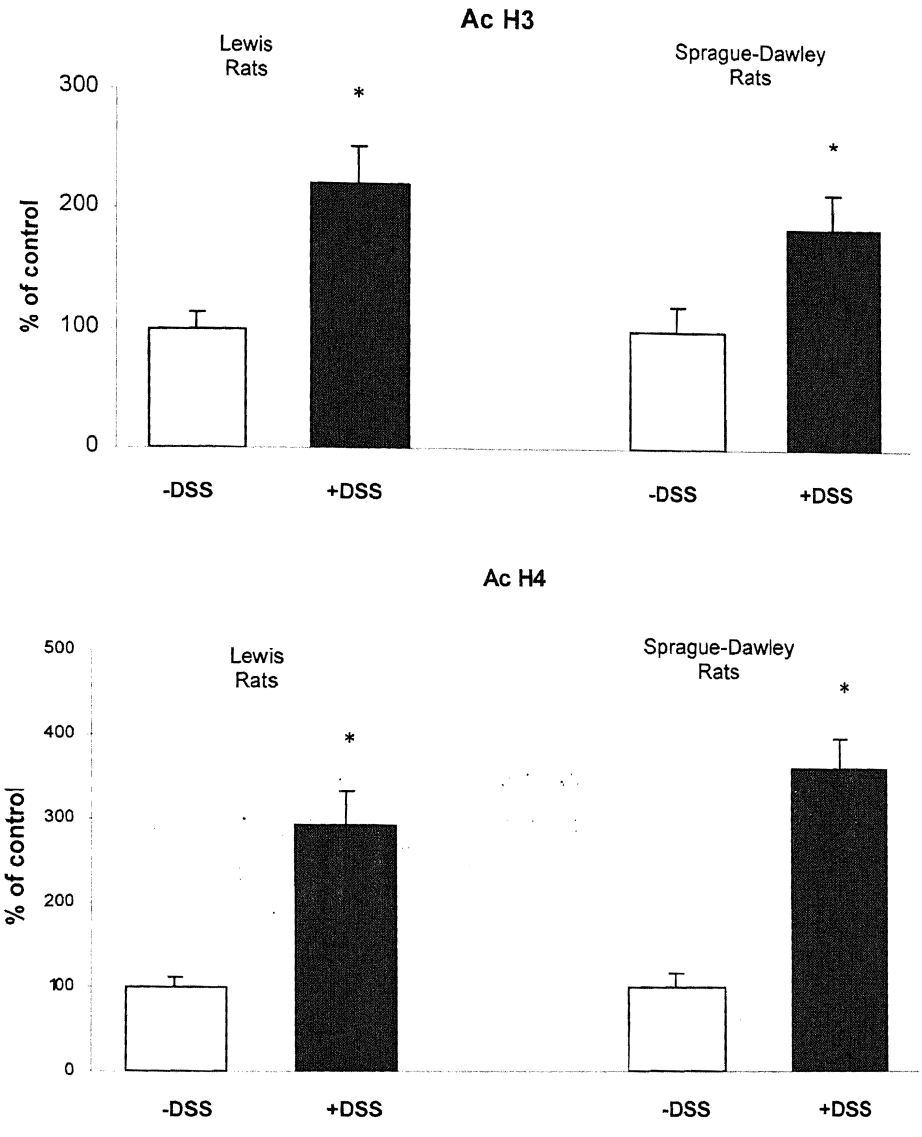


Figure 6-2 Acetylation on histones 3 (H3) and 4 (H4) in Lewis and Sprague-Dawley dextran sulfate sodium (DSS) treated rats (5% of DSS was added to drinking water). The results were obtained by Western blotting. In both strains of non-DSS treated animals acetylation was not induced. Acetylation was increased significantly in Lewis and Sprague-Dawley rats when treated with DSS to induce inflammation. Columns represent the mean ± SEM of three independent experiments. (*$p<0.05$).

6.2.2 Localisation of histones 4 and 3 in Peyer's patches

In Chapter 4 it was showed that acetylation on histones 4 and 3 was increased in cell lines stimulated with inflammatory stimuli, with histone 4 being the predominantly acetylated histone. After demonstrating that acetylation on both histones was upregulated in the Peyer's patches of DSS treated animals, as measured by Western blotting, this investigation was continued by localising the activation of those two histones in the Peyer's patches from the same animals. The results (Fig. 6-3) revealed an increase in the acetylation of both histones during inflammation of the bowel, regardless of the fact that no apparent signs of inflammation were present in the Peyer's patches of both animals macroscopically. It was also apparent as shown in figure 6-3 that different cell types were acetylated in Peyer's patches obtained from the DSS treated animals. Light microscopy revealed that in the Peyer's patches of both DSS treated rats, only cells situated in the mantle zone of the Peyer's patches stained positive for acetylated histone 3. This pattern of staining was consistent in both Lewis and Sprague-Dawley rats as seen in Peyer's patches from two separate animals. Immunocytochemical results showed a more uniformed staining for acetylated histone 4 throughout the surface of the Peyer's patches i.e. all cells present were stained positive for acetylated histone 4. Again the results were similar for both animal strains and were consistent in two individual experiments performed.

6.2.3 Acetylation on histone 4 specific lysine residues in the Peyer's patches of two DSS models of inflammation

The results on the acetylation on histones 4 and 3 showed an upregulation of acetylation on both histones in the Peyer's patches of both DSS treated rat strains. However, similarly to results shown in chapter 4, acetylation on histone 4 was significantly higher (3 fold) compared to upregulation on histone 3 (2 fold elevation on acetylated histone levels). Thus, to continue this investigation acetylation on histone 4 specific lysine residues 5, 8, 12 and 16 was determined in both DSS models of inflammation (Lewis and Sprague-Dawley strains)(Fig. 6-4 and 6-5). In this instance, the investigation into the activation on histone 4 lysine acetylation studying the DSS animal model was expanded by the addition of metronidazole. Metronidazole (MTD) is a synthetic 5-nitroimidazole, has an antibiotic action that is based on the modification of the genetic substance of microorganisms and been reported to be effective in the treatment of ulcerative colitis and Crohn's disease (Sartor, 1995). In experiments as described in Chapter 2, increased doses of MTD were administered in the DSS treated rat models.

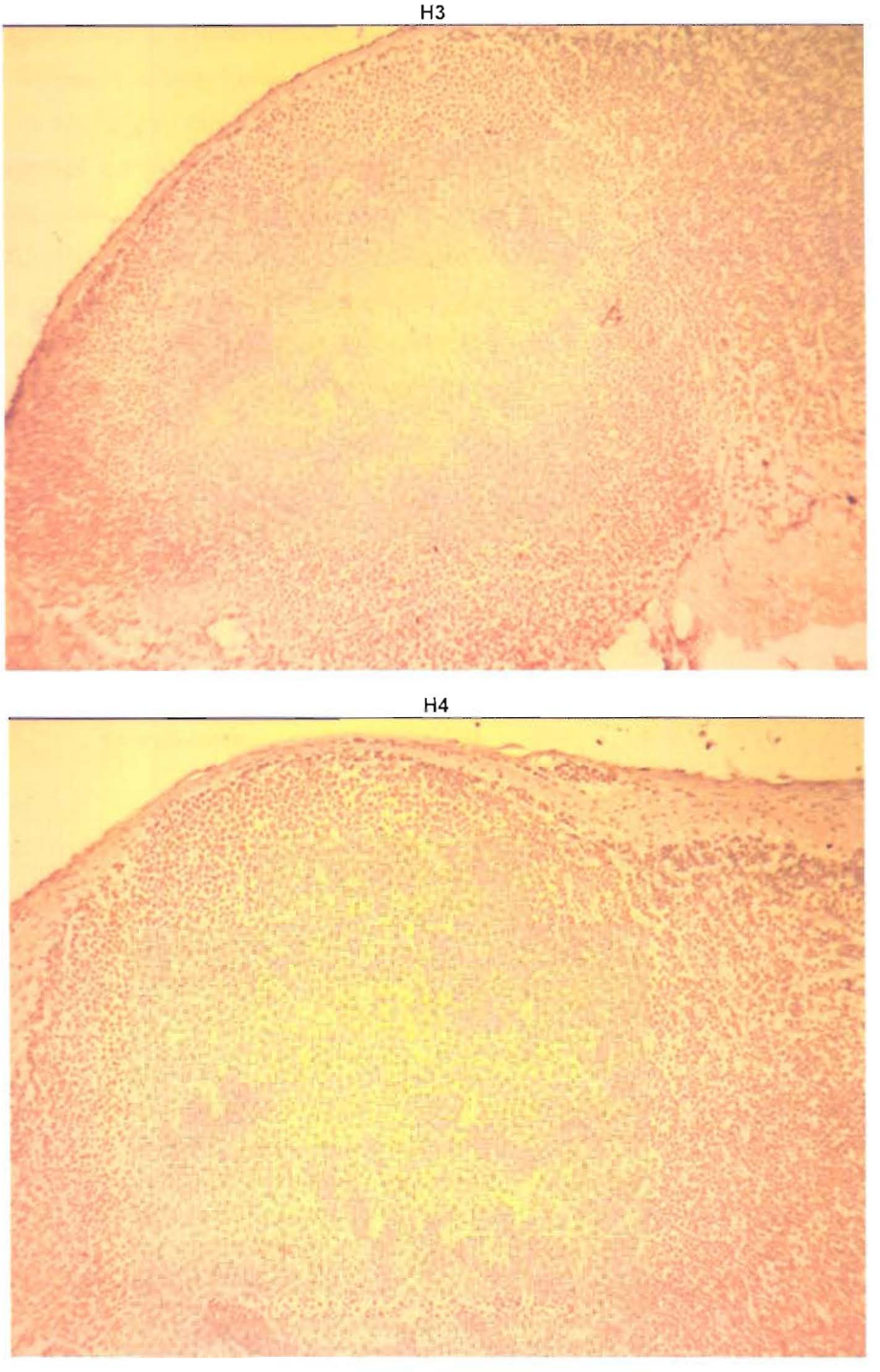


Figure 6-3 Immunocytochemistry of dextran sulfate sodium (DSS) treated Lewis rats using anti-histone 3 (H3) and 4 (H4) antibodies. The micrographs show sections of Peyer's patches. Histone 3 was acetylated only in the mantle zone of the Peyer's patch, while histone 4 appears to be acetylated throughout the surface of the Peyer's patch to both mantle zone and germinal centre cells. Similar results were obtained from Sprague-Dawley DSS-treated cells. In the Peyer's patches of untreated animals no acetylation on either histone 3 or 4 was apparent. The micrographs are representative of two individual experiments for each strain. Isotype controls show no staining.

Figure 6-4 shows representative bands obtained by Western blotting of the acetylation on lysine residues 5 and 8 of histone 4 in both Lewis and Sprague-Dawley DSS treated rat models. Figures 6-5a and 6-5b show histograms on the acetylation on lysine residues 5, 8, 12 and 16 of histone 4 in both Lewis and Sprague-Dawley DSS treated rat models. Acetylation on H4 K5 in the Lewis DSS models increased by 3 fold as shown in figure 6-5.a. Similarly administration of 0.1% MTD to the same strain induced K5 acetylation. However, when the administered dose of MTD was increased to 0.2%, acetylation levels were reduced to control (untreated) levels. Acetylation levels on H4 K8 were also elevated in the DSS treated animal. In animals treated with 0.1% MTD, acetylation remained at similar levels as with the DSS treated animals whilst administration of 0.2% MTD again reduced K8 acetylation back to control levels. The effect of MTD on DSS-treated Lewis rats was not determined.

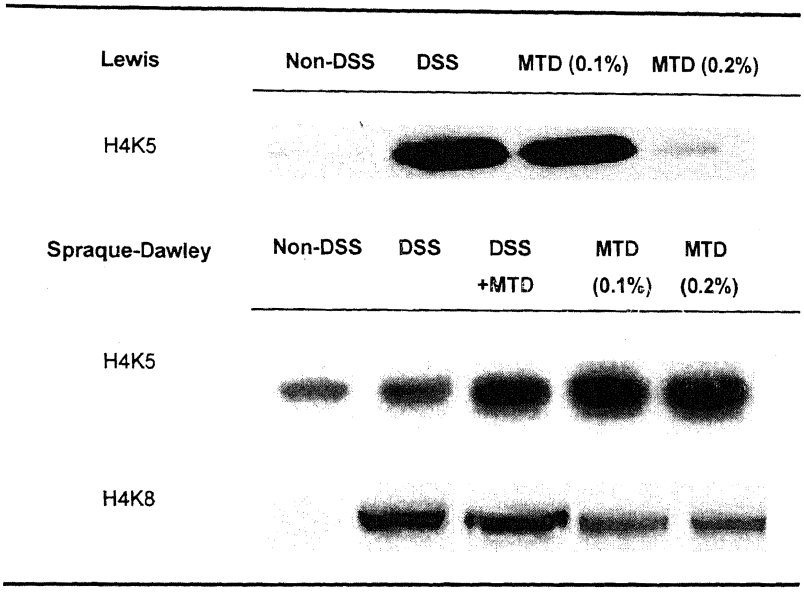


Figure 6-4 Representative bands of histone 4 (H4) lysine 5 (K5) and 8 (K8) acetylation as obtained by Western blotting in Lewis and Sprague-Dawley dextran sulfate sodium (DSS) and Metronidazole (MTD) treated rats. Lanes for Lewis rats represent: non-DSS treated rats (control), DSS-treated rats, rats treated with 0.1% MTD rats treated with 0.2% MTD. Lanes for Sprague-Dawley rats represent: non-DSS treated rats (control), DSS-treated rats, rats treated with DSS and 0.1% MTD rats treated with 0.1% MTD rats treated with 0.2% MTD. Due to the similar patterns of acetylation on K5, K8, K12 and K16 in the Lewis rats strains one representative gel is illustrated. Likewise representative bands are illustrated for the Sprague-Dawley rats. The results are representative of two independent experiments. Due to lack of tissue β-actin loading controls were not performed. 20μg protein was loaded onto each well.

In the Sprague-Dawley rats, the pattern of acetylation was different compared to the Lewis model. Treatment with DSS upregulated H4 K5 acetylation two-fold. Treatment with 0.1% MTD enhanced K5 acetylation four-fold, an effect that was not modified by co-treatment with DSS. Treatment with MTD was dose-dependent with a seven-fold increase in K5 acetylation seen with 0.2% MTD (Fig. 6-5.a). Similar results were obtained for both Lewis and Sprague-Dawley, for K8 acetylation induced by DSS treatment. DSS induced an increase in K8 acetylation (414 ± 51% versus 100 ± 23% non-DSS treated Lewis rats, 1275 ± 123% versus 100 ± 13% non-DSS treated Sprague-Dawley rats). In contrast, MTD (0.1%) enhanced K8 acetylation but higher doses (0.2%) reduced this increase. This effect was more marked in Lewis rats. Combined MTD and DSS treatment did not alter K8 acetylation.

K12 and K16 acetylation (Fig. 6-5.b) revealed similar patterns to those seen for K8 and K5 acetylation respectively in both rat models of inflammation. DSS treatment of both animal strains induced acetylation on lysine residues 12 and 16. The acetylated levels of K12 were markedly higher than those of lysines 16 and 5 (in Lewis rats: 703 ± 64% versus 100 ± 14 control; in Sprague-Dawley rats: 1117 ± 113 versus 100 ± 27 control). In the Lewis rats administration of MTD had a dose-dependent bell shaped response curve with an initial induction on K12 and K16 acetylation at 0.1% followed by repression at 0.2%. In Sprague-Dawley rats, administration of MTD in two increasing doses results in the dose-dependent downregulation of acetylation on K12 similarly to the effect observed in the Lewis rats. The same treatment however, resulted in the elevation of K16 acetylated levels in a dose-dependent manner.

The additional effect of DSS and MTD studied in the Sprague-Dawley strain, resulted in highly acetylated K12 levels. The same effect was seen on K16 acetylation (high levels of acetylated K16 but not as high as the levels noted for K12). Limited supply of tissue allowed only two independent experiments to be conducted on these models. It was therefore not possible to perform any statistical analysis of these results.

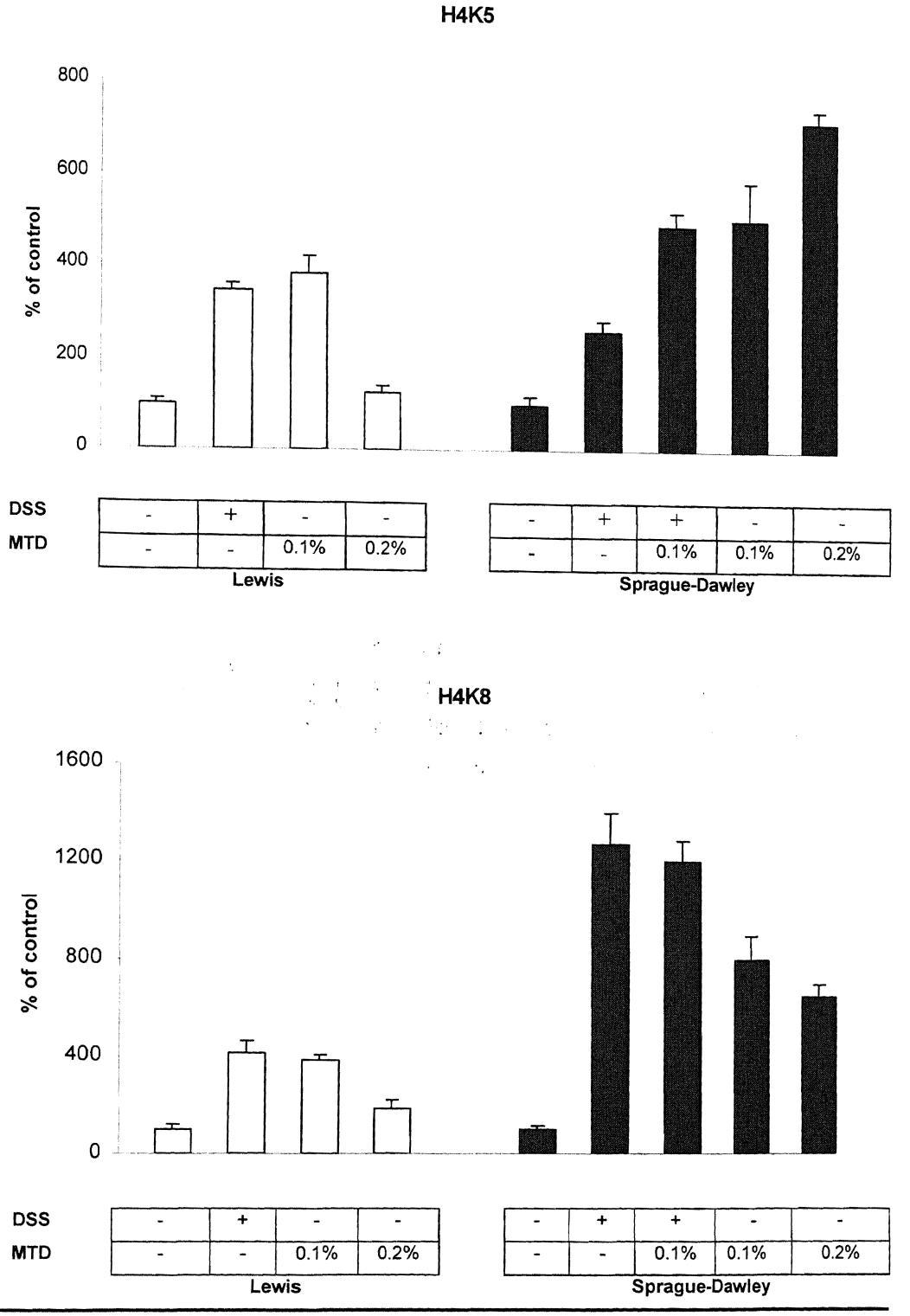


Figure 6-5.a Acetylation on histone 4 (H4) specific lysine residues 5 (K5) and 8 (K8) in Lewis and Sprague-Dawley dextran sulfate sodium (DSS) (5% of DSS was added to drinking water) and Metronidazole (MTD) treated rats. The results were obtained by Western blotting. DSS induced acetylation on both K5 and K8 lysine residues in both rat models. The lysine acetylation pattern that MTD administration induced appeared to be dose- and lysine residue-dependent in both rat strains. Columns represent the mean ± SEM (bar) of two independent experiments.

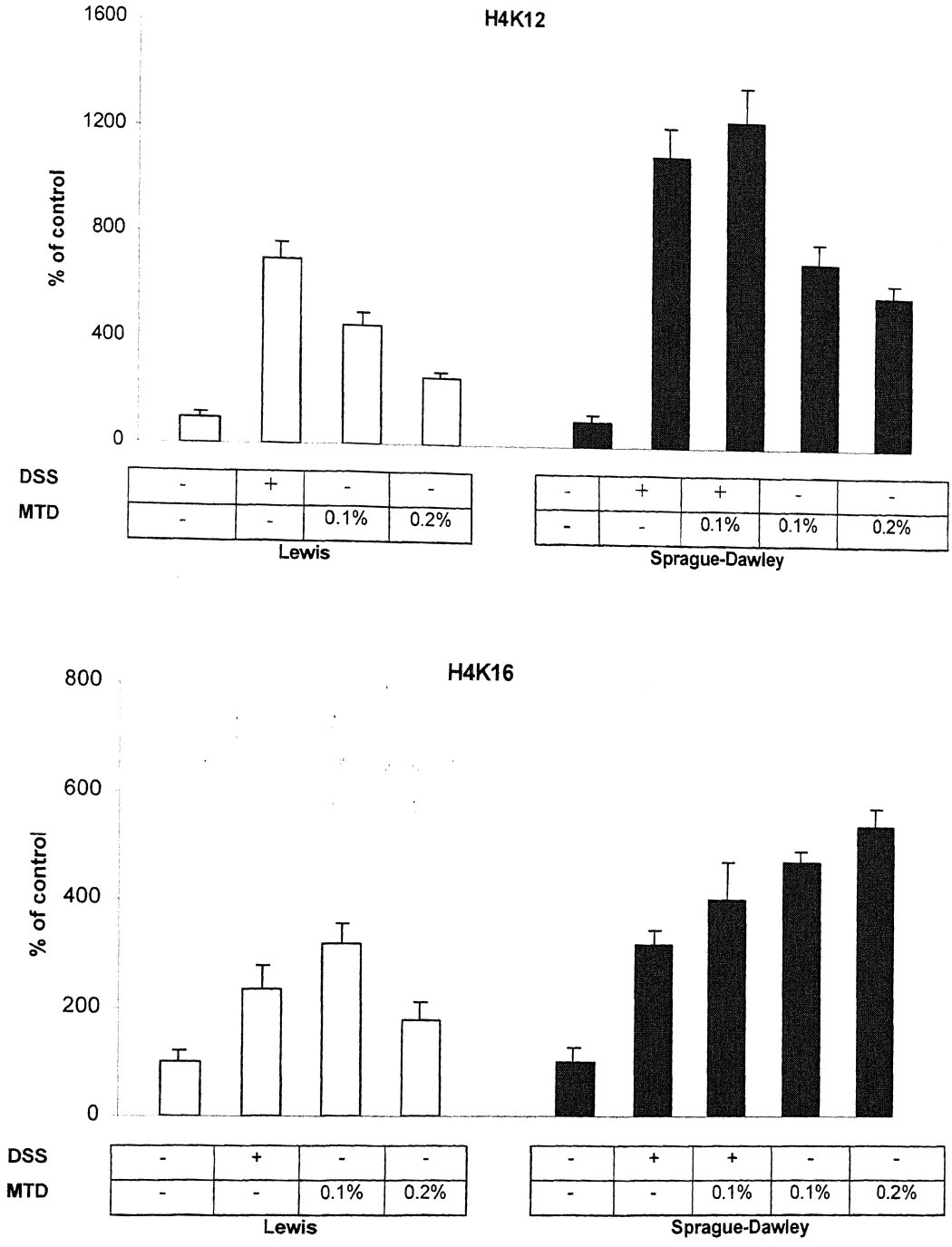


Figure 6-5.b Acetylation on histone 4 (H4) specific lysine residues 12 (K12) and 16 (K16) in Lewis and Sprague-Dawley dextran sulfate sodium (DSS) (5% of DSS was added to drinking water) and Metronidazole (MTD) treated rats. The results were obtained by Western blotting. DSS induced acetylation on both K12 and K16 lysine residues in both rat models. The lysine acetylation pattern that MTD administration induced appeared to be dose- and lysine residue-dependent in both rat strains. Columns represent the mean ± SEM (bar) of two independent experiments.

6.2.4 Macroscopical characterisation of the intestine in a rat TNBS model of colitis

The TNBS model of colitis was compared to a Sham operated group (saline treated animals) and initial investigations were made macroscopically comparing newly operated colon tissue from both animal groups. Visual observation of the samples showed that the distal colon in the TNBS model was significantly inflamed compared to the control Sham group (Fig. 6-6). Comparing the two samples from proximal to the distal region of the colon also revealed that in the distal region of the TNBS model the inflammatory process was quicker as inflammation was well advanced, indicated by the dark reddish colouration of the tissue also indicative of necrosis.

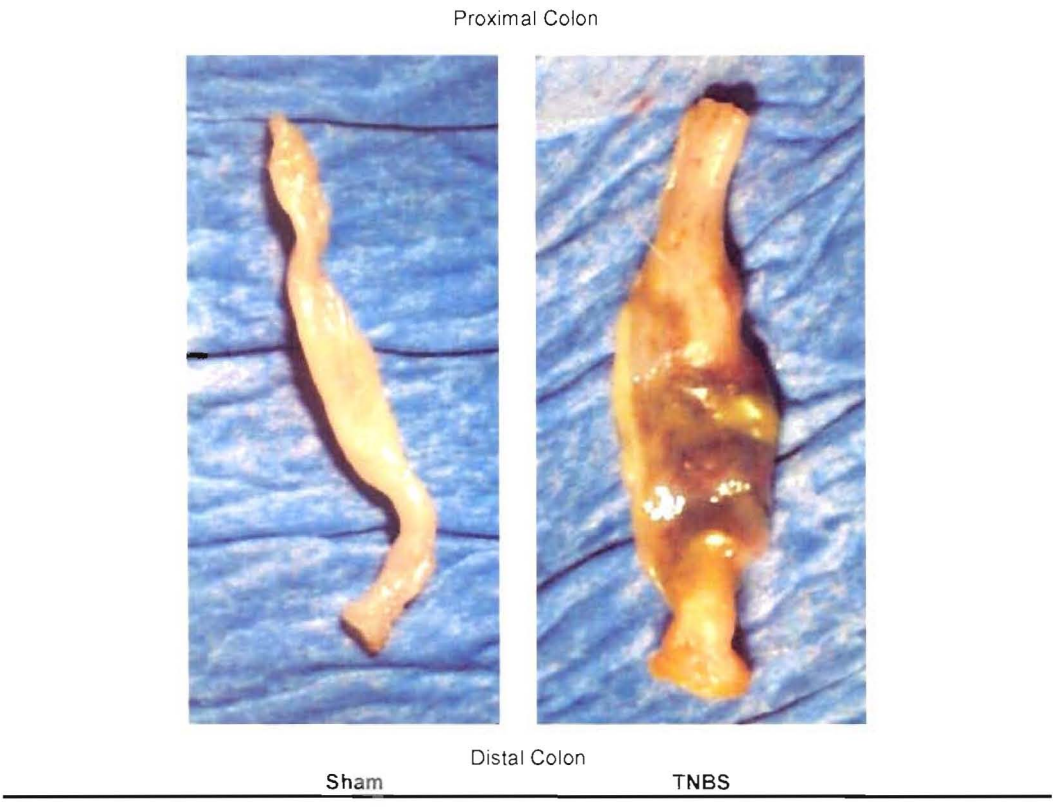
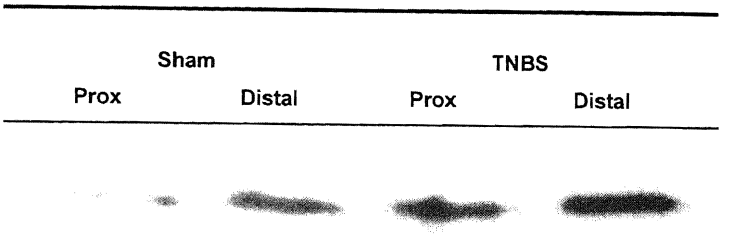


Figure 6-6 Sham (saline treated) operated and trinitrobenzene sulfonic acid (TNBS) treated rat large intestine. Rats were Sham or TNBS treated for 7 days before sacrifice. The large intestine was removed and photographed. Well-advanced inflammation is apparent in the colon of the TNBS rat model.

6.2.5 Histone acetylation in inflamed and non-inflamed regions of the colon in the rat TNBS model of colitis

Histone 4 acetylation was assessed by Western blotting in the Sham (non-treated) animal as well as in macroscopically inflamed and non-inflamed tissue of the TNBS rat model. Rats were Sham operated or TNBS treated seven days prior to sacrifice. The proximal (non-inflamed) and distal (inflamed) regions of the colon were removed and homogenised followed by histone isolation as described in chapter 2. Initially pan acetylation on histone 4 was investigated (Fig. 6-7).



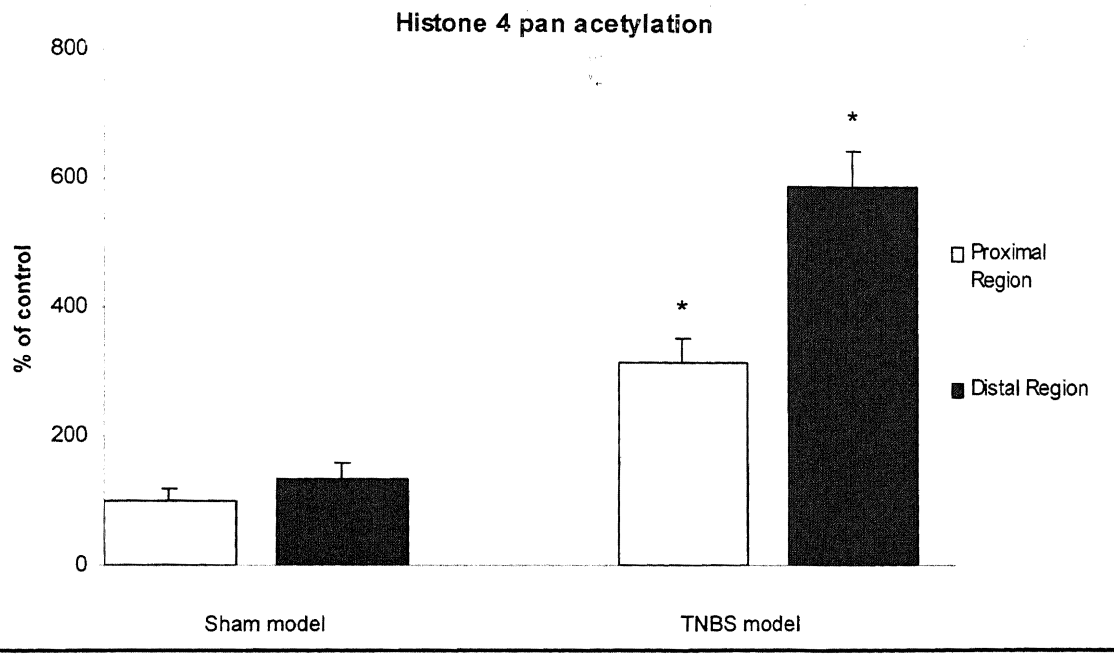


Figure 6-7 Pan acetylation on histone 4 (H4) in the trinitrobenzene sulfonic acid (TNBS) rat model of inflammation. The Sham model represents saline and therefore non-inflamed (control) treated mice. In the TNBS model, animals were treated with TNBS seven days prior to sacrifice. The proximal region of the TNBS model macroscopically showed no signs of inflammation while the distal region was severely inflamed. The results were obtained by Western blotting. In both regions of the TNBS model histone 4 was induced with the most significant elevation of acetylation on histone 4 revealed at the distal (inflamed) regions of the colon. A representative example of the bands obtained is shown on top of the graph. Columns represent the mean ± SEM of three independent experiments. (*$p<0.05$ vs Sham proximal or Sham distal respectively).

Figure 6-7 illustrates acetylation on H4 in the Sham and TNBS rat models. In the Sham treated animals, both proximal and distal regions showed low levels on pan histone 4 acetylation. Following TNBS treatment the proximal (non-inflamed) regions showed significant upregulation on pan histone 4 acetylation (315 ± 39% versus 125 ± 19% sham operated animals, $p<0.05$). Histone 4 acetylation of the distal regions of the colon (inflamed) of the TNBS model, were also significantly elevated five-fold compared to the Sham model (592 ± 54% versus 135 ± 24 Sham operated animals, $p<0.05$). The level of elevation was almost twice that compared to the proximal region of the TNBS treated animals.

Following confirmation that H4 is acetylated in the TNBS rat model of colitis, it was further investigated whether specific lysine residues of H4 are acetylated in the colon and whether acetylation on these specific lysine residues was associated with inflamed and non-inflamed regions in induced colitis. Figures 6-8.a and 6-8.b illustrate acetylation on specific histone 4 lysines in the Sham and the TNBS models. In the TNBS model acetylation on K5 was induced in the proximal (255 ± 39% versus 100 ± 15% Sham operated animals, $p<0.05$) region of the colon. The levels of acetylated K5 are higher to the non-inflamed (proximal) region compared to the inflamed region. In addition, acetylated levels of K8 were significantly induced in the TNBS model compared to Sham. In the proximal region of the TNBS model acetylation was significantly upregulated (546 ± 50% versus 100 ± 21% Sham operated animals, $p<0.05$). Acetylated K8 levels were also upregulated six-fold in the inflamed (distal) region (818 ± 111% versus 138 ± 19% sham operated animals, $p<0.05$).

The graphs illustrated in figure 6-8.b illustrate acetylation on histone 4 residues K12 and K16. The patterns on K12 and K16 acetylation were similar to K8 and K5 acetylation respectively. K12 acetylation was increased in the proximal region (533 ± 69% versus 100 ± 26% Sham operated animals, $p<0.05$) and the distal (741 ± 64% versus 121 ± 34% Sham operated animals, $p<0.05$) region of the colon compared to the same region in the Sham operated animals. K12 acetylation was higher in the inflamed region of the colon. K16 acetylation was very similar to that for K5. TNBS induced K16 acetylation significantly in the proximal (300 ± 63% versus 100 ± 29% Sham operated animals, $p<0.05$) colon, albeit to a reduced extent. In summary, TNBS induced the greatest increase in K8 and K12 acetylation in both the proximal and distal colon with highest levels seen in the distal (inflamed) colon. Smaller increases were seen with K5 and K16 acetylation with greater acetylation seen in the non-inflamed (proximal) colon.

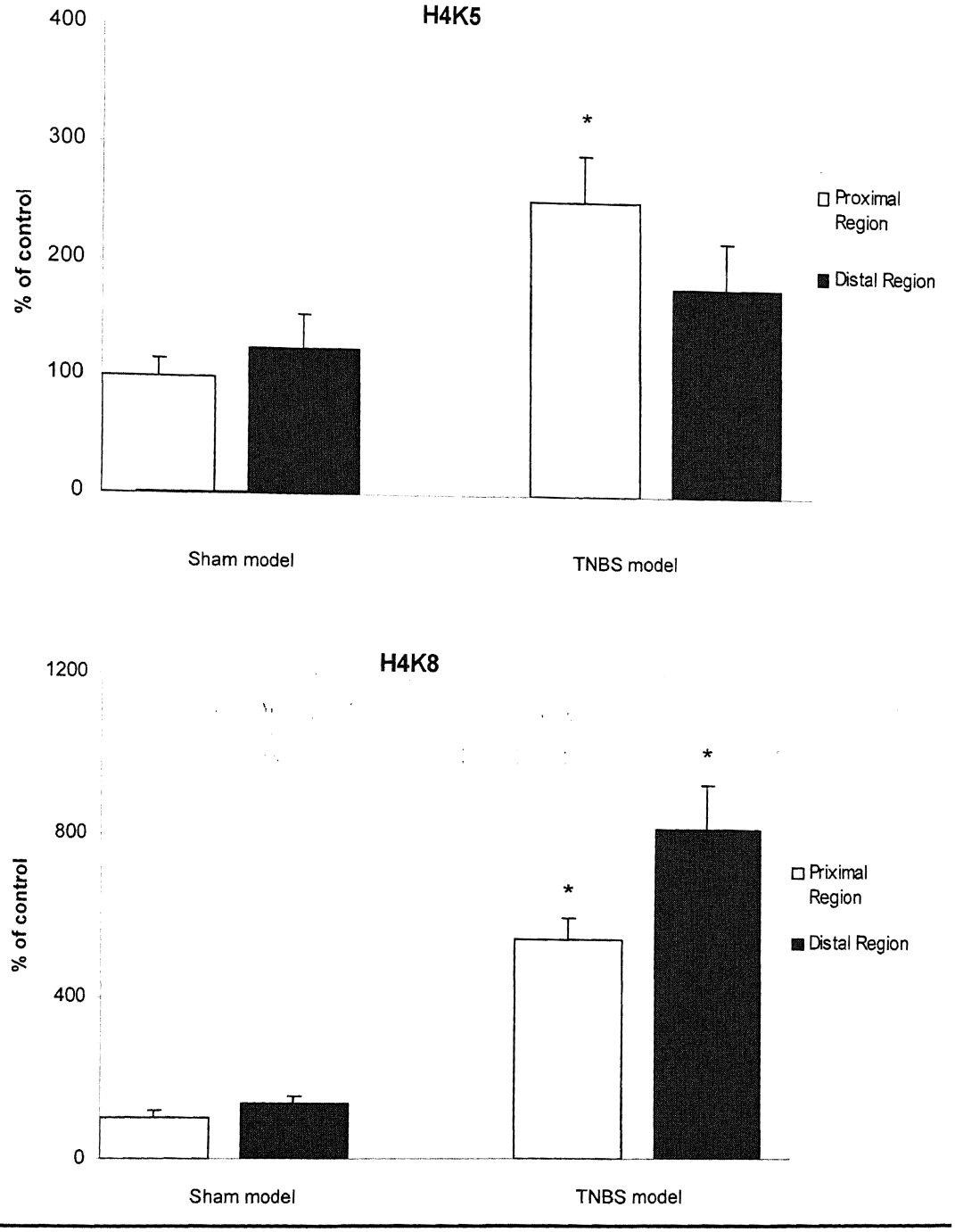


Figure 6-8.a Acetylation on histone 4 (H4) specific lysine residues 5 (K5) and 8 (K8) in Sham (control) and trinitrobenzene sulfonic acid (TNBS) rat model of colitis. The results were obtained by Western blotting. TNBS induced acetylation on both lysine 5 and 8 residues in the rat model. In the proximal (non-inflamed) and distal (inflamed) regions of the colon of the TNBS model acetylation on lysine 8 was significantly upregulated. Columns represent the mean ± SEM of three independent experiments. (*$p<0.05$ vs Sham proximal or Sham distal respectively).

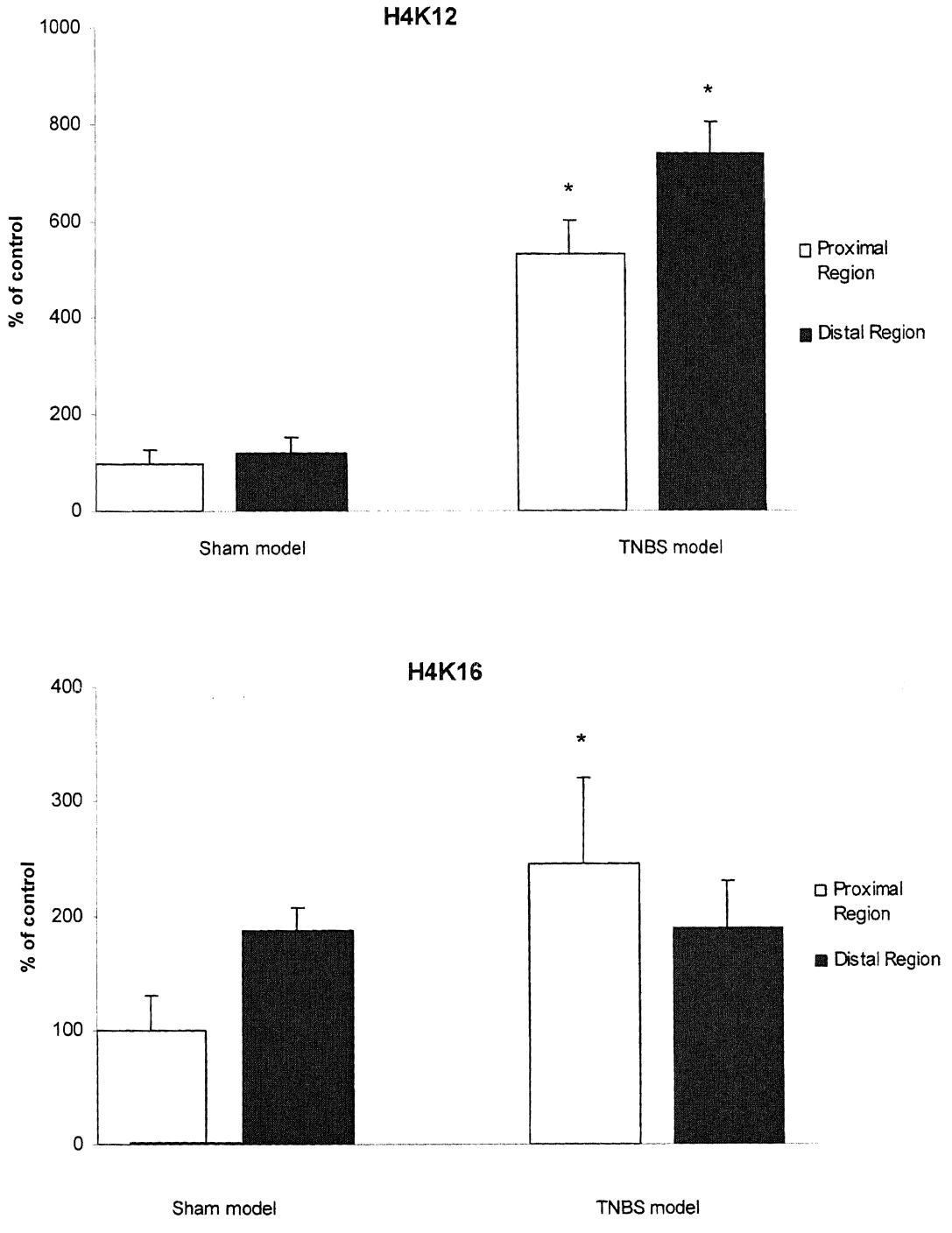
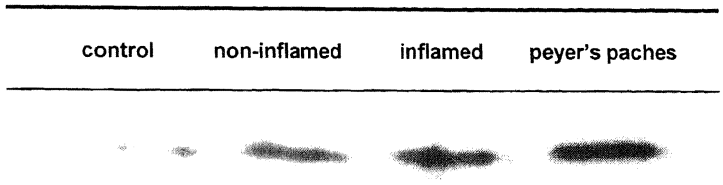


Figure 6-8.b Acetylation on histone 4 (H4) specific lysine residues 12 (K12) and 16 (K16) in Sham (control) and trinitrobenzene sulfonic acid (TNBS) rat model of colitis. The results were obtained by Western blotting. TNBS induced acetylation on both lysine 12 and 16 residues in the rat model. In the proximal (non-inflamed) and distal (inflamed) regions of the colon of the TNBS model acetylation on lysine 12 was significantly upregulated. Columns represent the mean ± SEM of three independent experiments. (*$p<0.05$ vs Sham proximal or Sham distal respectively).

6.2.6 Histone acetylation in inflammatory bowel disease

Histone 4 acetylation was finally assessed in twelve patients with Crohn's disease. The age range was 18-57 years old. Because no connection has been previously made as to the susceptibility of sex and Crohn's disease, no distinction was made between males and females, in grouping the samples. The biopsies all came from the ileum, or were isolated Peyer's patches and were grouped to inflamed and non-inflamed based on macroscopic examination by a surgeon. Control, non-inflamed biopsies from non-IBD patients who had a colonoscopy for other reasons (i.e. suspicion of cancer or other bowel diseases) were also examined. The biopsies collected at routine endoscopy were homogenised and histones were isolated as described in chapter 2.



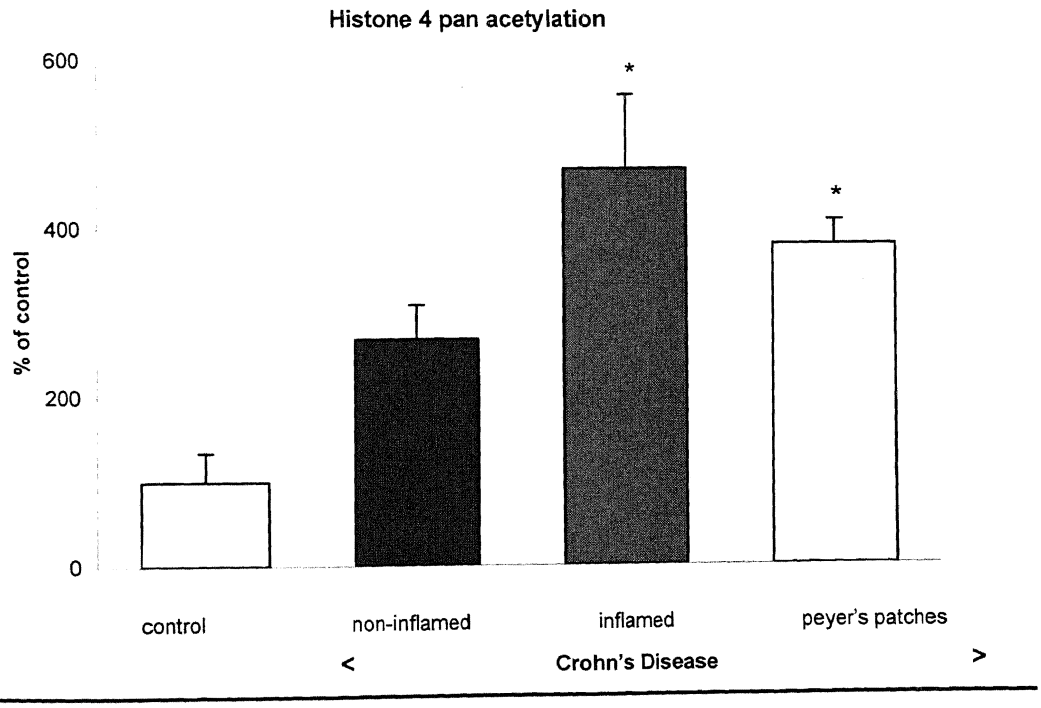


Figure 6-9 Pan acetylation on histone 4 (H4) in Crohn's disease. Acetylation on histone 4 was investigated by Western blotting. Histone 4 acetylation was slightly increased in the non-inflamed regions of Crohn's disease patients. In inflamed regions acetylation on histone 4 was significantly upregulated. Similarly in Peyer's patches of Crohn's disease patients histone 4 acetylation was upregulated. Columns represent the mean ± SEM of three independent experiments. A representative example of the bands obtained is shown on top of the graph. Four biopsies were pooled to obtain sufficient protein for one experiment. (*$p<0.05$ vs control).

In figure 6-9, pan acetylation on H4 in Crohn's disease is illustrated. Four biopsies were homogenised to collect enough protein for one sample, due to the very small size of the samples (approximately 0.2 g/biopsy). Acetylation on H4 was slightly induced in the non-inflamed ileum of Crohn's disease patients. In the inflamed regions, however, acetylation on H4 was significantly elevated. The Peyer's patches of Crohn's disease patients also showed a significant increase on H4 acetylation compared to the control non-inflamed, non-IBD tissue.

Figures 6-10.a and 6-10.b illustrate further investigation on H4 specific lysine residue acetylation in Crohn's disease. In figure 6-10.a levels of acetylated lysines 5 and 8 detected by Western blotting are shown. Levels of acetylated K5 were not significantly upregulated compared to the control.

Acetylation on K8 was not upregulated in the non-inflamed CD areas, however, acetylation on K8 was significantly induced compared to control samples in the inflamed regions (527 ± 44% versus 100 ± 25% control tissue, $p<0.05$) and the non-inflamed CD samples (527 ± 44% versus 195 ± 42% non-inflamed CD, $p<0.05$). In the Peyer's patches of CD patients, K8 was also significantly upregulated compared to the control (488 ± 52% versus 100 ± 25% control tissue, $p<0.05$) and the non-inflamed CD tissue (488 ± 52% versus 195 ± 42% non-inflamed CD tissue, $p<0.05$).

Enhanced acetylation on K12 was detected in inflamed regions of CD compared to control (442 ± 54% versus 100 ± 29% control tissue, $p<0.05$) and non-inflamed CD tissue (442 ± 54% versus 223 ± 38% non-inflamed IBD tissue, $p<0.05$). Similarly, enhanced acetylation on K12 was detected in Peyer's patches compared to control (429 ± 65% versus 100 ± 29% control tissue, $p<0.05$) and non-inflamed CD tissue (429 ± 65% versus 223 ± 38% non-inflamed IBD tissue, $p<0.05$). Acetylation on lysine 12 was not significantly increased in non-inflamed tissue compared to control.

Finally, investigation of lysine 16 acetylation showed no increase in the non-inflamed or inflamed tissue of Crohn's disease patients. In the Peyer's patches, however, a significant elevation of acetylation on K16 was observed.

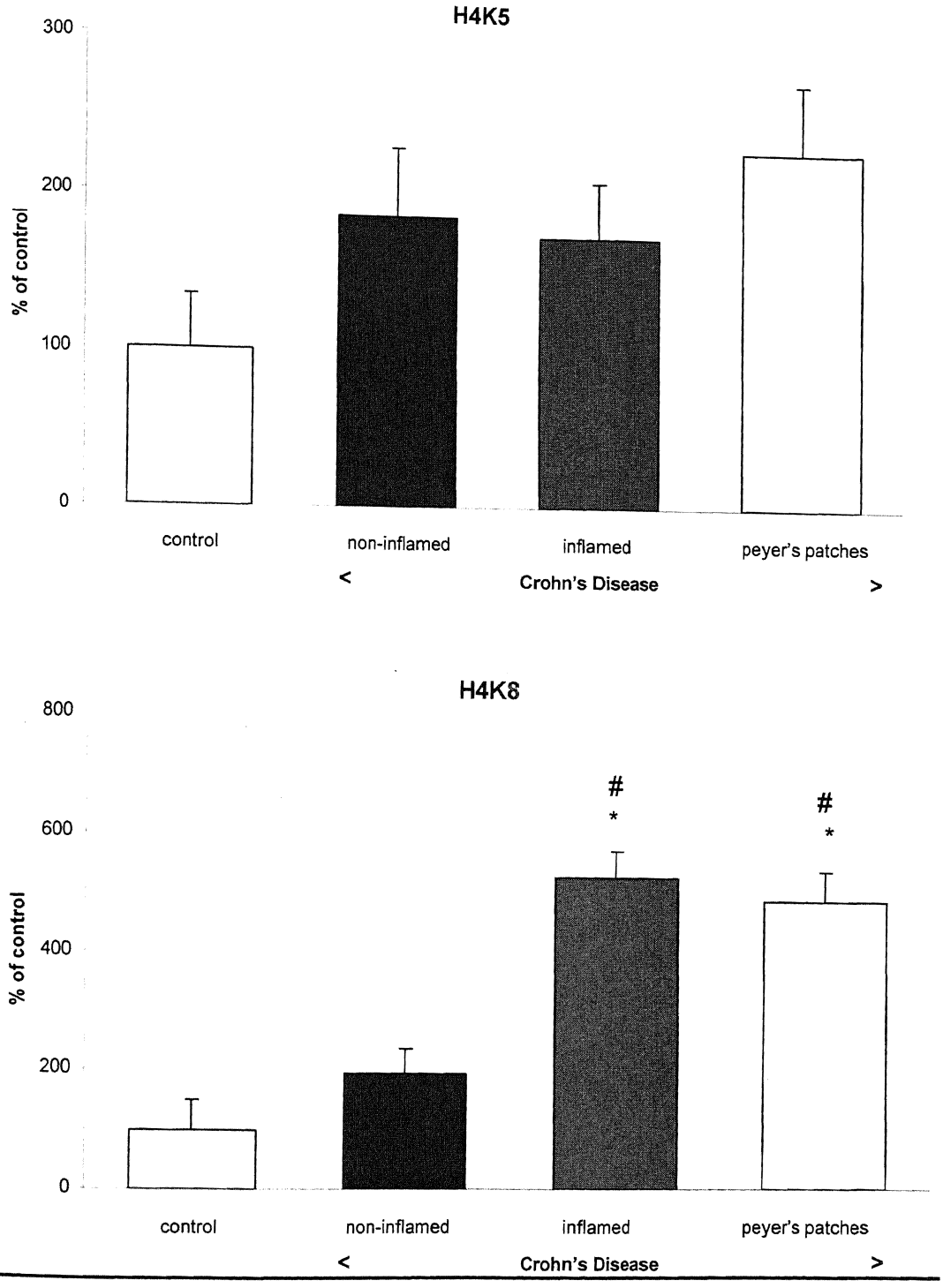


Figure 6-10.a Acetylation on histone 4 (H4) specific lysine residues 5 (K5) and 8 (K8) in non-inflamed, inflamed tissue and Peyer's patches of Crohn's disease patients. The results were obtained by Western blotting. Lysine 8 acetylation was significantly induced in the inflamed tissue as well as in the Peyer's patches of CD patients. Acetylation on K5 was not significantly increased in the non-inflamed nor the inflamed regions of CD patients. Columns represent the mean ± SEM of three independent experiments. (*$p<0.05$ vs control) (#$p<0.05$ vs non-inflamed CD).

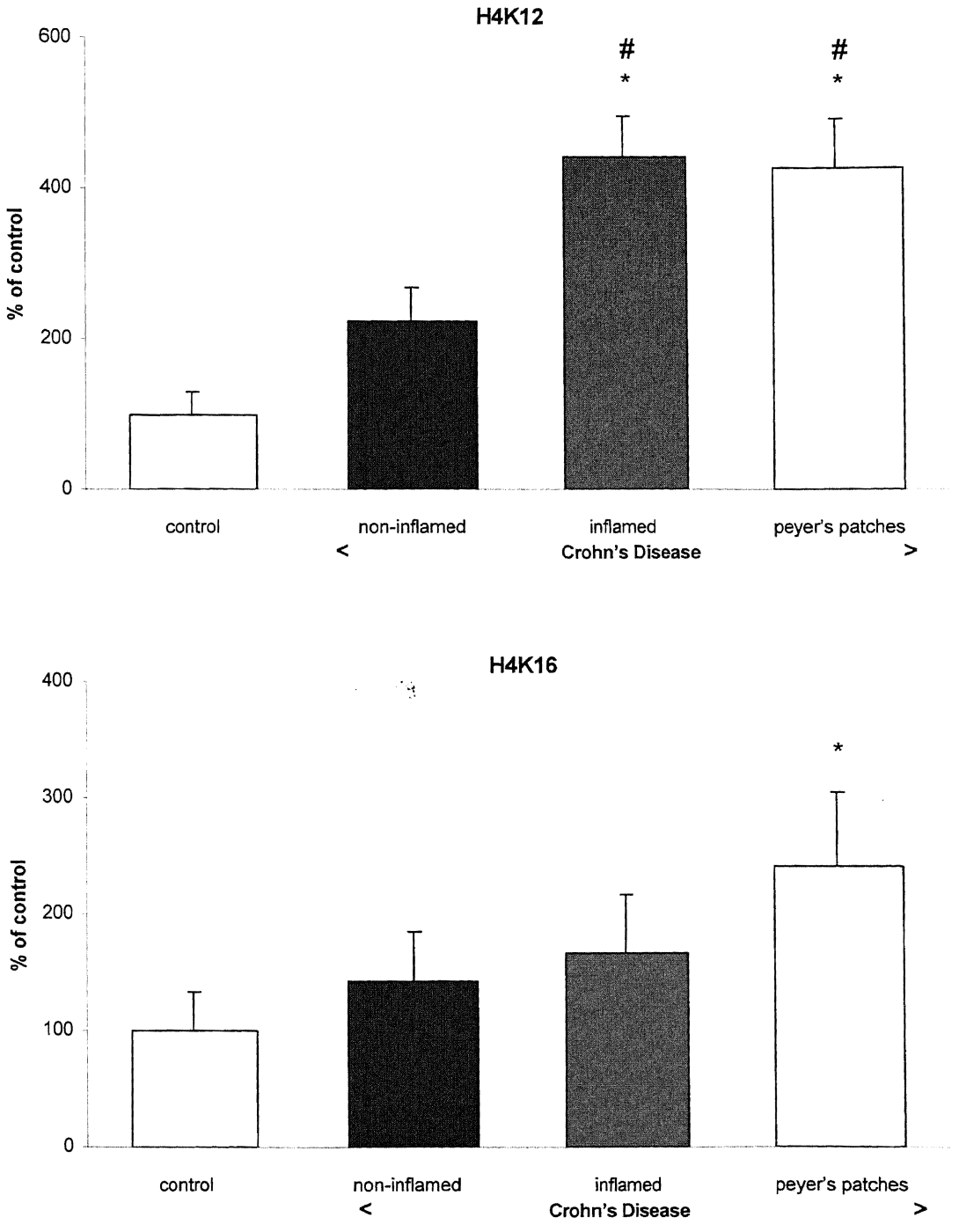


Figure 6-10.b Acetylation on histone 4 (H4) specific lysine residues 12 (K12) and 16 (K16) in non-inflamed, inflamed tissue and Peyer's patches of Crohn's disease patients. The results were obtained by Western blotting. Lysine 12 acetylation was significantly induced in the inflamed tissue as well as in the Peyer's patches of CD patients. Acetylation on lysine 16 was not significantly increased in the non-inflamed nor the inflamed regions, but it was noted to be slightly upregulated in the Peyer's patches of CD patients. Columns represent the mean ± SEM of three independent experiments. (*$p<0.05$ vs control) (#$p<0.05$ vs non-inflamed CD).

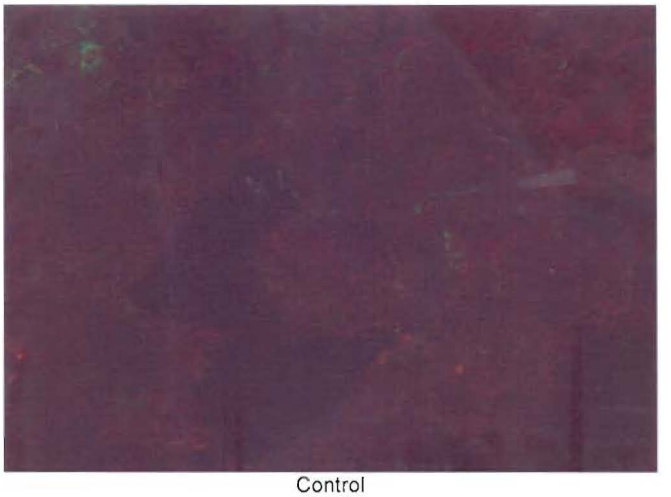
6.2.7 Localisation of H4 acetylation and associated lysine residues in Crohn's disease

The investigation on the activation of histone acetylation in Crohn's disease was concluded by localising immunohistochemically H4 and its specific lysine residues acetylation in illeal biopsies and Peyer's patches of Crohn's disease patients.

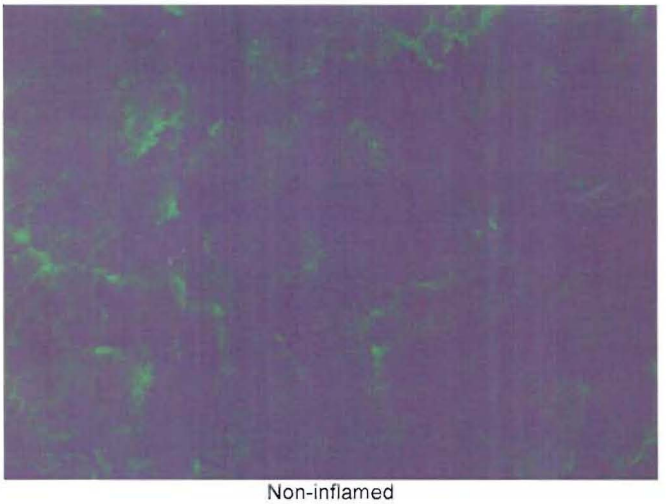
First, control non-inflamed non-IBD tissue was compared to non-inflamed and inflamed Crohn's disease tissue by staining against a pan-histone 4 antibody. Figure 6-11 illustrates the representative micrographs obtained from three individual experiments. Histone 4 was not demonstrably pan-acetylated in the control tissue by this technique, and only weak non-specific staining could be visualised. In the non-inflamed CD tissue however, positive cells containing pan-acetylated H4 could be localised. In the inflamed tissue, the very strong staining indicated activity of pan-acetylated H4. Similarly, in the control Peyer's patches H4 pan-acetylation could not be localised as shown in figure 6-12. In the Peyer's patches of Crohn's disease patients, however, H4 was pan-acetylated, localised throughout their surface as evidenced by very strongly stained cells.

Acetylation on the specific H4 lysine residues in the illeal biopsies of Crohn's disease patients was also investigated immunohistochemically (Fig. 6-13). None of the lysine residues was highly acetylated in the non-inflamed, non-IBD biopsies. Immunohistochemical staining against the H4 lysine residues revealed that specific lysines were acetylated differently in the non-inflamed and inflamed tissue. Acetylation on H4 K5 residue produced a very weak staining in both non-inflamed and inflamed tissue sections. K8 was acetylated in both inflamed and non-inflamed biopsies but the signal was much stronger in the inflamed tissue. In contrast to K8 acetylation, acetylated K12 showed strong nuclear staining in both the non-inflamed and inflamed tissue. Acetylation on K16 was very weakly localised in both non-inflamed and inflamed biopsies of the Crohn's disease patients.

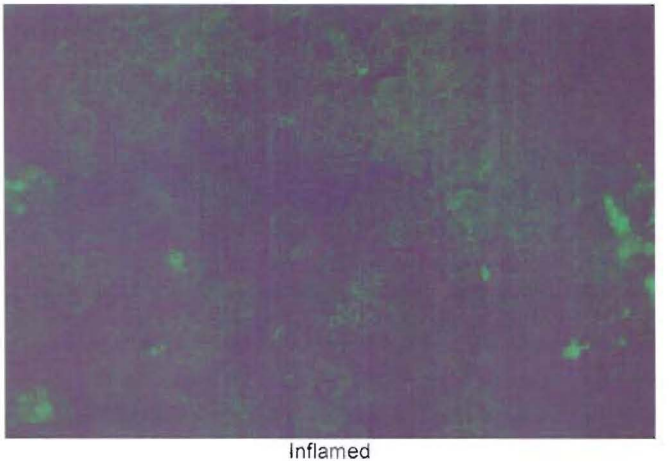
Finally acetylation on the specific H4 lysine residues was investigated in the Peyer's patches of Crohn's disease patients, shown in figure 6-14. The pattern of acetylation on lysine residues was very similar to that observed in the Peyer's patches of the animal models shown previously. K5 and K16 appeared to be acetylated at the periphery of the Peyer's patches within the mantle zone. Acetylation on K8 and K16 could be localised not only at the periphery but also in the germinal centre of the Peyer's patches and the nuclear staining produced was very strong. Due to the very similar patterns formed by the two groups of lysine residues (K5 and K16, K8 and K12) one set of micrographs is only shown in figure 6-14 as representative of the results obtained.



Control

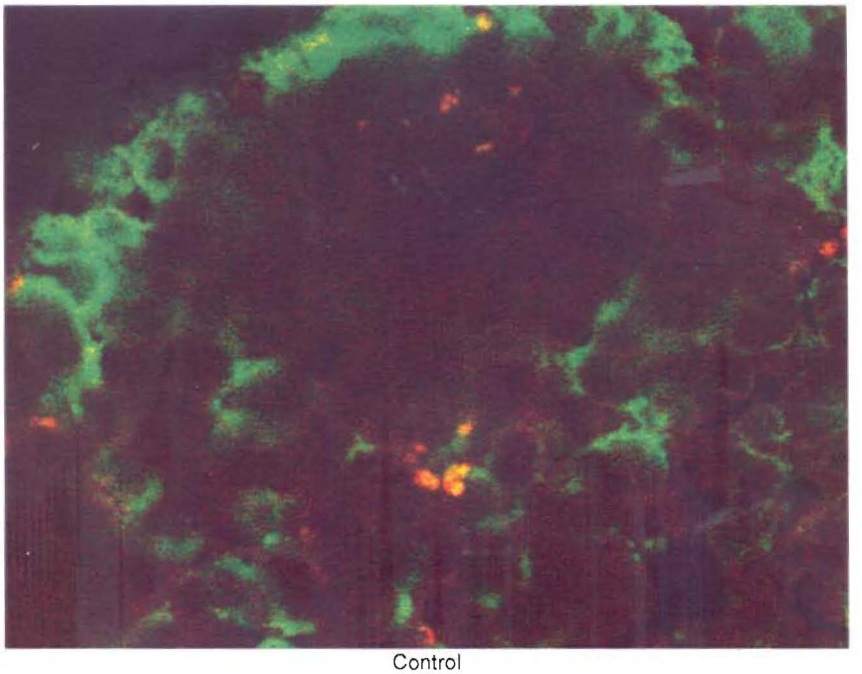


Non-inflamed

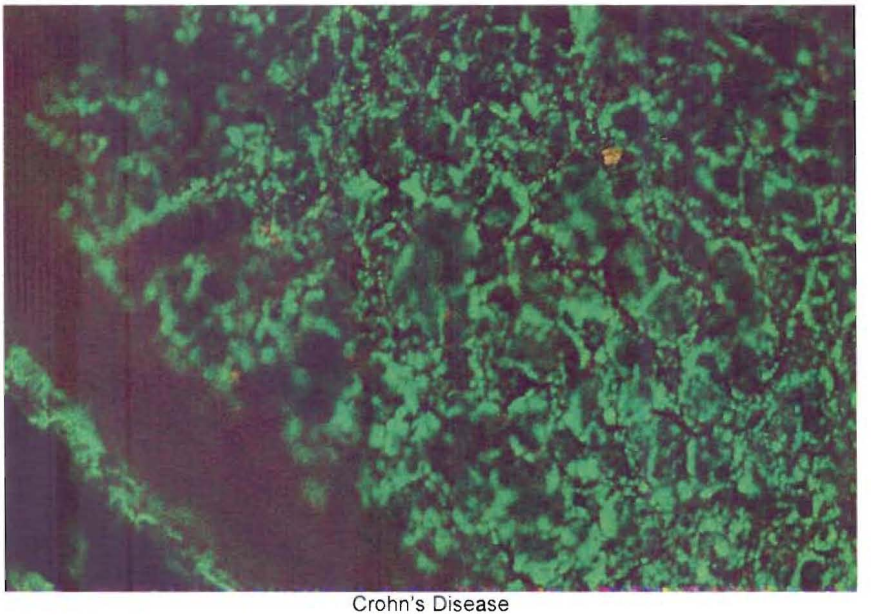


Inflamed

Figure 6-11 Immunocytochemical analysis for acetylation on histone 4 (H4) in inflamed and non-inflamed biopsies of Crohn's disease patients. Histone 4 was not acetylated in the control (non-IBD). Acetylated histone 4 could be localised in the non-inflamed tissue. Activation on histone 4 acetylation was higher in the inflamed biopsies. Counterstaining with DAPI showed that staining was nuclear and not cytoplasmic. Micrographs are representative of 3 independent experiments.



Control



Crohn's Disease

Figure 6-12 Immunocytochemical analysis for acetylation on histone 4 (H4) in the Peyer's patches of Crohn's disease patients. Histone 4 was not acetylated in the control (non-IBD). Increased activation on acetylated histone 4 could be localised in the Peyer's patches of Crohn's disease patients. Counterstaining with DAPI showed that staining was nuclear and not cytoplasmic. Micrographs are representative of 3 independent experiments.

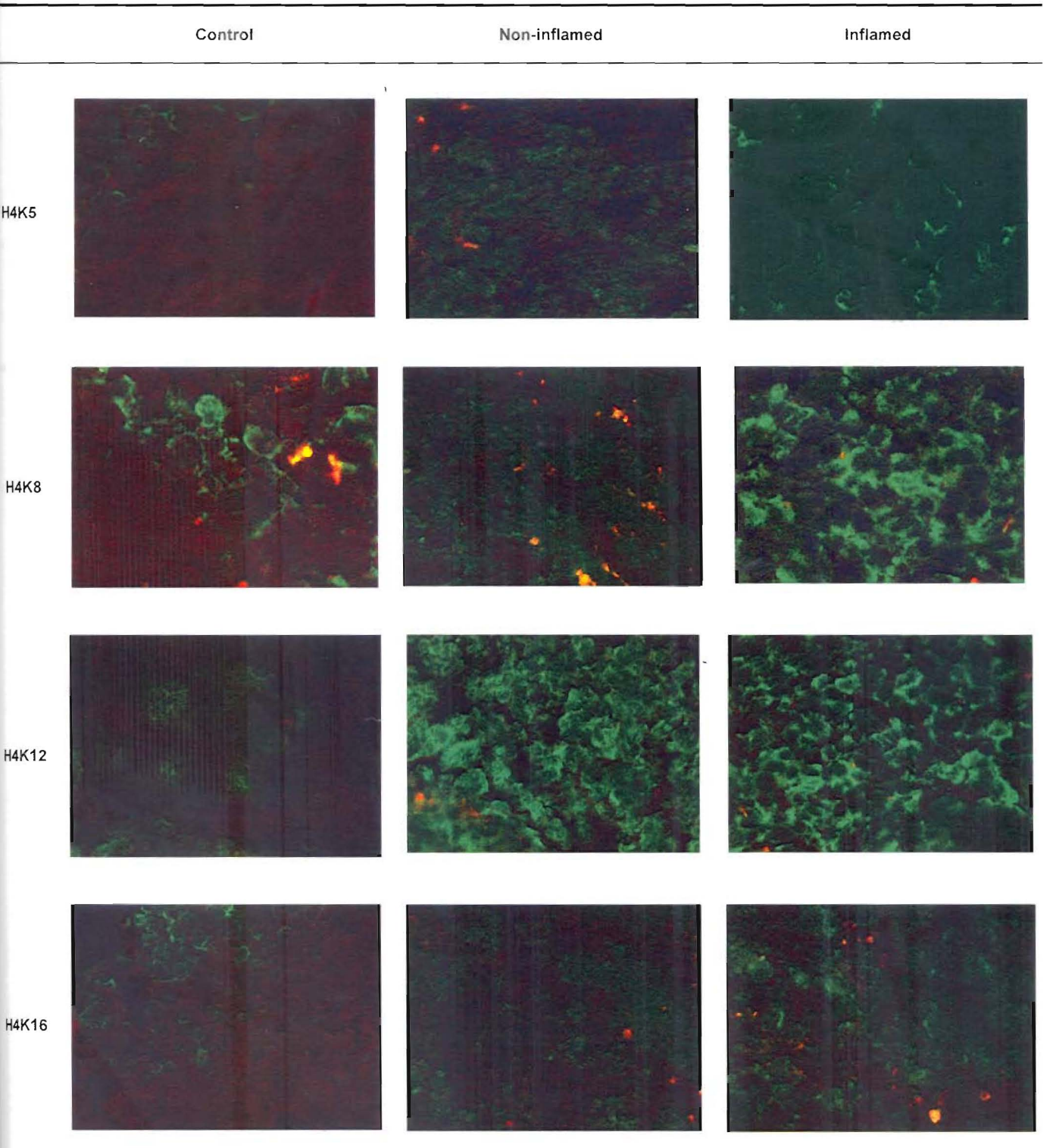
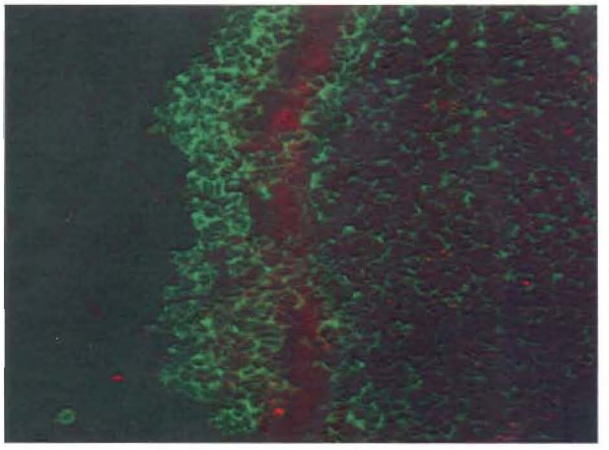
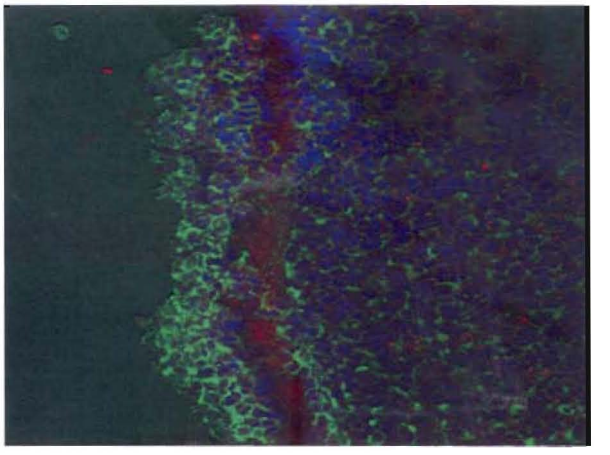


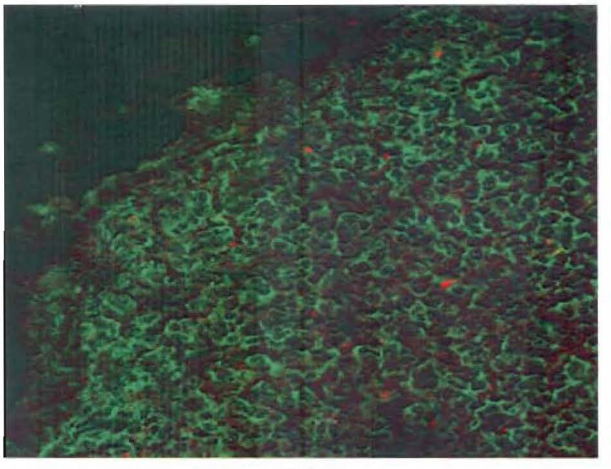
Figure 6-13 Immunocytochemical analysis for acetylation on histone 4 (H4) specific lysine residues in the illeal biopsies of Crohn's disease patients. Increased activation on acetylated histone 4 lysine residues 8 (K8) and 12 (K12) is observed in the inflamed tissue of Crohn's disease patients. K12 was also highly acetylated in the non-inflamed mucosa of CD patients. Counterstaining with DAPI showed that staining was nuclear and not cytoplasmic. Micrographs are representative of 3 independent experiments.



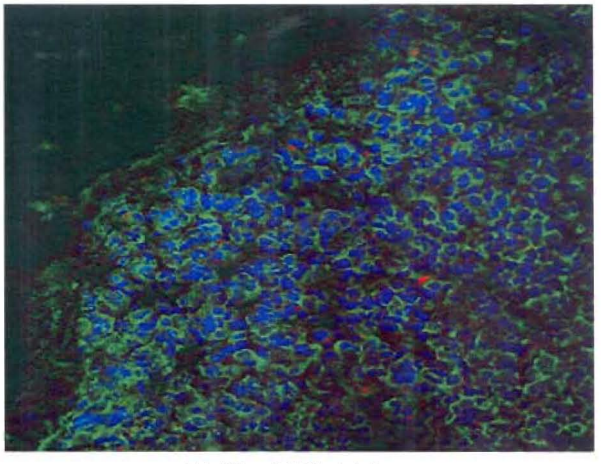
H4 K5



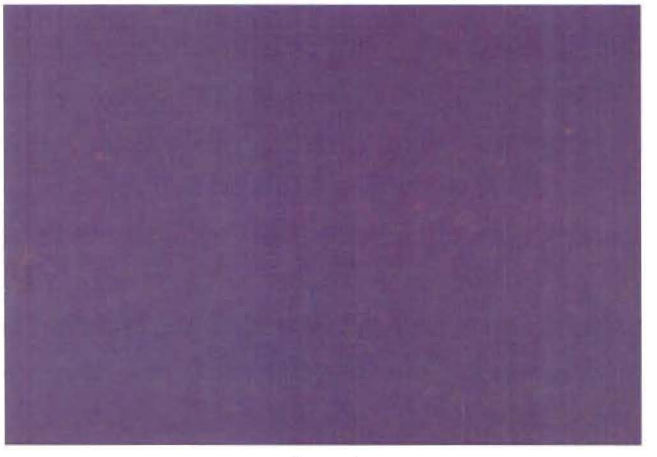
H4 K5 + DAPI staining



H4 K8



H4 K8 + DAPI staining



Control

Figure 6-14 Immunocytochemical analysis for acetylation on histone 4 (H4) lysine residues 5 (K5) and 8 (K8) in Peyer's patches biopsies of Crohn's disease patients. H4 K5 (and K16 not shown) was only acetylated in the mantle zone of the Peyer's patches. Acetylated K8 (and K12 not shown) was localised throughout Peyer's patches. Acetylated lysines were not localised specifically in control Peyer's patches. Due to the similarities of acetylation patterns on K5 and K16 and K8 and K12 micrographs from acetylation on K16 and K12 are not shown. DAPI nuclear staining is also shown for comparison. Micrographs are representative of 3 independent experiments.

6.3 Discussion

In this chapter, the possible involvement of histone acetylation in inflammatory bowel disease *in vivo* was investigated. Initially, widespread use of sodium butyrate, a histone deacetylase inhibitor in the treatment of IBD, lead to the hypothesis that its histone deacetylation inhibitory activity (and not only its anti-proliferative action) could be associated with its therapeutic effects. However due to the pleiotropic effects of butyrate its therapeutic role may be attributed to properties distinct from its HDAC inhibitory actions. This theory is supported by a study in human umbilical vein endothelial cells (HUVEC), showing induction of tissue-type plasminogen activator (t-PA) transcription by butyrate and Trichostatin A was found to be preceded by histone 4 acetylation (Arts *et al.,* 1995). Additionally, recent findings report that histone 1 bears a recurring COOH-terminal epitope recognised by monoclonal Ulcerative colitis-associated perinuclear anti-neutrophil cytoplasmic (pANCA) marker antibodies (Eggena *et al.,* 2000).

The diversity of IBD and the difficulty in successfully distinguishing between Ulcerative colitis and Crohn's disease formed the criteria of employing two different animal models for studying histone acetylation, namely DSS and TNBS associated with Ulcerative colitis and Crohn's disease respectively. It is also known that naive T cells differentiate into effector cells upon stimulation with antigen, a process that is accompanied by changes in the chromatin structure of effector cytokine genes. It has been reported that TCR, in the presence of polarizing cytokines, established a selective pattern of histone acetylation on IL-4 and IFN-γ cytokine genes correlating with exclusive gene expression by the differentiated T cells (Avni *et al.,* 2002). The two models, as discussed before, show different T_H profiles (the DSS model involves T_H2 type inflammation while the TNBS model involves T_H1 type inflammation) and these types of studies could help to clarify whether histone acetylation is T_H dependent *in vivo* as well. In the DSS model, the acetylation activity on histones 4 and 3 was initially measured. Findings indicated that histone acetylation was upregulated in the two investigated rat strains (Lewis and Sprague-Dawley). Comparison of acetylated levels between histones 3 and 4 revealed that while both were acetylated, the latter reached significantly higher acetylation levels. Similarly, in the Peyer's patches of the DSS model, histone 4 acetylation was higher than histone 3. Localisation of histones in the Peyer's patches immunohistochemically revealed a pattern formation, which was noticeable in both Lewis and Sprague-Dawley strains. Histone types were acetylated forming a specific pattern. Acetylation on H3 was only detected in the mantle zone of the Peyer's patches, whilst acetylated H4 occurred in both the periphery and the germinal centre of the Peyer's patches. Therefore, it was concluded that acetylation on H3 could possibly be cell specific, whereas H4 is generally induced in all cell types present in the Peyer's patches (T-cells, B-cells, dendritic cells and macrophages). These results indicated a uniform mechanism of H4 acetylation in the regulation of inflammation in IBD. Paradoxically, HDAC inhibitors are used in the treatment of IBD. This may reflect either an anti-proliferative

effect seen with high, non-specific doses of HDAC inhibitors or an effect on the acetylation status of non-histone proteins e.g. tubulin and transcription factors such as NFκB and GATA (Adcock and Caramori, 2001 and references therein).

Previous findings described in Chapter 4, have suggested that H4 residues K8 and K12 are associated with the regulation of inflammatory gene expression. Conformation of the activation of H4 in the DSS model required further investigation as to whether histone acetylation is, as *in vitro*, *in vivo* lysine residue dependent. In the experimental models of colitis, H4 K8 and K12 were highly acetylated in the Sprague-Dawley rats. These findings were in agreement with previous results documented in this report *in vitro,* and with other reports that pro-inflammatory cytokines can cause acetylation on H4 lysines 8 and 12 (Ito *et al.,* 2000). Interestingly, in the Lewis rats, K5, K8 and K16 reached very similar levels. In contrast with results in the Sprague-Dawley strain, only K12 acetylation was strongly induced. In DSS treated Lewis rats, dose-dependent metronidazole (MTD) treatment resulted in the downregulation on all lysine residue levels. The same treatment in the Sprague-Dawley rats had equivalent effects on K8 and K12 acetylation, but upregulated the acetylation on K5 and K16. This could be attributed to the association of these lysine residues with cell proliferation and cell death (Zhu *et al.,* 2001). One report on the effect of the HDAC inhibitor, Trichostatin A (TSA) on hepatic stellate cells showed that TSA (inducing histone hyperacetylation) retarded the morphological changes, characteristic for activation of primary stellate cells, and that the proliferation rate of those cells was strongly suppressed (Rombouts *et al.,* 2001). These differences could be further attributed to genetic variances between the two rat strains, also discussed by other groups (Quary, *et al.,* 2000; Jurado *et al.,* 1999).

The combined effect of DSS and MTD in the Sprague-Dawley rat strain, did not lead to further elevation of acetylated lysine levels. These findings are in agreement with a report on the role of masalazine, another anti-inflammatory drug used widely in the treatment of Crohn's disease. The authors reported that levels of anti-histone antibodies, in a patient with lupus induced by mesalazine therapy (administered for over a year for Crohn's disease treatment), were considerably high and that discontinuation of mesalazine was followed by a rapid reduction of the anti-histone antibodies to almost undetectable levels (Timsit *et al.,* 1997).

Pro-inflammatory cytokines are key factors in the pathogenesis of Crohn's disease (CD). Activation of nuclear factor kappa B (NFκB), which is involved in pro-inflammatory cytokine gene transcription, is increased in the intestinal mucosa of CD patients (Schreiber, 2000). As discussed in Chapter 4, it has been reported that modulation of histone acetylation is involved in transcriptional regulation, associated with the NFκB pathway (Beato & Eisfeld, 1997; Ito *et al.,* 2000; Ashburner *et al.,* 2001). Previous work within the group (Jones *et al.,* 2002; Ellis *et al.,* 1998), suggest the existence of abnormalities in NFκB activity in the non-inflamed intestinal tissue of CD patients. This was attributed to the possibility that an inflammatory

state in one section of the intestine is able to trigger changes in the NFκB signaling pathway in another macroscopically non-inflamed section. Recent evidence revealed that butyrate decreases pro-inflammatory cytokine expression via inhibition of NFκB activation and IκBα degradation (Segain *et al.*, 2000) while it has also been demonstrated that NFκB induction of inflammatory gene expression is associated with histone acetylation (Ito *et al.*, 2000, Ashburner *et al*, 2001) and indeed with p65 acetylation (Chen *et al.*, 2002).

Therefore, experiments were carried out in to investigate whether acetylated histone 4 activity was altered in inflamed and non-inflamed tissue of a TNBS model of colitis. Pan acetylation on histone 4 was increased in lower levels in the non-inflamed mucosa and was significantly elevated in inflamed tissue. Results further revealed a general upregulation on all H4 lysine residues in the proximal (non-inflamed) and distal regions (inflamed) of TNBS treated animals. In both regions of the TNBS treated bowel, acetylation on K8 and K12 was significantly increased, specifically in the distal region where acetylated levels were also significantly higher compared to the proximal. This specificity on lysine acetylation could be explained by the reported NFκB selectivity of DNA binding in response to a large variety of stimuli leading to its activation. As a result, genes with promoters or enhancers, containing variant κB elements, have the potential to be regulated by specific NFκB complexes (Perkins, 1997). Therefore NFκB DNA binding does not necessarily correlate with transcriptional activation (Perkins, 1997). Although tempting to suggest a cause-and-effect model it is unclear whether increased inflammation leads directly to increased histone acetylation *in vivo* at specific gene promoters. Further studies will be needed to address this. Preliminary evidence suggests that this may be the case for the GM-CSF promoter in alveolar macrophages from smokers (Ito *et al.*, 2001).

The studies on the role of H4 acetylation in IBD were completed, by measuring its activity in Crohn's disease patient biopsies. As with the TNBS model, non-inflamed and inflamed biopsies were assessed as well as Peyer's patches. In preliminary experiments, it was observed that in the inflamed biopsies, levels of acetylated H4 were the most prominent, followed by those in Peyer's patches. Acetylation was also detectable in the non-inflamed mucosa of the Crohn's disease patients. The results for acetylation on H4 lysines in Crohn's disease were very similar to those obtained in the TNBS treated animals. K5 and K16 were only slightly activated in all samples, with the inflamed and non-inflamed samples presenting no significant difference in acetylation. Peyer's patches showed the highest levels of K5 and K16 acetylation. Finally, Western blotting indicated that in biopsies of inflamed bowel and in Peyer's patches of Crohn's disease patients, K8 and K12 were both significantly acetylated. Acetylation on lysine residues in the non-inflamed biopsies was only slightly upregulated. Interestingly, the immunohistochemical studies in Peyer's patches, revealed acetylation on K5 and K16 in the mantle zone with a similar pattern to histone 3 acetylation. In contrast, acetylation on K8 and K16 was localized in both mantle zone and germinal center. These

latter results suggested that although pan acetylation on H4 in the Peyer's patches is probably not cell specific, it is possible that acetylation of its specific lysine residues is cell type dependent. They could also explain the significant increase of lysine 8 and 12 acetylation revealed by Western blotting as immunocytochemistry showed acetylation of these lysine residues in abundance throughout the surface of the Peyer's patches. Cell-dependent acetylation on histone lysine residues has not been suggested before. However, it is possible that these results may, at least in part, be explained by a recent study, which stated that the regulatory T-cells involved in active inflammatory suppression are primarily induced in the Peyer's patches (Tsuji *et al.*, 2001).

Studying histone acetylation in inflammatory bowel diseases concluded this investigation in the role of histone acetylation in inflammatory processes. The results presented in this chapter are indicative of the importance of histone 4 acetylation in the expression of inflammatory genes in inflammatory diseases, such as IBD. Albeit, an increase of the n numbers in the animal model and subjects used, would be appropriate to confirm these findings and an examination of the effects of various deacetylase inhibitors *in vivo* would be suitable. In general the present preliminary studies aim to provide further understanding in the role that histone acetylation plays in the regulation of inflammation. It is, therefore, possible to speculate that further understanding of the role of histone modifications in IBD may lead to new therapeutic strategies in the treatment of IBD and explain the therapeutic utility of current treatment regimes.

Chapter 7

Conclusions and Further Work

7.1 Concluding remarks & further work

Current research in the field of inflammation and inflammatory responses has focused in certain cell models, and the cytokines produced by these cells following inflammatory stimuli. However, the mechanisms underlying the transcriptional control of inflammatory gene expression, mechanisms of fundamental importance to biomedical research, are still not well understood. One of the most exciting advances has been the identification that chromatin remodeling is linked with transcriptional regulation. More specifically, levels of histone post translational modifications are associated with transcriptionally active/inactive chromatin. Early stages of the present research revealed increased histone acetylation, in various cell types (epithelial cells [A549 cells] and macrophages [U937 cell]), stimulated with inflammatory mediators. This suggested that ongoing gene transcription occurs during the early inflammatory responses of the cells. Cause and effect for the role of histone modification in inflammatory gene transcription is yet to be conclusively established. Nevertheless, it is now clear that modified histones have an essential function in establishing and maintaining stable states of gene activity. In this thesis, an explanation on the mechanism by which the chromatin remodeling machinery regulates inflammatory gene expression is attempted.

A number of cytokines play a significant role in the development of an acute or chronic inflammatory response. One of the best characterized cytokines is interleukin-8 (IL-8) produced by a variety of cells including monocytes/macrophages, neutrophils and endothelial cells. This investigation was initiated by monitoring the effects of pro-inflammatory stimuli LPS and TNF-α on the production of IL-8 in a macrophage cell line (U937 cells) as well as in two T-cell lines (Jurkat and HUT-78 cells) as a marker of NF-κB-directed inflammatory gene expression. LPS and TNF-α caused an increase in IL-8 expression. The histone deacetylase inhibitor Trichostatin A (TSA) alone, produced a concentration-dependent increase in IL-8 release. In addition, LPS and TNF-α -stimulated release of IL-8 was further enhanced by TSA, suggesting a role for histone acetylation in IL-8 production in these cells.

It has well been established that glucocorticoids downregulate the transcription of proinflammatory genes (van de Stolpe *et al.*, 1993) but the mechanism by which glucocorticoids preclude activation has been a matter of debate. Here, the synthetic glucocorticoids, dexamethasome (Dex) and triamcinolone acetonide (TA), repressed LPS and TNF-α -induced IL-8 expression in U937, HUT-78 and Jurkat cells. TA was significantly more potent than Dex in all cell lines studied. In addition, the effect of Dex was significantly less in Jurkat cells compared to HUT-78 cells. TSA attenuated the suppressive action of both Dex and TA on LPS- and TNF-α-induced IL-8 production, in all three cell lines. This variation in the

response of the two T-cell lines to Dex (and the slight difference in their response to TA) might reflect altered GR expression present in the two cell lines or a difference in the regulation of IL-8 in the two cell lines. To clarify these results, further studies are required to expand on other cell lines and in primary cells. Contrary to a previous report (Nissen and Yamamoto, 2000), where it was shown that in the transformed A549 lung epithelial cell line, GR repression of NF-κB activity is resistant to the effects of a single concentration of TSA, here it is showed that in U937, Jurkat and HUT-78 cells, TSA upregulated the production of LPS and TNF-α -induced IL-8 both in the presence and absence of glucocorticoids. In the non-transformed lung epithelial cell line A549 similar results have been reported for GM-CSF (Ito *et al.*, 2000). The present data suggest that different cells and genes are selectively responsive to various concentrations of TSA and that this might explain the reported discrepancies. Stimulus-dependent effects on gene expression may also be important in this respect. These contradictory findings also enhance the theory that pleiotropic transcriptional regulators such as GR are likely to exploit a diversity of mechanisms across different cellular and promoter contexts. The use of the histone deacetylase inhibitor shows that the actions of glucocorticoids may result from a functional reversal of the ability of CBP-associated HATs to regulate histone acetylation (Sheppard *et al.*, 1998) rather than a direct inhibition of CBP activity per se although these are not mutually exclusive.

The data presented in Chapter 3 indicate that LPS and TNF-α -induction of IL-8 requires histone modifications, and that this could be attenuated in the presence of glucocorticoids. .In addition, a direct effect of GR on inhibiting TNF-α and LPS –mediated histone acetylation at the IL-8 promoter is shown. In agreement with these results, a recent paper using chromatin immunoprecipitation showed that TSA rapidly induced H4 acetylation at the IL-8 promoter (Hoshimoto *et al.*, 2002). Interestingly, the present results also showed a decrease in acetylated histones within the IL-8 promoter when cells were stimulated in the presence of the glucocorticoids, with no apparent differences were detected between cell lines. HDAC1, HDAC2 and HDAC3 have been reported to participate in p-65 modulated activating complexes but it is possible that other HDACs may be activated (Zhong *et al.*, 2002; Ito *et al.*, 2002; Peterson, 2002, Ashburner *et al.*, 2001). The use of specific HDAC inhibitors or interference RNA could help to clarify which HDAC(s) are involved in the transcriptional regulation of cytokine release and whether their effect is cytokine or GR specific.

Attempting to determine the involvement of histone acetylation in the inflammatory gene expression and the mechanism through which the glucocorticoid receptor and chromatin activation interact, it was firstly required to determine whether a histone deacetylase inhibitor (TSA) would influence the release of inflammatory stimuli and glucocorticoid actions *in vitro*. The blockade of glucocorticoid (Dex and TA) effects by TSA in macrophages as well as T-lymphocytes, indicated a role of histone deacetylases in inflammatory gene expression in these cells. Having established this role, the work presented in Chapter 4 demonstrates a

direct link between histone acetylation and inflammatory regulation. In the U937 cells, as well as in the T-cell lines Jurkat and HUT-78, both LPS and TNF-α induced histone acetylation. Acetylation of histones (H) 2A, 2B and 3 increased by a small factor. Histone 4 acetylation was significantly upregulated (seven-fold increase in the monocytic cell line and six-fold increase in the T-cell lines), suggesting a role of H4 in inflammatory transcriptional regulation, while the rest of the core histones did not seem to be as important. Further investigation into the acetylation of specific H4 lysine residues showed that lysines (K) 8 and 12 were significantly upregulated in all three cell lines suggesting a common pathway occurring in the inflammatory gene expression process. LPS and TNF-α did not show any significant differences in the upregulated levels of histone acetylation in any of the cell lines in question. Ito et al. (2000) reported an upregulation of the same lysine residues in A549 cells. The same paper showed that p65 mediated activation of the GM-CSM promoter *in vitro* is concomitant with the acetylation of histone H4 K8 and K12 residues.

The effect of TSA in the activation of H4 lysines revealed that the actions of TSA were not lysine residue specific. It was however shown that TSA significantly upregulated acetylation of all H4 lysine residues in both monocytes and T-cells. Results presented in this chapter showed a K5 and K16 specificity for glucocorticoids alone in all three cell lines. The effect of the glucocorticoids on pro-inflammatory stimuli LPS and TNF-α induced cells was again lysine residue specific, but the pattern revealed was different. Both Dex and TA decreased acetylation of the lysine residues (K8 and K12) that were upregulated by inflammatory stimuli. It is also shown that TSA attenuated the inhibitory effect of steroids on histone acetylation in all cell lines. It is noteworthy that in the U937 cells the effect of TSA in cells stimulated with TA was not as potent as expected compared to the T-cell lines.

The data to this point suggested that TA was a more effective glucocorticoid than Dex and therefore it would be interesting to study the effect of other more specific histone deacetylase inhibition by using RNAi to study TA actions. Interestingly, TA and Dex induced an increase in whole cell HDAC activity with TA being the more potent than Dex. The increase was significant in all three cell lines, again indicating a similar mechanism of cross talk between GR and pro-inflammatory transcription factors in these cells. The repressive action of glucocorticoids may therefore, at least in part, result from recruitment of activated HDACs to sites in the promoters of inflammatory genes regulated by transcription factors, including AP-1 and NF-κB as well as the induction of HDAC expression (Adcock, 2001). Alternatively, activated GR could bind to one of the several transcription corepressor molecules, such as RIP140, which associate with proteins that have a differing histone deacetylase activity (Adcock and Caramori, 2001). Addition of TSA repressed the effect of the steroids and resulted in a downregulation of HDAC activity reaching almost pre-steroid treatment levels in all cell lines.

Most important, however, was the suggestion that monocyte and lymphocyte gene expression follows a similar and distinct pattern of histone acetylation. This leads to the conclusion that a more general pharmacological manipulation of specific histone acetylation status is a potentially useful approach for the treatment of inflammatory diseases. Extensive knowledge of the exact mechanism by which activated GR recruits HDACs may reveal new targets for the development of drugs that may dissociate the anti-inflammatory actions of glucocorticoids from their side effects that are largely due to gene induction. Alternatively, it could assist in the development of drugs that prevent K8 and K12 acetylation. So far, available drugs are non-specific in targeting histone and non-histone lysine residue acetylation. The finding that K12 is less sensitive to GC actions than K8 could hint in the development of new drugs that will be lysine residue specific and therefore target lysine K8 acetylation rather than acetylation of both K8 and K12 lysine residues. Interestingly, pCAF/GCN5 is known to be a potent activator of K8 acetylation following IL-1β stimulation (Ito *et al.*, 2000). Recent evidence suggests that the development of specific HAT inhibitors may prove to be important pharmacological tools (Workman and Kaye, 2002). The crystallisation of the GR LBD band to Dex indicates a distinct dimmer interface. Mutation of I628A prevents dimerisation and produced contrasting effects on transactivation and transrepression suggesting that the monomer and dimmer forms of GR may regulate distinct signalling pathways possibly through recruitment of distinct co-activators/repressors and subsequent changes in the ability to acetylate histone residues.

Controlled cell suicide (apoptosis) has long been associated with inflammation. Recent evidence on the effects of TSA and butyrate (both histone deacetylase inhibitors) on cell death and proliferation (Dangond *et al.*, 1998) have been the initiators for the debate on whether histone acetylation can directly, or indirectly, be linked with the regulation of apoptosis. Having established from previous work that increased histone acetylation occurs in cells when induced with inflammatory stimuli, this work focused in the role of histone acetylation in apoptosis. TSA is now in phase I and II clinical trials for breast cancer and acute myeloid leukaemia.

Preliminary studies on the initiation of cell death following stimulation with inflammatory mediators showed that in LPS stimulated U937 cells, cell death was not detectable whereas in both T-cell lines apoptosis was induced. Stimulation of the cells with a known inducer of oxidative stress and consequently apoptosis (H_2O_2) and also a toxic, high concentration of TSA (100 ng/ml) induced cell death in all three cell lines. U937 and Jurkat cells were prone to cell death when stimulated with TNF-α whereas this was not demonstrated in HUT-78 cells where cell death was not detected. Finally, a low concentration of TSA (10 ng/ml) that induced histone acetylation did not appear to induce cell death in any of the cell lines investigated.

Studies into programmed cell death showed that in U937 cells apoptosis accounted for over 50% of cell death following TNF-α and TSA (100 ng/ml) treatment. Despite its toxicity, H_2O_2 did not induce apoptosis in U937 cells. In Jurkat cells H_2O_2, TNF-α and TSA caused more than

60% of the cells to undergo apoptosis. Similarly in HUT-78 cells, H_2O_2 and TSA also induced apoptosis. Interestingly, LPS also induced more than half the cell population to undergo apoptosis. TNF-α treatment however did not induce apoptosis in these cell lines. Similar findings have been reported previously where in contrast to Jurkat cells, HUT-78 cells were resistant to the apoptotic effects of TNF (Giri *et al.*, 1998). In U937 cells activation of Bcl-2 was only significantly downregulated following treatment with LPS, and slightly downregulated with the high concentration of TSA. Studies in the activation of Bcl-2 in T-cell lines showed a similar pattern of activation for both Jurkat and HUT-78 cells. Bcl-2 levels were activated in all stimulations with the exception of the highest concentration of TSA. The complexity of apoptotic signalling in these cells warrants further investigation into the effects of TSA on the expression of patterns such as $p21^{CIP}$, which are upregulated by HDAC inhibitors and control cell cycle proliferation. The effects of HDAC inhibitors on proliferation and apoptosis may however be divorced from HDAC inhibition since concentrations required to inhibit HDAC activity are generally 10 to 100-fold lower than those required to modify cell proliferation (Paul *et al.*, 2002).

In U937 cells, H_2O_2 and TNF-α significantly upregulated annexin V. In Jurkat cells LPS, H_2O_2 and TNF-α stimuli also upregulated annexin V expression but in HUT-78 cells TNF-α did not induce annexin V. Low concentrations of TSA induced annexin V despite the fact that high levels of apoptosis were not detectable at this concentration. This suggests that analysis of apoptosis by a single factor could be misleading in these cells. Interestingly, 100 ng/ml of the inhibitor did not upregulate annexin V levels. This might be due to the fact that high concentrations of TSA cause immediate apoptosis to the cells and therefore changes in annexin V, which is elevated at the first apoptotic stages, may have been missed. Studies to this point showed that TSA and presumably histone acetylation did not affect induction of apoptosis in cells since only high concentrations of the inhibitor caused cells to undergo apoptosis. This, however, is attributed to the toxic effect of TSA rather than its inhibitory effects on histone deacetylation. Investigation into the effects of the glucocorticoids Dex and TA in the induction of apoptosis in cells alone failed to show any significant changes in the activation of Bcl-2 in all three cell lines. The levels of the protein following treatment were only slightly upregulated with the exception of Dex treatment to U937 cells were Bcl-2 levels were significantly elevated. In the U937 cells the addition of glucocorticoids to treated cells also did not lead to any significant activational changes. In the T-cell lines only the addition of glucocorticoids to H_2O_2 treated cells caused a noteworthy upregulation of Bcl-2. However, investigation in the activation of annexin V in stimulated cells in the presence of glucocorticoids revealed an upregulation of the protein in all cell lines in all treatments with the exception of the U937 cells were the presence of both steroids in TSA stimulated cells did not induce upregulation of annexin V.

Studies into the activation of Caspase 3 under the same conditions revealed that the combinatory effect of steroids and TSA in stimulated cells significantly enhanced this marker of apoptosis in all three cell lines. It was also significant that co-stimulation of the cells with glucocorticoids and TSA only, downregulated caspase 3 levels and therefore suggested an inhibitory effect on apoptosis in all three cell lines. TA was again more potent than Dex. Results showed that TSA modifies Caspase 3 expression due to its histone deacetylase inhibitory action and not due to its toxicity. This is also demonstrated in LPS- and TNF-α-stimulated cells in combination with the glucocorticoids. TSA acted synergistically with glucocorticoids to enhance apoptosis in stimulated monocytes and lymphocytes while preventing programmed cell death alone. These findings support the notion that histone acetylation regulates apoptosis as part of the transactivation and trans-repression ability of glucocorticoids.

A recent report offers an explanation on the mechanism by which histone acetylation could regulate apoptosis through the role of the nuclear, growth inhibitory proteins ING1 (Vieyra *et al*., 2002). The authors showed that human ING1 proteins interact with proteins associated with histone acetyltransferase (HAT) activity such as TRAP, CBP, PCAF and p300 and that overexpression of ING1 induced hyperacetylation of H3 and H4 *in vitro* and *in vivo*. Overall their data suggested that human ING1 proteins provide a direct linkage between DNA repair, apoptosis and chromatin remodelling via multiple HAT:ING1:pCNA protein complexes. Further studies as to whether these complexes are HAT specific and whether they are part of the same complexes formed with CBP to regulate inflammatory gene expression could help to paint a wider picture on the role of histone acetylation in the regulation of apoptosis in monocytes and T-cells.

Expanding on the possible role of histone 4 acetylation and deacetylation in disease, specifically in IBD and Peyer's patches, completed this thesis. Two animal models and samples from IBD patients were used to investigate the activation of histone 4 in inflamed and non-inflamed diseased biopsies. In the dextran sodium sulphate (DSS) rat model of inflammation, the acetylation activity of H4 and H3 was initially measured. Findings indicated that histone acetylation was upregulated in the two investigated rat strains (Lewis and Sprague-Dawley). Comparison of acetylated levels between H3 and H4 revealed that while both were acetylated, the latter reached significantly higher acetylation levels. Similarly, in the Peyer's patches of the DSS model, H4 acetylation was higher than H3. Localisation of histones in the Peyer's patches immunohistochemically revealed a pattern formation, which was noticeable in both Lewis and Sprague-Dawley strains. Acetylation of H3 was only detected in the mantle zone of the Peyer's patches, whilst acetylated H4 occurred in both the periphery and the germinal centre of the Peyer's patches. It was therefore concluded that acetylation of H3 could possibly be cell specific, whereas H4 is generally induced in all cell types present in the Peyer's patches (T-cells, B-cells, dendritic cells and macrophages). These results indicated

a uniformed mechanism of H4 acetylation in the regulation of inflammatory gene activation in IBD.

In the experimental models of colitis, H4 K8 and K12 were highly acetylated in the Sprague-Dawley rats. These findings were in agreement with findings presented previously *in vitro,* and with other reports that pro-inflammatory cytokines can cause histone acetylation of H4 lysines 8 and 12 (Ito *et al.*, 2000). In contrast to the data obtained in Sprague-Dawley rats, only K12 acetylation was strongly induced in Lewis rats with K5, K8 and K16 acetylation reaching similar levels. In non-treated Lewis rats, dose-dependent metronidazole (MTD) treatment resulted in the downregulation of all acetylated lysine residue levels. The same treatment in the Sprague-Dawley rats had equivalent effects on K8 and K12 acetylation, but upregulated the acetylation of K5 and K16. The combined effect of DSS and MTD in the Sprague-Dawley rat strain, did not lead to further elevation of acetylated lysine levels. Intriguingly, these are the residues targeted by glucocorticoids.

Histone H4 lysine acetylation was also studied in inflamed and non-inflamed tissue of the trinitrobenzene sulfonic acid (TNBS) model of colitis. Pan acetylation of histone H4 increased slightly in the non-inflamed mucosa and was significantly elevated in inflamed. Results further revealed a general upregulation of all histone H4 lysine residues in the proximal (non-inflamed) and distal regions (inflamed) of TNBS treated animals. In both regions of the TNBS treated bowel, K5 and K16 were elevated slightly. Acetylation of K8 and K12 however, was significantly increased, specifically in the distal region where acetylated levels were also significantly higher compared to the proximal. The results indicate that in both non-inflamed and inflamed regions of the bowel histone 4 lysine acetylation occurs with lysines 8 and 12 being predominantly acetylated whilst acetylated lysine levels in the non-inflamed regions were less than those recorded in inflamed regions. Ellis *et al.,* (1998) have described a selectivity of NFκB to DNA binding concluding that this could possibly explain how an inflammatory state in one section of the intestine could trigger changes in the NFκB signaling pathway in another macroscopically non-inflamed section. It is possible that a similar or the same process could be involved in the upregulation of acetylated lysine levels in the non-inflamed regions of the bowel. A direct link between NFκB activation and histone acetylation in these tissues needs to be confirmed.

The role of histone H4 acetylation in IBD was finally investigated by measuring H4 acetylation in Crohn's disease patient biopsies. Non-inflamed, inflamed and Peyer's patches biopsies were assessed. Initial experiments, showed that in the inflamed biopsies, levels of acetylated H4 were highest, followed by Peyer's patches. Acetylation was also detectable in the non-inflamed mucosa of the Crohn's disease patients. The results for acetylation of histone H4 lysines in Crohn's disease were very similar to those obtained in the TNBS treated animals. K5 and K16 were only slightly activated in all samples, with the inflamed and non-inflamed samples

presenting no significant difference in acetylation status. Peyer's patches showed the highest H4 acetylation activity for K5 and K16. Finally, in biopsies of inflamed bowel and in Peyer's patches of Crohn's disease patients, K8 and K12 acetylation was equally elevated. Acetylation of lysine residues in the non-inflamed biopsies was only slightly upregulated. Interestingly, immunohistochemical studies of Crohn's disease Peyer's patches, localized acetylation of K5 and K16 in the mantle zone with a similar pattern to histone H3 acetylation. In contrast, acetylation of K8 and K16 was localized in both mantle zone and germinal center. These latter results suggest that although pan acetylation of H4 in the Peyer's patches is probably not cell specific, it is possible that acetylation of its specific lysine residues is cell type dependent.

Appreciation of the structural and biochemical properties of nucleosomes and chromatin containing histone modifications in human diseases is still elementary and further work is needed to understand it. The work described throughout this thesis have the ultimate objective of pointing out the importance of the expanding need to fully understand the complicated chromatin remodelling machinery and the mechanisms by which it regulates inflammatory gene expression. With the extensive developments in linking covalent histone modifications to biological processes acetylation of histones has acquired clinical importance. It is likely that novel drugs directed in selective HAT/HDAC modulation will lead to novel types of anti-inflammatory drug that will result to fewer side effects in the future. The ultimate objective is the complete understanding of the mechanisms of how deregulation of chromosome structure leads to diseases leading to the development of new therapeutic approaches.

In conclusion, the following list assembles a summary of some of the most important points of interest that future work could be directed to based on this thesis:

- Increased numbers of animal models in order to establish the preliminary data presented here
- Use of IKK2 inhibitors and RNAi to confirm whether the effects observed are NFκB mediated
- Use of specific HATs and HDACs to determine whether, similarly to previous reports, GC induced histone acetylation is modulated by specific HAT/HDAC complexes and to assess their role in inflammatory gene expression
- Finally, to ascertain the molecular mechanism of TSA-induced cell death or its effects on cell proliferation

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Appendix A

Name:	**Jurkat E6.1**	**ECACC Ref No:**	**88042803**
Comments	Derived from Jurkat FHCRC. An IL-2 producing cell line, derived by incubating the cells at 41°C for 48h followed by a limiting dilution cloning over macrophages.		
Morphology	Suspension	Description Key Words:	Human leukaemic T cell lymphoblast
Tissue	Blood	Species:	Human
Medium	RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS).		
Sub Culture Routine	Maintain cultures between 3-9x100,000 cells/ml; 5% CO_2; 37°C. On resuscitation single cells can be observed, during culture most cells will grow as aggregates.	R.C.D:	No
Karyotype	Pseudodiploid, modal No 46	Passage Number:	
Receptors		Histocompatability:	
Products	Interleukin 2 (IL-2), human alpha interferon	Reference:	J Immunol 1984;133:123; J Immunol Meth 1993;157:203-207

Name:	**HUT-78**	**ECACC Ref No:**	**88041901**
Comments	Derived from peripheral blood of a 50 year old male patient with Sezary syndrome. Cells exhibit the features of a mature T cell line with inducer/helper phenotype. Biologically active IL-2 can be eluted from the cell surface.		
Morphology	Lymphoblast	Description Key Words:	Human T cell lymphoma
Tissue	Blood	Species:	Human
Medium	RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS).		
Sub Culture Routine	Maintain cultures between 3-9x100,000 cells/ml; 5% CO_2; 37°C.	R.C.D:	No
Karyotype	Not Specified	Passage Number:	
Receptors		Histocompatability:	
Products	Interleukin 2 (IL-2)	Reference:	J Exp Med 1981;154:1403

Name:	**A549**	**ECACC Ref No:**	**86012804**
Comments:	Derived from a 58 year old Caucasian male. The cells can synthesise lecithin utilising the cytidine diphosphocholine pathway. Occasional cells may also contain inclusion bodies although they are not known to carry any human pathogen.		
Morphology:	Epithelial	Description Key Words:	Human Caucasian lung carcinoma
Tissue:	lung	Species:	Human
Medium:	Ham's F12K or DMEM + 2mM Glutamine + 10% Foetal Bovine Serum (FBS).		
Sub Culture Routine:	Split confluent cultures 1:3 to 1:6 i.e. seeding at 2-4x10,000 cells/cm² using 0.25% trypsin or trypsin/EDTA; 5% CO2; 37°C.	R.C.D:	No
Karyotype:	Hypotriploid	Passage Number:	85
Receptors:		Histocompatability:	
Products:	Lecithin, High amounts of de-saturated fatty acids.	Reference:	J Nat Cancer Inst 1973;51:1417; Int J Cancer 1967;17:62

Name:	**U937**	**ECACC Ref No:**	**85011440**
Comments:	Derived from malignant cells of a pleural effusion of 37 year old caucasian male with diffuse histiocytic lymphoma. One of only a few human lines still expressing many of the monocytic like characteristics exhibited by cells of histiocytic origin.		
Morphology:	Lymphoblast	Description Key Words:	Human Caucasian histiocytic lymphoma
Tissue:	lung	Species:	Human
Medium:	RPMI 1640 + 2mM Glutamine + 10% Foetal Bovine Serum (FBS).		
Sub Culture Routine:	Maintain cultures between 2-9x100,000 cells/ml; 5% CO2; 37°C. Cells may take up to 72 hours until confluent.	R.C.D:	No
Karyotype:	Not Specified	Passage Number:	
Receptors:		Histocompatability:	
Products:		Reference:	Int J Cancer 1976;17:565; J Exp Med 1976;143:1528; Nature 1979;279:328; J Immunol 1980;125:463

Details of the patients used in Chapter 6

AGE	18-57 yrs
NO OF PATIENTS	12
STATE	Crohn's disease
CONTROL	Other disease, samples obtained from routine examination
TREATMENT	Sulfasalazine and/or antibiotics (ampicillin, tetracycline)

Appendix B

TRIAMCINOLONE ACETONIDE AND DEXAMETHASOME SUPPRESS TNF-α INDUCED HISTONE H4 ACETYLATION ON LYSINE RESIDUES 8 AND 12 IN MONONUCLEAR CELLS.

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Introduction

The anti-inflammatory properties of Glucocorticoids (GC) result from transcriptional repression of pro-inflammatory genes. In the inactive state DNA exists as condensed chromatin composed of nucleosomes, with the DNA tightly wrapped around a octameric protein complex composed of two copies each of four core histones, H3, H4, H2A and H2B. During the induction of gene transcription modification of these histones, e.g. by acetylation and methylation, results in local relaxation of the DNA/protein complex, permitting access of DNA-binding proteins and transcription[1]. Thus, Trichostatin A (TSA), a potent and specific inhibitor of histone deacetylase, has effects on cell proliferation and differentiation[2]. Recently, alterations in chromatin structure have been shown to play an important role in modulation of GC activity, for example the histone deacetylase (HDAC) inhibitor, sodium butyrate, interferes with GC receptor activated transcription[2, 3]. Here, we have studied the effects of the GCs, Dexamethasome (Dex) and Triamcinolone acetonide (TA), on production of the inflammatory cytokine IL-8 and regulation of histone acetylation in a macrophage cell line stimulated with TNF-α.

Experimental Procedures

U937 cells (40-60% confluent), cultured under standard conditions in the presence of antibiotics, were synchronised by FCS starvation for 48 hrs (0.25% FCS in RPMI), prior to 24h incubation (non-stimulated; NS) or stimulation with TNF-α (10ng/ml) in the presence, or absence, of Dex (10^{-6} M) or TA (10^{-8} M). IL-8 was measured in culture supernatants by ELISA (Amersham). Following SDS-polyacrylamide gel electrophoresis of extracted histones[4] the separated proteins were transferred to

nitrocellulose membranes. After blocking, membranes were exposed to antibodies to acetylated H4 (lysines 5, 8, 12 and 16). Subsequently, membranes were washed and treated with horseradish peroxidase-conjugated secondary antibody and binding of secondary antibody detected using enhanced chemiluminescence (ECL) and exposure to film, followed by densitometry.

Results

The HDAC inhibitor TSA enhanced TNFα-induced IL-8 release and partially blocked GC-inhibition of this release (results not shown) suggesting that histone acetylation plays role in IL-8 production and the actions of GCs. TNF-α (Fig.1) increased production of IL-8 as expected. Addition of Dex (1μM) and TA (1μM) significantly repressed the levels of IL-8 to almost control levels (Fig. 1). Stimulation with TNF-α increased acetylation of all histone H4 lysine residues (K5, 8, 12 and 16), the highest elevation of which was for lysines 8 and 12 (Fig.2). Addition of Dex or TA reduced the levels of acetylation of K8, K12 and K16 residues, the greatest effect being with lysines 8 and 12, without affecting K5 levels.

Discussion

The effects of Dex and TA on histone acetylation are in agreement with previous findings observed at A549 lung epithelial cells[1]. Thus the TA and Dex reduction in TNF-α induced IL-8 release appears to involve inhibition of K8 and K12 H4 acetylation. This possible mechanism for GC repression is novel and establishes inhibition of histone acetylation as an additional level of control of inflammatory gene expression suitable for drug intervention.

Acknowledgements

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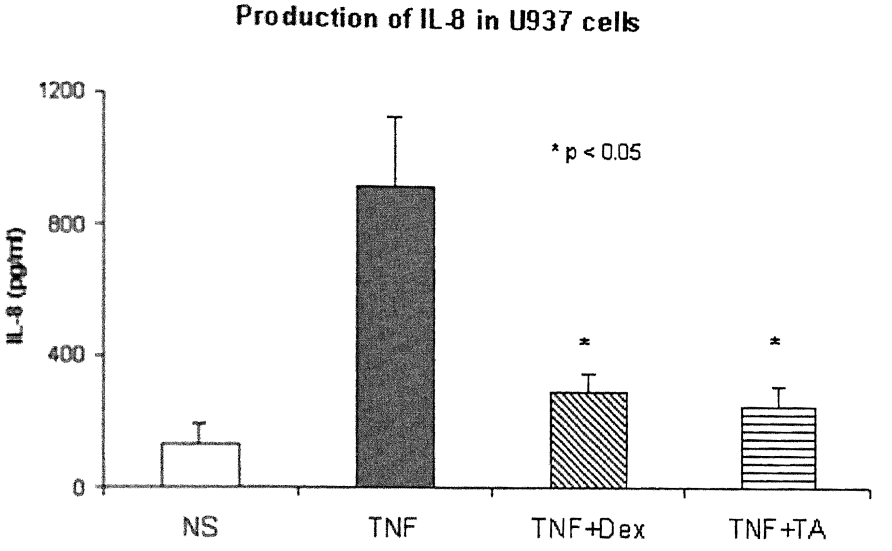

Figure 1. Production of IL-8 in macrophages stimulated with TNF-α (10ng/ml) in the absence and presence of steroids. Stimulation with TNF-α induced production of the inflammatory cytokine Il-8. Addition of the steroids Dexamethasone (Dex) and Triamcinolone acetonide (TA) significantly (n=6, * = $p<0.05$) down regulated IL-8 production approximately two fold.

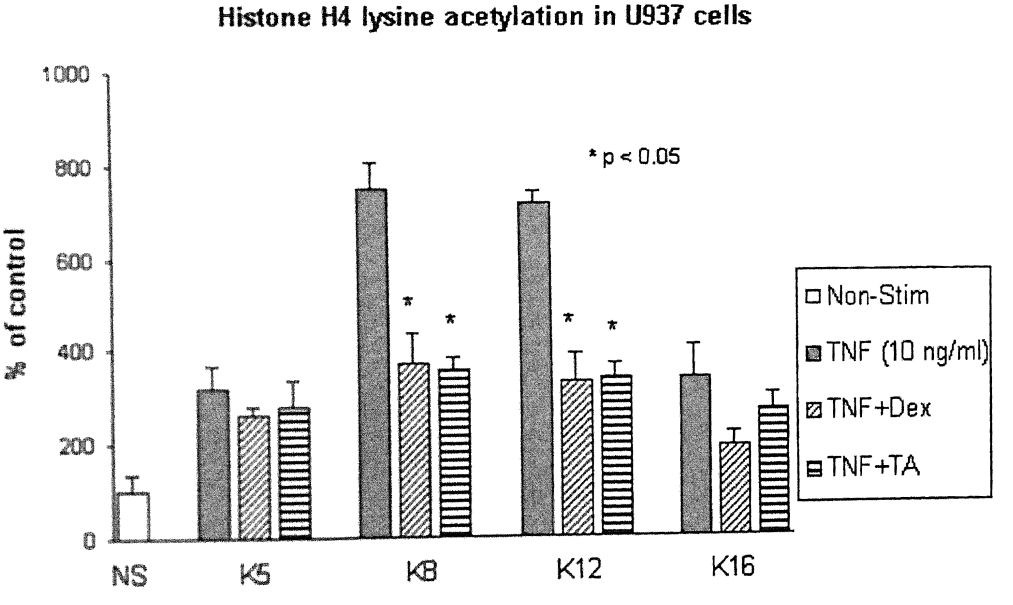

Figure 2. Histone H4 acetylation of lysine residues K5, K8, K12 and K16 in U937 cells. Increased levels of acetylation were observed in all lysine residues of U937 cells treated with TNF-α. Most notable was with lysine residues K8 and K12, where acetylation was elevated by almost a factor of 7. Both TA and Dex significantly (n=6, * = $p<0.05$) decreased acetylation levels in lysines K8 and K12.