„The role of nucleosome-induced neutrophil activation in systemic lupus erythematosus“

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„Blut ist ein ganz besonderer Saft.“

(Johann Wolfgang von Goethe, 1749-1832)

“Many roads lead to Rome,

— and many more to SLE.”

To I. W. and E. R.

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Abbreviations

2-ME	2-Mercaptoethanol
7-AAD	7-amino-actinomycin D
Ab	Antibody
ACR	American College of Rheumatology
Ag	Antigen
ANA	Antinuclear autoantibodies
APC	Antigen-presenting cells
autoAb	Autoantibody
autoAg	Autoantigen
BCR	B cell receptor
BM	Bone marrow
BMDC	Bone marrow-derived DC
BMN	Bone marrow-derived neutrophils
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
CMV	Cytomegalovirus
CpG	Cytosine-phospho-guanine
CpG motif	Unmethylated CpG dinucleotide
CpG ODN	ODN containing CpG motif
CRP	C-reactive protein
DAI	DNA-dependent activator of IFN-regulatory factors
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
ds	Double-stranded
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
GBM	Glomerular basement membrane
G-CSF	Granulocyte colony-stimulating factor

GM-CSF	Granulocyte/macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HMGB-1	High-mobility group box-1
HRP	Horseradish peroxidase
IC	Immune complex
IFN	Interferon
Ig	Immunglobulin
IL	Interleukin
KO	Knockout
LE	Lupus erythematosus
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
mAb	Monoclonal antibody
MBL	Mannose-binding lectin
MDA5	Melanoma-differentiation-associated gene 5
mDC	Myeloid DC
MDDC	Monocyte-derived DC
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor κ of B cells
NK cells	Natural killer cells
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
ODN	Oligodeoxynucleotide
PAMP	Pathogen-associated molecular pattern
PB	Polymyxin B
PBS	Phosphate-buffered saline
pDC	Plasmacytoid DC
PE	Phycoerythrin
PEC	Peritoneal exudate cells
PIC	Protease inhibitor cocktail
PMN	Polymorphonuclear neutrophils

P-ODN	ODN with phosphodiester backbone
poly(I:C)	Polyinosine-polycytosine
PRR	Pattern-recognition receptor
PS-ODN	ODN with phosphorothioate backbone
PTO	Phosphorothioate
RBC	Red blood cell
RF	Rheumatoid factor
RIG-1	Retinoic-acid inducible gene I
ROS	Reactive oxygen species
SAP	Serum amyloid P
SDs	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
ss	Single-stranded
TCR	T cell receptor
Th	T helper
TIR	Toll/IL1-receptor domain
TLR	Toll-like receptor
TNF	Tumour-necrosis factor

1 Introduction

1.1 Immune system

The defence against infectious diseases is provided by the immune system. The immune system is a network of tissues, cells and molecules that protects the body from invading viruses, bacteria, fungi and parasites.

The landmark discovery in immunity is attributed to Edward Jenner (1749-1823) - a country doctor who discovered in 1798 that cowpox (vaccinia) induced protection against human smallpox. He observed that milkmaids, who developed cowpox lesions on the fingers from infected dairy cattle, were subsequently immune to smallpox. Therefore, he inoculated non-immune individuals with cowpox vesicle fluid and demonstrated that they later became resistant to smallpox. He called this procedure 'vaccination'. This term is still used to describe the inoculation of healthy individuals with attenuated strains of disease-causing agents to provide protection against the disease. The vaccination with vaccinia virus has resulted in the worldwide eradication of smallpox as of 1980. Prior to Jenner's studies, the only method for preventing smallpox was by 'variolation', practiced in China in the 15th century. Here, lesional fluid from mild case was inoculated onto nasal mucosa of the non-immune. Unfortunately, this practice resulted in an occasional smallpox death. In 1885, Louis Pasteur (1822-1895) prepared a rabies vaccine from desiccated brain and spinal cord of laboratory-infected rabbits and successfully vaccined dog-bite victims.

Fifteen years after Pasteur's rabies vaccination, Robert Koch (1843-1910) proved that microorganisms cause infectious diseases. Emil von Behring (1854-1917) received the first Nobel Prize for Physiology or Medicine in 1901 for his discovery of antitoxic immunity and the application of this therapeutic modality in diphtheria and tetanus (1).

A specific immune response with the production of antibodies against a particular pathogen (antigen) is known as an adaptive immune response. Adaptive immune responses depend upon lymphocytes, which provide lifelong protective immunity to reinfection with the same pathogen. In contrast, innate immunity is a first line of defence against invading pathogens. Innate immune recognition relies on a limited number of germline-encoded receptors and largely involves phagocytic cells (2).

The innate and adaptive immune systems together provide an effective defence system against all kinds of potentially pathogenic microorganisms.

1.1.1 Innate immune system

The innate immune system is an evolutionary ancient part of host defence mechanisms. In contrast to the adaptive immunity, the innate pattern recognition receptors are encoded in the germ line DNA and do not require gene rearrangements (see (2) for review). The innate immune system is made of leukocytes that are not B lymphocytes or T lymphocytes. All different types of blood cells derive from pluripotent hematopoietic stem cells in the bone marrow. The myeloid progenitor is the precursor of the main cellular components of the innate immune system: macrophages, granulocytes, dendritic cells and mast cells. Monocytes circulate in the blood stream and differentiate into mononuclear phagocytes or macrophages upon migration into body tissue. Granulocytes (polymorphonuclear leukocytes) have irregularly shaped nuclei and cytoplasmic granules, which give them their prominent staining pattern. Granulocytes are short-lived cells and produced in huge numbers during immune responses. There are three types of granulocytes: Eosinophils and basophils are thought to be involved in the defence against parasitic infection and play a role in allergic inflammation. **Neutrophils** are phagocytic and the most numerous and important cellular component of innate immune response. Here, neutrophils will be described in more detail, because they are of particular interest in the present work.

Paul Ehrlich first described neutrophils as polymorphonuclear leukocytes (3) in 1905. Later Elie Metchnikoff discovered the function of neutrophils and macrophages as migrating and phagocytosing cells (4).

Neutrophils are the first cells recruited from the bloodstream to sites of infection and inflammation. They provide a first line of defence against invading pathogens and can potentially kill any nucleated and non-nucleated host cell, as well as connective tissue. Acquired or inherited neutropenia as well as neutrophil malfunction result in recurrent, life-threatening infections (5). Neutrophils are terminally differentiated cells and have a short life span of only a few hours. Leaving the bone marrow, they are equipped with an intimidating array of antimicrobial weaponry, including toxic metabolites, antimicrobial proteins and proteolytic enzymes (6).

Upon contact via direct recognition or after opsonization of pathogens, neutrophils engulf the microbes into a phagocytic vacuole, called a phagosome. Subsequently, intracellular granules fuse with the phagosome and discharge their contents to form a phagolysosome. In these phagolysosomes, microbes are killed by a combination of non-oxidative and oxidative mechanisms (7). The oxygen-independent effectors are stored in different granule subsets, whereas the oxygen-dependent mechanism involves an abrupt, non-mitochondrial generation of reactive oxygen species (ROS) and is known as respiratory burst (8). In addition to the intracellular killing mechanisms, neutrophils bind and kill microorganisms via fibres called neutrophil extracellular traps (NETs) (9). Neutrophil recruitment to inflammatory sites is mediated by chemoattractants such as interleukin-8 (IL-8) (10). The rate of neutrophil production in the bone marrow is controlled by the hematopoietic growth factor granulocyte colony-stimulating factor (G-CSF) (11). As a key component of the inflammatory response, neutrophils recruit and activate antigen-presenting cells (APC). They are able to interact with monocytes, dendritic cells, T cells and B cells through cell-cell contact and secreted products. They produce tumour-necrosis factor (TNF) and other cytokines that induce DC and macrophage differentiation and activation (12, 13, 14). Activated neutrophils interact with immature DCs by binding of the β_2-integrin Mac-1 to the DC-specific C-type lectin, DC-SIGN (14).

Although neutrophils are known to rely on “non-specific” host defence mechanisms (2), a T cell receptor-based variable immunoreceptor was recently discovered in a subpopulation of human neutrophils (15).

In contrast to macrophages and neutrophils, which primarily act as phagocytic cells, immature **dendritic cells** (**DCs**) are both phagocytic and macropinocytic. They circulate in the blood to enter peripheral tissues.

After encountering a pathogen, DCs rapidly mature, express co-stimulatory molecules (CD80, CD86, MHC II molecules), secrete cytokines and migrate to the draining lymph node where they present pathogen-derived peptide antigens to naïve T lymphocytes. Consequently, DCs have a key role in coupling innate and adaptive immune responses.

Mast cells also mature in tissues and are mainly important in allergic responses and play a key role as mediators of inflammation.

Pathogens (viruses, bacteria, parasites) that overcome the epithelial barriers of the body are immediately faced by tissue macrophages. The binding of specific components of pathogens to their surface receptors and phagocytosis rapidly elicit an inflammatory response that leads to the recruitment of neutrophils and additional macrophages to sites of infection.

The receptors of the innate immune system are germline-encoded pattern-recognition receptors (PRR), that recognize pathogen-associated molecular patterns (PAMP), which are only found in/on microbes and (usually) not on host cells. The principal functions of PRRs include opsonization, activation of the complement and coagulation cascades, phagocytosis, activation of proinflammatory signalling pathways and induction of apoptosis (2).

Some of these cell surface receptors, like the macrophage mannose receptor (MMR), directly stimulate the ingestion of the recognized pathogen. The Toll-like receptors (TLRs) are a family of evolutionary highly conserved transmembrane receptors and function exclusively as signalling receptors. Each of the known 11 TLRs recognizes one or more distinct PAMP, by direct interaction with external and internal molecules of the pathogen. TLRs activate the ubiquitous transcription factor NFκB, which induces the transcription of several genes for cytokines, chemokines and co-stimulatory molecules that help initiating adaptive immune responses later in the course of infection. TLRs will be described in more detail in 1.1.1.1. Pattern recognition in the innate immune system.

NK cells develop in the bone marrow from the common lymphoid progenitor and circulate in the blood. They represent 5 to 10 % of human peripheral blood mononuclear lymphocytes. They are larger than T and B lymphocytes and have distinctive cytoplasmic granules. NK cells belong to the innate immune system and have two types of invariant cell surface receptors that control their cytotoxic activity: ‘activating receptors’ (e.g. NKG2D), which trigger the release of cytokines (e.g. IFNγ) and direct killing of target cells. ‘Inhibitory receptors’ which are specific for MHC class I molecules and prevent NK cells from killing host cells.

NK cells are activated in response to interferons (IFN-α, IFN-β) and macrophage-derived cytokines (IL-12), which are produced early during infections. Thus, activated NK cells serve to contain infections while the adaptive immune response is generated (16).

NKT cells are a class of T lymphocytes that express an invariant αβ-T cell receptor, as well as cell surface markers of NK cells. They are known as innate-like lymphocytes (ILLs) and recognize glycolipid antigens presented by CD1, a molecule related to MHC class IB (17).

In addition to cellular innate immune responses act soluble PRRs like the recognition molecules of the complement system, which was discovered by Jules Bordet (1870-1961). The **complement system** consists of more than 20 plasma proteins that react with one another to kill target cells by the formation of transmembrane pores. Furthermore, complement components opsonize pathogens and induce a series of inflammatory responses that help to fight infection. The binding of C1q to antibody-antigen complexes or other structures activates the classical complement pathway. Recognition of microbial saccharides by mannose-binding lectin (MBL) initiate the lectin pathway (18). The spontaneous hydrolysis of the thioester bond of native C3 causes the initiation of the alternative pathway, which provides an amplification loop for the two other pathways (19). Each pathway follows a series of cleavage reactions to generate a protease called C3 convertase, which cleaves complement component C3 into C3b and C3a. C3b, the main effector molecule of the complement system, binds covalently to pathogens, opsonizes bacteria and thereby target it for the engulfment by phagocytes. C3a is a peptide mediator of local inflammation. C3b also binds to the C3 convertase to form a C5 convertase that produces C5a, a powerful peptide mediator of inflammation, and C5b. C5b triggers the late events in which the terminal components of complement (C6 - 9) interact to form a membrane-attack complex (MAC), which generates a pore in the bacterial cell membrane to kill the bacterium.

Because of the amplifying capacity of complement and the production of several inflammatory mediators, control mechanisms exist to prevent ongoing activation that could result in host tissue damage (20).

The role of complement in the development of systemic lupus erythematosus will be discussed in 1.2.1.7 Pathogenesis of SLE.

In response to infection, various immune cells release chemokines and cytokines.

Cytokines are small proteins (25 kD) that act via cytokine receptors and affect the behaviour of other cells.

Cytokines can be grouped by their structure into families: the hematopoietin family, the interferons and the TNF family, which function in both innate and adaptive immunity. After encountering the antigen (e.g. LPS, CpG DNA), mature DCs migrate to the draining lymph node. The secretion of IL-12 and IL-18 induces the differentiation of CD4 T helper cells into T_H1 cells (21,22). The selective production of T_H1 cells induces cell-mediated immunity and the production of IgG antibodies by B cells. Extracellular antigens (e.g. parasites) induce the secretion of IL-4, which favours the differentiation of CD4 T helper cells into T_H2 cells. The secretion of T_H2 cytokines by T_H2 cells leads to the stimulation of humoral immunity by aiding B cell activation and class swichting (23). Immature DCs produce the anti-inflammatory cytokines IL-10 and TGFβ.

T_H1 cells secrete predominantly IL-2, IL-3, TNFα and most notably IFNγ, which is the main macrophage-activating cytokine. T_H2 cells secrete B-cell-activating cytokines (IL-4, IL-5, IL-9, IL-13) and IL-10, which inhibits macrophage activation.

Chemokines are chemotactic cytokines, which induce cell migration and activation by binding to specific G-protein-coupled cell surface receptors on nearby target cells in response to pathogens (24,25).

Chemokines are 8-to-10 kD proteins that have been subdivided into families according to the relative position of their amino terminal cysteine residues (26,27). The CC-chemokines – two invariant cysteines adjacent - bind to CC-chemokine receptors, whereas CXC-chemokines – an amino acid residue (X) between two invariant cyteines - bind to CXC-chemokine receptors.

Chemokines often act in concert with other cytokines to cause tissue infiltration by increasing the circulating pool of a given leukocyte and up-regulating particular adhesion molecules as well as increasing leukocyte responsiveness to a chemokine (10).

1.1.1.1 Pattern recognition in the innate immune system

The innate immune system is highly developed in its ability to discriminate between self and pathogens, a process that relies to a great extent on an evolutionary conserved family of **Toll-like receptors** (**TLR**) (28). Toll receptors were originally identified in the fruit fly *Drosophila melanogaster*, where they were found to play a major role in dorso-ventral polarization in fly embryos and host protection from fungal infections (29-31).

Soon after, a mammalian homolog of the Toll receptor (now known as TLR4) was shown to induce expression of genes involved in inflammatory responses (32). The first evidence that TLR4 functions as a signal-transducing receptor for LPS came from a mouse strain (C3H/HeJ), which is unresponsive to LPS due to a point mutation in the *TLR4* gene (33,34). To date, ten human TLRs have been identified that target predominantly PAMPs, a limited set of conserved molecular patterns that are unique to microorganisms and absent from host cells (35). The basic structural features of the TLR family include an extracellular leucine-rich repeat (LRR) domain, a transmembrane region and a highly conserved cytoplasmic Toll/IL-1 receptor domain (TIR), which is significantly similar to the intracellular portion of the IL-1 receptor (29,32,36). Activation of the signal transduction pathways by TLRs leads to the induction of various genes that are involved in host defence, including inflammatory cytokines, chemokines, MHC and costimulatory molecules.

The recognition of PAMPs by TLRs stimulates the recruitment of a set of intracellular TIR-domain containing adaptors, including MyD88 (myeloid differentiation factor 88), TIRAP, Trif, IRAK and TRAM via TIR-TIR interactions (37). MyD88 is a universal adaptor that activates inflammatory pathways. MyD88 is shared by all TLRs with the exception of TLR3. Recruitment of MyD88 leads to the activation of MAP kinases (MAPKs) (ERK, JNK, p38) and the transcription factor NFκB to control the expression of inflammatory cytokine genes. The TIR-containing adaptor protein TIRAP (or MAL) mediates the activation of a MyD88-dependent pathway downstream of TLR2 and TLR4. The TIR-containing adaptor inducing IFNβ (TRIF) is recruited to TLR3 and TLR4, and activates an alternative pathway (TRIF-dependent pathway) that culminates in the activation of NFκB, MAPKs and the transcription factor IRF3 (see (38) for review).

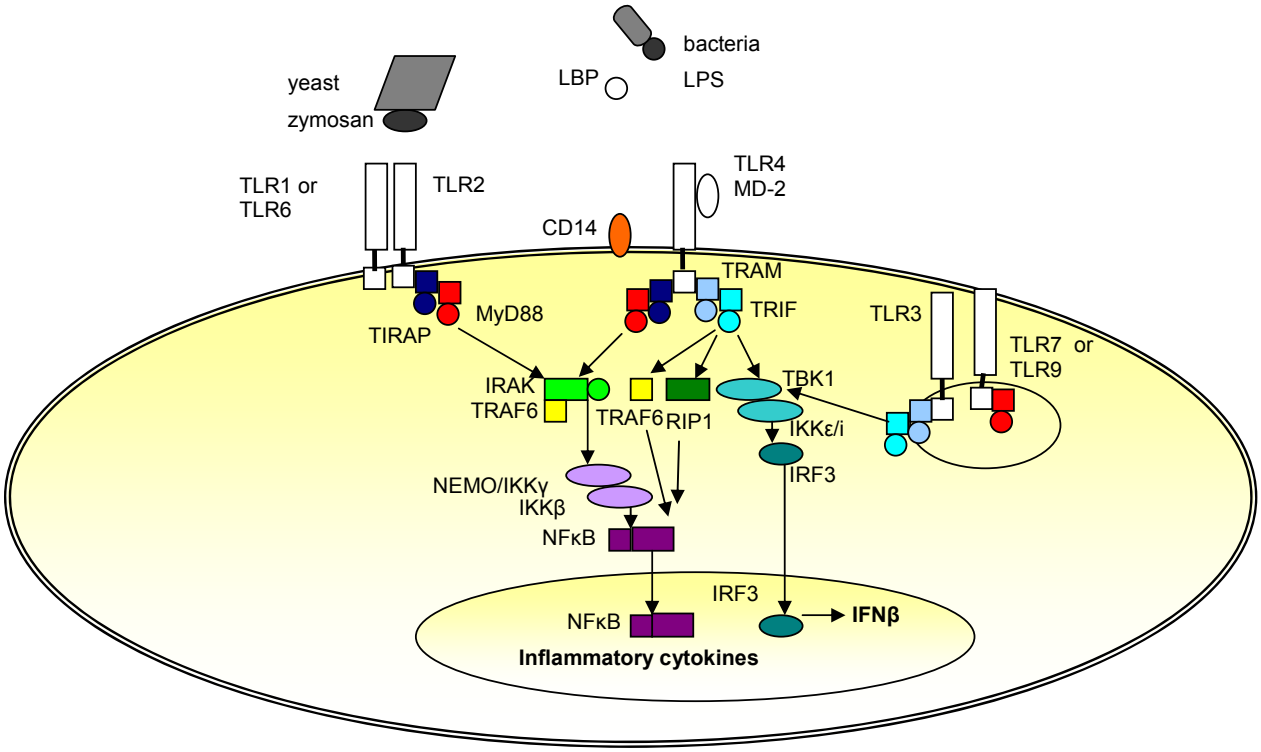


***Figure 1**. **TLR signalling pathway**. Adapted from (39). MyD88 associates with the cytoplasmic TIR domain of TLRs and recruits IRAK to the receptor upon ligand binding. IRAK activates TRAF6, leading to the activation of IKK complex. The IKK complex phosphorylates IκB, resulting in the nuclear translocation of NFκB which induces the expression of inflammatory cytokines. TIRAP, a second TIR domain-containing adaptor, is involved in the MyD88-dependent signalling pathway via TLR2 and TLR4. In TLR3- and TLR4-mediated signalling pathways, activation of IRF3 and the induction of IFNβ are observed in a MyD88-independent manner. A third TIR domain-containing adaptor, TRIF, is important for the MyD88-independent pathway. Non-typical IKKs mediate the activation of IRF3 downstream of TRIF. A fourth TIR domain-containing adaptor, TRAM, is specific for the TLR4-mediated MyD88/TRIF-dependent pathway.*

TLR1, -2, -4, -5 and -6 are expressed on the cell surface, where they recognize bacterial products (40). Whereas TLR3, -7, -8 and -9 are localized in intracellular compartments, such as endosomes, and are specialized in viral detection or recognition of nucleic acids (41-43).

Human TLR	**Ligand**
TLR1/TLR2 heterodimer	triacyl lipopeptides
TLR2	lipoprotein/lipopeptides peptidoglycan (Gram+) LTA (Gram+) lipoarabinomannan (Mycobacteria) zymosan (fungi)
TLR2/TLR6 heterodimer	diacyl lipopeptides
TLR3	dsRNA poly(I:C)
TLR4	LPS (Gram-) LTA (Gram+) HSP60/70 Taxol (*Taxus brevifolia*)
TLR5	flagellin
TLR7	ssRNA Imidazoquinolines Resiquimod (R848)
TLR8	ssRNA Resiquimod/Imidazoquinolines (*not mouse TLR8*)
TLR9	Unmethylated CpG DNA
TLR10 (mouse)	?
TLR11 (mouse)	?

***Table 1**. **Toll-like receptors and ligands.** Adapted from (39) (Gram-, Gram-negative; Gram+, Gram-positive; HSP60/70, heat shock protein 60/70; LPS, lipopolysaccharides; LTA, lipoteichoic acid; poly(I:C), polyinosine-polycytosine)*

Thus, TLRs sense the presence of infection through recognition of PAMPs. Innate immune cells, such as DCs or macrophages, engulf pathogens by phagocytosis and present pathogen-derived peptide antigens to naïve T cells.

Phagocytosis-mediated antigen presentation together with TLR-dependent gene expression of inflammatory cytokines and co-stimulatory molecules instruct the development of antigen-specific acquired immunity (39).

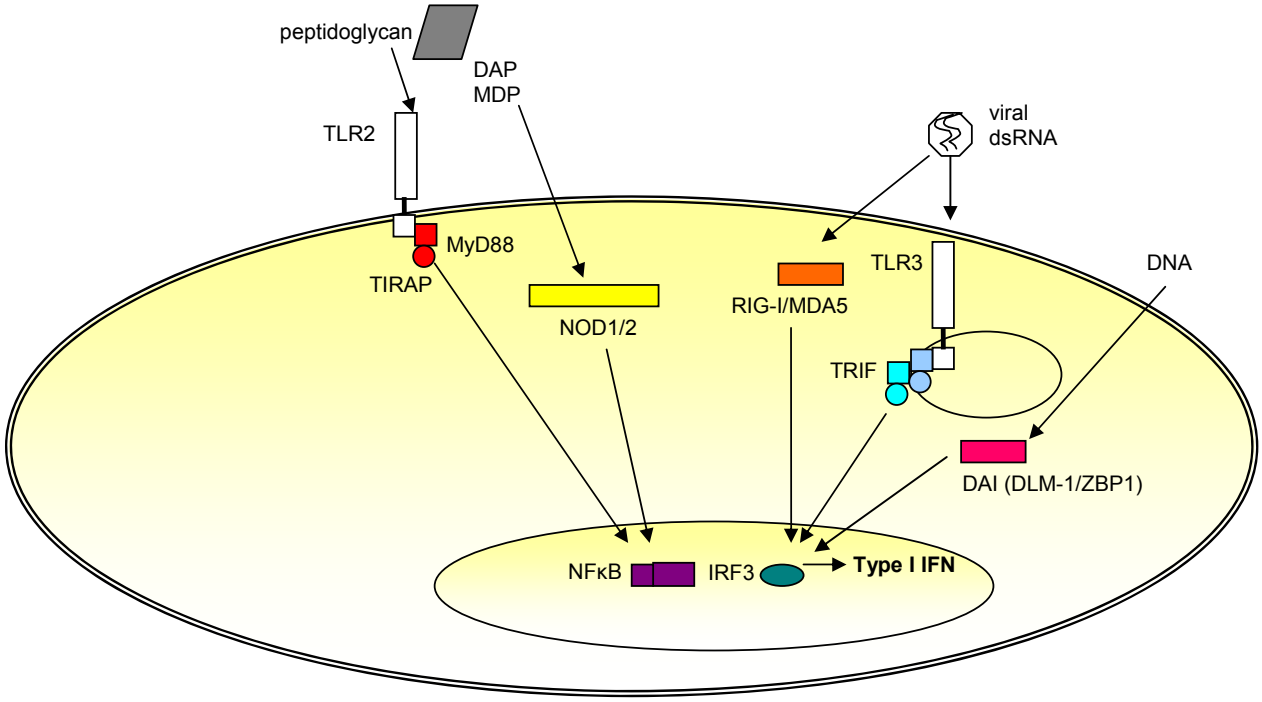


Figure 2. TLR dependent and independent recognition of microbial components*. Adapted from (39,44). TLR2 mediates peptidoglycan (PGN) recognition. NOD1 and NOD2 have recently been shown to recognize motifs found in the layer of PGN. TLR3-mediated recognition of viruses or dsRNA results in the TRIF-dependent activation of IRF3 and NFκB. However, viruses or dsRNA are recognized in a TLR3-independent manner, since the recognition of viruses and dsRNA is only partially impaired in TLR3-deficient mice. RIG-I and MDA5 are identified as molecules for viral recognition, resulting in the activation of IRF3. DAI (DLM-1/ZBP) is a cytoplasmic recognition receptor that senses and is activated by DNA from different sources, leading to type I IFN gene induction through the activation of IRF3, and probably IRF7. (See text for abbreviations.)*

Another group of proteins likely to be involved in intracellular pattern recognition is the family of nucleotide-binding oligomerization domain (**NOD**) proteins. NOD proteins contain an N-terminal caspase-recruitment domain (CARD), a NOD domain and a C-terminal LRR region (45-47). NOD1 and NOD2 activate NFκB in response to bacterial peptidoglycan (see (48) for review).

Recently, three homologous DExD/H box RNA helicases were identified as cytoplasmic sensors of virus-derived dsRNA. Retinoic-acid inducible gene I (**RIG-I**) and melanoma-differentiation-associated gene 5 (**MDA-5**) have two N-terminal CARDs and a RNA helicase domain. Upon dsRNA stimulation, RIG-I and MDA-5 trigger the activation of NFκB and IRF3/7, which cooperate in the induction of antiviral type I IFNs. A third member of the helicase family, LGP2 lacks the CARD domain and functions as an endogenous negative feedback regulator of RIG-I- and MDA-5-mediated signalling (49-52).

In addition to the membrane-associated TLR9, a cytosolic DNA sensor, **DAI** (DNA-dependent activator of IFN-regulatory factors (53)) has been recently identified. DAI (previously named DLM-1 or ZBP1) recognizes and is activated by DNA from a variety of sources, resulting in the induction of type I IFN gene through IRF3, and probably IRF7 (44,54,55).

	PRR	**Family**	**Ligands**
Soluble PRRs	MBL	Collectin-family C-type lectin	Terminal mannose residues
	CRP, SAP	Pentraxins	Phosphorylcholine on bacterial surfaces
	LBP	Lipid-transfer protein family	LPS
Cell surface PRRs	CD14	GPI-linked protein	LPS, peptidoglycan
	Dectin-1	C-type lectin	β-glucan
	Macrophage Mannose receptor	C-type lectin	Terminal mannose residues
	Macrophage scavenger receptor	Scavenger receptor type A (SR-A)	LPS, dsRNA, LTA

	MARCO	(SR-A)	Bacterial cell walls, LPS
Intra-cellular PRRs	Protein kinase PKR	dsRNA-binding domain, PK domain	dsRNA
	OAS/RNaseL pathway	IFN-inducible antiviral enzymes	dsRNA
	NODs	NOD family	LPS
	RIG-I MDA-5	DEXD/H box RNA helicase family	dsRNA
	DAI (DLM-1/ZBP1)	N-terminal Z-DNA binding domain (56,57)	DNA
Complement/ Opsin	FcR		IgG-opsonized particles
	CR1		C1q, C4b, C3b, MBL
	CR2		iC3b, C3d, C3dg
	CR3		iC3b
	CR4		iC3b

***Table 2**. **Pattern-recognition receptors** others than TLRs. Adapted from (2,6,35,55) (CR, complement receptor; CRP, C-reactive protein; DAI, DNA-dependent activator of IFN-regulatory factors; FcR, Fc receptor; GPI-linked protein, glycosylphosphatidylinositol-linked protein; LBP, LPS-binding protein; LRR, Leucine-rich repeats; LTA, lipoteichoic acid; MARCO, macrophage receptor with collagenous structure; MBL, Mannose-binding lectin; MDA-5, melanoma-differentiation-associated gene 5; NOD, nucleotide-binding oligomerization domain; OAS, 2'5'-oligoadenlyate synthase; PKR, dsRNA-activated protein kinase; PRRs, pattern-recognition receptors; RIG-I, retinoic-acid inducible gene I; SAP, serum amyloid P).*

1.1.2 Adaptive immunity

The adaptive immune system uses somatically rearranged antigen receptor genes to create receptors for virtually any antigen. In contrast to the innate immunity, the onset of the adaptive immune system is slower and delayed, but it provides specific recognition of foreign antigens, immunological memory of infection and pathogen-specific adaptor proteins (2). Adaptive immune responses depend upon lymphocytes, which derive from a common lymphoid progenitor in the bone marrow. (In addition, it gives also rise to NK cells and to a minority of immature DCs.) There are two major types of lymphocytes: **T and B lymphocytes**, which are distinguished by their sites of differentiation. B lymphocytes mature in the bone marrow and precursor T lymphocytes migrate to the thymus to undergo maturation.

Mature T and B lymphocytes circulate between the blood and peripheral lymphoid tissues (e.g. lymph nodes, spleen).

The antigen recognition receptors of B cells (**BCR**) are membrane-bound immunoglobulins (Ig). Ig of the same antigen specificity are secreted as antibodies (Ab) by terminally differentiated B cells, the plasma cells. These antibodies specifically recognize the three dimensional structures of native antigens.

Each Y-shaped Ig molecule is made of two identical light chains and two identical heavy chains joined by disulfide bonds. Each of the four chains has a constant region and a variable region at its amino terminus, which presents the antigen-binding site. The nature of the heavy chain determines the isotype (IgM, IgG, IgE, IgA, IgD). The great diversity of the Ig repertoire is achieved in several ways: Separate gene segments, which are brought together by somatic recombination, encode the variable (V) regions. Then, the association of different light and heavy chain V regions to form the antigen-binding site, as well as somatic hypermutation of the coding sequences for its V regions upon stimulation of the B cells by the antigen, contribute to further diversity.

The receptor for antigen on most T cells, α:β T cell receptor (**TCR**), consists of a two-chain heterodimer, TCRα and TCRβ, that is noncovalently complexed to the signal-transducing CD3 structure. The TCR is structurally similar to a Fab fragment of the Ig. TCR genes are assembled by somatic recombination from sets of gene segments in the same way that the Ig genes are.

During thymocyte development, signals through the pre-TCR induce proliferation and differentiation at early stages of maturation. Thereafter, signals through the α:β TCR complex mediate positive and negative selection and the commitment to the CD4 or CD8 lineage. Thus, the TCR repertoire is selected during the T cell-development in the thymus in such a way that T cells of each individual recognize an antigen only in conjunction with their own **major histocompatibility complex (MHC) molecules**. TCRs are never secreted and always membrane-bound. T cells recognize short peptide fragments, produced by the proteolytic cleavage of antigens and presented by MHC molecules.

The MHC molecules are polymorphic glycoproteins encoded in a large cluster of genes, the major histocompatibility complex. (In humans these genes are called human leukocyte antigen (HLA) genes.) In general, the MHC molecules on the cell surface display peptide fragments of antigens, which are derived from proteins that are degraded inside the cell. The peptides are then bound in a pocket cleft formed by the outer extracellular domain of the MHC molecule.

There are two types of MHC molecules: MHC class I molecules are expressed on all nucleated cells and platelets, whereas only APCs express MHC class II molecules. MHC class I molecules bind peptides from proteins degraded in the cytosol by a multicatalytic protease complex, the proteasome. Peptides produced by the proteasome are transported into the endoplasmic reticulum (ER) by a heterodimeric ATP-binding protein, TAP. Once the peptide has bound to the MHC class I molecule, the peptide:MHC complex leaves the ER and is transported through the Golgi complex to the cell surface. In contrast, MHC class II molecules bind peptides from proteins that are degraded in intracellular vesicles of DCs, macrophages and B cells.

Among APC, DCs are particularly efficient in presenting antigens to T cells (58). Tissue DCs ingest antigens by phagocytosis or macropinocytosis at sites of infection and migrate to local lymphoid tissues, where they differentiate into mature DCs expressing co-stimulatory molecules. The activation of naïve T cells by APC leads to their proliferation and differentiation into armed effector T cells.

T lymphocytes can be subdivided into three main types of armed effector T cells, which produce distinct sets of effector molecules. CD8 cytotoxic T lymphocytes (CTL) are predominantly killer T cells, which recognize pathogen-derived peptides bound to MHC class I molecules.

They release perforin, granzymes and the cytokine IFNγ. CD8 T cells express the membrane-bound Fas ligand (CD178), which binds to Fas (CD95) on target cells to induce apoptosis.

Peptides from extracellular proteins generated inside intracellular vesicles are presented together with MHC class II molecules to CD4 T helper cells. As mentioned before, the differentiation of CD4 T cells into T_H1 and T_H2 cells is dependent upon the production of cytokines by APC. While IL-12 and IL-18 induce the differentiation of CD4 T cells into T_H1 cells, IL-4 promotes the differentiation into T_H2 cells. T_H1 cells activate CTL and macrophages, enabling them to destroy intracellular pathogens (cell-mediated immunity). T_H2 cells are specialized for B cell stimulation inducing humoral immune responses (59-61).

A T_H0 cell has the potential to become either a T_H1 cell or a T_H2 cell. The secretion of TGFβ by APCs supports the formation of regulatory T cells (T regs). However, the additional presence of IL-6 results in the production of T_H17 cells, which are considered to be the pathogenic T cell population during autoimmunity. The APC-derived cytokine IL-23 is critical for the maintenance and survival of these inflammatory T_H17 cells (see (62) for review). Other T cell-subsets bearing suppressive capacity are T_H3 cells and type 1 regulatory T cells (Tr1).

Adaptive immune responses cannot only eliminate a pathogen, but also generate increased numbers of differentiated memory lymphocytes through clonal selection, allowing a rapid and effective response upon reinfection with the same pathogen.

Thus, adaptive immunity provides the key elements of specificity and memory, but it also carries a risk of inducing maladaptive immune responses (e.g. autoimmunity, allergy and allograft rejection). Invertebrates and plants lack adaptive immunity and therefore do not show evidence of any inadequate immune responses (2).

1.2 Autoimmunity

Autoimmune diseases are characterized by the presence of autoreactive lymphocytes in affected tissues and/or circulating autoantibodies against self-antigens. Likewise, autoimmunity represents adaptive immune responses directed against self-antigens. In organ-specific autoimmune diseases, the effector functions target autoantigens that are restricted to particular organs: insulin-producing β cells of the pancreas (type I diabetes mellitus), myelin sheath of the central nervous system (multiple sclerosis), nicotinic acetylcholine receptor in muscles (myasthenia gravis) and the thyroid-stimulating hormone receptor (Graves' disease). In contrast, systemic autoimmune diseases such as systemic lupus erythematosus (SLE), primary Sjögren's syndrome and rheumatoid arthritis cause inflammation in various tissues, because some autoantigens are ubiquitous and abundant. All of these diseases tend to become chronically active, because their autoantigens cannot be cleared (63).

1.2.1 Systemic lupus erythematosus (SLE)

1.2.1.1 History of Lupus erythematosus (LE)

The name Lupus [Latin: wolf] erythematosus [Latin: redness] was given to the disease in the middle age, because it was thought that the skin damage resembled the bite of a wolf. LE was first described in 1828 by the French dermatologist Laurent T. Biett (1761-1840). However, it was first published in 1851 by Biett's student Pierre L. Cazenave (1795-1877). Early studies were simply descriptions of the disease with emphasis on the skin manifestations. Forty five years later, a dermatologist named Moritz Kaposi (1837-1902) noted that some patients with LE skin lesions showed signs that the disease affects internal organs. In the 1890s, Sir William Osler (1849-1919) observed that systemic LE (SLE) could affect internal organs without occurrence of skin changes. In 1948, Malcom M. Hargraves, a haematologist at the Mayo Clinic, described the LE cell, which is particularly found in the blood of SLE patients, but not in healthy individuals. His discovery made it possible to identify many more cases of SLE by using a simple blood test.

Since 1954, various proteins or antibodies that act against the patient's own tissue have been shown to be associated with SLE (see (64) for review).

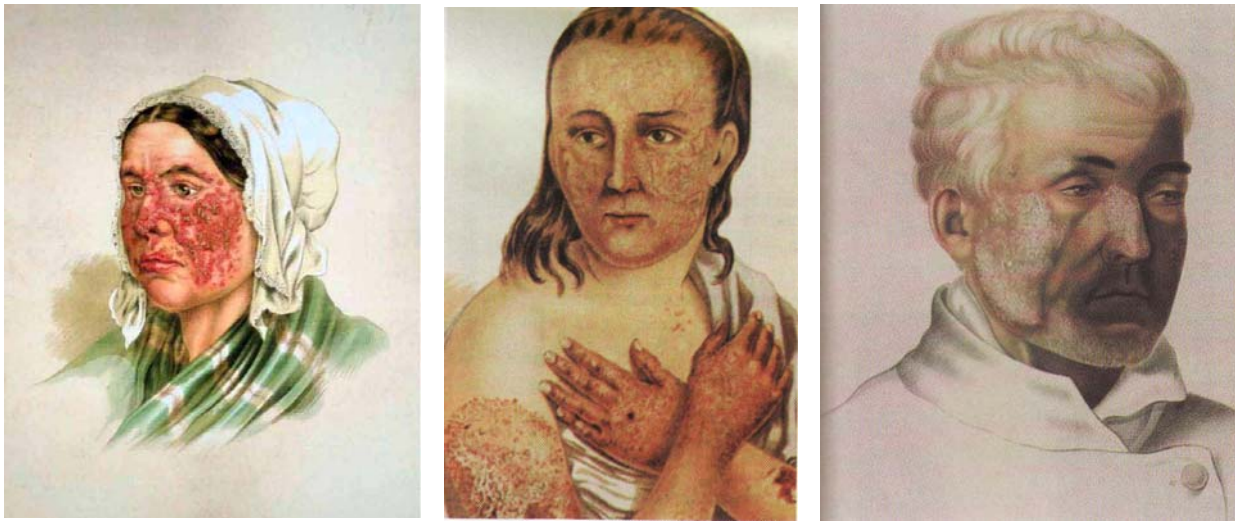


Figure 3. Lupus erythematosus*. All pictures from (65). Left and middle panel: Disseminated lupus erythematosus (From Kaposi M. Neue Beiträge zur Kenntnis des Lupus erythematosus. Arch Dermatol Syph 1872; 4: 36-78); Right panel: The first modern illustration of cutaneous lupus in 1856 (From Wallace DJ, Lyon I. Pierre Cazenave and the first detailed modern description of lupus erythematosus. Sem Arthritis Rheum 1999; 28:305-313.)*

1.2.1.2 Different forms of Lupus erythematosus (LE)

(A) Systemic lupus erythematosus (SLE)

SLE is a rheumatic inflammatory and multifactorial autoimmune disease with systemic manifestations that is caused by autoantibody production against cytoplasmic and nuclear autoantigens and immune complex deposition resulting in tissue damage.

In the following chapters, SLE will be explained in more detail.

(B) Types of skin lupus

Chronic cutaneous lupus (Discoid lupus)

The skin form of lupus erythematosus shows a particular skin rash with raised, red, scaly areas, often with healing in the centres or with scars. These eruptions typically occur on the face and other light-exposed areas.

Subacute cutaneous lupus erythematosus (SCLE)

SCLE is a non-scarring subset of lupus that is characterized by distinct immunologic abnormalities and some systemic features.

Papular lupus erythematosus

Papular lupus erythematosus is a less emphasized form of cutaneous lupus, which also occurs predominantly in light exposed regions. It is characterised histologically by a dense T lymphocyte infiltrate with relatively minor epidermal changes.

(C) Drug-induced lupus (DIL)

Several drugs, such as procainamide, hydralazine, phenytoin, chlorpromazine, α-methyldopa and others have been reported to induce a “lupus-like disease”. Drug-induced lupus is associated with the presence of anti-histone antibodies. Improvement and permanent resolution of symptoms often occur within days or weeks after discontinuation of the offending drug (66).

(D) Neonatal lupus

Neonatal lupus reflects a passively acquired autoimmunity, in which the serum of the pregnant woman contains specific antibodies against 52 or 60 kD SSA/Ro and/or 48 kD SSB/La. These autoantibodies cross the placenta and are associated with the development of congenital heart block in the foetus and/or a transient lupus rash and various liver and blood cell abnormalities in the newborn (67).

1.2.1.3 Epidemiology of SLE

The overall prevalence of SLE in the United States and Hawaii has been reported to range between 14.6 and 122 cases per 100,000 persons. Moreover, the average annual incidence of SLE in the USA has been estimated to vary from 1.8 to 7.6 cases per 100,000 persons per year. In general, prevalence and incidence rates are higher in females compared to males, and higher in African Americans, Afro-Caribbeans, and Asians than in the Caucasian population.

The mean age of diagnosis for white females ranged from 37 to 50 years. Clinical studies have revealed a female predominance approaching 90 % of SLE cases (see also (68)).

The Centres for Disease Control and Prevention (CDC) monitored SLE deaths between 1979 and 1998 and reported an increase in the annual number of deaths from 879 (1979) to 1,406 (1998) (69). Among black women, death rates were highest and increased most among those aged between 45 and 64 years.

1.2.1.4 Genetic and environmental aspects of lupus

Compared to the general prevalence of SLE, the disease is much more prevalent among family members of SLE patients (70,71). The risk for siblings of SLE patients to develop the disease is 29 times increased in comparison to the general population (72). The concordance rate in monozygotic twins (24 % to 58 %) is approximately ten times higher than the rate in dizygotic twins or in siblings (2 % to 5 %). This difference in disease concordance rates between identical and fraternal twins suggests that several genes influence the susceptibility to SLE. In addition this rather low disease concordance rate in genetically identical persons indicates that non-genetic factors also contribute to the pathogenesis of SLE (see (73) for review).

SLE represents an array of heterogeneous phenotypes and this further increases its complexity. The first evidence for linkage of 1q41-42 to SLE was first shown in targeted genome screen of human chromosome 1 region corresponding to the identified murine SLE susceptibility loci (74). Interestingly, one member of the TLRs, TLR5, maps to chromosome 1q41 (74-76), supporting a potential role for flagellated bacteria and innate immune responses in the pathogenesis of SLE.

Linkage analyses of families containing multiple members affected with SLE have further revealed several chromosomal regions that are likely to contain SLE susceptibility genes (1q23, 1q31, 2q37, 4p16, 6p21, 12q24, 16q12-13).

Moreover, within these regions many immunologically relevant genes have been associated with SLE, including MHC class II alleles, hereditary deficiencies of complement components, Fcγ receptors and cytokines. MHC class II alleles may be more strongly associated with autoantibody subsets than with the disease status of SLE because of their pivotal role in T cell dependent antibody responses. Thus, anti-dsDNA antibodies may contribute to the development of glomerulonephritis and have been associated with HLA-DR3 (77), DR2 (78,79) and DR7 (80).

The identification of specific SLE susceptibility genes will provide better understanding of the pathogenesis and will enable the development of more specific therapies in the future (see (81) for review).

In addition to inherited risk factors, there is evidence supporting the role of environmental agents in the pathogenesis of SLE. Non-infectious agents such as silica, pesticides, tobacco, solvents and sex hormones have been associated with lupus (see (82) for review). Based on animal models and limited human data, infectious agents are likely to induce autoimmune diseases (83). Thus, a number of studies evaluated the possible association of Epstein-Barr virus (EBV) with SLE. Consequently, some SLE patients showed elevated titres of anti-EBV antibodies compared to controls (84,85) and some lupus animal models can be induced by EBV antigens (86-88). There are several findings suggesting that early responses to EBV antigens lead to antibodies that are cross-reactive with lupus autoantigens (87,89). Of note, all viruses that have been associated with lupus such as EBV, Cytomegalovirus (CMV) and Varizella-zoster virus are all herpetoviruses, which persist in a latent state for years before reactivation and development of clinical disease. Finally, a variety of other agents, including allergens, drugs, bacteria, selected foods, stress, ultraviolet B radiation, vaccines, retroviruses and metals, have been proposed to be possible risks factors for SLE (82). Photosensitivity is one of the characteristics of SLE, affecting 10 % to 50 % of patients (90). There is a clear relationship between sunlight exposure and the manifestation of cutaneous lupus erythematosus and cutaneous lesions tend to occur in sun-exposed skin. Sunlight exposure, especially ultraviolet B light (UVB), can induce systemic disease. UV light may be an important initiator of apoptosis and possibly necrosis. Apoptotic cells are the principal source for the nuclear autoantigens that are immunogenic in SLE. The role of apoptosis in SLE (as well as the clearance of apoptotic cells) will be described in 1.2.1.7 Pathogenesis of SLE.

1.2.1.5 Clinical manifestations and diagnosis of SLE

Each patient with SLE has (slightly) different symptoms that can range from mild to severe. Almost all patients show musculoskeletal manifestations, such as arthritis, arthralgia or myalgia and are seropositive for antinuclear autoantibodies (ANA). About 71 % of SLE patients show skin lesions of different types.

Approximately 30 % of the patients have elevated titres for anti-dsDNA antibodies, which are a diagnostic marker of the disease, as well as low complement, cognitive dysfunction, fever, leukopenia and pleuritis. To a lesser extent, patients show central nervous system manifestations, renal involvement with proteinuria, anemia, antiphospholipid antibodies (91). The great difficulty in diagnosing SLE is to distinguish SLE from closely related connective tissue disorders. In addition to a detailed clinical history and physical examination, a careful laboratory survey has to be made including determination of the presence of ANA, rheumatoid factor (RF), anti-cyclic citrullinated peptide (CCP), creatine phosphokinase (CPK), C3 complement, Westergren sedimentation rate and a complete blood count. To define SLE according to the American College of Rheumatology (ACR) classification, patients must fulfil four or more of the eleven criteria (92), as shown in **Table 3**.

ACR criterion	**Definition**
1. Malar rash	Fixed malar erythema, flat or raised
2. Discoid rash	Raised patches with keratic scaling and follicular plugging
3. Photosensitivity	Skin rash as an unusual reaction to sunlight
4. Oral ulcers	Oral or nasopharyngeal ulcers
5. Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterized by tenderness and swelling
6. Serositis	Pleuritis or pericarditis
7. Renal disorder	Persistent proteinuria (>0,5g/d or >3+) cellular casts of any type
8. Neurologic disorder	Seizures or psychosis
9. Hematologic disorder	Hemolytic anemia or leukopenia or lymphopenia or thrombocytopenia
10. Immunologic disorder	Anti-ds DNA or anti-Sm or positive finding of antiphospholipid antibodies
11. Antinuclear antibody	Abnormal titre of antinuclear antibody (ANA)

Table 3. The 1997 revised eleven criteria for the classification of SLE from the ACR*. Adapted from (93). For identifying patients in clinical studies, a person shall be said to have SLE if any four or more of the eleven criteria are present.*

1.2.1.6 Animal models of SLE

In the past, mouse models have provided valuable insights concerning the pathogenesis of lupus. Based on how they were generated, these mouse models can be divided into three groups: (1) spontaneous lupus models, (2) induced models, and (3) engineered models of lupus.

The spontaneous mouse models have been the earliest to be reported. Several inbred and hybrid strains were shown to develop elevated levels of ANA with varying degree of lupus-like glomerulonephritis. The F1 generation of the hybrid cross between New Zealand white (NZW) and New Zealand black (NZB), (NZB/NZW) F1, is considered to be the mouse model that closely resembles human SLE. These mice develop fatal glomerulonephritis mediated primarily by immune complexes with IgG antibodies directed against dsDNA that is more severe in females (94-96). The (SWRxNZB) F1 (SNF1) mouse model is another example of a female-dominant, T-cell-dependent lupus nephritis in a hybrid mouse strain on a NZB background (97,98). The MRLlpr/lpr mice carry an lpr mutation of Fas on the lupus-prone MRL background. The massive lymphoproliferation that is associated with the autosomal-recessive lpr gene almost surely results from defective apoptosis (see (99) for review). The BXSB strain has a single genetic alteration in the *Yaa* gene and is unique in that lupus nephritis is more severe in males than in females (100). In the induced mouse models, SLE-like disease is induced in healthy mice with genetic backgrounds that do not predispose to autoimmunity by the induction of chronic graft-versus-host disease (101), injections of hydrocarbon oils, such as pristane (102), or exposure to heavy metals, such as mercury (103).

Finally, engineered mouse models, such as knockout mice, provide a unique tool for studying the potential role of individual genes in the development of lupus-like phenotypes. Typically, the inactivation of genes that downregulate accumulation and/or activation of B or T cells (e.g. Lyn, Fyn, CD22, PD-1), as well as the deletion of genes that regulate normal degradation and clearance of DNA, immune complexes or apoptotic cells (e.g. SAP, DNase I, C1q, c-mer) result in the development of lupus-like disease (see (104)).

1.2.1.7 Pathogenesis of SLE

SLE is characterized by the loss of self-tolerance and the consequent appearance of autoantibodies directed against nuclear autoantigens, most notably DNA, histones, and their complexes (105). Two major theories have been proposed to account for the correlation between infection and autoimmunity. The “antigen-specific hypothesis” is defined by the concept of molecular mimicry. Here, microbial antigens and self-antigen share structural similarities that facilitate the loss of self-tolerance. The “antigen non-specific hypothesis” points out a primary role to bystander activation, in which the immune system is activated by either an abnormal release of endogenous proteins as a consequence of pathogen-induced cell death, or by self-antigen presentating APC. In addition, a direct engagement of TLRs by inappropriately released or modified self-antigens may be sufficient to induce autoimmunity in the absence of infection (see (106)).

In SLE, there is growing evidence for infections as initial events triggering lupus-specific autoimmune responses in genetically predisposed individuals. **Bacterial or viral infections** may activate the innate immune system via PRR to promote activation of autoreactive T and B cells. Recent data supports the role of Epstein-Barr virus (EBV) as initial trigger for SLE (107,108). There is considerable evidence for molecular mimicry between EBV and regions of the Sm nucleoprotein recognized by autoantibodies from many SLE patients (109,110). Furthermore, bacterial infections have also been implicated in triggering lupus, as immunization of healthy mouse strains with bacterial DNA induced the production of anti-dsDNA antibodies (111). Bacterial DNA, but also viral DNA, differs from vertebrate DNA in a higher frequency of unmethylated CpG dinucleotides in DNA (CpG DNA) (112). Nevertheless, vertebrate DNA is not completely methylated: only 70 -80 % of the CpG dinucleotides in the vertebrate genomes are actually methylated. The immunostimulatory properties of unmethylated CpG DNA were first discovered in the context of bacterial DNA, based on the observation that bacterial DNA induces B cell proliferation *in vivo*, while vertebrate DNA does not (113). Interestingly, DNA methylation is decreased in cells from autoimmune mice and humans, which supports the hypothesis that unmethylated self-DNA may be a pathogenic factor in autoimmunity (114).

Several drugs can induce “lupus-like disease” in humans by causing DNA damage and releasing altered nucleosomes (115-118), in addition to inhibiting CpG methylation (119).

The innate immune system uses **TLR9** to detect the presence of unmethylated CpG dinucleotides as a signal for infection. Upon detection of CpG motifs, B cells are stimulated to proliferate and secrete Ig, whereas DCs release cytokines, interferons and chemokines that promote T cell help (120). Although its activity is not clear, chloroquine was accidentally found to be an effective therapy for systemic autoimmunity. Chloroquine and other compounds that interfere with endosomal acidification and maturation specifically block all CpG-mediated signals in leukocytes (121).

Studies of AM14 transgenic mice, whose B cells express antibodies specific for self-IgG, also known as rheumatoid factor (RF), revealed a link between nucleic acid-sensing TLRs and B-cell activation (122). It was shown that immune complexes containing self-DNA activate RF-positive autoreactive B cells via dual engagement of the BCR through the Ig portion of the immune complex and activation of TLR9 through the DNA portion of the immune complex. Furthermore, this stimulation was DNase sensitive and MyD88 dependent.

In the following, it was demonstrated that hypomethylated CpG motifs in mammalian DNA help activating chromatin-specific autoreactive B cells in a TLR9-dependent manner (123). Likewise, chromatin-containing immune complexes activate DC. The importance of DNA as an autoimmune stimulus was also supported by the finding that DNase I knockout mice develop a lupus-like syndrome (124).

Other endosomal TLRs, such as **TLR3, TLR7 or TLR8**, recognize viral ssRNA or dsRNA. Importantly, RNA and RNA-associated autoantigens can activate autoreactive B cells through sequential engagement of TLR7 and the BCR (125).

Consistent with a role for TLRs in autoimmune disease, **MyD88**, an adaptor protein, involved in the signalling of all TLRs (except TLR3), plays an important role in autoantibody production and antibody class switching (126,127) in MRL-lpr/lpr and FcγRIIB-deficient mice (125,127).

Although, in one study TLR9 protected MRL-lpr/lpr from the disease (128), Christensen et al. confirmed that the formation of anti-chromatin and anti-dsDNA autoantibodies was impaired in the absence of TLR9 (129).

In agreement with these results is the finding that the genetic absence of TLR9 prevented the formation of anti-nucleosome antibodies (130).
Thus, TLR9 is required for the generation of high-titer antibodies to DNA-containing antigens in murine lupus (131).
Among many early clues, it was noted that **type I interferons** (IFNα and IFNβ) might have pathogenic importance in lupus (132,133).
Subsequently, IFN therapy of certain malignancies and persistent viral infections was shown to induce autoimmunity (134). Furthermore, elevated levels of IFNα have been observed in SLE patients (135), and IFNα in the serum of lupus patients induced normal monocytes to differentiate into DCs (136). **Plasmacytoid DCs** (pDCs) have the remarkable capacity to secrete large amounts of IFNα and IFNβ upon exposure to viral infections (137), especially upon TLR7/9 triggering.
Although pDC numbers are reduced in SLE blood, these cells strongly infiltrate inflamed tissues, lupus skin lesions and lymphoid organs (138,139). Chromatin-containing and snRNPs-containing immune complexes and Ig-opsonized apoptotic cells are internalized by pDCs via FcγRIIa and reach the endosomal compartment where they activate TLR9 and TLR7, respectively, leading to the secretion of cytokines including **IFNα and IFNβ** (140-144).
In the steady state, immature **myeloid DCs** (mDCs) capture self-antigens (e.g. apoptotic cells) and migrate, without maturing, to the draining lymph node. There, they present self-peptide-MHC complexes in the absence of costimulation signals to naïve autoreactive T cells, resulting in their anergy or deletion. In addition, they may control peripheral tolerance through the induction of regulatory T cells. Such tolerance mechanisms prevent the development of autoimmunity when dying cells are generated or processed during infections. Accordingly, the sustained overproduction of IFNα/β or pathogens triggering TLRs may induce the activation of immature DCs, which control the peripheral tolerance by deleting autoreactive lymphocytes. These mature DCs now express co-stimulatory molecules and activate autoreactive T cells, both helping autoreactive B cell development (145,146). The production of pathogenic autoantibodies in SLE is T cell-dependent (147-152). In mouse models of SLE, depletion of **CD4+ T cells** blocks the disease onset (153), and athymic mice do not develop lupus (154,155).

Thus, polarized CD4+ T cells can provide help to autoreactive B cells, completing an activation cycle between B cells, DCs and T cells. Certainly, the provision of T cell help may be the limiting step in the alteration from initial B cell activation to autoimmunity and autoantibody production (156,157).

Some autoantibodies fix directly to target organs, such as platelet surface molecules or α-actinin in the glomeruli (158). Other autoantibodies form pathogenic immune complexes (IC) of correct size, charge, conformation and antigen-reactivity to bind to tissues (platelets, glomeruli, skin, blood vessels). Antibody-dependent cell cytotoxicity or complement activation with subsequent inflammation can result, leading to clinical disease and to tissue damage.

IFNα/β-stimulated mDCs trigger B cell growth and differentiation through IL-12 and IL-6 (159), as well as B cell-activating factor (BAFF/BlyS) (160). Importantly, mDCs stimulated with chromatin-containing immune complexes secrete BAFF (142), and excess levels of BAFF have been implicated in systemic autoimmune disease (161,162). pDCs are considered the main producers of IFNα/β in SLE. Upon viral infection, healthy pDCs secrete IFN followed by the production of other cytokines, including TNF, which terminates the release of IFNα/β. Genetic alterations in this dynamic system might prevent the shutdown of IFN production, as demonstrated by the SLE-like syndrome in mice deficient for the suppressor of cytokine signalling-1 (SOCS-1) (163). Furthermore, increased levels of soluble TNF receptors in SLE may also contribute to the sustained IFN production by blocking TNF (164). And finally, the above-mentioned presence of DNA- or RNA-containing immune complexes may sustain IFN production by pDCs through triggering TLR9 and TLR7 (125,140,165).

Despite the clinical heterogeneity, different lupus mouse models and SLE patients have autoantibodies specific for ubiquitous self-antigens in common. Indeed, the repertoire of these autoantigens is unlimited and antibodies directed against nuclear components are prominent. A breakthrough in determining the probable source of autoantigens in SLE was provided by Rosen and co-workers, who established that autoantigens targeted in SLE are clustered in blebs at the surface of apoptotic cells (166,167). This led to the assumption that dying cells provide the source of autoantigens responsible for the autoantibody production in SLE.

Apoptosis or programmed cell death is an active process that leads to the ordered destruction of cells, avoiding the release of intracellular contents into extracellular microenvironment, where they could have inflammatory effects. Apoptotic cells undergo a series of morphological and biochemical changes, including alteration of the phospholipid membrane, cytoskeletal disruption, cell shrinkage and a characteristic pattern of DNA fragmentation.

During apoptosis, lupus autoantigens undergo post-translational modifications including cleavage, phosphorylation and oxidation. This autodigestion could generate “neoepitopes”, which might appear as non-self to the immune system (168). Interestingly, injection of apoptotic cells into nonautoimmune mouse strains induced an autoantibody response (169). SLE patients and lupus mice were shown to have an increased rate of apoptosis *in vitro* (170-172).

Additional evidence implicating the products of dying cells in the immunization of SLE patients includes the presence of nucleosomes in the circulation of lupus patients with active disease (173). Oligo- and mononucleosomes are generated by internucleosomal cleavage of chromatin during apoptosis (174) and packed in apoptotic blebs along with other nuclear components (166). As a result of clearance defects, apoptotic cells can become late apoptotic and enter secondary necrosis, where the nuclear membrane becomes permeable causing the release of intranuclear components, such as nucleosomes, which favours inflammation (175). Like several spontaneous lupus mouse models, SLE patients are reported to have impaired clearance of apoptotic cells (176). Thus, increased apoptosis of mononuclear cells and a failure to clear them, results in the persistent T cell exposure to nucleosomal antigens (166,177). Defects in T cell activation result in a decreased threshold for T cells to respond to the antigen (178). In addition, DCs and B cells have an enhanced capacity to present nucleosomal autoantigens (179). Accordingly, it was demonstrated that the Fc-mediated clearance of immune complexes is defective in patients with SLE (180).

Furthermore, decreased macrophage phagocytosis (181-183), decreased levels of the chromatin-binding C-reactive protein (CRP), and decreased levels of the early complement components (184,185) result in persistent high concentrations of potentially pathogenic autoantigens.

CRP and serum amyloid P (**SAP**) are the major acute-phase serum proteins found in mice and humans, respectively. They share the ability to selectively recognize dying cells and thereby mediating their uptake by phagocytic cells (186,187).

Interestingly, the administration of CRP to lupus-prone NZBxNZW F1 mice has been shown to reduce autoantibody levels and to prolong survival (188). SAP-/- mice spontaneously develop autoimmunity and glomerulonephritis (189).

Decreased activity of **deoxyribonuclease I** (**DNase I**), the main nuclease in serum, secretions and urine, was also reported in SLE patients, particularly in patients with active nephritis (190). Serum DNase I is responsible for the degradation of nuclear material, which is released by primary and secondary necrotic cells. In humans, DNase I acts together with C1q and the plasminogen system to efficiently digest chromatin derived from necrotic cells (191,192). DNase-/- mice show the classical symptoms of SLE, namely the presence of ANA, the deposition of immune complexes in glomeruli and full-blown glomerulonephritis (124).

Mice with mutations in the structurally related tyrosine kinases Mer and Tyro3 show an impaired apoptotic cell clearance and an increased incidence of autoimmunity (193,194). Mice deficient for the secreted form of **IgM** develop lupus-like autoimmunity (195,196).

Complement was implicated in the clearance of apoptotic cells by the observation that **C1q** could bind *in vitro* directly and specifically to surface blebs on apoptotic keratinocytes (197). Furthermore, it was shown that the opsonization of apoptotic cells by C1q allows and facilitates their uptake by macrophages or DCs (198). C1q, C4 and C3 serum concentrations are reduced in SLE patients, resulting in an impaired uptake of apoptotic cells by macrophages (199).

Homozygous deficiency of any of the early components of the classical pathway of the complement cascade (C1q > C4 > C2) predisposes to the development of SLE. In fact, inherited deficiencies of C1q and C4 are invariably associated with the development of severe, lupus-like disease in early life, whereas C2 deficiency is only weakly associated with a milder form of SLE (184). For example, mice lacking the functional complement protein C1q develop significant titres of antinuclear antibodies and proliferative glomerulonephritis, characterised by an increased number of uncleared apoptotic cell bodies (200).

Paradoxically, the complement system plays a role in maintaining immune tolerance to prevent the development of SLE (201-203), but it also participates in tissue-destructive inflammatory processes once SLE is established in a patient (204,205). Finally, alterations of complement receptors and Fcγ receptors may also contribute to clearance defects in patients with SLE.

1.2.1.8 Autoantigens and autoantibodies in SLE

Autoimmunity in SLE is directed to some highly conserved and ubiquitously expressed, intracellular molecules, particularly against nuclear and cell membrane phospholipid components (206 and **Table 4**). Indeed, autoantigens detected in SLE are mostly macromolecules with protein and nucleic acid constituents such as snRNP, Ro, La, nucleosomes or ribosomes. An important aspect of SLE is the multiplicity of the autoantibodies. Recently, a summary of “all” 116 autoantibodies so far reported in SLE has been published (207).

High-affinity **antibodies to dsDNA** are a characteristic hallmark of mouse and human SLE. Some subsets of these antibodies cause renal and vascular injury (208,209). DNA as antigen might occur as dsDNA or as ssDNA, however, it is present *in vivo* in the form of circulating nucleosomes (173). (Nucleosomes and anti-nucleosome antibodies will be the key issue of the next chapter and is therefore not discussed here.) Interestingly, some non-pathogenic anti-DNA antibodies can be also found in normal healthy individuals. SLE pathogenic anti-DNA antibodies, however, are thought to be high-avidity IgG reacting with dsDNA and are somatically mutated as a result of an antigen-driven selection process (210).

Autoimmune responses towards DNA or RNA, characteristic of SLE, require T cell-help for the production of high-affinity antibodies. In contrast to bacterial DNA, human DNA is poorly immunogenic and does not bear T cell epitopes. Interestingly, mammalian DNA becomes strongly antigenic when combined with a highly charged fusion protein, as for example histones. Therefore, it has become widely accepted that DNA-binding protein in complex with DNA is required to overwhelm tolerance to DNA (211).

SLE is typically considered as an immune complex-mediated disease. In this concept, anti-dsDNA antibodies bind nucleosomal DNA released from apoptotic cells and induce classical immune complex-related damage in glomeruli, skin and blood vessels. Anti-dsDNA antibodies might also cross-react with non-DNA kidney-specific antigens inducing antibody-mediated injury. Finally, anti-dsDNA antibodies bind to planted antigens, either DNA or nucleosomes, previously bound to glomerular basement membrane, leading to *in situ* immune complex formation (212).

Anti-Sm antibodies are directed against seven proteins (B/B', D1, D2, D3, E, F, G) that constitute the common core of U1, U2, U4 and U5 small nuclear ribonucleoprotein (snRNP) particles. **Anti-RNP antibodies** react with proteins (70 kD, A, C) that are associated with U1 RNA and form U1SnRNP (213,214). The snRNP are RNA-protein complexes that are abundant in the nuclei of all eukaryotic cells and play a role in RNA splicing. Anti-Sm and anti-RNP antibodies are both detectable in some SLE patients. The measurement of these antibodies is more important in the diagnosis of SLE, than in the follow-up of the patients. While anti-RNP antibodies are more prevalent in patients with Raynaud's phenomenon and associated with milder renal involvement, anti-Sm antibodies are linked to severe and active renal disease (see (215) for review).

Anti-Ro/SSA and **anti-La/SSB antibodies** are also directed against RNA-protein complexes. Anti-Ro/SSA antibodies are most prevalent among many autoimmune diseases (SLE, Sjögren's syndrome/SLE overlap syndrome, subacute cutaneous lupus erythematosus, neonatal lupus and primary biliary cirrhosis) (see (216) for review). In contrast, anti-La/SSB antibodies are more closely associated with Sjögren's syndrome. Anti-La/SSB antibodies are usually accompanied by anti-Ro/SSA antibodies and more frequently detected in patients with rheumatoid factor, polyclonal hypergammaglobulinemia and cryoglobulinemia (217).

Anti-ribosomal P antibodies are of particular importance, because they are highly specific for SLE (218,219). They recognize three specific ribosomal proteins, termed P0, P1 and P2 (220). Elevated titers of anti-P antibodies are mainly detected during active disease and may be associated with lupus nephritis, chronic active hepatitis (221-223) and neuropsychiatric manifestations (see (224) for review).

Importantly, it has been demonstrated, indeed, that the penetration of anti-ribosomal P Abs into living cells leads to the inhibition of protein synthesis (225-227), suggesting a possible pathogenic mechanism for these autoAbs. As anti-ribosomal P Abs are linked to neuropsychatric manifestations in SLE, intracerebroventricular injection of these antibodies induced autoimmune depression in mice via the limbic system (228). Interestingly, ribosomal P proteins are not only the antigenic target for anti-ribosomal P autoantibodies, but also for immune responses against some parasitic infections, such as Chagas disease triggered by Trypanosoma cruzi (229,230).

Type of autoantibody	**Target autoantigen**	**Frequency in SLE (%)**
Antinuclear antibody (ANA)	Nuclear antigens	98
Anti-nucleosome	Nucleosome	56 - 70
Anti-dsDNA	dsDNA	40 - 60
Anti-ssDNA	ssDNA	70
Anti-histone	H1, H2A, H2B, H3, H4	70
Anti-Sm	snRNP proteins B/B', D1, D2, D3, E, F, G	20 - 40
Anti-U1RNP	snRNP proteins A, C, 70kD	30 - 40
Anti-A/Ro SSA	52 and 60 kD proteins complexed with Y1-Y5 RNAs	30 - 45
Anti-La/SSB	48 kD protein complexed with various small RNAs	10 - 15
Anti-ribosomal P	38, 17, 19 kD ribosomal phosphoproteins	10 - 12
Antiphospholipid	Various phospholipid-binding proteins, phosphotidylserine, cardiolipin	30

Table 4. Autoantibodies in SLE*. Adapted from (231 and (232-235)). Prevalence (in % of patients) of the most frequent autoantibodies in SLE. (See text for abbreviations.)*

1.2.1.9 Therapy of SLE

Current therapies for SLE are broad spectrum and include non-steroidal anti-inflammatory drugs (NSAIDs), antimalarials, corticosteroids and immunosuppressive drugs. Patients with mild lupus can generally be maintained on a combination of NSAIDs and antimalarials (e.g. hydroxychloroquine). Relatively low doses of corticosteroids are used when NSAIDs and antimalarials have failed to control symptoms of arthritis or rash sufficiently. In the case of aggravation of symptoms with potential life threatening sequelae, corticosteroids are used at higher doses. The onset of active lupus with major organ involvement is often rapid and requires a prompt and aggressive therapy with immunosuppressive agents, such as cyclophosphamide, azathioprine, cyclosporine or methotrexate. A relatively new immunosuppressive drug is Mycophenolate mofetil in the treatment of severe lupus nephritis, refractory to other cytotoxic agents. Other standard treatments are intravenous high doses of immunglobulins in lupus patients with severe thrombocytopenia or neutropenia (see (236) for review). In addition there are potential novel therapies in development or at clinical trial phase, which have B cells, T cells, complement or cytokines as targets (237,238). The treatment with the B-cell depleting CD20 antibody (rituximab), for instance, improved clinical symptoms in lupus patients (239).

Indeed, weighing potential benefits of treating the active disease against the risks of therapy induced side effects, often represent a considerable challenge in the treatment of SLE.

1.2.2 Nucleosome

In this part, the particular role of nucleosomes in SLE is going to be discussed in addition to the more common facts about lupus described above.

1.2.2.1 The nucleosome structure

Nucleosomes are the basic packing unit of eukaryotic DNA. Unfolded chromatin can be seen under the electron microscope as a series of “beads on a string”. The string represents DNA, and each bead stands for a nucleosome particle (relative molecular mass of 260 kD) that consists of DNA wound around a protein core, formed of histones.

Each individual nucleosome consists of a complex of eight histone proteins - two molecules each of histones H2A, H2B, H3 and H4 – assembled in an octamer around which 146 bp of dsDNA are wound, forming the core particle. This complex is stabilized by one copy of histone H1, outside the core in contact with DNA, to form a mononucleosome with 180 bp DNA.

The nucleosomes are separated from each other by a region of linker DNA, which can vary in length from a few nucleotide pairs up to about 80. The formation of nucleosomes converts a DNA molecule into a chromatin thread about one third of its initial length, and this provides the first level of DNA packing (**Figure 4**).

The high-resolution structure of the nucleosome core particle was solved in 1997 (240). It revealed a disc-shaped histone core around which the DNA was wrapped 1.65 turns in a flat, left-handed coil. All core histones are relatively small proteins (102–135 amino acids), rich in lysine and arginine and positively charged. The histone octamer is divided into histone folds, one time H3–H4 tetramer and two times H2A-H2B dimmers (**Figure 5**).

Histones are present in such large amounts in the cell that their total mass in chromatin is about equal to that of the DNA. As might be expected from their fundamental role in DNA packaging, the histones are among the most highly conserved eukaryotic proteins.

Besides its function in DNA packaging, nucleosomes are involved in gene expression, DNA replication and DNA repair (see (241)).

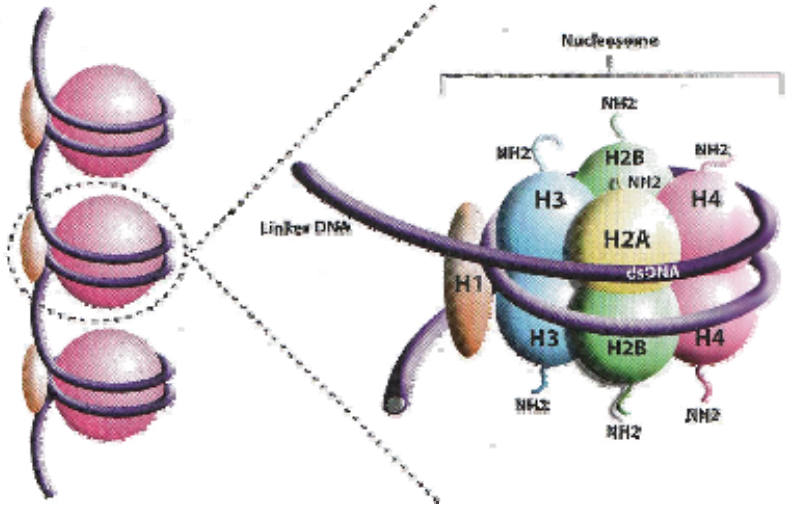


***Figure 4**. Schematic representation of the **nucleosome core particle**. From (242).*

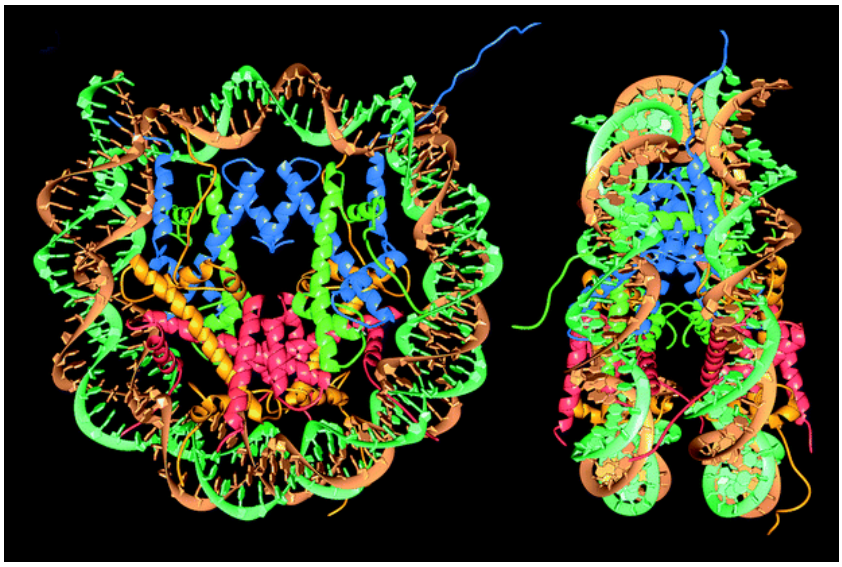


***Figure 5**. **Nucleosome core particle**. Adapted from (240). 146 bp DNA phosphodiester backbones (brown and turquoise) and eight histones (H3, blue; H4, green; H2A, yellow; H2B, red). The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle.*

1.2.2.2 Circulating nucleosomes: abnormal apoptosis and clearance deficiency

During the process of **apoptosis**, oligo- and mononucleosomes are generated by internucleosomal cleavage of the DNA. The nucleosomes, along with other nuclear components, become clustered and concentrated within and on the surface of apoptotic blebs (166,243).

In vivo, these blebs are released into the circulation and rapidly cleared by several mechanisms, including direct complement binding, complement binding through CRP, SAP, MBL and IgG binding, followed by phagocytosis (244-250).

However, in patients with SLE, free nucleosomes are found in the circulation (173) and the plasma concentration of nucleosomes is significantly higher in patients than in normal healthy individuals.

As mentioned above, accelerated apoptosis and impaired clearance of apoptotic cells might be a reason of the pathogenesis of SLE, since nuclear autoantigens are exposed and then released during late phase of apoptosis and secondary necrosis, respectively.

Deficiencies in serum proteins that assist in the recognition and clearance of apoptotic cells are associated with the development of SLE. Likewise, alteration of cell surface receptors involved in the recognition of apoptotic cells or apoptotic cell material might impair the clearance of dying cells.

The **complement system** plays an important role in the clearance of immune complexes and dying cells and is therefore considered to be involved in the pathogenesis of SLE. Numerous patients with SLE develop hypocomplementemia as a secondary event, which is often associated with antibodies against C1q (anti-C1q) (251). Furthermore, 90 % of patients with homozygous C1q deficiency develop a lupus-like syndrome (184,201,202). Interestingly, our group has recently found that free nucleosomes interact with **C1q**. This interaction resulted in an enhanced nucleosome uptake by human PMN, which express C1q receptors on the cell surface (Erbacher et al., manuscript in preparation).

Members of the collectin family (C1q, MBL) and the pentraxin family (CRP, SAP) bind to apoptotic blebs at the cell surface (187,249,252,253). **SAP** and **C1q** are both thought to be important in the clearance of chromatin exposed on apoptotic cells (189,203,254).

Interestingly, both SAP and CRP recognize chromatin as well as purified nucleosome core particles *in vitro* (189,255).

Since nucleosomes were found to be exposed on apoptotic blebs, they might become accessible for interactions with the immune system (243). Interestingly, nucleosomes from cells undergoing apoptosis were shown to have an unusual histone composition (256), as well as an abnormal DNA methylation and deoxycytosine-deoxyguanine content (257).

In MRL lupus-prone mice, nucleosomes are persistently present in the circulation. However, LPS administration was shown to increase the systemic nucleosome release due to an enhancement of apoptosis and a decrease in the clearance of apoptotic cells (258).

As aforementioned, abnormalities of phagocyte function in patients with lupus have been reported (259). Thus, monocyte-derived macrophages from lupus patients showed an impaired uptake of apoptotic cell material *in vitro* (176,260). The reduced phagocytic ability of macrophages from patients with SLE was confirmed in another study, showing also an increase in the number of apoptotic PMNs and macrophages (261). Phagocytosis of autologous apoptotic cells by macrophages is impaired in the presence of sera from diseased lupus mice (262). Evidence exists to suggest that high levels of free nucleosomes contained in the sera (258) may inhibit the uptake of apoptotic cell material (263).

DNase I may be responsible for the removal of DNA from circulating nuclear autoantigens at sites of high cell turnover. Therefore, the activity of DNase I might prevent the development of SLE through the clearance of circulating nucleosomes, whereas the reduction or loss of DNase I activity may result in autoimmune responses in genetically predisposed individuals. Thus, serum DNase I activity was reported to be decreased in patients with SLE, being lowest in patients with active nephritis (190). Furthermore, DNase I knockout mice develop lupus-like symptoms, namely presence of ANA and glomerulonephritis (124).

After all, the elimination of circulating DNA-protein complexes seems to be a crucial event in the prevention of SLE.

1.2.2.3 Nucleosome: major autoantigen in SLE

The combination of several deficiencies, genetic predisposition and/or environmental triggers may lead to the presence of circulating nucleosomes in patients with SLE and lupus mice. These circulating nucleosomes, free or in the form of immune complexes, have certain effects on immune cells and may be involved in the pathogenesis of SLE. Thus, the levels of circulating nucleosomes were elevated in SLE patients with active central nervous system and renal involvement (264). And both anti-nucleosome and anti-dsDNA IgG antibody activities were found to correlate with SLE disease activity index (SLEDAI) scores (265).

Although it is believed that nucleosomes give rise to the production of anti-dsDNA antibodies, anti-dsDNA are a hallmark of SLE (266,267). The notion that nucleosomes may be the *in vivo* targets of some anti-dsDNA antibodies was supported by specificity studies of lupus polyclonal sera or monoclonal antibodies by ELISA, with nucleosomes as substrates (268). The nucleosomal antibody reactivity was first detected in monoclonal antibodies that were isolated from lupus-prone MRL/Mp(-)+/+ mice (269) Nucleosome-specific autoantibodies were then described in lupus mice and later in SLE patients, showing that early autoantibodies recognized discontinuous epitopes on native chromatin and the (H2A-H2B)-DNA subnucleosome (115,147). Importantly, it has been shown that a large part of anti-nucleosome activity is really due to nucleosome-restricted autoantibodies (115,270,271) and was proven to be distinct from anti-dsDNA and anti-histone reactivities. These nucleosome-restricted autoantibodies react against quaternary nucleosomal epitopes, meaning that they recognize the whole nucleosome structure and not its individual components (DNA and histones). It thus seems that the nucleosome is the autoantigen leading to the production of anti-dsDNA, anti-histone and nucleosome-restricted antibodies, which compose the broad family of antinucleosome antibodies (see (272) for review).

The development of nucleosome-specific antibodies during the disease supports the key role of nucleosomes in the pathogenesis of SLE.

In addition to nucleosome-restricted antibodies, nucleosome-specific Th lymphocytes are detected in lupus patients (117,273). Anti-nucleosome antibodies were shown to arise before anti-dsDNA and anti-histone antibodies emerge and they are thought to be nephritogenic in murine lupus (270).

In contrast to anti-dsDNA antibodies, anti-nucleosome antibodies are present in SLE patients with active and inactive disease, suggesting a more sensitive marker for SLE. Moreover, anti-nucleosome antibodies are found in anti-dsDNA negative patients (232,265,271). Anti-nucleosome IgG were found to be only present in a restricted set of connective tissue disease, namely SLE, systemic sclerosis and mixed connective tissue disease. Of note, anti-nucleosome antibodies of the IgG3 subclass are present at high levels in active SLE and the levels were shown to positively correlate with the disease activity. Besides, the levels of IgG3 anti-nucleosome antibodies are increased during flares and this increase was closely associated with active nephritis (232).

Nucleosome-specific CD4+ T cells have been identified in lupus-prone mice long before disease development (274). Interestingly, it was demonstrated that a single nucleosome-specific Th clone could provide help to dsDNA-, histone- or nucleosome-specific B cells, which results in the cognate interaction between B cells and nucleosome-specific T cells. Nucleosome-specific Th cells only recognize some major autoepitopes in the core histones of nucleosomes (117,275), and a more recently found histone H1' epitope (22-42), which is also recognized by autoimmune B cells (276). These nucleosome epitopes may be important for the disease process, because their injection induced the development of severe glomerulonephritis (275,276).

In agreement, long-term therapy with nucleosomal peptides was found to prolong survival and halt progression of renal disease in (SWR x NZB)F1 mice (277). Finally, chronic nasal instillation of a peptide containing an autoreactive Th cell epitope of histone H4 suppressed the development of autoantibodies and reduced the incidence of glomerulonephritis in lupus-prone mice (278).

In another study, Th cells from normal BALB/c mice immunized with purified nucleosomes, recognize a histone peptide from the C-terminal region of histone H4, suggesting that histone-specific Th cells are not deleted in the normal repertoire of naïve BALB/c mice (279). This observation implies that large amounts of autoantigen, when above the threshold tolerated by T cells for activation, might bypass peripheral tolerance.

As discussed above, alterations in the functions of DCs may be involved in the development of SLE (136). Thus, genomic dsDNA released from dying cells was shown to induce the maturation of APC after transfection (280). Furthermore, free nucleosomes directly induced DC maturation *in vitro* (281). Interestingly, monocyte-derived DCs (MDDCs) from SLE patients were found to be pre-activated in contrast to MDDCs from normal healthy individuals (282). Alternatively, autoreactive nucleosome-specific B cells also play a role as APC in SLE. Although B cells are believed to be less efficient in priming naïve T cells, they might be involved in recall autoimmune T cell stimulation (see (283) for review).

1.2.2.4 Nucleosome in the pathogenesis of SLE

Nucleosome-specific and anti-DNA antibodies were shown to be present in kidney eluates of proteinuric lupus mice (270), suggesting a potential nephritogenic role. Furthermore, antinucleosome antibodies were isolated from renal eluates of SLE patients suffering from nephritis (284). It is noteworthy, that some monoclonal nucleosome-specific antibodies isolated from young, preautoimmune mice have been found to deposit in the glomeruli *in vivo*, meaning that nucleosome-specific antibodies may initiate kidney lesions early in the disease before anti-DNA antibodies arise (285). Taken together, anti-nucleosome antibodies are believed to be pathogenic in the form of immune complexes upon deposition in kidneys, causing inflammation and tissue damage in the form of a type III hypersensitivity reaction (283).

Nucleosomes may mediate binding of antinucleosome antibodies to form immune complexes that deposit or form *in situ* in the kidney. Thus, it was observed that some anti-dsDNA and anti-histone antibodies cross-react with heparan sulfate, an intrinsic component of the glomerular basement membrane (GBM) (286). It was demonstrated that nucleosome-restricted mAb bind to GBM *in vivo*, partly via heparan sulfate, and activate the complement system, but only when complexed with nucleosomes (287), suggesting that nucleosomes bridge antibodies to target cells via the positively charged histone tails, which bind to heparan sulfate.

Experiments showing that monoclonal nucleosome-specific antibodies complexed to nucleosomes bound to rat GBM when perfused *in vivo*, while nucleosome-specific antibodies alone did not, provided the first evidence for nucleosome-mediated antibody deposition (287). This nucleosome-mediated binding was thought to occur through the interaction of the cationic N terminal tails of the core nucleosomal histones with GBM heparan sulfate. This binding decreased after renal perfusion of heparinase, and the staining with anti-heparan sulfate antibodies in the GBM was almost completely absent in both human and murine lupus with nephritis, probably due to immune complex deposition (287,288). In contrast to monoclonal anti-histone antibodies, immune complexes formed by anti-DNA or anti-nucleosome antibodies and nucleosomes are considered to be pathogenic, because they bind different nucleosomal epitopes and leave the histone tails exposed to bind anionic components of the heparan sulfate in the GBM (289). The latter study also showed that nucleosomes are present in nephritic kidneys.

Furthermore, prophylactic treatment of lupus-prone mice with heparin, which acts as heparan sulfate decoy, prevented the development of proteinuria and glomerulonephritis (290). Nevertheless, other studies with lupus mice and SLE patients revealed type IV collagen, another major component of the GBM, as potential ligand for nucleosome/antinuclear immune complexes (291,292).

Nucleosomal antigens were detected in the epidermal basement membrane of nonlesional skin of SLE patients, indicating that nucleosome-mediated binding of autoantibodies to basement membranes occurs at sites in the body other than in the glomerulus (293).

As mentioned above, chromatin-containing immune complexes activate rheumatoid factor-positive B cells via dual engagement of the BCR and TLR9. Furthermore, the activation of murine myeloid DCs by chromatin-containing immune complexes occurs through a combined involvement of FcγRIII and TLR9. Nevertheless, chromatin-containing immune complexes also activate murine mDCs in a TLR9-independent pathway, namely in conjunction with reagents that cross-link CD40 on DCs (see (106) for review).

Besides having a role in mediating autoantibody production and immune complex deposition, the nucleosome itself may be pathogenic through binding to cell-surface receptors on different cell types (294-298). It is noteworthy, that free nucleosomes might be pathogenic through direct interaction with target cells without requiring immune complex formation. Thus, nucleosomes can elicit various biologic activities *in vitro*, such as IL-6 secretion by mouse spleen cells, stimulation of lymphoproliferation and IgG production by splenic B cells (295,299,300). In agreement, nucleosomes were shown to inhibit NK cell-mediated toxicity *in vitro* (301).

Furthermore, it was shown that purified nucleosomes induced necrosis-like cell death of lymphocytes (302). Here, purified nucleosomes induced primary necrosis of lymphocytes from normal healthy donors and SLE patients *ex vivo*, in contrast to human DCs, which were relatively resistant. Finally, it was found that nucleosomes, as a mimic of increased cell apoptosis *in vivo*, specifically inhibited the phagocytosis of apoptotic cells by MRL+/+ macrophages (263) and influence the glomerular mesangial cell function (303), suggesting a pathogenic role for nucleosomes independent of their immune complex construct.

1.3 Aim of the study

SLE is a multifactorial, inflammatory autoimmune disease of unknown aetiology. Nucleosome is a major autoantigen frequently targeted in SLE. Of note, nucleosomes play a crucial role in the pathogenesis of SLE and were shown to elicit many biologic activities in various cell types.

Neutrophils - the most abundant type of leukocytes - are an essential component of the innate immune system. Circulating neutrophils are rapidly recruited to sites of infection and inflammation, where they bind and ingest microorganisms, which trigger the production of reactive oxygen species. Besides their protective functions, they also contribute to host cell and tissue damage and are known to link innate and adaptive immunity. Interestingly, sera from SLE patients have been shown to contain an activity responsible for neutrophil activation (304). In addition, the level of neutrophil activation is directly correlated with the disease activity (305).

Thus, it is likely, that nucleosomes in the serum of SLE patients may be responsible for neutrophil activation and that the level of circulating nucleosomes may be correlated to neutrophil activation and therefore to inflammation.

For these reasons, we aimed to determine if purified free nucleosomes were able to directly activate human and mouse neutrophils *in vitro*.

Subsequently, we wanted to compare nucleosome-induced activation of neutrophils from SLE patients and healthy volunteers in order to exclude any intrinsic differences.

Furthermore, we questioned if nucleosomes are able to recruit and activate neutrophils *in vivo* in mice.

We further wanted to investigate how this activation and recruitment of neutrophils is achieved and what kind of signalling cascade might be involved in this mechanism.

After all, the aim of this work was to evaluate the pathogenic role of free nucleosomes in the development of SLE, which might help to understand the crucial distinction between self and non-self in the innate immune system and to reveal novel therapeutic approaches for the treatment of SLE.

2 Materials and methods

Instruments and materials

- Tecnoflow 3F 120-II GS	Integra Bioscience
- Water Jacketed Incubator	Labotect Labortechnik
- Centrifuge, Megafuge 1.0	Heraeus Instruments
- Centrifuge, Labofuge 400	Heraeus Instruments
- Centrifuge, Biofuge pico	Heraeus Instruments
- Portable balance	Sartorius
- Stirring and hot plate RCT basic	IKA Labortechnik
- Water bath	Köttermann Labortechnik
- Shaker Rocky	Fröbel Laborgeräte
- Vortex Genie2	Scientific Industries
- Electrophoresis Power Supply EPS3500	Amersham
- Gel documentation system	Vilber Lourmat
- Thermomixer 5436	Eppendorf
- MilliQ ultra pure water purification system	Millipore
- Pharmacia Bio Tech Ultrospec 3000 UV/Visible Spectrophotometer	Amersham
- Leica DM IL inverted contrasting microscope	Leica
- Pipetman P2, P10, P20, P100, P200, P1000	Gilson
- Finnpipette Focu Multichannel Pipette	Thermo Fisher Scientific
- Pipetboy acu	Integra Biosciences
- Reagent stand	Carl Roth

Consumables

- 15 ml polystyrene conical tube	BD Falcon
- 50 ml PP-test tube	Greiner
- 2 ml, 5 ml, 10 ml, 25 ml, 50 ml pipettes	BD Falcon
- U-, V-shape, Flat-bottom TC-plate, 96 well	Greiner
- Millex GP/GV syringe driven filter unit (0.22 µm)	Millipore
- Acrodisc unit with Mustang E membrane	Pall

- 50 ml reagent reservoir — Corning
- 0.5 ml polystyrene tube 38x6.5 mm — Sarstedt
- 0.5 ml, 1.5 ml, 2 ml safe-lock tubes — Eppendorf
- Cell strainer, nylon (40 µm, 70 µm) — BD Falcon
- Surgical disposable scalpel, scissors, tweezers — Aesculap
- 5 ml, 14 ml polystyrene round-bottom tube — BD Falcon
- 10 ml, 20 ml, 30 ml, 50 ml syringe — BD
- Sterican hypodermic needle Gr.18, 26Gx1'' — Braun
- Multifly, 21Gx3/4" TW, 0.8x19 mm — Sarstedt
- Liquemin N25 000 (Heparin-Natrium) — Roche

Mice

BALB/c and C57BL/6 mice were purchased from Charles River Laboratories. TLR2-knockout mice on a 129/Sv background, nine times backcrossed with C3H/HeJ (mis-sense mutation in the cytosolic domain of TLR4, LPS-insensitive) were obtained from Prof. H. Schild (Johannes Gutenberg University, Mainz, Germany). TLR9-deficient mice, six times backcrossed to C57BL/6, were kindly provided by Prof. H. Wagner (Institute of Medical Microbiology, Immunology and Hygiene, Technische Universität München, Munich, Germany) and originally generated by S. Akira (Osaka, Japan). All animals were housed in our animal facility with free access to standard laboratory chow and water and were kept on a light-dark cycle of 12 hours. In all animal experiments, mice were anaesthetized with diethyl ether and sacrificed by cervical dislocation.

2.1 Preparation of (mono-) nucleosomes from calf thymus

a) Materials

- Centrifuge, Sorvall RC5C Plus Superspeed
  - o with SS34 Rotor — Dupont
- Centrifuge, Beckman L-80 Ultracentrifuge
  - o with SW 40 Ti rotor and buckets — Beckman Coulter
- Hand Homogenizer Dounce Cylinder — Sartorius
- Plunger (L/S) — Sartorius
- 30 ml Corex conical tubes — Thermo Fisher Scientific

- Peristaltic Pump P-1 Amersham
- Gradient makers SG15 Hoefer

b) Chemicals

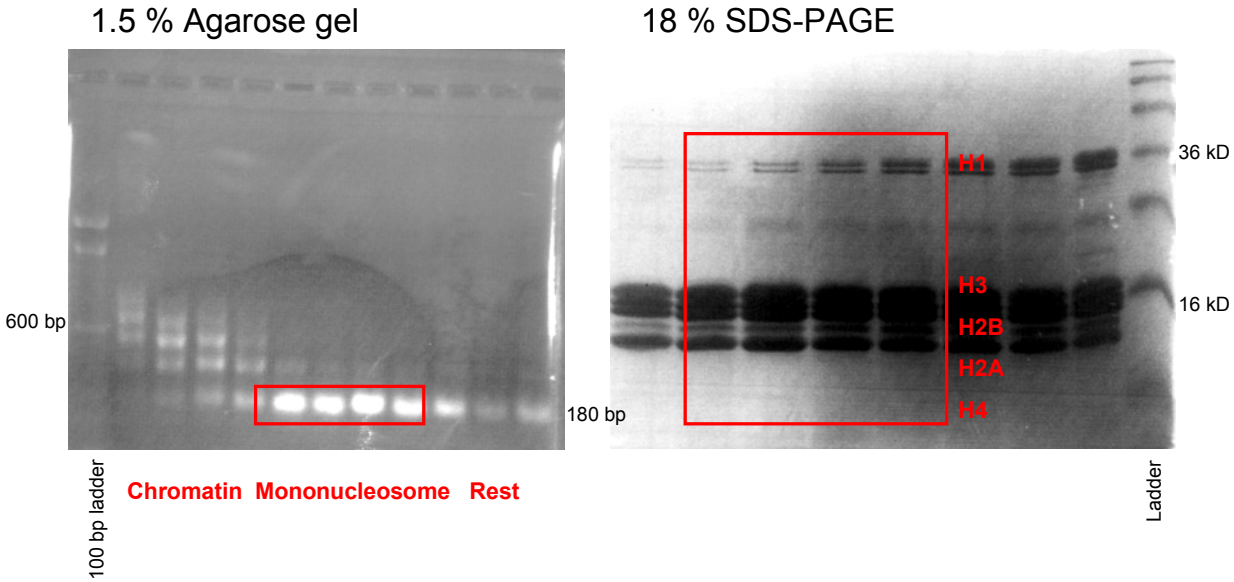
- D(+)-sucrose AppliChem
- 2-Mercaptoethanol (2-ME) Carl Roth
- Nuclease micrococcal from *Staphylococcus aureus* Sigma
- Protease inhibitor cocktail (PIC) Sigma
  (for use with mammalian cell and tissue extracts)
- Lysis buffer (in 50 ml MilliQwater, 0.22 µm filtered):
  Tris 1 mM pH 7,8
  EDTA 1 mM
- NaOH 0.3 M

Buffer 1	**Buffer 2**	**Buffer 3**	**Gradient 5%**	**Gradient 29%**
Tris 15 mM pH 7.4	Tris 15 mM pH 7.4	Tris 15 mM pH 7.4	Tris 15 mM pH 7.4	Tris 15 mM pH 7.4
NaCl 15 mM	NaCl 15 mM	NaCl 15 mM	-	-
KCl 60 mM	KCl 60 mM	KCl 60 mM	-	-
$MgCl_2$ 5 mM	$MgCl_2$ 5 mM	$MgCl_2$ 5 mM	-	-
-	-	$CaCl_2$ 1 mM	-	-
-	-	-	EDTA 0.2 mM	EDTA 0.2 mM
70 µl PIC	70 µl PIC	70 µl PIC	-	-
51 µl 2-ME	51 µl 2-ME	51 µl 2-ME	-	-
0.25 M sucrose	2.3 M sucrose	0.25 M sucrose	0.15 M sucrose	0.85 M sucrose
in 50 ml MilliQwater	in 50 ml MilliQwater	in 10 ml MilliQwater	in 50 ml MilliQwater	in 50 ml MilliQwater

As nucleosomes are evolutionary conserved among species, calf thymus (Schlachthof Rottenburg, Rottenburg, Germany) was used as source for the nucleosome purification. About 5 g of thymus were homogenized in Dounce tube with 20 ml of buffer 1, then 10 ml of buffer 2 were added.

The nuclei were isolated by centrifugation (20 minutes, 5,000 g, at 4°C) onto a bilayer composed of 2.5 ml of buffer 1 and 5 ml of buffer 2. After removing the supernatant, the pellet was washed with 10 ml of buffer 1 (2,500 g, 5 minutes, at 4°C).

The nuclei of the isolated cells were subjected to micrococcal nuclease (0.04 U/ 1 OD260 U) digestion for 10 minutes at 37°C, which causes internucleosomal cleavage. The reaction was immediately stopped by adding 100 µl of 200 mM EDTA and the mixture was centrifuged for 10 minutes at 4,000 rpm. The resulting supernatant (S1) mainly contained H1-depleted mononucleosomes and other contaminants. The pelleted and digested nuclei were resuspended in lysis buffer and incubated for 1 hour on ice. After centrifugation, the resulting supernatant (S2) contained mono- and oligonucleosomes, as well as free DNA, histones or other chromatin components. These contaminations can be removed by sucrose gradient ultra-centrifugation for 21 hours at 25,500 rpm and 4°C, without brake. Different fractions were collected and the quality and purity of the nucleosome preparation was checked by 18 % SDS-PAGE and 1.5 % agarose gel electrophoresis. An empty gradient (“Gdt Nuc”), consisting of lysis buffer instead of fraction S2, was used as a negative control for the nucleosome preparation. The fractions which only contained 180 bp of DNA (corresponding to mononucleosomes) and had all five histone proteins were pooled, filtered (0.2 µm) and used in the experiments. The corresponding empty gradients were also pooled. Nucleosomes were freshly prepared every 2 to 3 weeks and stored at 4°C. Nucleosome concentrations are expressed in µg/ml of DNA as estimated by OD260 nm measurement.



Reconstituted nucleosomes were provided from Prof. Dr. K. Luger (Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA) and prepared as described in (306).

2.2 Preparation of histone H1-depleted (mono-) nucleosomes from calf thymus

To deplete the nucleosome preparation obtained by nuclease digestion from any attached non-histone proteins, the preparation was additionally treated with a sodium chloride solution. The nucleosome purification was carried out following the classical protocol (see above). However, the S2 fraction was incubated with a sodium chloride solution for at least 20 minutes on ice, before loading on the sucrose gradient. The final concentration of the mixture was 0.5 M NaCl, 10 mM Tris-HCl pH 7.4 and 0.2 mM EDTA. The 5 % and 29 % gradients (see above) used here additionally contained 0.5 M NaCl, respectively.

2.3 SDS-PAGE (Polyacrylamide gel electrophoresis)

a) Materials

- Mini-Protean 2 Electrophoresis System	Bio-Rad

b) Chemicals

- Rotiphorese gel 30 (Acryl 30 %-Bisacryl 0.8 %)	Carl Roth
- Temed	Fluka BioChemika
- Ammonium persulfate (APS)	Sigma
- Seablue Prestained	Invitrogen
- Gel staining (in 100 ml bidistilled H_2O):	
0.55 g Coomassie Brilliant Blue R or G	Sigma
20 ml Acetic acid	Merck
100 ml Methanol	Merck
- Destaining for histones (in 250 ml bidistilled H_2O):	
58 ml Acetic acid	Merck
192 ml Methanol	Merck

Electrophoresis buffer 10x (in 1 l bidistilled H_2O):

144 g Glycine	Carl Roth
30.3 g Trizma Base	Sigma
100 ml SDS 10 %	Carl Roth

- Loading buffer (in 100 ml bidistilled H_2O):

50 mM Tris pH 6.8	
6 M Urea	AppliChem
6 % 2-ME	Carl Roth
0.003 % Bromphenol blue	Sigma
3 % SDS	Carl Roth
25 % Glycerol	Carl Roth
0.125 M EDTA	Carl Roth

Resoluting gel 18%	**Concentrating gel 6%**
3.75 ml Tris-HCl 1 M pH 8.8	500 µl Tris-HCl 1 M pH 6.8
100 µl SDS 10 %	50 µl SDS 10 %
6 ml Rotiphorese	800 µl Rotiphorese
60 µl APS	35 µl APS
15 µl Temed 5 %	7 µl Temed 5%
-	2.6 ml bidistilled H_2O

First, the 18 % resoluting gel was prepared by mixing the above-specified ingredients. The gel was then poured into the gel casting form. As soon as the gel was completely polymerized, the 6 % concentrating gel was prepared and poured on top of the resoluting gel. The combs were inserted before the gel polymerized. 4 µg of each sample and a molecular weight standard were prepared by adding 10 µl of the loading buffer and boiling for 4 minutes at 95°C. Gels were clamped in and buffer chambers were filled with 1x electrophoresis buffer. Samples were added to the wells and gel was run for 1 hour at 200 V. Finally, gels were stained with Coomassie Brilliant Blue G or R over night on a shaker and then destained for several hours using destaining buffer.

2.4 Agarose gel electrophoresis

a) Materials

- PerfectBlueGelsystem — Peqlab

b) Chemicals

- Ethidium bromide (10 mg/ml) — Carl Roth
- 100 bp DNA ladder — Invitrogen
- Agarose — Biozym
- TAE 50x (in 1 l bidistilled H_2O):
  - 242 g Trizma base — Sigma
  - 57.1 ml Acetic acid — Merck
  - 18.6 g EDTA — Carl Roth
- Special TE-buffer 5x (in Tris-HCl 1 M, pH 7.4):
  - 0.12 % Bromphenol blue — Sigma
  - 0.12 % Xylene cyanole FF — Sigma
  - 2 % SDS — Carl Roth
  - 25 % Glycerol — Carl Roth
  - 0.125 M EDTA — Carl Roth

1.5 % agarose gel was prepared by mixing 1.5 g agarose and 100 ml 1x TAE buffer in an Erlenmeyer flask, followed by heating in a microwave until the agarose was dissolved. After addition of 5 µl Ethidium bromide, the gel was poured onto a taped plate with combs in place. After polymerization, tapes and combs were removed and gel placed into the electrophoresis chambers. 1x TAE was added to the reservoirs. Samples and 100 bp DNA ladder containing special TE-buffer (1/5 dilution) were loaded into the wells. The gel was running for 60 minutes at 100 V. The DNA fragments were visualized on a UV light box and photographed.

2.5 Nucleosome-FITC conjugation

a) Materials

- Dialysis tubing visking — Carl Roth

b) Chemicals

- FITC (Fluorescein isothiocyanate isomer I) — Sigma
- $NaHCO_3$ — Merck

- DMSO Merck
- Sephadex G25 for gel filtration, superfine Sigma

Nucleosomes were dialyzed against 1 l PBS (without Ca^{++}, Mg^{++}) over night at 4°C. After that, nucleosomes were dissolved in 1 M $NaHCO_3$ buffer (pH 8 - 9) at the ratio of 20 to 1 and were then allowed to react with FITC at the ratio of 5 to 1 for 90 minutes at room temperature. The reaction was stopped with 1 M Tris-HCl (pH 8 - 9) at the ratio of 10 to 1 for 45 minutes at 4°C. Free FITC molecules were removed by gel filtration through a Sephadex G25-column. FITC and protein concentration were determined by measuring optical density (OD) at 495 nm and 280 nm against 0.3 M NaOH. The ratio (Q) between both values gives an indication of the conjugation level. Nuc-FITC concentrations were estimated by calculating (c) as follows:

Q = OD495/OD280	c = OD495 x dilution/ ε x L
(Q = 0.5 - 1.2)	(ε = 71,260.2 M/cm,
	L = length of cuvette (cm))

2.6 DNA purification from (mono-) nucleosomes

a) Chemicals

- Chloroform (24:1 Isoamyl alcohol) Merck
- Phenol Carl Roth
- NH_4O acetate (7.5 M)/Ethanol 1:6 Merck

DNA was purified from nucleosome fractions by phenol/chloroform extraction in three steps. Here, nucleosomes were mixed well with an equal volume of phenol, next phenol/chloroform (1:1), then chloroform and finally phenol/chloroform. After each step, the sample was centrifuged (3 minutes, 13,000 rpm, 15°C) and the upper, aqueous layer was transferred to a clean tube, carefully avoiding proteins at the interface. The concentration of DNA was performed by ethanol precipitation adding 3 volumes of NH_4O acetate/Ethanol to the DNA sample, followed by incubation over night at -80°C. After centrifugation (30 minutes, 13,000 rpm, 4°C), the DNA pellet was washed in ice cold 70 % Ethanol and finally resuspended in 0.22 µm filtered MilliQwater. OD was measured at 260 nm against 0.3 M NaOH.

2.7 Preparation of human polymorphonuclear neutrophils from whole blood and cell culture

a) Chemicals

- Polymorphprep	Axis Shield
- Dulbecco's phosphate buffered saline (PBS) without Ca^{++}, Mg^{++}, pH 7,2 (used in all experiments)	Institut für Zellbiologie, Abt. Immunologie
- RPMI 1640:	Cambrex
10 % Fetal calf serum (FCS), decomplemented	Cambrex
2 mM L-Glutamin in 0.85 % NaCl	Institut für Zellbiologie, Abt. Immunologie
100 U/ml Penicillin/100 µg/ml Streptomycin	Cambrex
50 µM 2-ME	Carl Roth
- ACK lysis buffer pH 7.3 (in MilliQwater, 0.22 µm filtered):	
150 mM NH_4Cl	Carl Roth
10 mM $KHCO_3$	Carl Roth
0.1 mM Na_2 $2H_2O$ EDTA	Carl Roth
- 0.05 % Trypan blue for microscopy	Merck

Blood was taken from normal healthy volunteers and SLE patients. Lupus blood and clinical data were provided from PD Dr. I. Kötter (Department of Internal Medicine II, University Hospital, Tübingen, Germany). SLE patients fulfilled the ACR criteria.

Polymorphonuclear neutrophils (PMN) were prepared from heparinized blood by dextran sedimentation using Polymorphprep. 6.5 ml of anticoagulated blood were carefully layered over 8.5 ml of Polymorphprep in a 15 ml Falcon tube, followed by centrifugation (498 g, 35 minutes, without brake). After the centrifugation, the lower band containing polymorphonuclear cells was harvested using a sterile Pasteur pipette. The PMN fraction was diluted by the addition of PBS and centrifuged (1,800 rpm, 7 minutes). One or two hypotonic lysis steps using ACK lysis buffer removed contaminating red blood cells (RBC). PMN were resuspended in complete RPMI containing 10 % FCS.

The purity of the cell preparation was assessed by flow cytometry using CD66b as a marker for PMN. Usually 85-90 % of the cells were CD66b-positive.

Purified neutrophils were cultured in RPMI 1640 supplemented with 10 % heat-inactivated FCS at 10^6 cells/ml in 96-well plates (round-bottom) at 37°C. Cells were incubated with medium alone or different stimuli. After over night culture (16 – 20 hours), cell culture supernatants (for IL-8 ELISA) were harvested and frozen at -80°C. PMN activation was also determined by flow cytometry. In some experiments, cells were pre-incubated for 30 minutes at 37°C with polymyxin B sulfate (12.5µg/ml, 25 µg/ml), ammonium chloride (5mM, 7.5 mM), chloroquine (5 µg/ml, 10 µg/ml) or phycoerythrin (PE)-labelled polystyrene microspheres (1/100) before cell culture.

- Reagents used for cell culture:
- Purified nucleosomes (Nuc) and corresponding empty gradients (Gdt Nuc)
- LPS *Salmonella typhimurium* Sigma
- LPS *Escherichia coli* 0111:B4 Sigma
- CpG 2006 ODN (CpG-B) Metabion
  5’ TCGTCGTTTTGTCGTTT GTCGTT 3’
- CpG 2216 ODN (CpG-A) Metabion
  5’ GGGGGACGATCGTCGGGG 3’
- CpG 1826 ODN Invivogen
  5’ TCCATGACGTTCCTGACGTT 3’
- Cytokine-quality high mobility group box-1 (HMGB-1) HMG-Biotech

Reconstituted nucleosomes (Rec Nuc) Prof. Dr. K. Luger

- Buffer corresponding to Rec Nuc (prepared by us)
  20 mM Tris pH 7.5
  1 mM EDTA
  1 mM DTT Sigma
- Imidazoquinoline resiquimod (R848) Invivogen
- ssRNA (Protamin stabilized) Curevac
- Genomic DNA from E.coli strain B Sigma
- Polymyxin B sulfate Fluka
- Ammonium chloride Sigma
- Chloroquine diphosphate salt Sigma
- Polystyrene microspheres-PE (ø1 µm, 1/100) Polysciences

2.8 Preparation of peritoneal exudate cells from mice

Mice were intraperitoneally (i.p.) injected with 100 µg nucleosomes, an equal volume of the purification buffer (Gdt Nuc) or 50 µg LPS. After 18 hours, mice were sacrificed to prepare peritoneal exudate cells (PECs). The peritoneal cavity was washed twice with 5 ml of PBS containing 1 mM EDTA. Cells were harvested from the peritoneum and any possible contaminating RBC were removed by a 30 seconds hypotonic lysis step. PECs were then washed with PBS and resuspended in PBS containing 1 % FCS. Neutrophil recruitment and activation was determined by flow cytometry measuring Ly-6G and CD11b expression. PECs were first incubated with an FcR-blocking mAb, washed and then stained with FITC-conjugated anti-Ly-6G and PE-conjugated anti-CD11b-specific mAb or the corresponding isotype controls. In a final step, 7-amino-actinomycin D (7-AAD) was added as a viability probe to exclude any dead cells. Among living cells (7AAD negative), Ly-6G^{high} and CD11b^{high} expressing cells were considered as mature activated neutrophils.

2.9 Preparation of mouse bone marrow-derived DCs

a) Chemicals

- IMDM:	Cambrex
10 % FCS, decomplemented	Cambrex
2 mM Glutamin	
100 U/ml Penicillin/100 µg/ml Streptomycin	Cambrex
50 µM 2-ME	Carl Roth
- Recombinant murine (rm) GM-CSF	Immunotools
- Chloroquine diphosphate salt	Sigma

Mice were sacrificed and bone marrow (BM) was flushed out from femur and tibia with a syringe and a 26G needle using 5 ml of IMDM containing 10 % FCS. The cell suspension was passed through a 70 µm cell strainer to remove cell debris and the cells were collected by centrifugation. Contaminating RBC were lysed for 30 seconds with 10 ml 0.2 % sodium chloride.

To restore osmolarity, 10 ml of 1.6 % sodium chloride were added. $10x10^6$ of BM cells were cultured in a Petri dish in 10 ml IMDM supplemented with 20 ng/ml rmGM-CSF for 6 days at 37°C. Usually, the cultures were fed every 2 days by aspirating half of the medium and adding fresh medium containing 20 ng/ml rmGM-CSF.
At day 6, immature bone marrow-derived DCs (BMDCs) were analyzed by flow cytometry for cell surface markers using mAb specific for CD11c, CD86 and CD14. The BMDCs were cultured in IMDM containing 10 % heat-inactivated FCS at 10^6 cells/ml in 96-well plates (round-bottom). Here, immature BMDCs were pre-treated with chloroquine (0.001 mM, 0.01 mM, 0.1 mM) for 2 hours at 37°C, and then incubated with nucleosomes or different stimuli. After over night culture (about 16 hours), cell culture supernatants were harvested and frozen at -80°C (for IL-12 ELISA). At day 8, mature BMDCs were analyzed by flow cytometry for cell surface expression of CD86 and CD80.

2.10 Preparation of mouse bone marrow-derived neutrophils

a) Material

- LD MACS Separation columns (Capacity of up to $1x10^8$ cells magnetically labelled cells from up to $5x10^8$ total cells) — Miltenyi Biotec
- MidiMacs Separator — Miltenyi Biotec

b) **Chemicals**

- Mouse, StemSep, Negative Selection Kit — StemCell
    - o Rat serum
    - o Antibody cocktail: anti-CD4, anti-F4/80, anti-CD45R, anti-CD5, anti-Ter119
    - o Anti-biotin TAC (tetramer antibody complex)
    - o Magnetic colloid

To obtain bone marrow cells, femur and tibia of a mouse were flushed with RPMI 1640 containing 1 % FCS. Tissue fragments were removed by passing through a 70 µm cell strainer and cells were collected by centrifugation.
Bone marrow cells were prepared at a concentration of $5x10^7$cells/ml in separation medium (PBS containing 2 % FCS).

50 µl per ml of cells of normal rat serum was added to the cell suspension and incubated for 15 minutes at 4°C. Then cells were incubated with 35 µl per ml of cells of primary antibodies specific for cell surface markers F4/80, CD4, CD45R, CD5 and Ter119 for 15 minutes at 4°C. After that, cells were washed and resuspended in separation medium at the concentration of $5x10^7$cells/ml. Anti-biotin TAC was added at 100 µl per ml of cells and incubated for 15 minutes at 4°C. Finally, 60 µl per ml of cells of colloidal magnetic dextran iron particles were added to the suspension and incubated for 15 minutes at 4°C. The entire cell suspension was placed into an LD column surrounded by a magnet. T cells, B cells, RBC, monocytes and macrophages were captured in the column, allowing the neutrophils to pass through by negative selection. Neutrophil purity, as determinded by flow cytometry using Ly6G and CD11b, was >80 %. BM-derived neutrophils were cultured in RPMI containing 1 % FCS for about 14 hours at 37°C.

2.11 Enzyme-linked immunosorbent assay (ELISA)

a) Materials

- Molecular Devices Spectra Max 340 (Software SOFTmax PRO 2.1) — GMI
- Nunc Maxisorp, 96 well plate — Nunc

b) Chemicals

- BD OptEIA™ Set Human IL-8 — BD
    - o Purified anti-human IL-8 mAb (1/250)
    - o Biotinylated anti-human IL-8 mAb (1/250)
    - o Streptavidin-horseradish peroxidase conjugate (HRP) (1/250)
    - o Recombinant human IL-8 (10 ng/ml)
- Streptavidin-HRP (1/800) — BD
- Purified anti-mouse IL-12 (p40/p70) (5 µg/ml) — BD
- Biotin anti-mouse IL-12 (p40/p70) (2 µg/ml) — BD
- Recombinant mouse IL-12 (p40/p70) (10 ng/ml) — BD
- Tween-20 — Carl Roth
- Albumin Fraction V Protease free (BSA) — Carl Roth

- Coating buffer pH 9.6 (in 1 l bidistilled H_2O):
  15 mM Na_2CO_3 Merck
  35 mM $NaHCO_3$ Merck
  0.02 % NaN_3
- ABTS-solution pH 4.35 (in 500 ml bidistilled H_2O): Sigma
  150 mg ABTS (2,2'-Azino-bis(3-ethylbenz-thiazoline -6-sulfonic acid)
  0.1 M Citric acid Serva
- H_2O_2 (30 %) Sigma

Wells were coated with 50 µl/well capture antibody diluted in coating buffer and incubated over night at 4°C. The following steps were conducted at room temperature. Then, wells were aspirated and three times washed with 200 µl/well wash buffer (PBS/0.05 % Tween). Plates were blocked with 200 µl/well for 2 hours with PBS/Tween containing 1 % BSA. After washing the plate, diluted samples and standard (dilution series) were added and incubated for 2 hours. After several washes, 50 µl/well of detection antibody and Streptavidin-HRP in PBS/0.05 % Tween/1 % BSA were added together (or in two separate steps) to the plate and incubated for 1 hour (respectively). After a final wash step, 100 µl/well of ABTS substrate solution was added and incubated in the dark. The optical density was determined using an ELISA plate reader set at 405 nm.

2.12 Flow cytometry

a) Materials

- BD FacsCalibur system BD
  (Software: CellQuestPro)

b) Chemicals

Antibodies (all from BD) (except*)	**Species**	**Clone**	**Isotype**	**Dilution**
Anti-human CD66b-FITC	mouse	G10F5	IgM1,k	1/75
Anti-human CD11b-PE	mouse	ICRF44 (44)	IgG1,k	1/75
Anti-mouse Ly-6G-FITC	rat	1A8	IgG2a,k	1/200
Anti-mouse CD11b-PE	rat	M1/70	IgG2b,k	1/200

Anti-mouse CD80-FITC	hamster	16-10A1	IgG2k	0.6 µg/ml
Anti-mouse CD86-PE	rat	GL1	IgG2a,k	0.3 µg/ml
Anti-mouse CD11c-FITC	hamster	HL3	IgG1,λ	0.6 µg/ml
Anti-mouse CD14-PE	rat	rmC5-3	IgG1,k	0.3 µg/ml
Annexin V-PE				1/75
7-AAD				0.16 µg/test
Propidium iodide (PI) (*Sigma)				0.13 µg/test
$(Fab')_2$ anti-mouse IgG-FITC (*Jackson ImmunoResearch)	goat			1/100
$(Fab')_2$ anti-mouse IgG-PE (H+ L) (*Jackson ImmunoResearch)	goat			1/100
Anti-mouse CD16/CD32 (FcγIII/II receptor)	rat	2.4G2	IgG2b,k	1 µg/1x10^6 cells
Purified anti-histone (*Roche)	mouse	H11-4	IgG1	5 µg/ml
Purified anti-human CD289 (TLR9) (*AbD Serotec)	mouse	5G5	IgG2a	5 µg/ml
Purified IgG2a,k	mouse	G155-178		5 µg/ml

- Polystyrene microspheres-PE (ø1 µm, 1/100) Polysciences
- <u>Apoptosis binding buffer 1x</u>:
  10 mM Hepes/NaOH pH 7.4
  140 mM NaCl
  2.5 mM $CaCl_2$
- Cytofix/Cytoperm BD
- Human Serum AB CCPro
- FCS Cambrex
- Mouse serum (from BALB/c or C57BL/6)
- <u>Permwash buffer</u> (in 500 ml PBS):
  0.1 % Saponin from Quillaja Bark (S7900) Sigma
  0.5 % BSA Carl Roth
  0.02 % NaN_3

Activation of human PMN was estimated by flow cytometry. Up-regulation of cell surface molecules was measured using FITC-conjugated anti-CD66b and PE-conjugated anti-CD11b-specific mAb (and the corresponding isotype controls). Therefore, 10^5 cells/150 µl/well were (after over night culture or at time point 0) transferred to a 96-well plate (V-shape), centrifuged and washed with 1 % FCS/PBS. Then, the cells were stained with either primary antibody or conjugated antibodies for 30 minutes at 4°C. After 2 washes with 1 % FCS/PBS, cells were either incubated with a secondary antibody or fixed with 1.5 % formaldehyde/PBS or directly used for flow cytometry. The PMN population was defined through a gate according to the forward- (FSC) and side-scatter (SSC) plot, which are proportional to size and cell granularity/internal complexity.

In binding (and endocytosis) assays, PMN were incubated with FITC-conjugated nucleosomes for 30 minutes at 4°C or 37°C in PBS containing 10 % heat-inactivated FCS. The cells were washed and used for flow cytometry in the presence of propidium iodide (PI) to exclude any dead cells.

In apoptosis assays, PMN were first incubated with PE-conjugated annexin V for 20 minutes in apoptosis binding buffer at room temperature, followed by a co-staining with 7-AAD. PMN were then immediately used for flow cytometry.

Cell activation was also determined by measuring the phagocytic activity. Therefore, freshly purified PMN were pre-incubated with PE-conjugated polystyrene microspheres (1/100 dilution) for 30 minutes at 37°C, followed by an over night incubation with different stimuli. Thereafter, cells were washed and fixed with 1.5 % formaldehyde/ PBS.

For the detection of TLR9 expression at the cell surface of human PMN, 10^5 cells/well were incubated with PBS containing 5 % human Serum AB for 20 minutes at 4°C. Then, cells were centrifuged and incubated with purified anti-CD289 (/TLR9) mAb in 1 % FCS/PBS for 30 minutes at 4°C. After two washes, PMN were incubated with PE-conjugated goat anti-mouse IgG (1/100) for 25 minutes at 4°C. After two washes and incubation with 5 % decomplemented mouse serum for 20 minutes at 4°C, cells were stained with FITC-conjugated anti-CD66b Ab for 30 minutes at 4°C. In order to examine intracellular TLR9 expression, membrane permeabilization was required. Therefore, cells were first incubated with Cytofix/Cytoperm for 20 minutes at 4°C and then washed with Permwash buffer.

It was then proceeded as described for cell surface staining with the exception that all incubation steps were done in Permwash buffer. Finally cells were fixed with 1.5 % formaldehyde/ PBS and TLR9 expression was estimated by flow cytometry. Similar protocols were used for flow cytmetric analysis of mouse BMDC.

2.13 Confocal laser scanning microscopy

a) Materials

- Laser Scanning Microscope LSM510 (Software LSM510 3.2 SP2) — Carl Zeiss
- Lab-Tek Chambered cover glass — Nunc

b) Chemicals

Probes (all from Molecular Probes) (except*)	**Absorption**	**Emission**	**Dilution**
Nucleosome-FITC (*self-made)	495 nm	525 nm	10 - 50 µM
LysoTracker Red DND-99	577 nm	590 nm	75 - 100 nm
Carboxylated-modified microspheres Crimson (0.02 µm in diameter)	625 nm	645 nm	1/2,500,000
Cholera toxin subunit B Alexa Fluor 555	555 nm	565 nm	20 µg/ml
Transferrin-Alexa Fluor 633	632 nm	647 nm	25 µg/ml
To-Pro-3	642 nm	661 nm	1/1,000
Propidium iodide (*Sigma)	535 nm	617 nm	1.5 µM
7-AAD (*BD)		650 nm	32 µM
Dextran Alexa Fluor 647	650 nm	668 nm	10 µM
CpG 2006-FITC (*InvivoGen)	495 nm	520 nm	2 µM

- Methyl-β-cyclodextrin (MβCD) 5 mM — Sigma
- Chlorpromazine 10 µg/ml — Calbiochem
- Ammonium chloride 7.5mM — Sigma
- Human complement C1q 50 µg/ml — Sigma

In all life cell imaging experiments, freshly purified PMN were cultured at a density of $2.2x10^5$/well in 250 µl RPMI 1640 containing 10 % autologous, heat-inactivated serum.

FITC-conjugated nucleosomes alone or in combination with other fluorophore-labelled reagents were directly added to the chambered cover glass and immediately analyzed by confocal laser scanning microscopy. In some experiments, PMN were pre-treated with indicated inhibitors for 30 minutes at 37°C.
Microscope cover glasses were mounted and analyzed using a Zeiss LSM510 confocal microscope. In all experiments, a Plan-Apochromat 63x/1.4 Oil DIC objective was used and the pinhole was set to scan layers < 1 µm. The experiments were performed with either living or fixed cells placed in a 8-well chambered cover glass, which was previously blocked with 0.1 % BSA for 3 hours at 37°C. Life cell microscopy was performed at room temperature.
For the simultaneous detection of FITC-labelled nucleosomes and Alexa Fluor 633 or 647 (or Alexa Fluor 555) the 488 nm line of an argon ion laser and the light of a 633 nm helium neon laser (or 543 nm helium neon laser) was directed over an HFT UV/488/(543)/633 beam splitter. The fluorescence was detected using a NFT 545 beam splitter in combination with a BP505-530 band pass filter for the FITC detection and LP650 long pass filter for the Alexa Fluor 633 and 647 detection (BP560-615 band pass filter for the Alexa Fluor 555 detection). All other probes were detected similarly according to their absorption and emission spectra.

Two-dimensional gel electrophoresis, as well as **mass spectrometry and analysis of spectrometric data** were performed by Annika Erbacher in collaboration with the Proteom Center Tübingen (Institute for Cell Biology, University of Tübingen, Tübingen, Germany)

List of manufacturers

AbD Serotec, Düsseldorf, Germany

Aesculap AG & Co.KG, Tuttlingen, Germany

Amersham Pharmacia Biotech, Uppsala, Sweden

AppliChem GmbH, Darmstadt, Germany

Axis Shield PoC AS, Oslo, Norway

B. Braun Melsungen AG, Melsungen, Germany

BD Biosciences, Bedford, MA, USA

Beckman Coulter, Inc., Krefeld, Germany

Bio-Rad Laboratories, GmbH, München, Germany

Biozym Scientific GmbH, Hess. Oldendorf, Germany

Calbiochem, Bad Soden, Germany

Cambrex Bio Science, Verviers, Belgium

Carl Roth GmbH & Co.KG, Karlsruhe, Germany

Carl Zeiss MircoImaging GmbH, Jena, Germany

CCPro, Neustadt, Germany

Charles River Laboratories, Inc., Wilmington, MA, USA

Corning Incorporated, Corning, NY, USA

Curevac GmbH, Tübingen, Germany

Dupont, Bad Homburg, Germany

Eppendorf AG, Hamburg, Germany

Fluka Biochemika, Buchs, Switzerland

Fröbel Laborgeräte, Lindau, Germany

Gilson Inc., Middleton, WI, USA

GMI Global Medical Instrumentation, Ramsey, MN, USA

Greiner bio-one, Frickenhausen, Germany

Heraeus, Hanau, Germany

HMG Biotech S.r.l., Milano, Italy

Hoefer, Inc., San Francisco, CA, USA

IKA Labortechnik, Staufen, Germany

ImmunoTools GmbH, Friesoythe, Germany

Integra Biosciences AG, Chur, Switzerland

Invitrogen, Paisley, UK

InvivoGen, San Diego, CA, USA
Jackson ImmunoResearch Europe Ltd., Newmarket, UK
Köttermann Labortechnik, Uetze, Germany
Labotect Labortechnik, Göttingen, Germany
Leica Microsystems, Wetzlar, Germany
Merck KG aA, Darmstadt, Germany
Metabion international AG, Martinsried, Germany
Millipore, Billerica, MA, USA
Miltenyi Biotec, Bergisch Gladbach, Germany
Molecular Probes Inc., Eugene, OR, USA
Nalge Nunc International, Rochester, NY, USA
Pall Life Sciences, Ann Arbor, MI, USA
Peqlab Biotechnologies GmbH, Erlangen, Germany
Polysciences Europe GmbH, Eppelheim, Germany
Roche, Grenzach-Wyhlen, Germany
Sarstedt, Nümbrecht, Germany
Sartorius, Göttingen, Germany
Scientific Industries, Inc., Bohemia, NY, USA
Serva, Heidelberg, Germany
Sigma-Aldrich, Inc., Saint Louis, MO, USA
StemCell Technologies Inc., Vancouver, BC, Canada
Thermo Fisher Scientific GmbH, Schwerte, Germany
Vilber Lourmat GmbH, Eberhardzell, Germany

3 Results

3.1 Nucleosome-induced neutrophil activation

Nucleosome plasma levels in SLE patients were reported to be about 100 ng/ml (265), but can reach concentrations of 1.2 µg/ml in some patients. In another study, nucleosome plasma concentrations were shown to be 10 µg/ml and can reach values of 100 µg/ml to 200 µg/ml in some patients (264). Thus, it might be difficult to determine the true physiological nucleosome concentration in SLE patients. Since, we do detect *in vitro* PMN activation (see below) using 1 µg/ml to 35 µg/ml purified nucleosomes (25 µg/ml to 200 µg/ml for mouse BMDCs), we are in the range of nucleosome concentrations measured *in vivo*.

3.1.1 Purified (mono-) nucleosomes directly induce PMN activation without the requirement of immune complex formation

Freshly isolated human polymorphonuclear neutrophils (PMN) from healthy volunteers were cultured with purified nucleosomes or the same volume of either chromatin or “rest” (degraded mono-nucleosomes, which contain DNA smaller than 180 bp; see Materials and methods) in order to determine if poly-, mono-nucleosomes or degraded nucleosomes can induce PMN activation. Since their concentrations strongly varied, the same volume was used, theoretically containing the same amount of endotoxins.

In general, medium supplemented with the empty gradient (Gdt Nuc) from the nucleosome preparation, and not medium alone, was used as appropriate negative control for nucleosomes. The empty gradient corresponds to a sucrose gradient unloaded with chromatin (see Materials and methods). Accordingly, the empty gradients corresponding to the chromatin and “rest” fractions were used as appropriate negative control for chromatin and “rest”. LPS (from *Salmonella typhimurium*) was used as positive control in all experiments (except experiments with mouse bone marrow-derived neutrophils). LPS was used in relatively low concentrations to be comparable to nucleosome-induced activation.

PMN activation was estimated by measuring IL-8 secretion. Although PMN secreted IL-8 spontaneously, only nucleosomes and LPS induced strong cytokine secretion in comparison to the empty gradient (Gdt Nuc) and medium, whereas chromatin and "rest" did not (**Figure 6**).

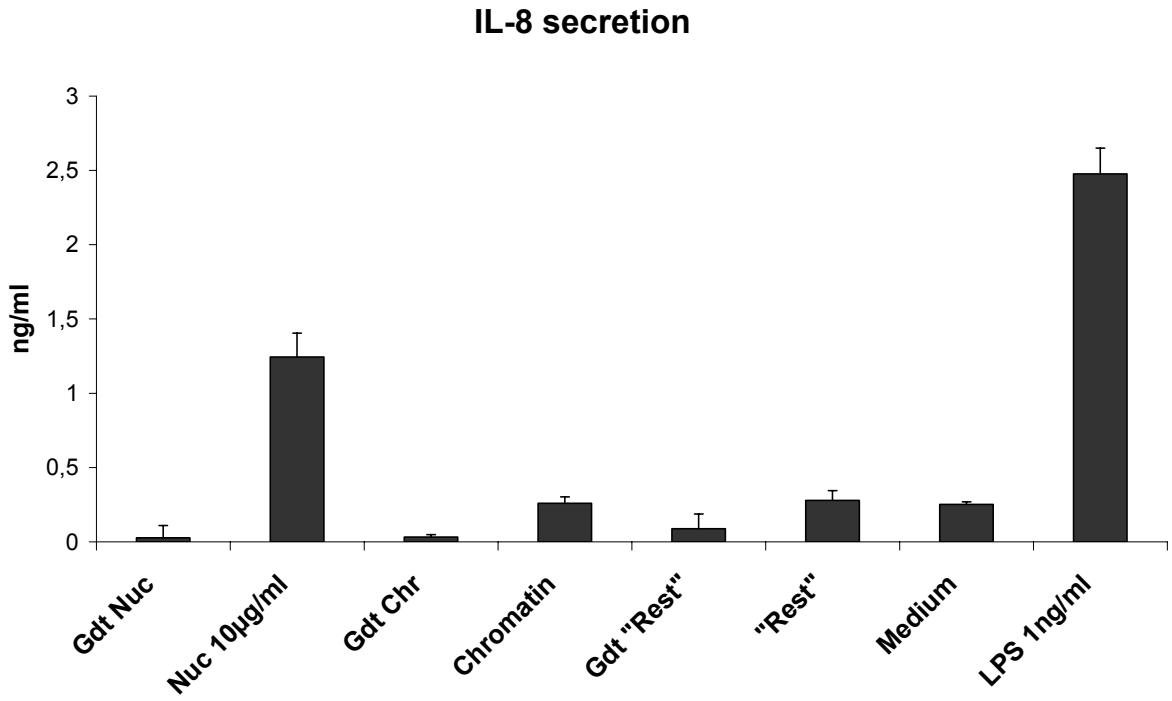


***Figure 6**. (Mono-) Nucleosomes induce activation of human PMN. Freshly isolated PMN were cultured with the same volume of either nucleosomes, chromatin or "rest" and the corresponding gradients for 16 hours. Cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. Gdt, corresponding purification buffers from the nucleosome preparation; Nuc, nucleosomes; LPS, lipopolysaccharides. IL-8 concentrations are expressed in nanograms per millilitre. Shown is 1 of 3 representative experiments. SDs are indicated.*

In the following, human PMN from healthy donors were cultured with purified nucleosomes and analyzed for cell activation. First, CD66b and CD11b up-regulation was determined by flow cytometry. CD66b is a granulocyte-specific and neutrophil-activation marker. CD11b/Mac-1 is expressed on myeloid cells and NK cells and up-regulated upon activation. As shown in **Figure 7A** and **B**, nucleosomes clearly induced CD66b and CD11b up-regulation in PMN, as compared to the isotype controls and to non-activated cells. PMN are sensitive to LPS, because LPS strongly induced PMN activation (**Figure 8 A** and **B**, without polymyxin B).

The nucleosome-induced CD66b up-regulation was as strong as LPS-induced CD66b expression (**Figure 8 A, without PB**), whereas LPS induced a stronger CD11b up-regulation than nucleosomes (**Figure 8 B, without PB**). Nevertheless, nucleosomes induced a two-fold increase in CD11b cell surface expression (**Figure 8 B, without PB**) in comparison to the empty gradient.

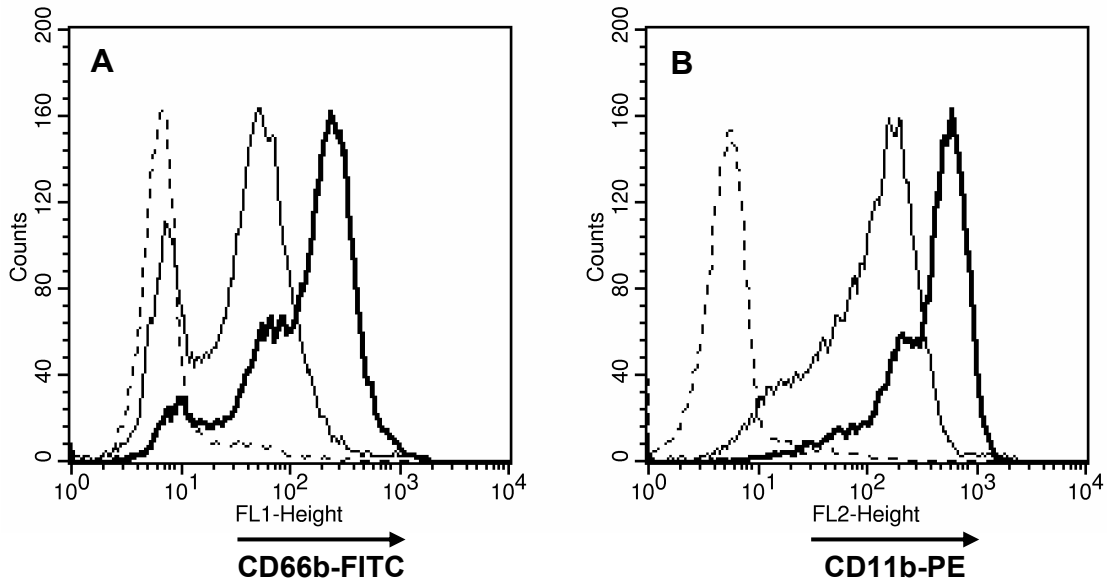


***Figure 7**. Human PMN up-regulate cell surface molecules after stimulation with nucleosomes. Freshly isolated PMN were cultured with nucleosomes for 16 hours and analyzed by flow cytometry using specific mAb. Representative up-regulation observed after activation with nucleosomes. (**A)** CD66b expression (**B)** CD11b expression. Bold line, Nucleosome-activated PMN stained with specific mAb; thin line, non-activated PMN stained with specific mAb; dashed line, corresponding isotype control.*

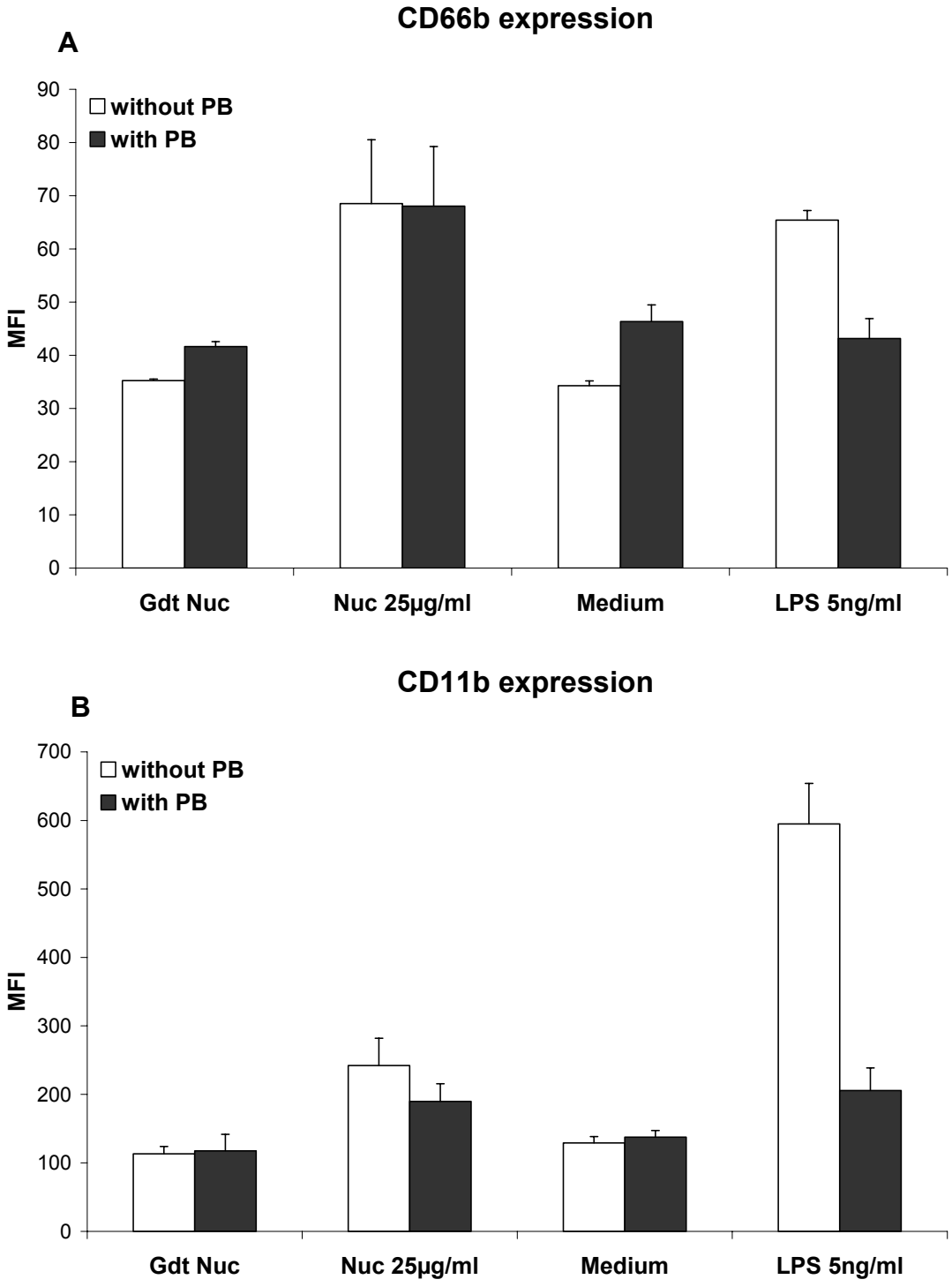
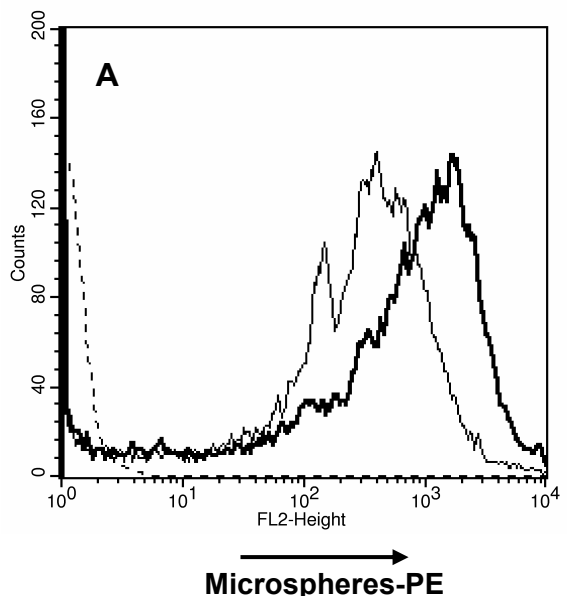


***Figure 8**. Human PMN up-regulate cell surface molecules after stimulation with nucleosomes. Freshly isolated PMN were cultured with different stimuli for 16 hours and analyzed by flow cytometry using specific mAb. Comparative CD66b and CD11b cell surface expression in the absence or presence of LPS inhibitor polymyxin B (PB; 25 µg/ml). CD11b expression was analyzed gating on CD66b-positive cells. MFI, mean fluorescence intensity; Gdt Nuc, empty gradient; Nuc, nucleosomes; LPS, lipopolysaccharides. Shown is 1 of 10 representative experiments. SDs are indicated.*

The nucleosome-induced CD66b and CD11b up-regulation occured in a dose- and time-dependent manner (starting at 10 µg/ml and after 4 hours). Indeed, nucleosome-induced PMN activation was best after over night culture (16 to 18 hours) at 37°C and at concentrations of 15 to 25 µg/ml (data not shown). In all experiments, the empty gradient (Gdt Nuc), used for the nucleosome purification, did not induce cell activation. The empty gradient contained a similar level of endotoxin as the nucleosome concentrations used in the experiments, as once determined by Limulus amebocyte lysate assay. Thus, nucleosomes did increase the percentage of CD11b- and CD66b-positive cells, as well as the level of their cell surface expression on PMN, whereas the empty gradient did not.

PMN activation was further investigated by measuring the phagocytic activity as determined by flow cytometry using PE-labelled polystyrene microspheres. At the site of infection, PMN bind and ingest microorganisms by a process known as phagocytosis. The increase of phagocytic activity can be used as an activation marker of PMN. As shown in **Figure 9 A**, PMN displayed a basal phagocytic activity when incubated in medium alone at 37°C. However, nucleosomes induced an increased phagocytic activity in comparison to the empty gradient. In agreement with the previous results, the nucleosome-induced increased phagocytic activity was comparable, although lower, to LPS-stimulated phagocytosis (**Figure 9 B**, without polymyxin B) and occurred in a time-, temperature- and dose-dependent manner (data not shown).



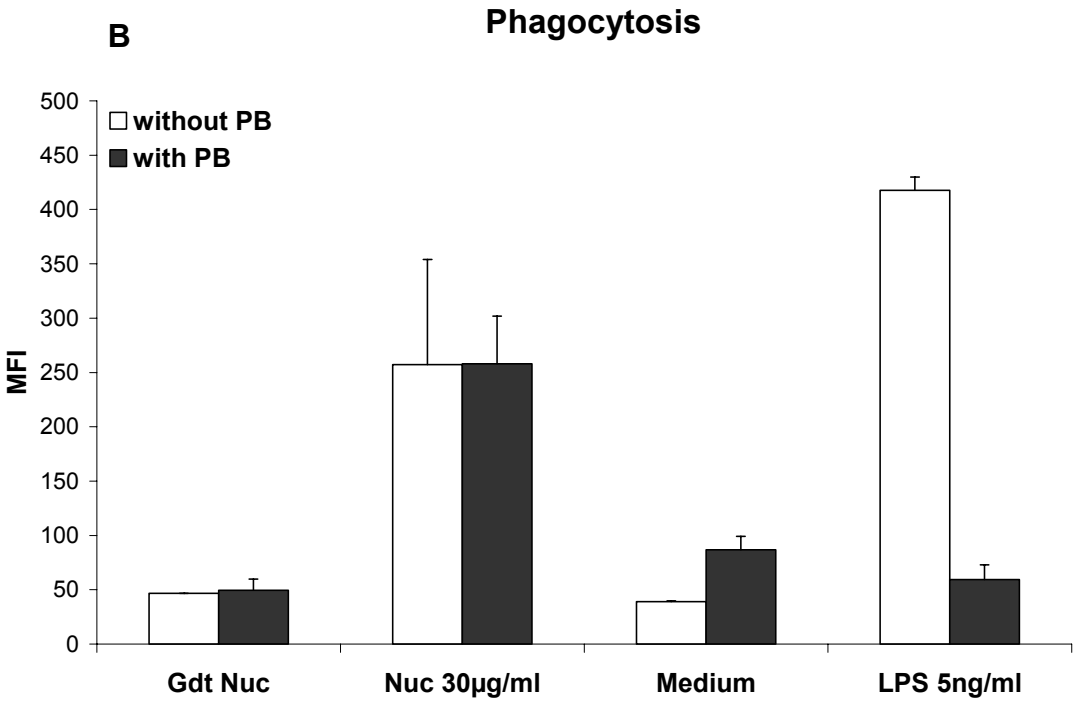


***Figure 9**. Nucleosome-induced PMN show increased phagocytic activity. Freshly isolated PMN were pre-treated with PE-conjugated microspheres for 30 minutes at 37°C, and then incubated with different stimuli for 16 hours and analyzed by flow cytometry. (**A**) Representative nucleosome-induced phagocytic activity. Bold line, Nucleosome-activated PMN pre-incubated with microspheres; thin line, non-activated PMN pre-incubated with microspheres; dashed line, PMN without microspheres (**B**) Comparative phagocytic activiy in the absence or presence of LPS inhibitor polymyxin B (PB; 25 µg/ml). See **Figure 8** for abbreviations. Shown is 1 of 6 representative experiments. SDs are indicated.*

Next, nucleosome-induced PMN activation was confirmed by cytokine ELISA, measuring IL-8 secretion. Although PMN secreted spontaneously IL-8, nucleosomes and LPS induced a strong IL-8 secretion, as compared to cells incubated with empty gradient or in medium alone (**Figure 10**, without polymyxin B).

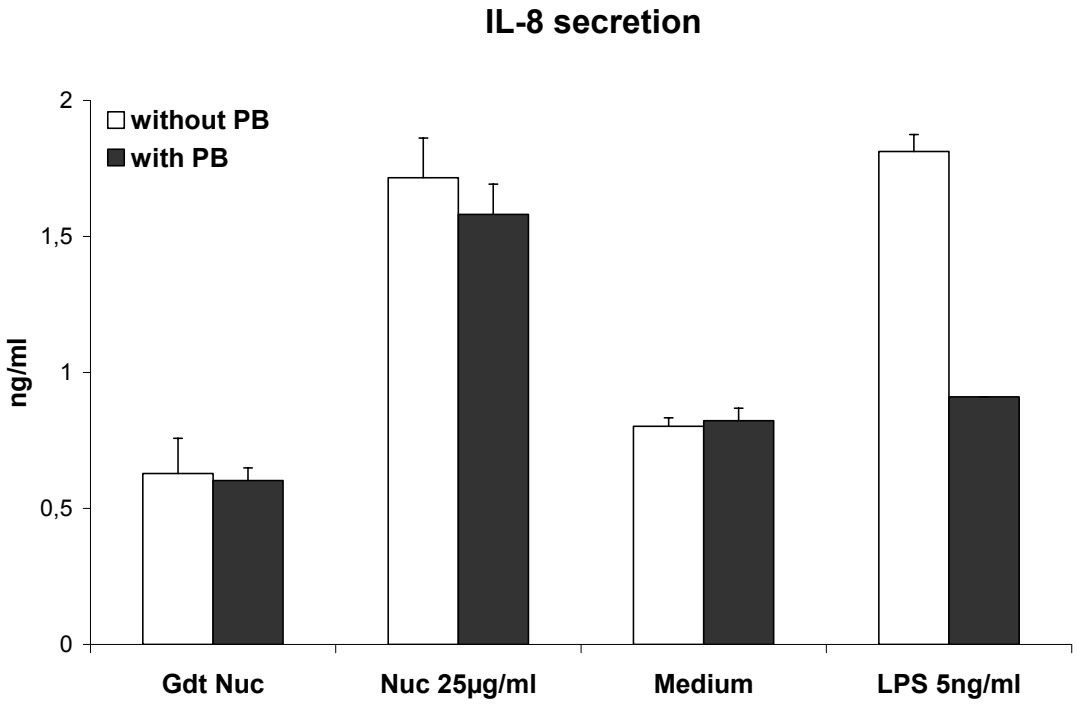


***Figure 10**. Nucleosome-activated PMN secrete IL-8. Freshly isolated PMN were cultured with different stimuli for 16 hours in the absence or presence of polymyxin B (PB; 25 µg/ml). Cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. Gdt Nuc, empty gradient; Nuc, nucleosomes; LPS, lipopolysaccharides. IL-8 concentrations are expressed in ng/ml. Shown is 1 of 10 representative experiments. SDs are indicated.*

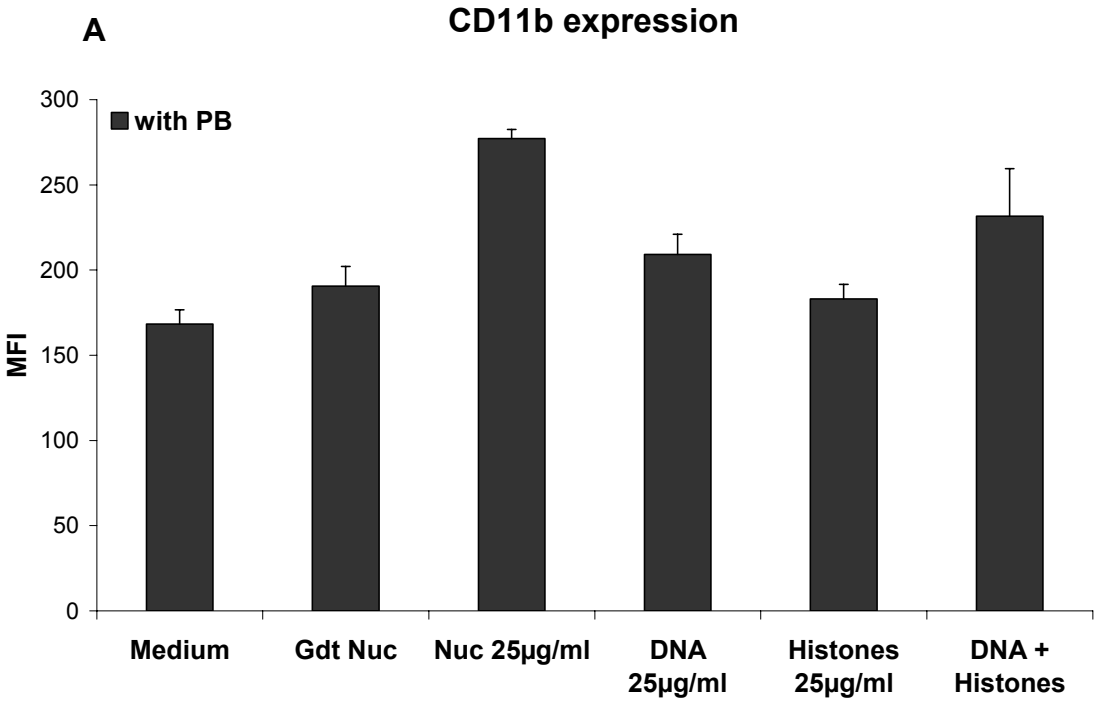
Of note, all these experiments were performed with purified nucleosomes in the absence of anti-nucleosome antibodies, indicating that free nucleosomes and not nucleosome-containing immune complexes induced PMN activation.

3.1.2 Nucleosome-induced PMN activation is not due to endotoxin contamination

PMN activation was then determined in the absence or presence of polymyxin B, an antibiotic which inhibits LPS-induced activity. As shown in **Figure 8**, **Figure 9 B** and **Figure 10** (with polymyxin B), the nucleosome-induced activation (as determined by either CD66b/CD11b up-regulation, phagocytic activity or IL-8 secretion) was not inhibited by the LPS-inhibitor polymyxin B. In contrast, LPS-induced PMN activation was almost completely inhibited, indicating that nucleosome-induced activation was not due to endotoxin contamination. Likewise, nucleosome-induced partial inhibition of cell death was polymyxin B-independent and nucleosome-induced neutrophil recruitment and activation was observed *in vivo* in TLR2/TLR4-deficient mice (see below).

3.1.3 The nucleosome core structure is crucial for PMN activation

In order to investigate if nucleosome integrity is important for PMN activation, PMN were incubated with either nucleosomes, commercial histones, DNA purified from nucleosomes or a mixture of the latter two. PMN activation was determined by measuring CD11b up-regulation and IL-8 secretion. Since the endotoxin content in the commercial histone preparation was not indicated, PMN were cultured in the presence of polymyxin B. As shown in **Figure 11 A**, nucleosomes induced CD11b up-regulation in comparison to the empty gradient. The individual components of nucleosome, namely histones and DNA, alone or in combination did not induce a clear CD11b up-regulation. Accordingly, nucleosomes strongly induced IL-8 secretion in the presence of polymyxin B, whereas neither histones nor DNA nor the histone-DNA mixture did induce IL-8 secretion by human PMN, in comparison to the empty gradient (**Figure 11 B**), indicating that the nucleosome structure is required for PMN activation.



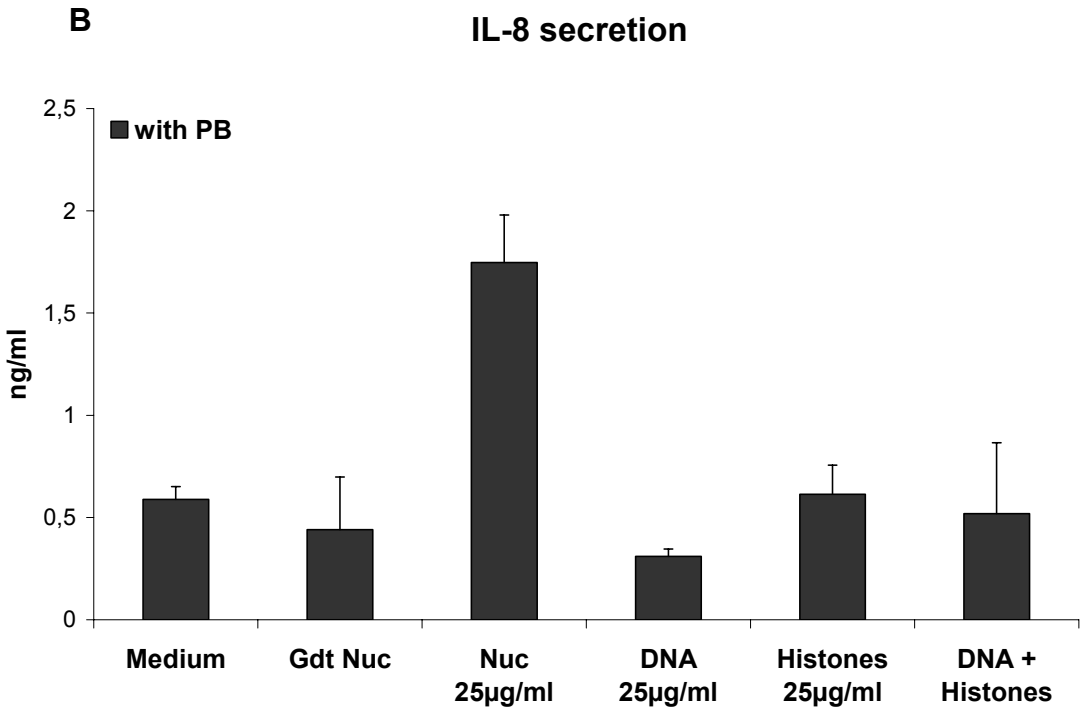


***Figure 11**. The nucleosome structure is important for PMN activation. Freshly isolated PMN were incubated with nucleosomes or its individual components (DNA, histones) in the presence of polymyxin B (PB; 25 µg/ml). (**A)** After 16 hours, CD11b expression was analyzed gating on CD66b-positive PMN by flow cytometry using specific mAb. And (**B)** cell culture supernatants were harvested and IL-8 concentrations (expressed in ng/ml) were determined by ELISA. Gdt Nuc, empty gradient; Nuc, nucleosomes; DNA, DNA purified from nucleosomes; histones, commercial purified histones. Shown is 1 of 4 experiments. SDs are indicated.*

The nucleosomes used in all experiments were purified from calf thymus (as described in Materials and methods). Calf thymus can be used for the preparation of nucleosomes, because histones are among the most conserved proteins. Thus, there is no difference in the protein sequence between histone H4 from calf, human and mouse. There are several different protocols to prepare nucleosomes. Reconstituted nucleosome core particles can be prepared from recombinant histones and DNA by dialysis against decreasing salt concentration, as described in (306). Thus, we wanted to compare PMN activation induced by our calf thymus-derived nucleosomes and reconstituted nucleosomes obtained from Prof. Dr. K. Luger (Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA). In theory, reconstituted nucleosomes have the advantage of containing only DNA and histones.

In cell activation studies, the use of such nucleosomes rules out the stimulation by any contaminants. As shown in **Figure 12** (with polymyxin B), nucleosomes induced CD11b up-regulation as determined by flow cytometry using specific mAb.

In contrast, neither purified DNA nor histones induced CD11b up-regulation by human PMN (**Figure 12**, without and with polymyxin B). However, reconstituted nucleosomes stimulated CD11b up-regulation, which was inhibited in the presence of polymyxin B, indicating the presence of contaminating LPS. Consequently, no conclusion could be drawn from the use of reconstituted nucleosomes.

The elaborate protocol for the preparation of reconstituted nucleosomes may make it more difficult to achieve low endotoxin concentrations. Nevertheless, *in vitro* reconstituted nucleosomes were expected to activate PMN in the absence or presence of polymyxin B as compared to the purification buffer (Buffer Rec Nuc; which was prepared by us under sterile conditions).

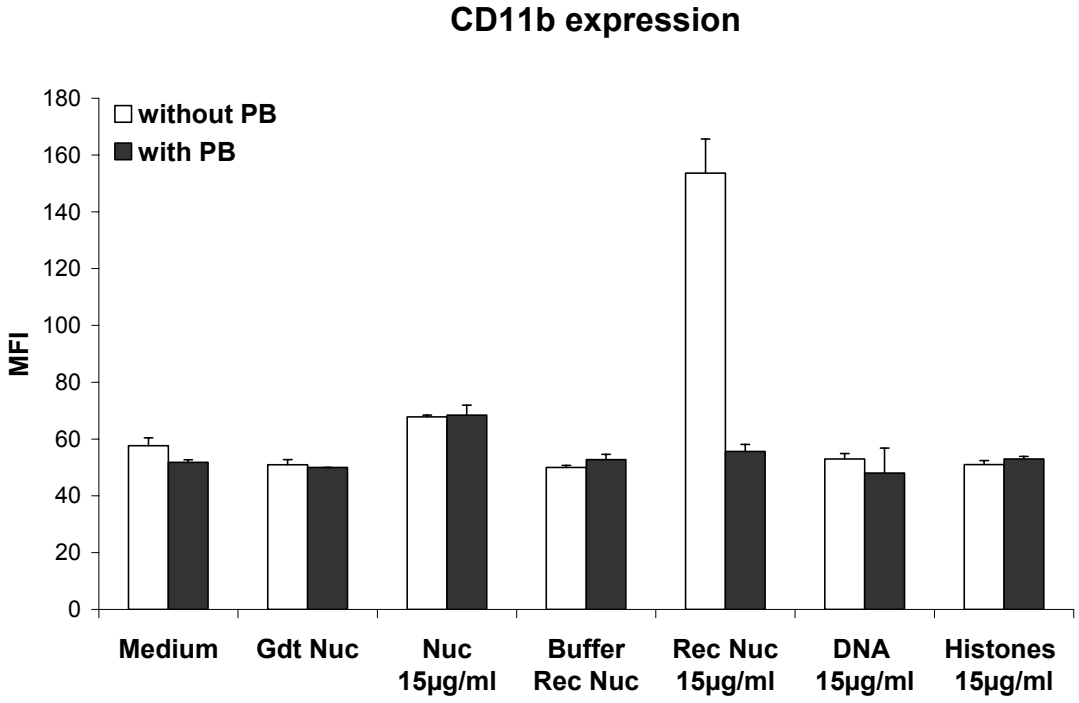


***Figure 12**. The nucleosome structure is important for PMN activation. Freshly isolated PMN were incubated with nucleosomes, its individual components or reconstituted nucleosomes in the absence or presence of polymyxin B (PB; 25 µg/ml). After 16 hours, CD11b expression was analyzed gating on CD66b positive PMN by flow cytometry using specific mAb. Gdt Nuc, empty gradient; Nuc, nucleosomes; Buffer Rec Nuc, buffer prepared according to the protocol for Rec Nuc; Rec Nuc, reconstituted nucleosomes; DNA, DNA purified from nucleosomes; histones, commercial purified histones. Shown is the result from one experiment. SDs are indicated.*

The results from **Figure 11** and **Figure 12** indicated that the integrity of the nucleosome structure is necessary for PMN activation. The previous experiments revealed that the nucleosome-induced PMN activation occured independently of the presence of unmethylated CpG motifs, since nucleosomal DNA did not activate the cells. Finally, it was shown that not all proteins were able to activate PMN.

In general, nucleosomes always induced increased PMN activation in comparison to the empty gradient. In some experiments, nucleosome-stimulated PMN activation was lower than expected, depending on the sensitivity of the purified neutrophils, cell culture conditions, the quality of the nucleosome preparation and the test carried out. However, in most cases the measurement of increased IL-8 secretion was a more sensitive activation marker as compared to the up-regulation of cell surface molecules.

High mobility group box-1 (HMGB-1) is an abundant and conserved component of vertebrate nuclei and has been proposed to play a structural role in chromatin organization, possibly similar to that of histone H1 (307). Although purified mono-nucleosomes usually contain only trace amounts of HMGB-1, some HMGB-1 may be copurified with nucleosomes from calf thymus. Since HMGB-1 has been shown to activate neutrophils (308), we aimed to exclude any possible HMGB-1 contamination in our nucleosome preparations, verifying that the detected PMN activation was not due to contaminating HMGB-1.
For this reason, freshly isolated PMN were incubated in the presence of nucleosomes, Histone H1-depleted nucleosomes (with a lower non-histone protein content and thus less HMGB-1) or cytokine-quality HMGB-1 and cell activation was estimated by IL-8 secretion. **Figure 13** shows the comparative result of PMN activation induced by each stimulus, normalized to the positive control, LPS, in the absence of polymyxin B.

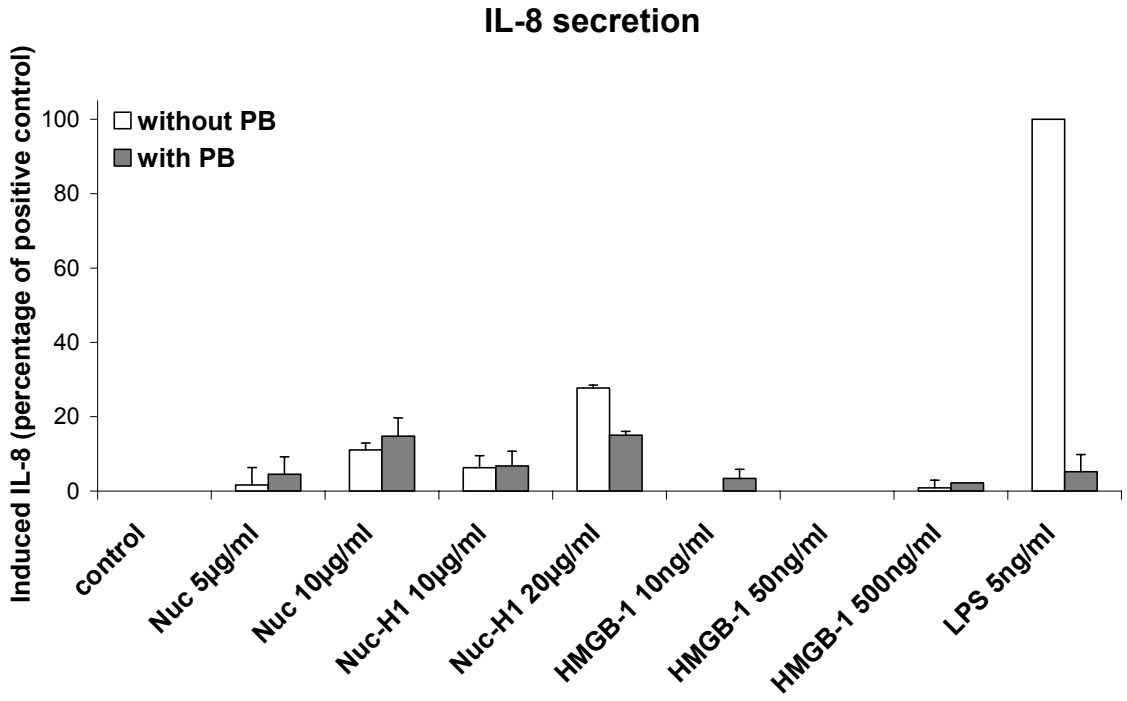


***Figure 13**. The core nucleosome structure is important for PMN activation. Freshly isolated PMN were cultured with either nucleosomes or H1-depleted nucleosomes or HMGB-1 or LPS in the absence or presence of polymyxin B (PB; 25 µg/ml). After 16 hours, cell culture supernatants were harvested and IL-8 concentrations were determined by ELISA. Induced IL-8 secretion was measured for each stimulus by subtracting baseline secretion (cells in medium) and referred to the positive control (LPS without polymyxin B) being 100 %. Nuc, nucleosomes; Nuc-H1, H1-depleted nucleosomes; HMGB-1, high-mobility group box-1; LPS, lipopolysaccharides. Shown is 1 of 3 representative results. SDs are indicated.*

While nucleosomes and H1-depleted nucleosomes clearly induced a dose-dependent IL-8 secretion (in the presence and absence of polymyxin B), there was no IL-8 secretion observed with HMGB-1- even at higher concentrations known to be biologically active (**Figure 13**). Thus, this result indicated that the core nucleosome structure (H1-depleted nucleosomes) was sufficient to induce PMN activation, and more importantly, that HMGB-1 did not activate PMN.

To confirm these results, we examined our nucleosome preparations for the presence of contaminating HMGB-1 by SDS-PAGE and two-dimensional gel electrophoresis.

Coomassie Brilliant Blue dyes are designed to stain proteins on SDS-PAGE gels. Coomassie G specifically stains basic proteins, such as histones, whereas Coomassie R stains all proteins. Here, we wanted to compare purified HMGB-1 and nucleosome, either with or without histone H1, on SDS-PAGE gels stained with either Coomassie Brilliant Blue G or R. As explained in Materials and methods, further treatment of the nucleosome preparation with a sodium chloride solution removed histone H1 and any other attached non-histone proteins (such as HMGB-1) from the nucleosomes (**Figure 14**, Nuc-H1).

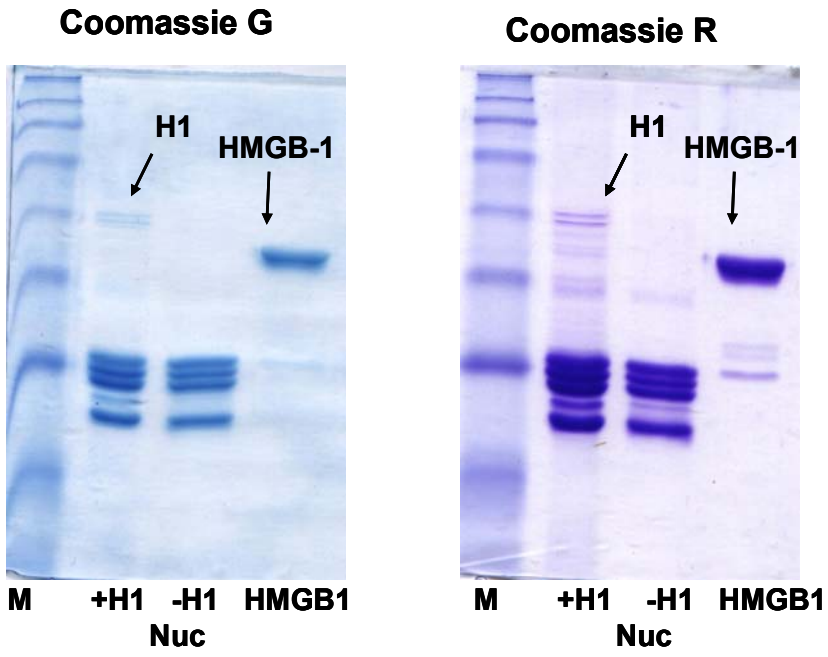


***Figure 14**. Nucleosome preparations were analyzed for HMGB-1 contamination by SDS-PAGE gel electrophoresis. An example of a SDS-PAGE gel showing nucleosome and H1-depleted nucleosome in comparison to purified HMGB-1 stained with Coomassie G (left panel) or Coomassie R Blue (right panel). (M, Marker; Nuc+H1, nucleosome with histone H1; Nuc-H1, histone H1-depleted nucleosome; HMGB-1, high-mobility group box-1)*

As shown in **Figure 14**, no contaminating HMGB-1 was detectable in our nucleosome-preparations (with or without histone H1) on the SDS gel stained with Coomassie G or R.

By comparing the reported molecular mass and pI of HMGB-1 from calf thymus (309) and purified HMGB-1 on two-dimensional gel with our nucleosome preparations, only two spots were likely to co-localize with HMGB-1 (**Figure 15**). These spots were cut out from two different gels, digested with trypsin and analyzed by nanoflow liquid chromatography tandem MS. Automated mascot software was used for searching NCBI.nr protein databases on all available mammalian proteins. And the proteins were finally identified as H2A histone family members.

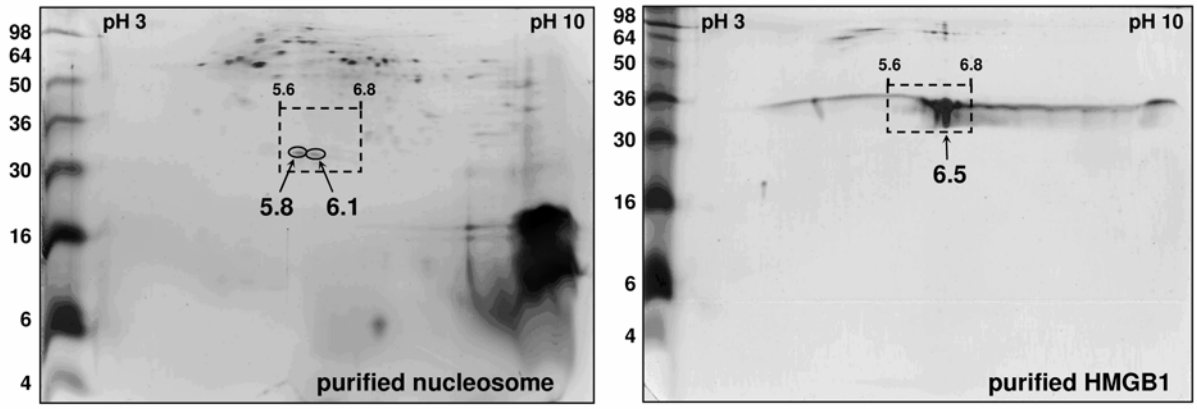


Figure 15. *Nucleosome preparations were analyzed for HMGB-1 contamination by two-dimensional gel electrophoresis. An example of a two-dimensional gel showing a nucleosome preparation (left panel) and the spot pattern of purified HMGB-1 (right panel). The position of HMGB-1 on the gel defined by its molecular mass and pI is marked (dashed box: pH 5.6 - 6.8, molecular mass 30 - 36kD). The two encircled spots of the corresponding area in the gel of the nucleosome preparation were identified by electrospray ionization-MS/MS as members of the H2A histone family.The prominent spots at pH 10 were shown to contain histone proteins. Shown is 1 of 3 representative experiments.*

These results revealed that there was no contaminating HMGB-1 detectable in our nucleosome preparations. Consequently, the nucleosome-induced PMN activation was not due to HMGB-1 contamination.

(SDS-PAGE gels, two-dimensional gel electrophoresis and MS were performed by Annika Erbacher in collaboration with Tobias Lamkemeyer and Johannes Madlung (Proteom Center Tübingen, Institute for Cell Biology, University of Tübingen, Tübingen).

3.1.4 Nucleosomes also activate PMN from SLE patients

We then compared the sensitivity of PMN from normal healthy donors and SLE patients to exclude any intrinsic difference. As expected, nucleosomes and LPS induced PMN activation in SLE patients, as estimated by IL-8 secretion (**Figure 16** and **Table 5**). The PMN activation observed with SLE patients was similar to the nucleosome-induced activation in normal healthy donors. Here, the aim was neither to test large patient cohorts nor to prove statistical differences between lupus patients and normal blood donors, but rather to verify that PMN from SLE patients are not defective or behave differently in nucleosome-induced activation. Thus, PMN from SLE patients responded to nucleosomes independently of gender, age, SLEDAI score, ANA and medication. By comparing nucleosome-induced activation, lupus PMN secrete comparable IL-8 levels (mean increase 4.49 fold; median 2.73 without PB; mean increase 3.26 fold; median 2.26 with PB) as PMN from healthy controls (mean increase 2.88; median 1.98 without PB; mean increase 3.7; median 2.35 with PB).

These results support the notion that such an activation mechanism exists *in vivo* in patients with SLE.

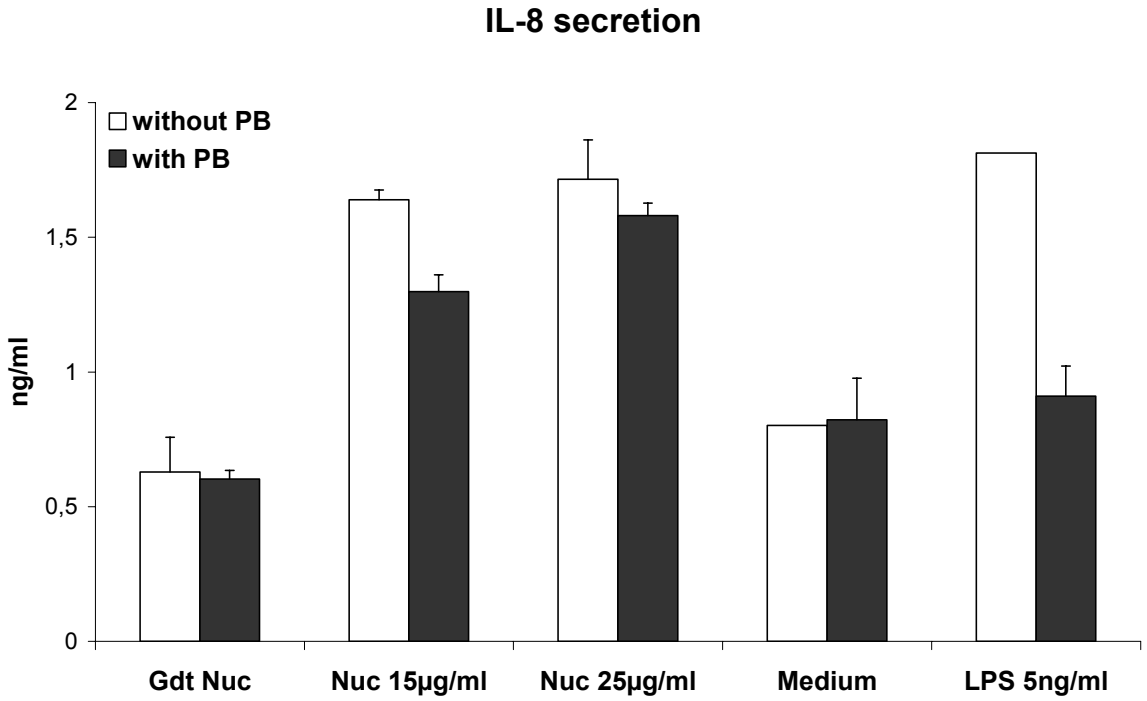


***Figure 16**. Nucleosomes also induce PMN activation in SLE patients. Freshly isolated PMN were prepared from **SLE patient 1** and cultured with nucleosomes or LPS in the absence or presence of polymyxin B (PB; 25 µg/ml). After 16 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. See **Figure 10** for abbreviations. Shown is 1 of 5 representative results (See **Table 5**). SDs are indicated.*

				ANA	Therapy			IL-8 secretion (ng/ml)							
								without Polymyxin B				with Polymyxin B 25µg/ml			
Patient No.	Sex	Age (years)	SLEDAI	Anti-DNA	PRED (mg/day)	AZA (mg/day)	CSA (mg/day)	Gdt Nuc	Nuc 25µg/ml	Medium	LPS 5ng/ml	Gdt Nuc	Nuc 25µg/ml	Medium	LPS 5ng/ml
1	M	57	6	neg.	0	0	0	0.628	1.716	0.802	1.813	0.603	1.581	0.823	1.183
2	F	61	2	pos.	3.5	50	0	0.021	0.227	0.102	0.156	0.215	0.409	0.296	0.479
3	F	26	0	pos.	0	0	0	5.850	12.258	6.931	19.011	2.754	4.337	6.174	6.623
4	F	26	0	neg.	0	0	0	0.41	0.738	0.693	3.407	0.264	0.523	0.384	0.857
5	F	29	0	n.d.	10	0	200	0.015	0.075	0.025	1.563*	0.025	0.158	0.071	0.167*

				ANA	Therapy			IL-8 secretion (ng/ml)							
								without Polymyxin B				with Polymyxin B 25µg/ml			
healthy control	Sex	Age (years)	SLEDAI	Anti-DNA	PRED (mg/day)	AZA (mg/day)	CSA (mg/day)	Gdt Nuc	Nuc 25µg/ml	Medium	LPS 5ng/ml	Gdt Nuc	Nuc 25µg/ml	Medium	LPS 5ng/ml
1	M	34	0	neg.	0	0	0	0.138	0.256	0.142	0.606	0.249	0.527	0.250	0.379
2	M	26	0	neg.	0	0	0	0.305	2.144	0.451	1.861	0.462	1.193	0.698	0.664
3	M	34	0	neg.	0	0	0	1.722	2.587	3.511	18.168	1.366	2.225	3.278	5.693
5	M	26	0	n.d.	0	0	0	0.229	0.608	0.285	2.225	0.053	0.450	0.175	1.061

Table 5. Nucleosomes induce PMN activation in normal healthy donors and SLE patients. *Freshly isolated PMN were prepared from SLE patients and healthy volunteers and cultured with nucleosomes or LPS in the absence or presence of polymyxin B (PB; 25 µg/ml). After 18 hours cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. Results are from four independent experiments. SLEDAI, disease activity index score; ANA, antinuclear autoAbs; Anti-DNA, anti-dsDNA Abs; PRED, prednisolone; AZA, azathioprine; CSA, cyclosporine A; Gdt Nuc, empty gradient used for nucleosome purification; Nuc, Nucleosome; LPS, lipopolysaccharides; neg., negative; pos., positive; n.d., not determined.*

3.1.5 Nucleosome-induced activation delayed PMN apoptosis

Prolonged survival or delayed cell death of PMN usually results from their prior activation. LPS strongly induced PMN activation and was demonstrated to prolong PMN survival. Therefore, the effect of nucleosomes on PMN cell death was investigated. Interestingly, the nucleosome-induced activation clearly prolonged the cell survival in human PMN, as compared to non-activated cells (**Figure 17**). This prolonged survival resulted from delayed early apoptosis, as shown in **Figure 18**. Here, early apoptotic PMN were defined as Annexin V-positive, but 7-AAD-negative cells among CD66b-positive cells. Although the positive effect of LPS on PMN survival was stronger, the nucleosome-induced delayed early apoptosis was polymyxin B-independent, as compared to LPS. Furthermore, nucleosome-induced activation delayed cell death after over night (20 hours) incubation, in contrast to non-activated cells when measuring total cell death (7-AAD-positive cells among CD66b-positive cells (**Figure 19**). LPS delayed cell death to a higher extent, but in a polymyxin B-dependent manner.

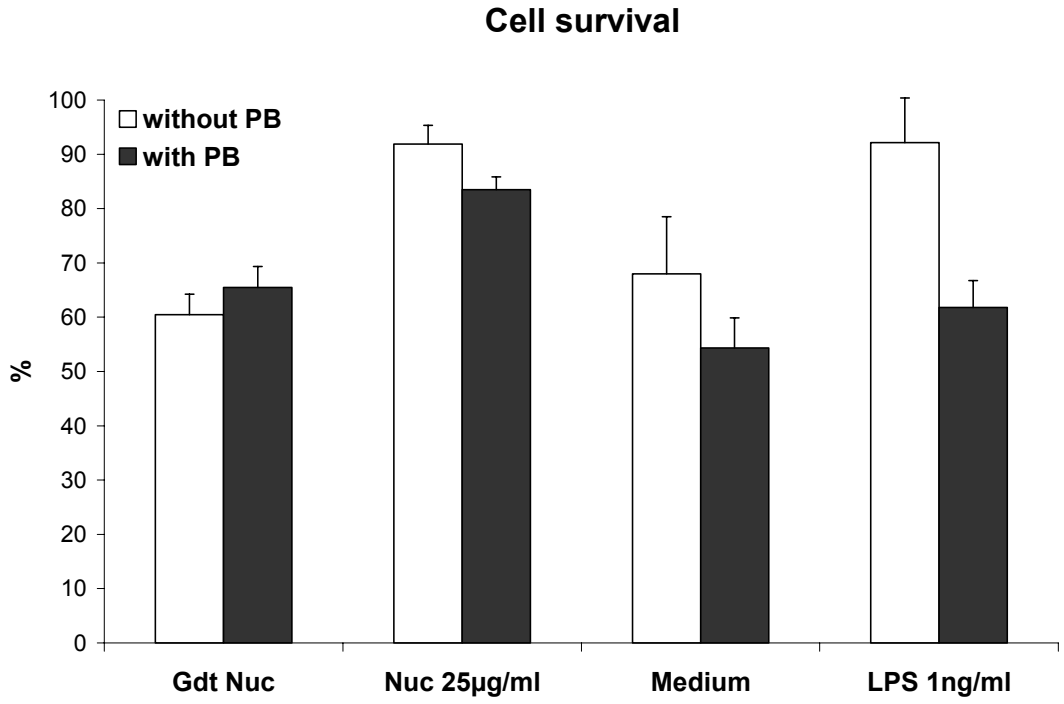


***Figure 17**. Nucleosome-induced PMN activation prolongs cell survival. Freshly isolated PMN were cultured over night with different stimuli in the absence or presence of polymyxin B (PB; 25 µg/ml) and analyzed by flow cytometry. PMN cell survival was estimated by measuring the percentage of Annexin V-negative, 7-AAD-negative cells among CD66b-positive cells. Gdt Nuc, empty gradient; Nuc, nucleosomes; LPS, lipopolysaccharides. Shown is the percentage of cells among CD66b-positive cells. Shown is 1 of 6 representative experiments. SDs are indicated.*

Importantly, an increased cell death was never observed when PMN were incubated in the presence of nucleosomes. These results revealed that nucleosome-induced activation prolonged the cell survival and delayed early apoptosis and cell death. Actually, the rate of *in vitro* apoptosis was found to be increased in patients with SLE when compared with normal healthy controls (261). However, in the present experiments the *in vitro* effect of nucleosomes on isolated PMN from healthy volunteers and SLE patients was examined.

In conclusion, nucleosome-induced stimulation transiently delayed the rate of *in vitro* apoptosis in human PMN. We do not claim that nucleosomes inhibit PMN cell death, but only that nucleosomes transiently delayed early apoptosis, as a result of PMN activation. In the present study, we did not compare PMN from normal donors and SLE patients for cell death rates, because human PMN usually start to die after several hours in cell culture.

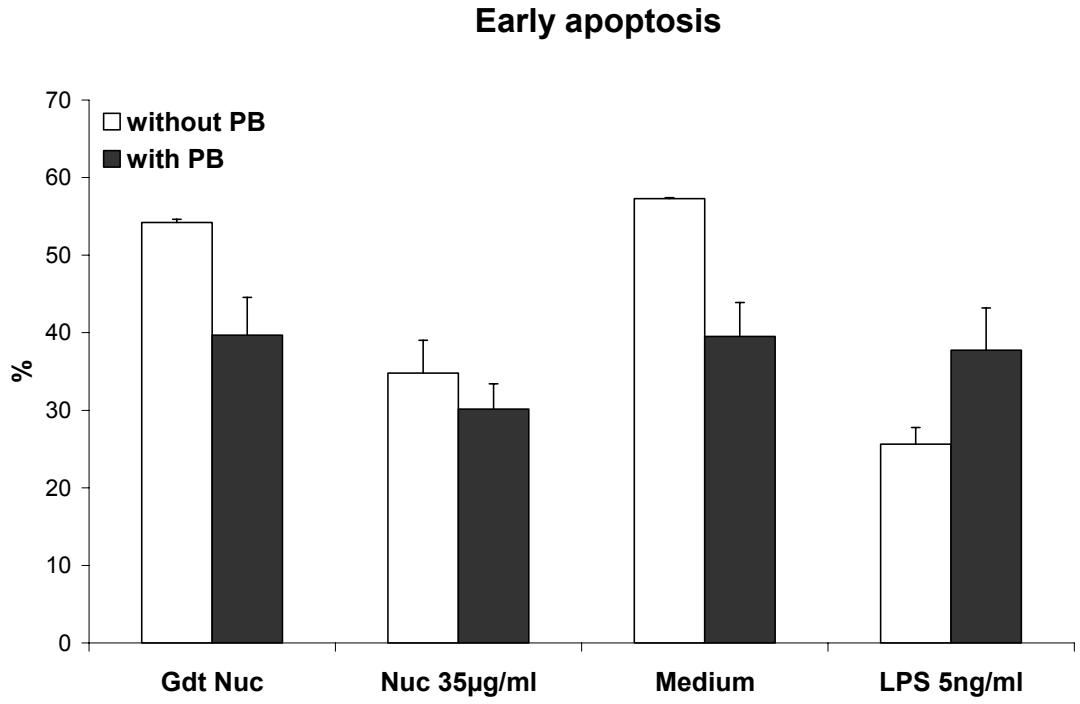


***Figure 18**. Nucleosome-induced PMN activation delays cell death. Freshly isolated PMN were cultured over night with different stimuli in the absence or presence of polymyxin B (PB; 25 µg/ml) and analyzed by flow cytometry. Early apoptosis was estimated by measuring the percentage of Annexin V-positive, 7-AAD-negative cells among CD66b-positive cells. See **Figure 17** for abbreviations. Shown is 1 of 6 representative experiments. SDs are indicated.*

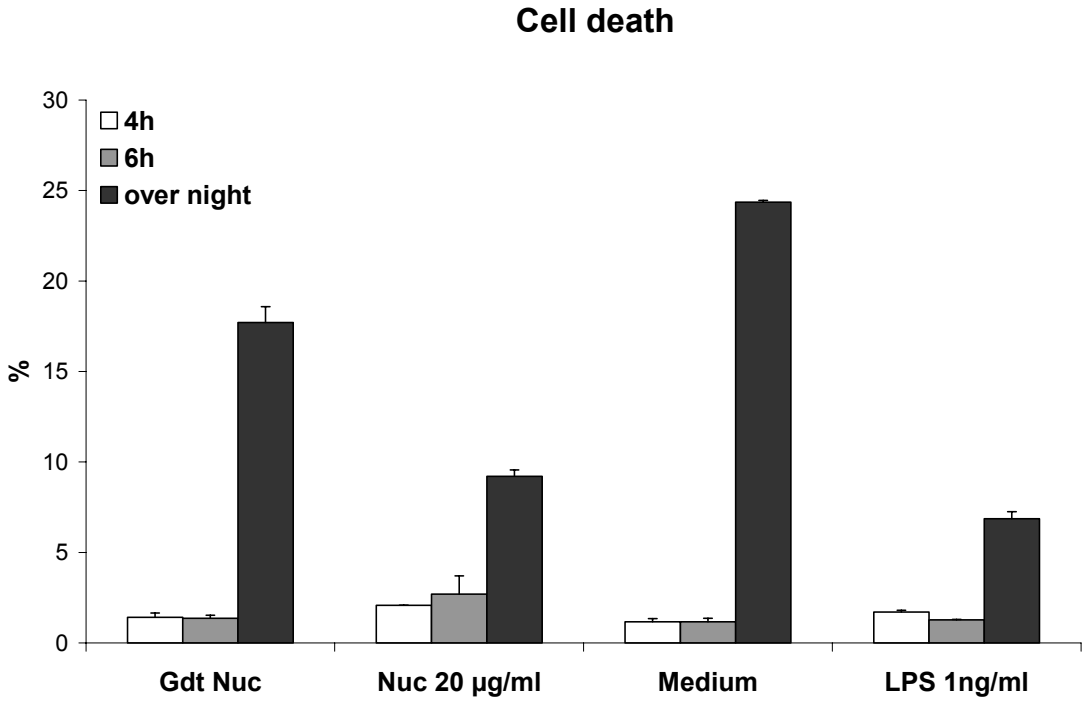


***Figure 19**. Nucleosome-induced PMN activation delays cell death. Freshly isolated PMN were cultured with different stimuli for 4 hours, 6 hours or 20 hours (over night) and analyzed by flow cytometry. Cell death was estimated by measuring the percentage of 7-AAD-positive cells among CD66b-positive cells. See **Figure 17** for abbreviations. Shown is 1 of 2 representative experiments. SDs are indicated.*

3.1.6 Nucleosomes activate and recruit neutrophils *in vivo* in a TLR2/TLR4-independent manner

In order to further support our *in vitro* results, we investigated nucleosome-induced neutrophil activation *in vivo* in mice. Therefore, mice were intraperitoneally (i.p.) injected with either purified nucleosomes or empty gradient or LPS (*S. typhimurium*). After 18 hours, mice were sacrificed and peritoneal exudates cells (PECs) were harvested. PEC activation and recruitment were analyzed by flow cytometry. The percentage of living activated neutrophils among peritoneal cells was defined as 7-AAD-negative, Ly-6G^{high} and CD11b^{high} expressing cells for each individual mouse. First, normal BALB/c mice were i.p. injected with different stimuli. As shown in **Figure 20**, the injection of nucleosomes and LPS resulted in activation and recruitment of neutrophils among peritoneal cells, in comparison to the empty gradient.

Although not all of the mice responded to the nucleosome- or LPS-injection, most of them had more neutrophils in the peritoneal cavity than the control mice. None of the empty gradient-injected mice showed increased activation or recruitment of neutrophils among PECs. This result supported the finding that nucleosomes may activate and recruit neutrophils *in vivo* in patients with SLE.

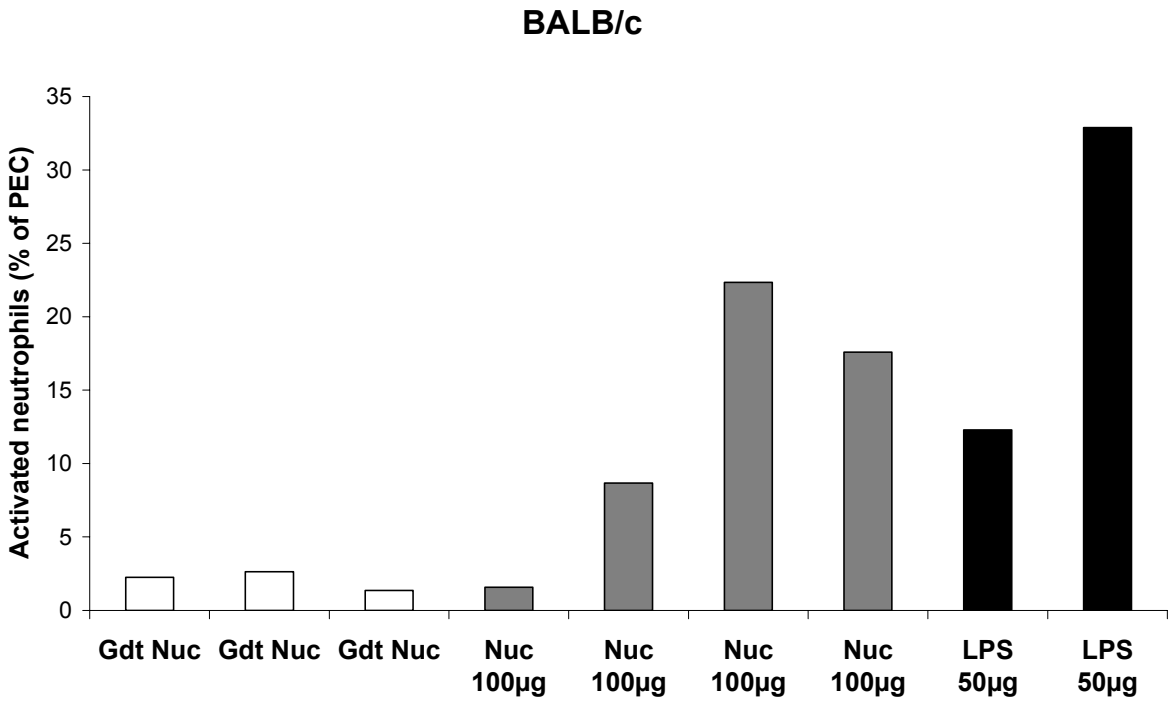


***Figure 20**. Nucleosomes activate and recruit neutrophils in vivo. Normal BALB/c mice were i.p. injected with purified nucleosomes (Nuc), empty gradient (Gdt Nuc) or LPS. After 18 hours, peritoneal exudate cells (PEC) were prepared and neutrophil recruitment and activation was estimated by flow cytometry measuring the percentage of living Ly-6G^{high} and CD11b^{high} cells among PEC. PEC were first incubated with an FcR-blocking mAb and then stained with FITC-conjugated anti-Ly-6G and PE-conjugated CD11b-specific mAb, followed by staining with 7-AAD. Shown is the percentage of neutrophils among PEC. Each bar represents an individual mouse. Shown is the result from one single experiment.*

In order to verify that the nucleosome-induced activation and recruitment of neutrophils in mice, was not due to endotoxin contamination, we repeated the previous experiment with TLR2/TLR4-deficient mice. For this purpose, C3H/HeJ TLR2-/- mice were i.p. injected with either nucleosomes, empty gradient or LPS.
Since these mice lack functional TLR2 and TLR4, it was expected that they did not show LPS-induced activation and recruitment of neutrophils, as shown in **Figure 21**.

On the contrary, the majority of nucleosome-injected mice clearly had increased numbers of activated neutrophils in the peritoneum, in comparison to the empty gradient-injected mice (**Figure 21**). None of the empty gradient-injected mice showed increased percentage of neutrophils in the peritoneum.

In fact, we did not expect to observe recruitment and activation in all nucleosome-injected mice. Besides, the aim was not to induce “lupus-like disease” in normal mice, but to demonstrate nucleosome-induced effects in normal mice, particularly in TLR2/TLR4-deficient mice, in order to exclude any endotoxin contamination.

This result indicated that nucleosomes induced recruitment and activation *in vivo* and in a TLR2/TLR4-independent manner.

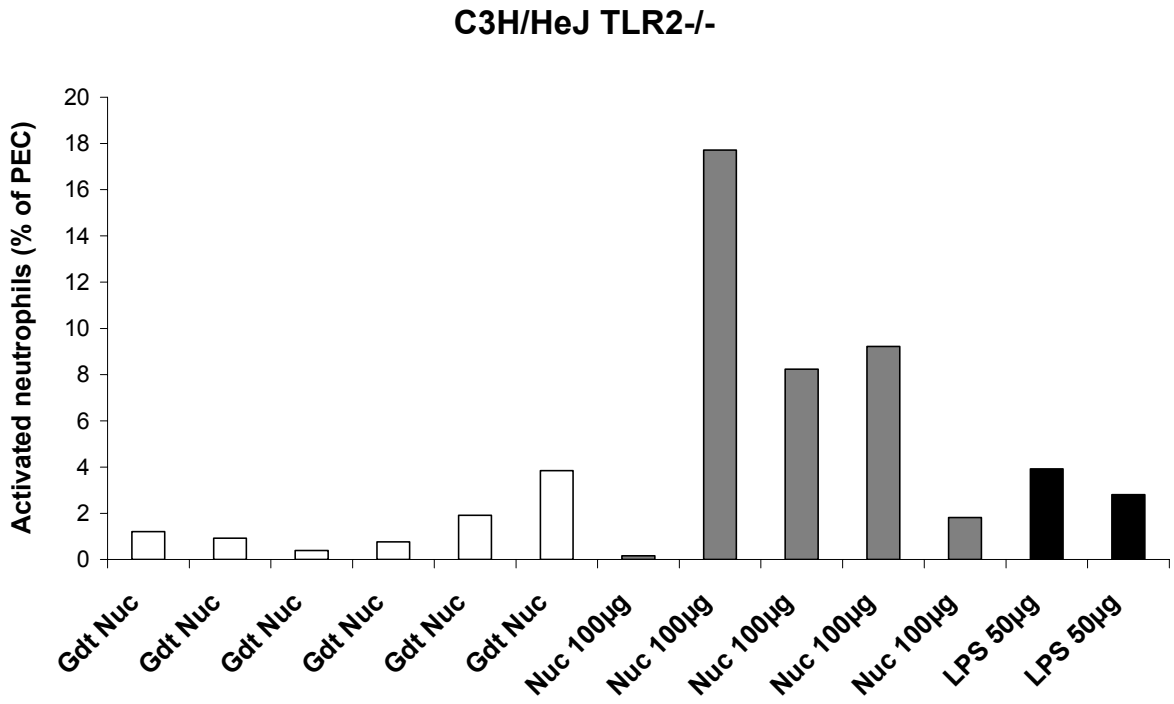


***Figure 21**. Nucleosomes activate and recruit neutrophils in vivo. TLR2/TLR4 deficient mice were i.p. injected with either purified nucleosomes (Nuc), empty gradient (Gdt Nuc) or LPS. After 18 hours, PEC were prepared and neutrophil recruitment and activation was estimated by flow cytometry measuring the percentage of living Ly-6G^{high} and CD11b^{high} cells among PEC. PEC were first incubated with an FcR-blocking mAb and then stained with FITC-conjugated anti-Ly-6G and PE-conjugated CD11b-specific mAb, followed by staining with 7-AAD. Each bar represents an individual mouse. See **Figure 20** for abbreviations. Shown are the results from two independent experiments.*

3.1.7 Nucleosomes are internalized by human PMN

To examine the mechanism involved in nucleosome-induced PMN activation, we first investigated the binding of FITC-conjugated nucleosomes to the cell surface of human PMN by flow cytometry. When PMN were incubated at 4°C, no cell surface binding was observed with FITC-conjugated nucleosomes in comparison to PMN alone (**Figure 22 A**). In contrast, a distinct FITC-signal was detected when PMN were incubated at 37°C in the presence of FITC-conjugated nucleosomes (**Figure 22 B**). These results suggested that FITC-conjugated nucleosomes hardly bound to the cell surface of PMN, but were rather internalized.

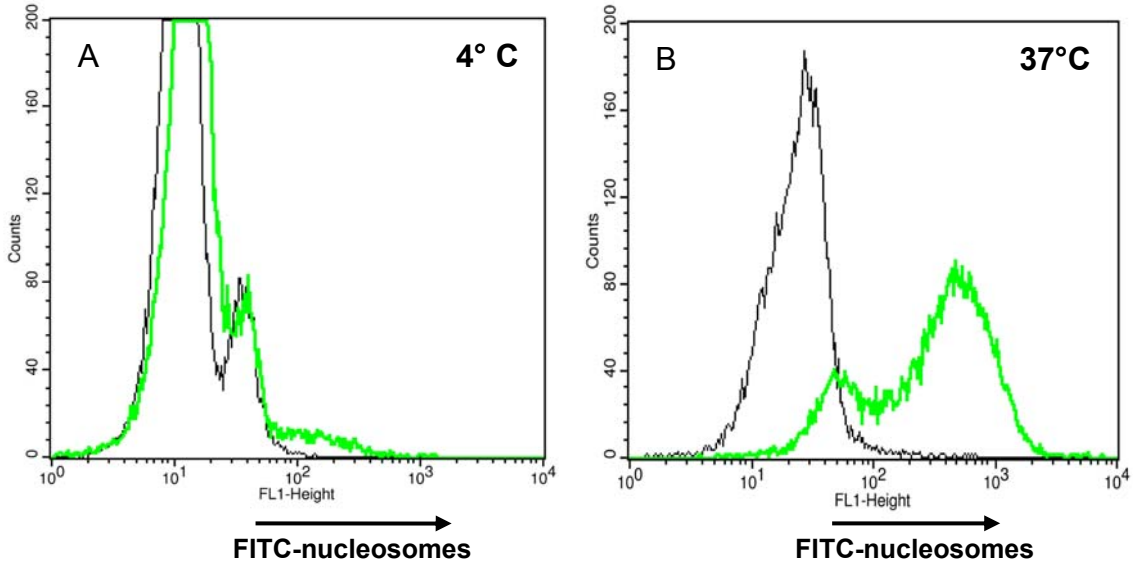


***Figure 22**. Nucleosomes are internalized by human PMN. Freshly isolated PMN were incubated with or without FITC-nucleosomes for 30 minutes at 4°C (**A**) or at 37°C (**B**) and analyzed by flow cytometry. Green line, PMN with FITC-nucleosomes; thin line, PMN alone (Propidium iodide-negative cells).*

To confirm these results, cell surface binding and endocytosis of nucleosomes by human PMN were further examined by confocal laser scanning microscopy. PE-conjugated anti-CD11b Ab was used as a cell surface marker for PMN. In contrast to the homogeneous cell surface staining with CD11b-specific Ab, the observed low binding of FITC-nucleosomes was different, namely more patchy and non-homogeneous (**Figure 23 A**).

In agreement with the result obtained by flow cytometry, FITC-nucleosomes were clearly detectable in the cytoplasm of PMN at 37°C (**Figure 23 B**), signifying that nucleosomes were taken up by human PMN.

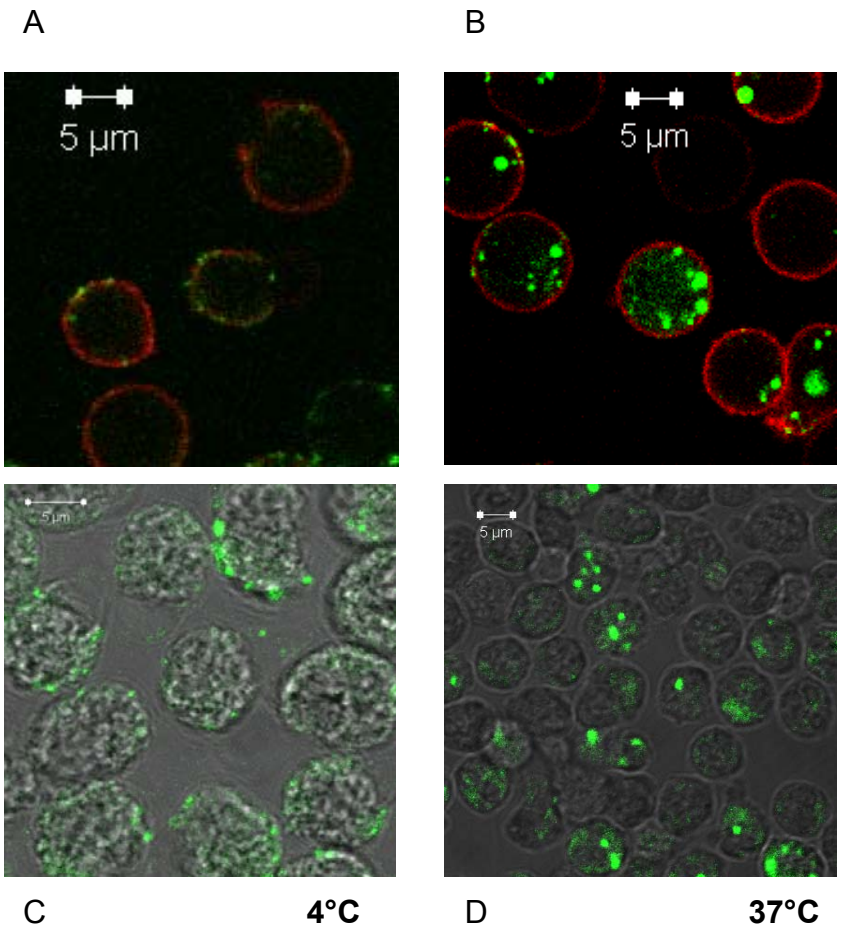


***Figure 23**. Nucleosomes are internalized by human PMN. Freshly isolated PMN were incubated with FITC-nucleosomes (10 µM) for 30 minutes at 4°C (**A**) or 37°C (**B**), washed, stained with PE-conjugated anti-CD11b mAb, fixed and analyzed by confocal microscopy. Green, FITC-nucleosomes; Red, anti-CD11b mAb (**A,B**). PMN were incubated with unconjugated nucleosomes (35 µg/ml) for 1 hour at 4°C (**C**) or 37°C (**D**), washed, permeabilized and stained with mouse anti-histone mAb (5 µg/ml).Then, cells were stained with FITC-conjugated goat anti-mouse secondary Ab (1/1,000) (green), fixed and analyzed by confocal microscopy (**C,D**). Representative slices are shown. The size is indicated.*

In order to exclude any structural differences between FITC-labelled nucleosomes and free nucleosomes due to the FITC-conjugation, which might influence cell behaviour, the above results were repeated using an indirect staining method.

For this purpose, PMN were incubated with unconjugated nucleosomes for 1 hour at 4°C or 37°C, washed, permeablilized and stained with a mouse anti-histone mAb. After two washes, cells were stained with a FITC-conjugated goat anti-mouse secondary Ab and analyzed by confocal microscopy.

In agreement with the previous results, a patchy cell surface staining of nucleosomes was observed at 4°C and a clear internalization at 37°C (**Figure 23 C** and **D**).

Likewise, the same results were also obtained when FITC-conjugated H1-depleted nucleosomes were used. As shown in **Figure 24**, freshly isolated PMN were incubated with either FITC-nucleosomes (**A**) or FITC-conjugated H1-depleted nucleosomes (**B**) for 30 minutes at 37°C (or 4°C, data not shown). The cells were then washed and stained with PE-conjugated anti-CD11b mAb, fixed and analyzed by confocal microscopy. Thus, FITC-conjugated nucleosomes as well as FITC-conjugated H1-depleted nucleosomes were taken up by human PMN at 37°C. (In agreement with the previous result, non-homogeneous cell surface binding was observed with FITC-conjugated H1-depleted nucleosomes at 4°C, data not shown.)

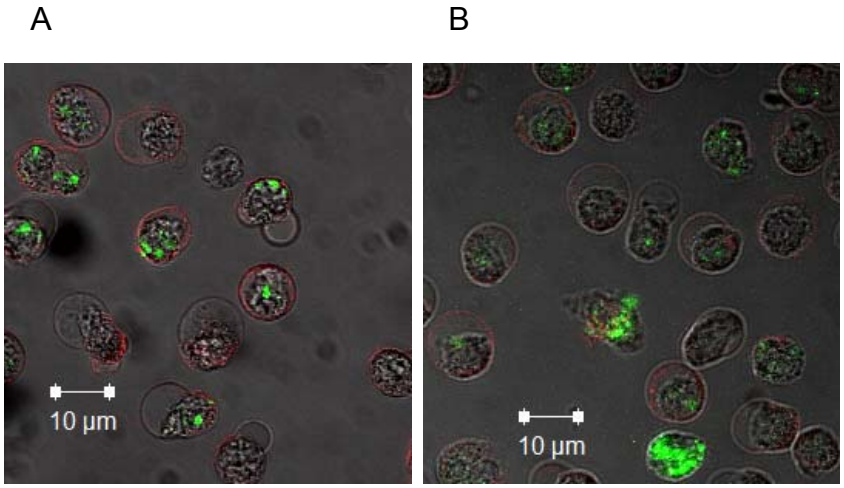


***Figure 24**. Nucleosomes are internalized by human PMN. Freshly isolated PMN were incubated with FITC-nucleosomes (10 µM) (**A**) or FITC-conjugated H1-depleted nucleosomes (10 µM) (**B**) for 30 min at 37°C, washed, stained with PE-conjugated anti-CD11b mAb, fixed and analyzed by confocal microscopy.Green, FITC-nucleosomes; Red, anti-CD11b mAb. Representative slices are shown. The size is indicated.*

All together, these results revealed that nucleosomes were endocytosed by human PMN independently of conjugated FITC molecules or any attached histone H1 or non-histone proteins.

Next, we wondered if the uptake of nucleosomes could be enhanced to some extent. Human complement component C1q is known to directly and specifically bind to apoptotic cells and isolated blebs from apoptotic cells, as well as to a variety of proteins in an antibody-independent manner (see (310) for review). Moreover, low serum C1q levels have been reported in SLE patients (199). Interestingly, our group has recently found that free nucleosomes directly interact with C1q (Erbacher et al., manuscript in preparation).

Therefore, freshly prepared human PMN were incubated for 30 minutes at 4°C or 37°C with FITC-nucleosomes in the absence or presence of human complement component C1q. The cells were then washed, fixed and analyzed by confocal microscopy. As shown in **Figure 25 A** and **C**, cell surface binding of FITC-nucleosomes at 4°C was slightly increased in the presence of C1q, although the signal was not strong. Likewise, C1q clearly enhanced the uptake of FITC-nucleosomes at 37°C (**Figure 25 B** and **D**). These results demonstrated that the binding of human complement component C1q to nucleosomes resulted in an increased cell surface binding and enhanced engulfment of nucleosomes by human PMN, which bear C1q-receptors at the cell surface.

Moreover, we observed by confocal microscopy that the endocytosis of nucleosomes was slightly enhanced, when PMN were incubated in medium containing 10 % decomplemented autologous serum (data not shown).

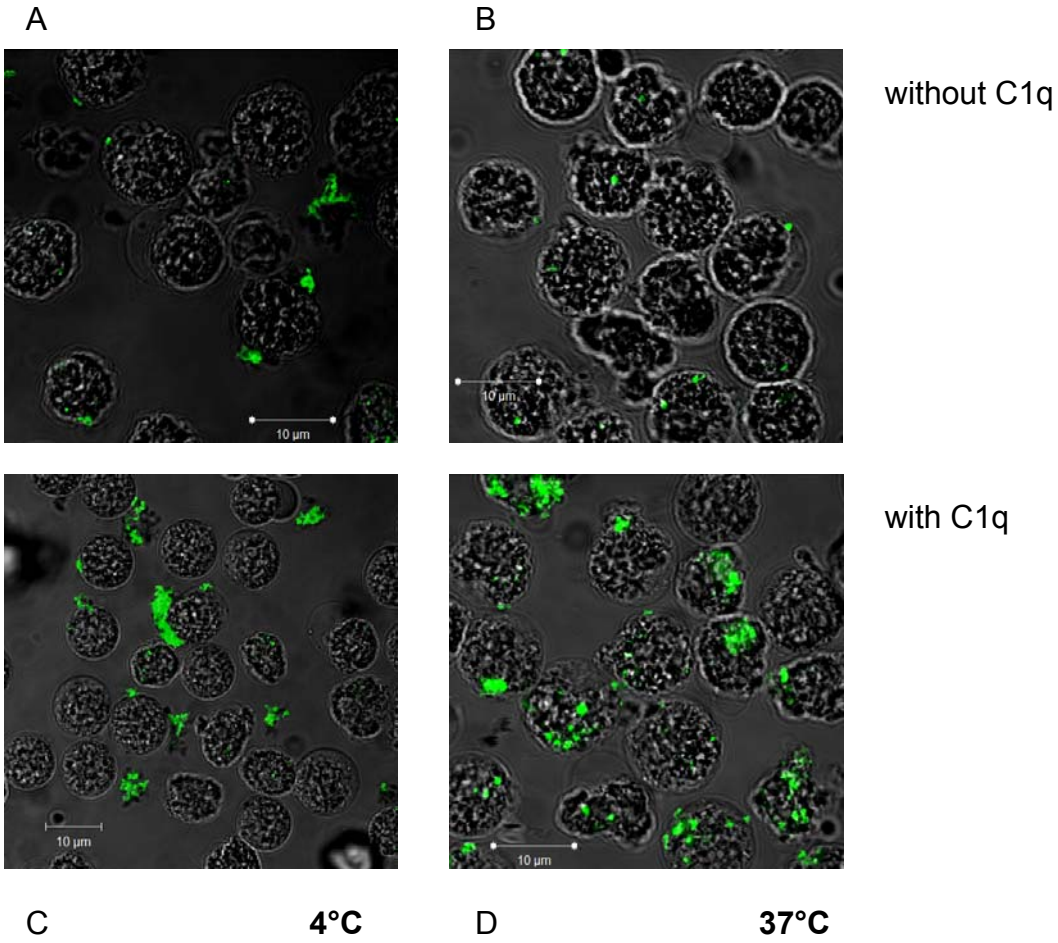


***Figure 25**. C1q enhances binding and uptake of nucleosomes by human PMN. Freshly isolated PMN were incubated with FITC-nucleosomes (30 µM) for 30 minutes at 4°C (**A, C**) or 37°C (**B, D**) in the absence (**A, B**) or presence (**C, D**) of human complement component C1q (50 µg/ml). Cells were then washed, fixed and analyzed by confocal microscopy. Green, FITC-nucleosomes. Shown is the fluorescein fluorescence. The size is indicated.*

3.2 The involvement of endosomal TLRs in nucleosome-induced neutrophil activation

In the first part, we have shown that free nucleosomes directly induced the activation of neutrophils *in vitro* and *in vivo*. Consequently, we wanted to find out more about the activation mechanism involved. For this purpose, we had two different approaches: On the one hand, we aimed to inhibit nucleosome-induced PMN activation, in order to indirectly gain knowledge about the putative receptors involved in the recognition of nucleosome. On the other hand, we further analyzed the internalization of nucleosome by confocal microscopy to get more information about its endocytic pathway.

3.2.1 Inhibition of endosomal acidification

Chloroquine and ammonium chloride are weak bases, which accumulate in any organelle or vesicle with an acidic pH. Both block endosomal acidification. Due to the elevated intravesicular pH, integral membrane proteins accumulate in endosomes, receptor-ligand interactions are disrupted and acid proteases in the endosome/lysosome degradative pathway are inhibited (311). Particularly, signalling through endosomal TLR is inhibited in the presence of chloroquine or ammonium chloride.

3.2.1.1 Chloroquine

SLE patients develop autoantibodies against self antigens, including nucleic acids. More precisely, they have antibodies that recognize the DNA backbone, which is common to the DNA of all species, including humans. The molecular events that give rise to these antibodies remain unknown, but signalling through nucleic acid-sensing TLRs, especially TLR9, might be involved. In addition, there are many publications, claiming that endosomal TLRs play a role in the development and pathogenesis of SLE. Since nucleosomes also consist of DNA, we wanted to investigate the engagement of intracellular TLRs in nucleosome-induced PMN activation.

By searching for studies about human neutrophil activation stimulated by bacterial DNA, we found two opposing publications:

Jozsef et al. (312) showed that endosomal acidification inhibitors (bafilomycin A, chloroquine) prevented CpG-DNA-induced cytokine release (IL-8, IL-6) from human PMN, suggesting an involvement of TLR9. In contrast, Trevani and collaborators (313,314) suggested that bacterial DNA activated human neutrophils through a TLR9-independent, but MyD88-dependent pathway. According to them, bacterial DNA-induced neutrophil activation was not inhibited by wortmannin or chloroquine and occurred in a CpG-independent manner. Interestingly, both groups have used E.coli DNA (strain B) from Sigma in their experiments. (As explained below, we suspect that the E.coli DNA from Sigma was likely to contain LPS.) Furthermore, Trevani's group also reported that only backbone-substituted phosphorothioate oligodeoxynucleotide (PS-ODN), such as CpG ODN 2006, induced the activation of human neutrophils in a CpG-independent manner, whereas native phosphodiester ODN did not (314).

Thus, freshly isolated human PMN were incubated in the absence or presence of chloroquine for 30 minutes at 37°C and then challenged with different stimuli. After 17 hours, nucleosome-induced PMN activation was determined by ELISA, measuring IL-8 secretion. As shown in **Figure 26**, pre-treatment of PMN with the endosomal acidification inhibitor chloroquine did not inhibit bacterial DNA-induced activation, as it has been claimed by Jozsef et al. Although the same E.coli DNA (strain B) from Sigma was used (dsDNA or heat-denaturated ssDNA), there was no inhibition of CpG DNA-induced IL-8 secretion observed. In addition, nucleosomes induced increased IL-8 secretion, as compared to the empty gradient, which was not prevented in the presence of chloroquine. LPS was used as a positive control and, as expected, not influenced by the chloroquine treatment. Thus, no conclusion could be drawn at that point on the role of endosomal TLRs in nucleosome-induced neutrophil activation.

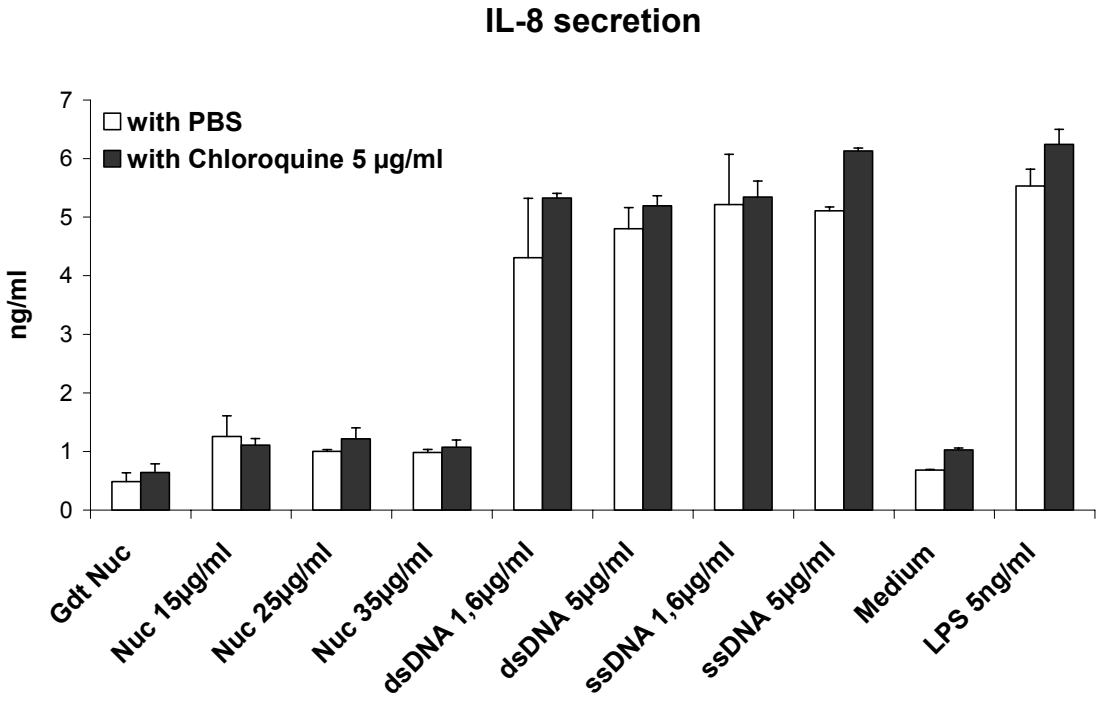


***Figure 26**. Bacterial DNA stimulation and inhibition of endosomal acidification. Freshly isolated PMN were incubated with or without chloroquine (5 µg/ml) for 30 minutes at 37°C and then cultured with different stimuli. After 17 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. Gdt Nuc, empty gradient used for the nucleosome purification; Nuc, nucleosomes; dsDNA, double-stranded DNA from E.coli; ssDNA, single-stranded DNA from E.coli; LPS, lipopolysaccharides. IL-8 secretion is expressed in ng/ml. Shown is 1 of 7 representative experiments. SDs are indicated.*

Although the incubation time with chloroquine was varied (30 minutes, 1 hour, 2 hours) and the concentration was titrated (from 0.2 to 20 µM, from 5 to 10 µg/ml), chloroquine never inhibited bacterial DNA-induced PMN activation. As shown in **Figure 27**, pre-treatment of PMN with different concentrations of chloroquine did not block PMN activation induced by neither bacterial DNA nor two different CpG PS-ODNs, although ODN were not potent activators.

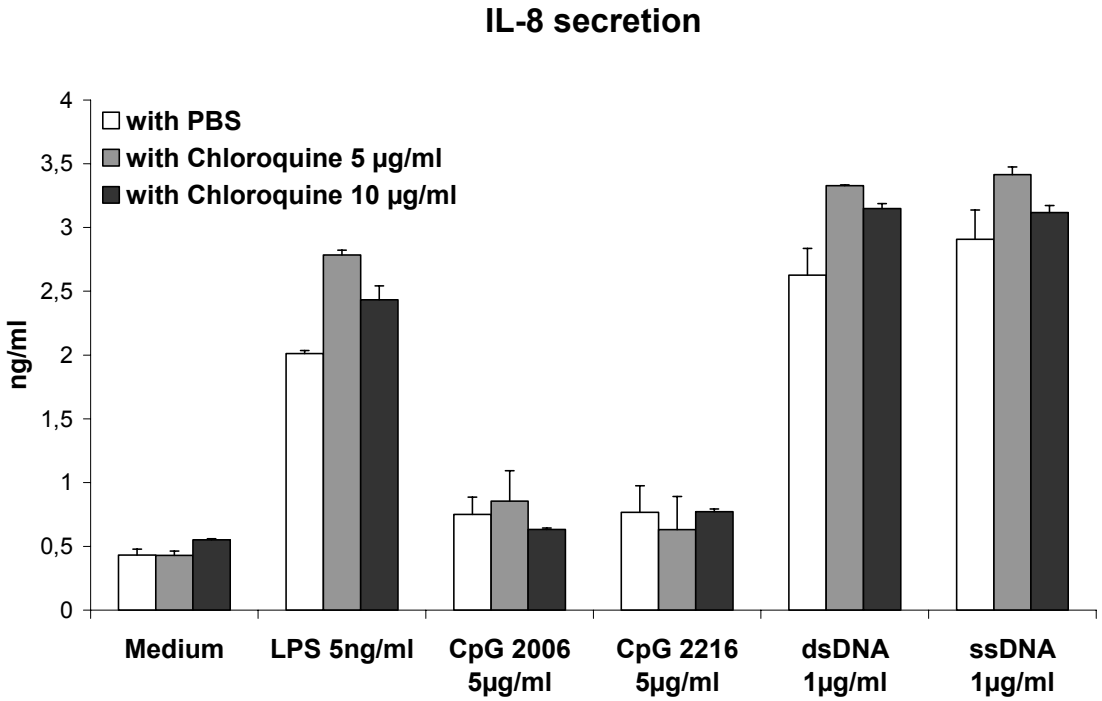


***Figure 27**. CpG PS-ODN and bacterial DNA stimulation and inhibition of endosomal acidification. Freshly isolated PMN were incubated with or without chloroquine (5 µg/ml, 10 µg/ml) for 1 hour at 37°C and then cultured with different stimuli. After 15 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. See **Figure 26** for abbreviations. Shown is 1 of 2 representative experiments. SDs are indicated.*

These results demonstrated that PMN activation stimulated by either CpG PS-ODN or bacterial DNA was not prevented, when endosomal acidification was blocked with chloroquine. Thus, Trevani and co-workers might be right when claiming that bacterial DNA stimulates neutrophils in a TLR9-independent manner. However, we have no control showing that chloroquine really inhibits endosomal acidification in these experiments. According to Trevani's group, bacterial DNA stimulated macrophages, monocytes, B lymphocytes, NK cells and DCs in a CpG-dependent manner, whereas human neutrophils were activated by a CpG-independent pathway.

To test this hypothesis, bone marrow-derived DCs (BMDCs) from normal C57BL/6 mice were prepared. At day 6 of cell culture, BMDCs were incubated in the absence or presence of chloroquine for 2 hours at 37°C and then challenged with different stimuli.

BMDC activation was determined by ELISA measuring IL-12 p40/p70 secretion. As shown in **Figure 28**, pre-treatment of BMDCs with chloroquine did almost completely inhibit IL-12 secretion induced by CpG ODN.

In contrast, dsDNA from E.coli as well as LPS stimulated IL-12 secretion, which was not inhibited by chloroquine, suggesting that this genomic DNA from E.coli strain B might be contaminated with other stimuli. In contrast to human PMN, the CpG ODN-induced cytokine secretion in mouse BMDCs was as strong as LPS-stimulated IL-12 release. Thus, ODNs seem to be more potent activators of BMDC than PMN. Interestingly, the chloroquine-mediated inhibition of endosomal acidification and the subsequent inhibition of cell activation was reversible at concentrations lower than 0.01 mM (data not shown). Nevertheless, these results support the hypothesis by Trevani. They also show that the inhibition of cell activation by chloroquine works in our experiments. However, the implications for the nucleosome-induced cell activation remained unsolved.

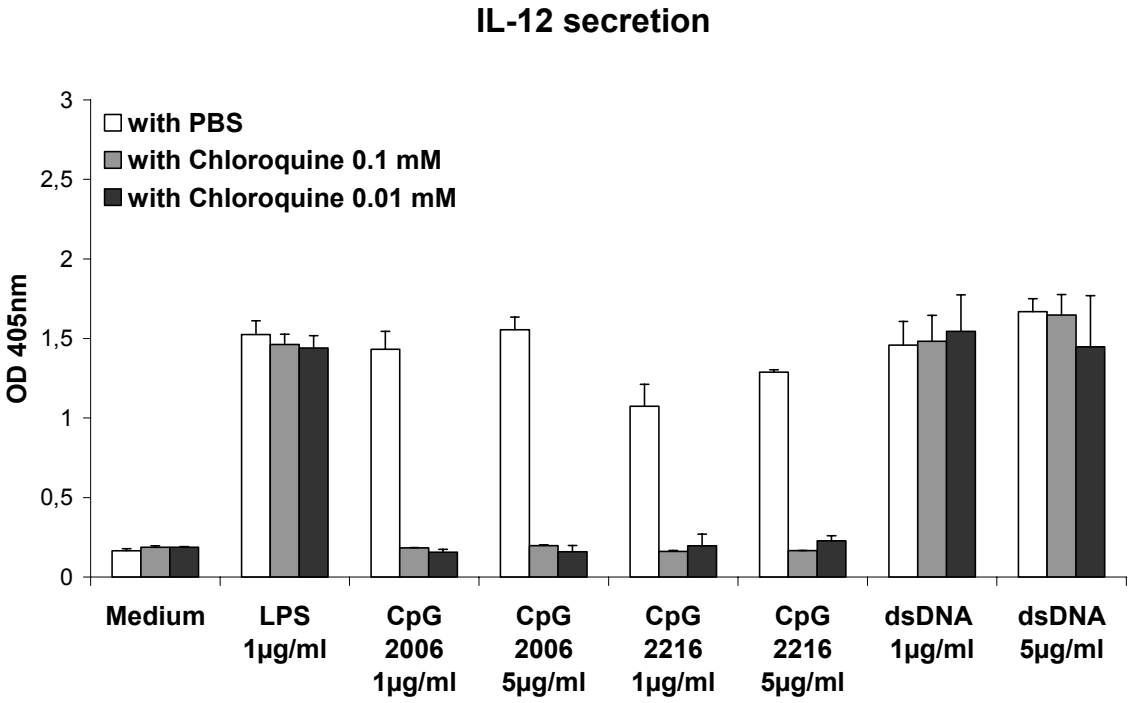


***Figure 28**. CpG ODN and bacterial DNA stimulation and inhibition of endosomal acidification. Mouse BMDCs were incubated in the absence or presence of chloroquine (0.1 mM, 0.01 mM) for 2 hours at 37°C and then cultured with different stimuli. After 18 hours, cell culture supernatants were harvested and analyzed for the presence of IL-12p40/p70 by ELISA. OD at 405 nm is displayed. See **Figure 26** for abbreviations. Shown is 1 of 6 representative experiments. SDs are indicated.*

Next, the effect of nucleosomes on BMDC was tested. As shown in **Figure 29**, nucleosome-induced IL-12 secretion was dose-dependent and not inhibited by chloroquine in BMDC. Nucleosomes induced a threefold increase in cytokine production in comparison to the empty gradient. Unlike nucleosomes, two different CpG ODNs strongly stimulated IL-12 secretion, which was blocked in the presence of chloroquine.

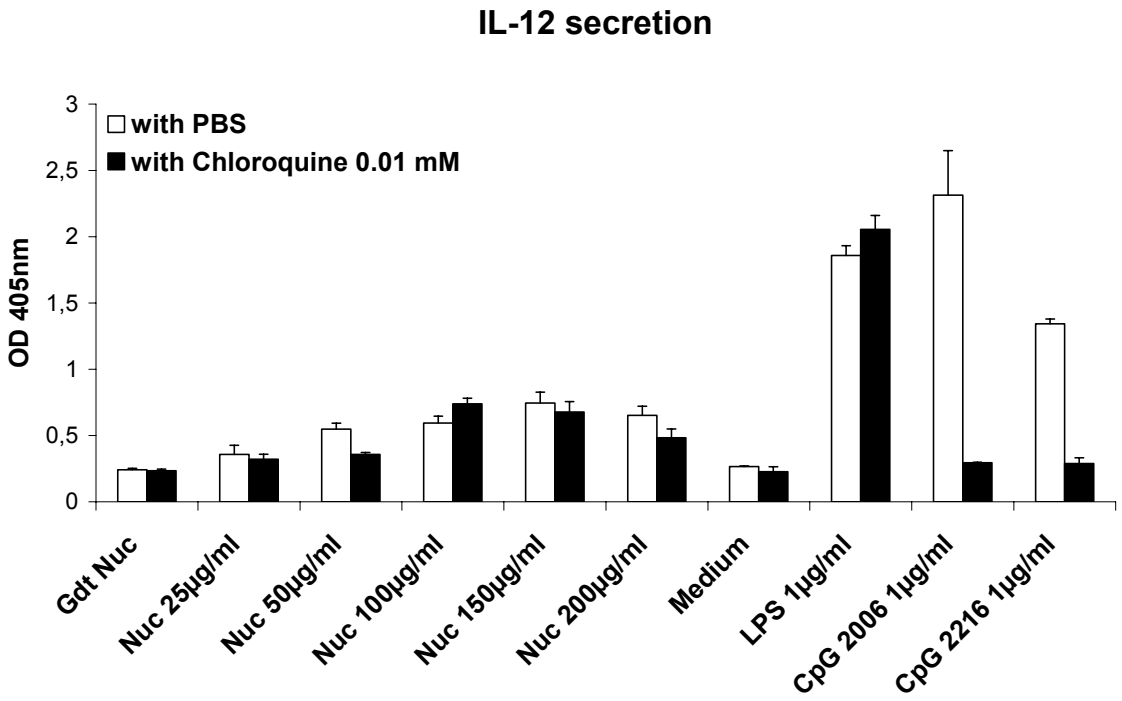


***Figure 29**. BMDC stimulation and inhibition of endosomal acidification. Mouse BMDCs were incubated with or without chloroquine (0.01 mM) for 2 hours at 37°C and then cultured with different stimuli. After 18 hours, cell culture supernatants were harvested and analyzed for the presence of IL-12p40/p70 by ELISA. OD at 405 nm is indicated. See **Figure 26** for abbreviations. Shown is 1 of 3 representative experiments. SDs are indicated.*

Of note, it was previously shown by Decker et al. (281) that nucleosomes induced BMDCs maturation and cytokine secretion. Here, we just wanted to compare the nucleosome-induced PMN activation with another cell type of the innate immune system expressing TLR9. Furthermore, we intended to confirm that chloroquine is effective in blocking endosomal acidification and thus in inhibiting receptor-ligand-interaction.

These results revealed that chloroquine was able to inhibit CpG-induced BMDC activation, whereas it did not prevent CpG- and bacterial DNA-stimulated activation of human PMN. They also indicate that nucleosome-induced BMDC activation occurs independently of endosomal acidification, suggesting a TLR9-independent pathway. (BMDC and PMN activation was determined by cytokine secretion and up-regulation of cell surface markers; data not shown.)

3.2.1.2 Ammonium chloride

In addition to chloroquine, there are several other inhibitors of endosomal acidification, such as ammonium chloride (NH_4Cl), Bafilomycin A and wortmannin. Thus, ammonium chloride was dissolved in PBS and passed through an Acrodisc unit with Mustang E membrane to reduce the endotoxin content.

Then, freshly prepared human PMN were incubated with or without ammonium chloride for 30 minutes at 37°C. The cells were challenged with different stimuli and activation was determined measuring IL-8 secretion. As shown in **Figure 30**, pre-treatment of PMN with ammonium chloride did not inhibit CpG ODN-induced IL-8 secretion, supporting a TLR9-independent pathway. Nucleosomes induced increased IL-8 secretion in a dose-dependent manner, as compared to the empty gradient, which was not prevented by ammonium chloride (see **Figure 30**, with NH_4Cl 1 mM). Unfortunately, higher NH_4Cl concentrations increased basal IL-8 levels (see **Figure 30**, Gdt Nuc, medium; with NH_4Cl 5 mM), decreasing the sensitivity for the detection of less potent activators, such as nucleosomes. At this point, we were still not able to conclude on the requirement of endosomal TLRs for nucleosome-induced PMN activation.

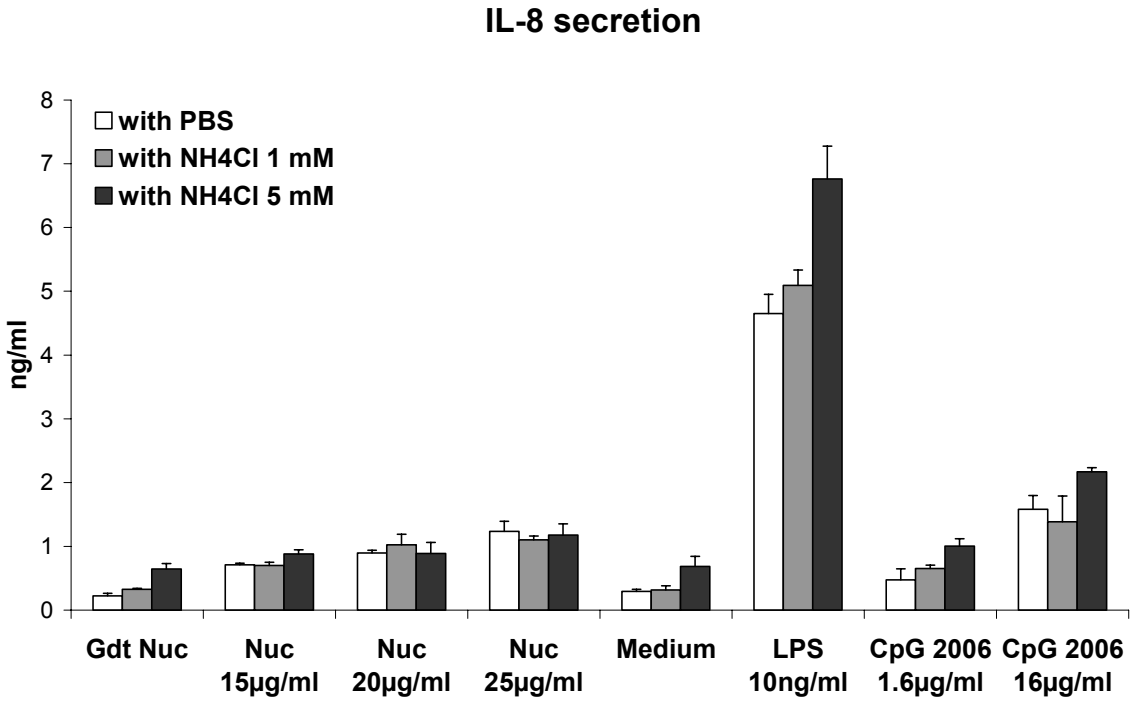


***Figure 30**. PMN stimulation and inhibition of endosomal acidification. Freshly isolated PMN were incubated in the absence or presence of ammonium chloride (NH_4Cl) (1 mM, 5 mM) for 30 minutes at 37°C and then cultured with different stimuli. After 16 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. Gdt Nuc, empty gradient used for the nucleosome purification; Nuc, nucleosomes; CpG 2006, CpG ODN 2006; LPS, lipopolysaccharides. Shown is 1 of 10 representative experiments. SDs are indicated.*

The imidazoquinoline resiquimod (R848) is an agonist of human TLR7 and TLR8. Just as TLR9, TLR7 and TLR8 are located in endosomal compartments and require endosomal acidification for signalling. Consequently, freshly isolated human PMN were incubated in the absence or presence of either chloroquine or ammonium chloride for 30 minutes at 37°C. Then, the cells were challenged with different stimuli and PMN activation was determined measuring IL-8 secretion by ELISA.

As shown in **Figure 31**, pre-treatment of PMN with chloroquine resulted in basal IL-8 secretion and did not inhibit R848- or RNA-stimulated activation. In contrast, pre-incubation with ammonium chloride did inhibit R848- as well as RNA-induced IL-8 secretion by human PMN.

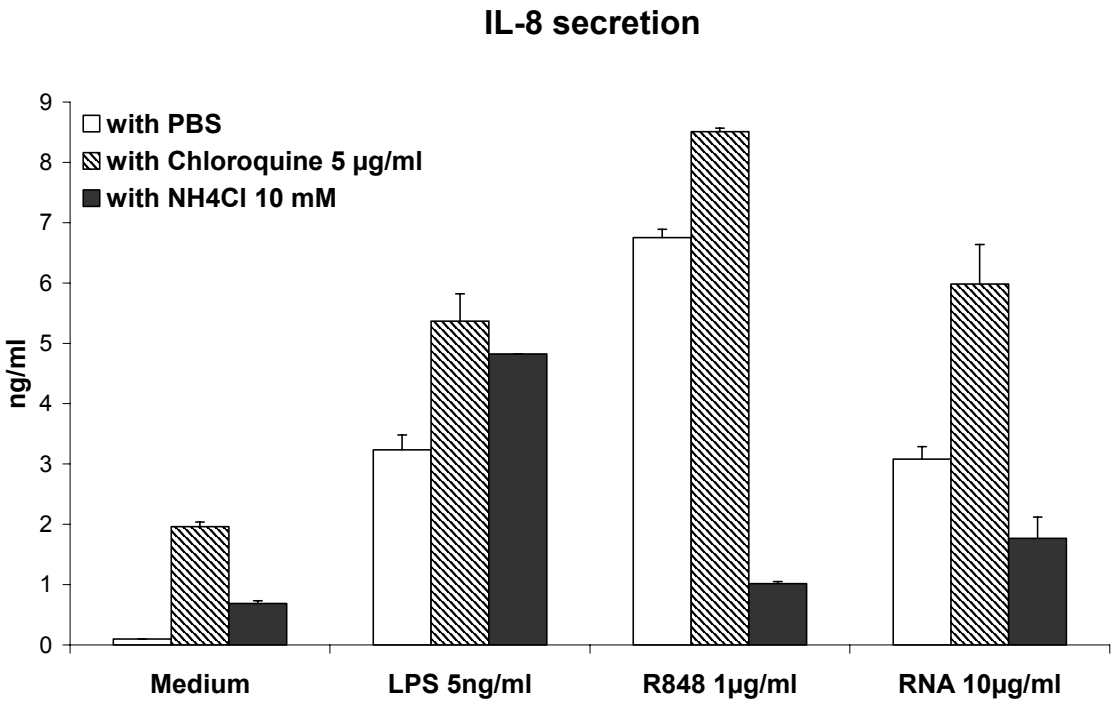


***Figure 31**. PMN stimulation and inhibition of endosomal acidification. Freshly isolated PMN were pre-incubated with either PBS or chloroquine (5 µg/ml) or ammonium chloride (NH_4Cl) (10 mM) for 30 minutes at 37°C and then challenged with different stimuli. After 16 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. See **Figure 30** for abbreviations. Shown is the result from one single experiment. SDs are indicated.*

This result revealed that chloroquine was ineffective in inhibiting endosomal acidification in human PMN. Hence, ammonium chloride was used instead in the following experiments.

Freshly isolated PMN were pre-treated with ammonium chloride for 30 minutes at 37°C and then challenged with different stimuli. PMN activation was estimated measuring IL-8 secretion by ELISA. As shown in **Figure 32**, the pre-incubation with ammonium chloride resulted in a slightly increased IL-8 secretion. Nevertheless, R848-induced PMN activation was prevented. In contrast, CpG ODN and nucleosomes still induced activation, in comparison to medium alone or the empty gradient. This result suggests that endosomal acidification is not required for PMN activation with nucleosomes.

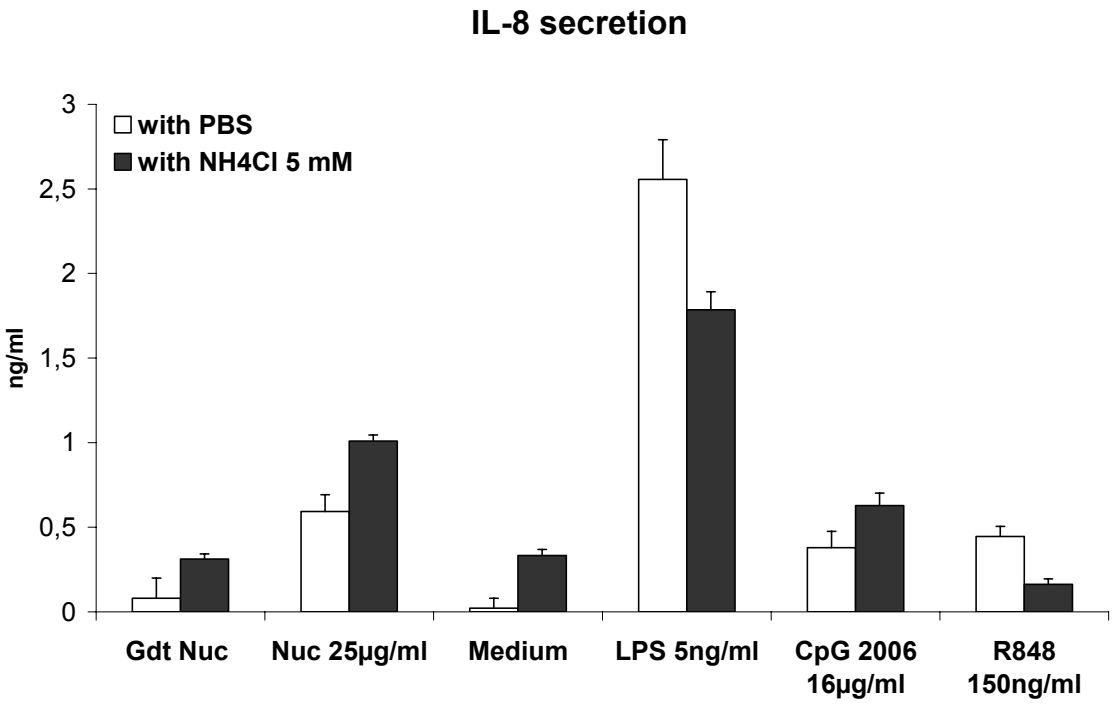


***Figure 32**. PMN stimulation and inhibition of endosomal acidification. Freshly isolated PMN were incubated in the absence or presence of ammonium chloride (NH_4Cl) (5 mM) for 30 minutes at 37°C and then cultured with different stimuli. After 16 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. See **Figure 30** for abbreviations. Shown is 1 of 4 representative experiments. SDs are indicated.*

In order to exclude any possible endotoxin contamination, freshly prepared human PMN were first incubated with the LPS inhibitor polymyxin B (12.5 µg/ml) for 30 minutes at 37°C, then with ammonium chloride (7.5 mM or 5 mM) for 30 minutes at 37°C to inhibit endosomal acidification and subsequently challenged with different stimuli. PMN activation was determined measuring IL-8 secretion by ELISA.

The viability of PMN preparation was controled by flow cytometry, using specific mAb for CD66b, CD11b and 7-AAD, in order to exclude any toxic side effects of ammonium chloride in combination with polymyxin B (data not shown).
Of note, the ammonium chloride concentrations applied in these experiments were found to be nontoxic for the cells.
As shown in **Figure 33**, nucleosomes induced increased PMN activation, in comparison to the empty gradient, in the presence of either PBS or ammonium chloride (5 mM, 7.5 mM) or polymyxin B or the combination of ammonium chloride (7.5 mM) and polymyxin B. Unfortunately, the latter combination slightly induced IL-8 secretion. In contrast, R848-induced PMN activation was inhibited in the presence of ammonium chloride (5 mM, 7.5 mM) and with the combination of ammonium chloride (7.5 mM) and polymyxin B. LPS-stimulated PMN activation was inhibited, when the cells were pre-treated with polymyxin B. In agreement with previous results, CpG ODN stimulation was not blocked in the presence of ammonium chloride (5 mM, 7.5 mM). (CpG ODN-induced activation in the presence of ammonium chloride and polymyxin B was not determined.)

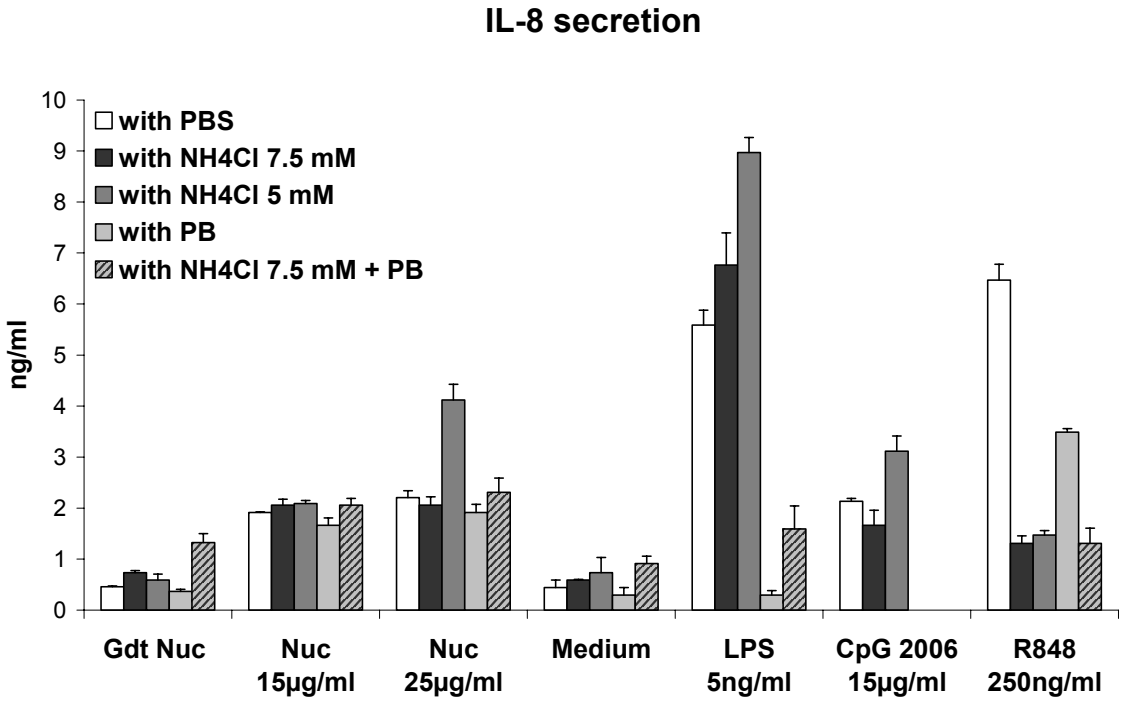


***Figure 33**. PMN stimulation and inhibition of endosomal acidification. Freshly isolated PMN were first incubated in the absence or presence of polymyxin B (PB) for 30 minutes at 37°C, next they were cultured with or without ammonium chloride (NH_4Cl) (7.5 mM or 5 mM) for 30 minutes at 37°C and then challenged with different stimuli. After 16 hours, cell culture supernatants were harvested and analyzed for the presence of IL-8 by ELISA. See **Figure 30** for abbreviations. Shown is 1 of 3 representative experiments. SDs are indicated.*

These results clearly demonstrated that nucleosome-induced PMN was not due to endotoxin contamination and occurred independently of endosomal acidification. In agreement with the finding that bacterial DNA stimulated human PMN activation in a TLR9-independent manner (313), CpG ODN-induced activation was not blocked in the presence of ammonium chloride. In contrast, the TLR7/8 ligand, R848, induced PMN activation, which was sensitive to the ammonium chloride treatment. Since nucleosome-induced PMN activation was not prevented by ammonium chloride, nucleosomes may stimulate human PMN independently of endosomal TLRs as well as endosomal maturation.

3.2.2 Expression of TLR9 (Preliminary results)

3.2.2.1 Expression of TLR9 in human PMN

A potential involvement of TLR9 in nucleosome-induced PMN activation is still unsolved. In order to confirm the expression of TLR9 by human PMN, intracellular and cell surface TLR9 expression was examined by flow cytometry using an anti-TLR9 (CD289) specific mAb. As shown in **Figure 34**, human PMN expressed TLR9 intracellularly at all three different time points. Surprisingly, TLR9 seem to be expressed at the surface of some CD66b-positive PMN (**Figure 35**). The TLR9 expression levels at the cell surface, however, were lower in comparison to the expression in subcellular compartments.

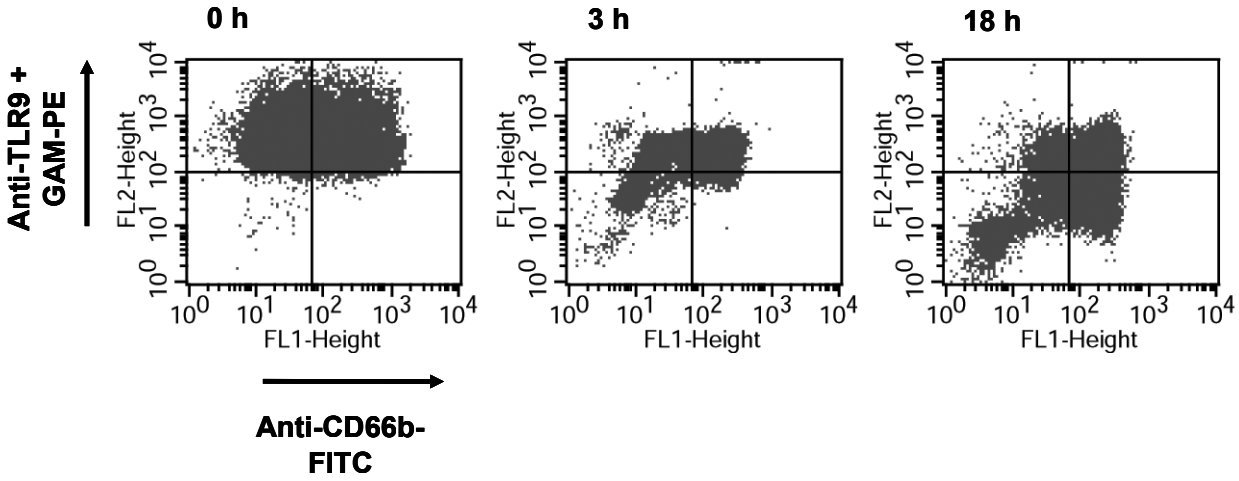


***Figure 34**. Intracellular TLR9 expression in human PMN. Freshly isolated human PMN were immediately (0 hours), after 3 hours or 18 hours of cell culture in RPMI, permeabilized and stained with a purified anti-TLR9 specific mAb, a PE-conjugated goat anti-mouse secondary Ab and a FITC-labelled anti-CD66b mAb. Intracellular expression of TLR9 and CD66b was measured by flow cytometry. Dot plots show TLR9 and CD66b expression levels in the gated granulocyte population. Representative of experiments performed on 3 donors are shown. Quadrants are set according to the corresponding isotype controls.*

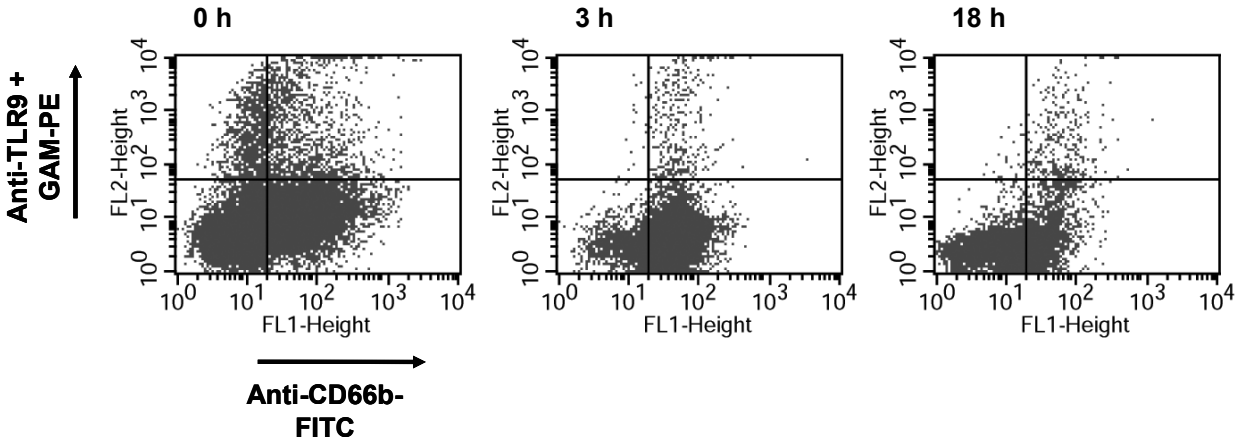


***Figure 35**. TLR9 cell surface expression in human PMN. Freshly isolated human PMN were immediately (0 hours), after 3 hours or 18 hours of cell culture in RPMI stained with a purified anti-TLR9 specific mAb, a PE-conjugated goat anti-mouse secondary Ab and a FITC-labelled anti-CD66b mAb. Expression of TLR9 and CD66b was measured by flow cytometry. Dot plots show TLR9 and CD66b expression levels in the gated granulocyte population. Representative of experiments performed on 3 donors are shown. Quadrants are set according to the corresponding isotype controls.*

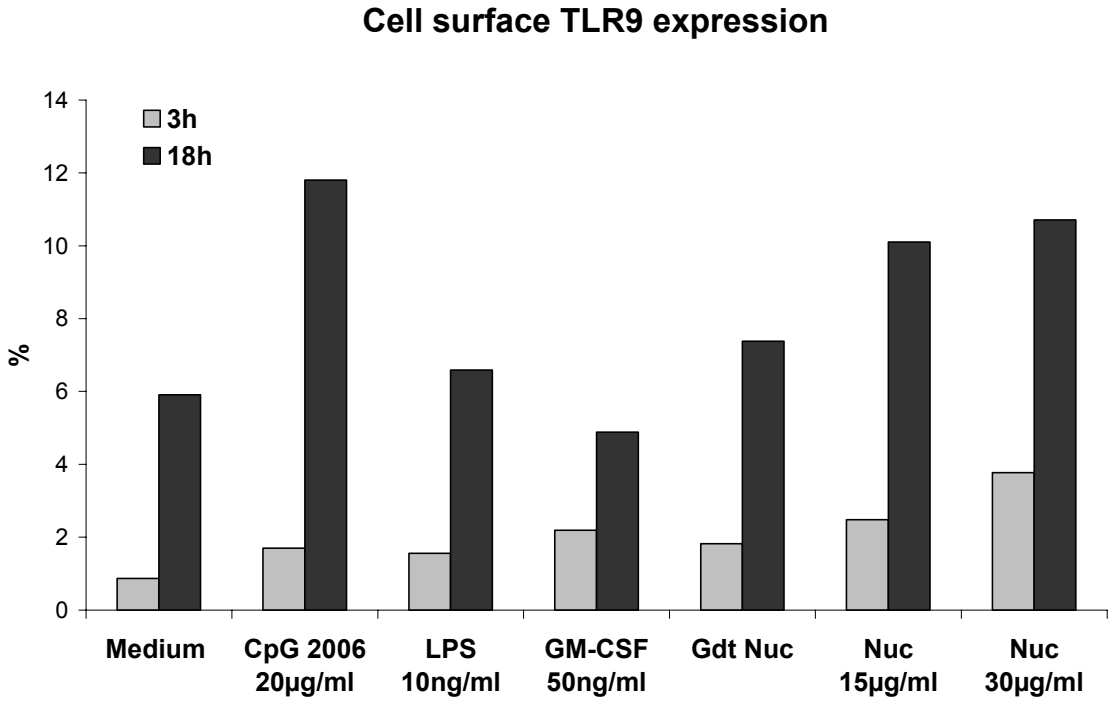


***Figure 36**. TLR9 cell surface expression in human PMN. Freshly isolated human PMN were incubated with different stimuli for 3 hours or 18 hours at 37°C and then analyzed by flow cytometry using a FITC-labelled CD66b, a purified anti-TLR9 specific mAb and a PE-conjugated secondary Ab. Shown is the percentage (%) of CD66b- and TLR9-positive granulocytes. Representative of experiments performed on 4 donors are shown.*

We further tested whether TLR9 expression could be modulated. As shown in **Figure 36**, the cell surface TLR9 expression was slightly increased upon stimulation with CpG 2006 ODN and nucleosomes after 3 hours and clearly increased after 18 hours, in comparison to cells in medium or the empty gradient. In addition, the percentage of CD66b-positive cells expressing TLR9 on the surface was significantly higher after over night incubation, as compared to 3 hours incubation. Of note, the expression of TLR9 on the cell surface of human PMN was detected in 8 healthy normal blood donors between the ages of 25 and 42.

Here, we show a yet unidentified expression of TLR9 on the cell surface of human PMN. In addition, we have demonstrated that the cell surface expression of TLR9 was increased after stimulation with unmethlyated CpG ODN and nucleosomes.
In view of the fact that TLR9 was also expressed at the cell surface of a human PMN sub-population, the inhibition of endosomal acidification was unlikely to prevent the interaction of the ligand and the receptor on the cell surface.
Thus, the presence of TLR9 at the cell surface of human PMN might explain, why CpG DNA-induced activation was not inhibited with ammonium chloride. This hypothesis is currently under investigation.

3.2.2.2 Role of TLR9 in nucleosome-induced activation of mouse bone marrow-derived neutrophils

To further investigate the putative role of TLR9 in nucleosome-induced neutrophil activation, neutrophils were purified from the bone marrow of TLR9-deficient (TLR9 KO) mice and C57BL/6 (wild type) mice by negative selection.
In a preliminary experiment using bone marrow-derived neutrophils (BMN) from wild type mice, it was determined that the activation of BMN was best after over night incubation (data not shown).
Thus, freshly purified BMN were challenged with different stimuli for at least 14 hours and Ly-6G and CD11b cell surface expression was examined by flow cytometry. In **Figure 37** and **Figure 38** are shown the preliminary results of three independent experiments, revealing a slight trend in Ly-6G and CD11b up-regulation on mouse BMN in the presence of nucleosomes. Interestingly, nucleosomes induced an increased expression of the cell surface molecules at lower concentrations in TLR9 KO mice and at higher concentrations in wild type mice, and the up-regulation of Ly-6G (**Figure 37**) was stronger as compared to the increased expression of CD11b (**Figure 38**). However, up-regulation of CD11b rather than Ly-6G is usually used as activation marker. Moreover, the activation was weak as compared to LPS-treated BMN. In contrast to the mouse specific CpG 1826 ODN, only CpG 2006 ODN induced a weak up-regulation of Ly-6G and CD11b in wild type mice. However, CpG ODN never induced the up-regulation of Ly-6G or CD11b in TLR9 KO mice.

This suggests that BMN are not sensitive to CpG ODN-induced activation and therefore it is difficult to conclude on the involvement of TLR9.

As aforementioned, E.coli DNA was suspected to contain contaminants, as it induced an increased up-regulation of Ly-6G and CD11b in TLR9 KO mice; although this was not clearly proved.

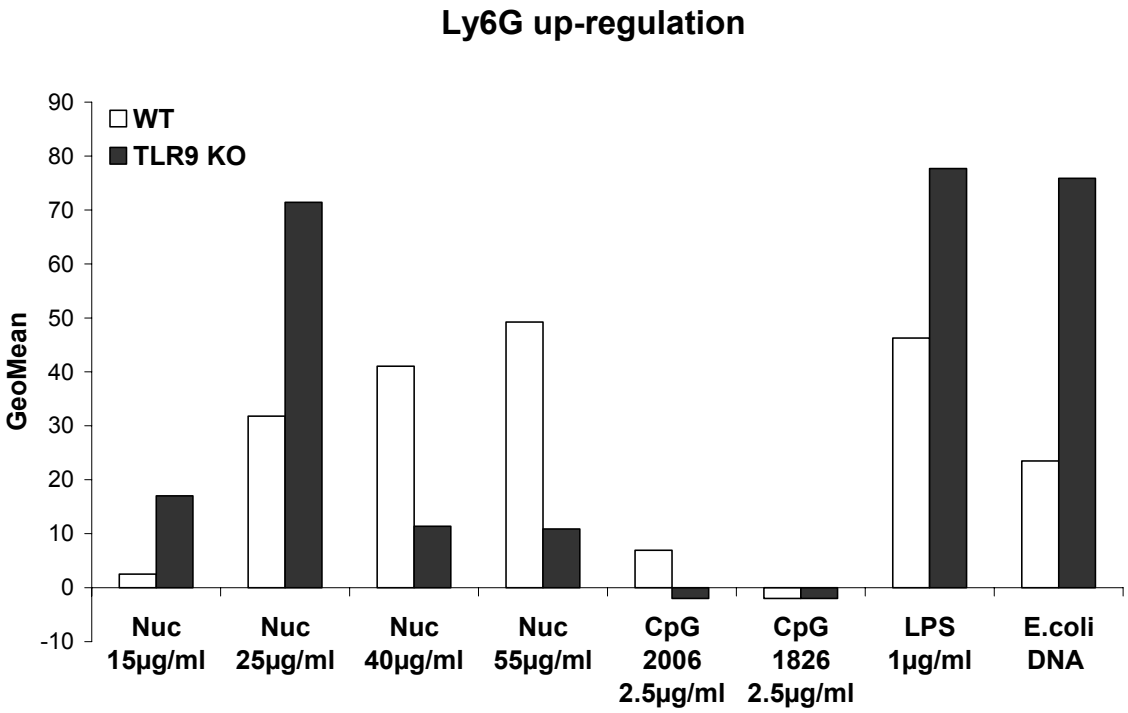


***Figure 37**. Ly-6G expression of activated mouse BMN. Freshly prepared BMN were incubated with different stimuli for 14 hours at 37°C and then Ly-6G and CD11b up-regulation was estimated by flow cytometry. Nuc, nucleosomes; CpG 2006, CpG 2006 ODN; CpG 1826, CpG 1826 ODN; LPS, LPS from E.coli; E.coli DNA, genomic dsDNA from E.coli. Shown is the comparative Ly-6G expression of wildtype (WT) and TLR9 knockout (KO) mice. Shown is the result of three independent experiments (number of mice per strain n = 5). Up-regulation of Ly-6G was determined by the subtraction of the corresponding negative controls (medium or Gdt Nuc).*

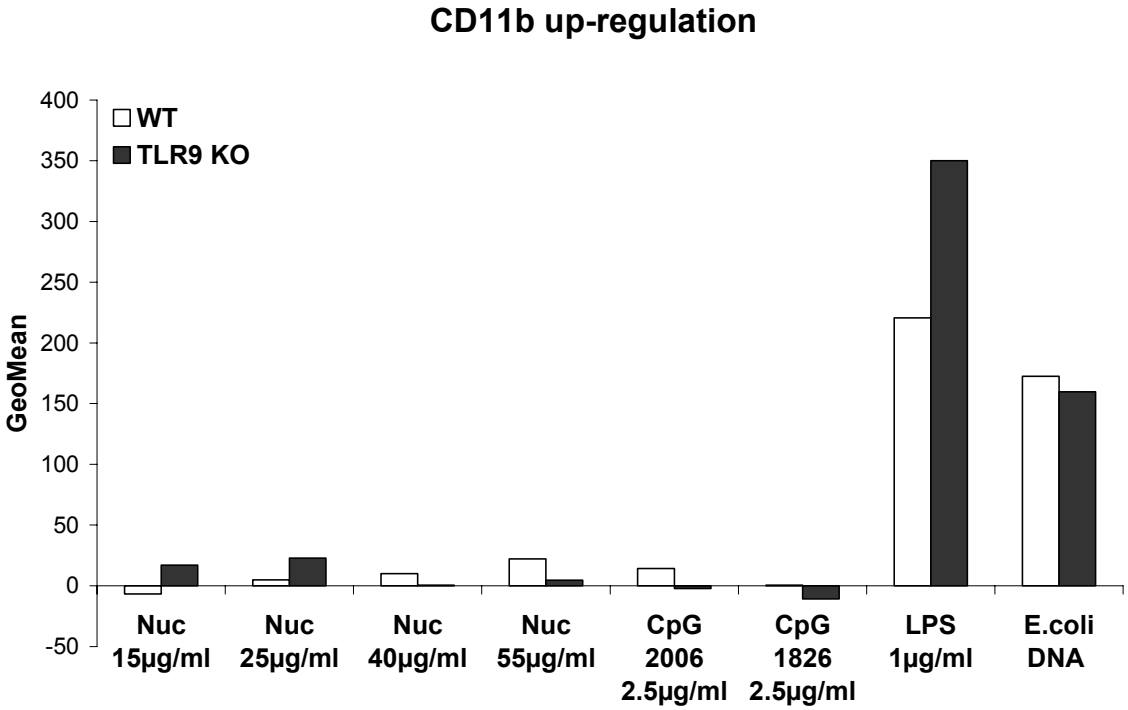


***Figure 38**. CD11b expression of activated mouse BMN. Freshly prepared BMN were incubated with different stimuli for 14 hours at 37°C and then Ly-6G and CD11b up-regulation was estimated by flow cytometry. (See **Figure 37** for abbreviations.) Shown is the comparative CD11b expression of wildtype (WT) and TLR9 knockout (KO) mice. Shown is the result of three independent experiments (number of mice per strain n = 5). Up-regulation of CD11b was determined by the subtraction of the corresponding negative controls (medium or Gdt Nuc).*

These preliminary data suggested that nucleosomes induced the activation of mouse BMN in a TLR9-independent manner.

However, the results have to be confirmed in further experiments, using more mice per group, possibly more cells per well as well as an appropriate positive control for the TLR9-mediated activation. It might still be possible, that mouse bone marrow-derived neutrophils behave differently than human PMN. For this reason, it has to be verified that BMN do express TLR9 and whether TLR9 is expressed in a subcellular compartment or at the cell surface.

3.2.3 Endocytic pathways of nucleosomes in human PMN

The plasma membrane is the interface between cells and their environment. Uptake and communication between cells and their environment occurs through this interface. Endocytosis encompasses several mechanisms by which cells internalize particles into transport vesicles derived from the plama membrane (315). Thus, diverse endocytic pathways were investigated in order to learn more about the uptake of nucleosomes by human PMN.

In most of the experiments, freshly prepared human PMN were incubated in medium containing 10% autologous decomplemented serum, then FITC-conjugated nucleosomes together with another fluorophore-labelled reagent were added. Live cell imaging was performed immediately using confocal laser scanning microscopy.

Phagocytosis is typically restricted to specialized mammalian cells, such as neutrophils. In order to investigate PMN phagocytosis low non-specific binding, carboxylate-modified fluorescent microspheres with a diameter of 0.02 µm (comparable to the diameter of nucleosome core particles) were used.

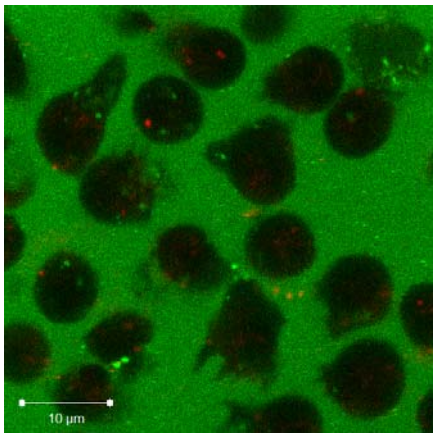


***Figure 39**. Endocytosis of nucleosomes by human PMN. Freshly isolated human PMN were cultured in medium containing 10 % autologous serum in a chambered cover glass previously coated with 0.1 % BSA. PMN were analyzed by confocal microscopy in the presence of 30 µM FITC-nucleosomes (Green) and crimson red fluorescent carboxylate-modified microspheres 1/2,500,000 (Red). Shown is the superposition of fluorescein and Crimson Red fluorescence channels. Size is indicated.*

As shown in **Figure 39**, FITC-nucleosomes and microspheres were (rapidly) taken up by human PMN. However, FITC-nucleosomes did not co-localize with crimson red fluorescent-microspheres, which are a marker of phagocytosis. Although neutrophils are professional phagocytes, nucleosomes may not be internalized by phagocytosis. In the following experiments, pinocytosis was examined.

Pinocytosis occurs in all cells by at least four basic mechanisms dependent on the size of particles: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis and clathrin- and caveolae-independent endocytosis.

Clathrin-mediated endocytosis (CME) (formerly: receptor-mediated endocytosis) occurs constitutively in all mammalian cells and ensures the continuous uptake of essential nutrients, such as iron-laden transferrin, which binds to the transferrin-receptor. The transferrin-receptor and bound transferrin are concentrated in coated pits at the plasma membrane and then internalized. The formation of coated pits is mediated by clathrin and actively supported by adaptor proteins (AP). The assembly of AP-2 and clathrin is selectively disrupted by chlorpromazine (see (316) for review). Thus, Alexa Fluor 633-labelled transferrin was used as a marker for CME. Freshly purified human PMN were incubated in medium containing 10 % autologous serum in the presence of FITC-nucleosomes and Alexa Fluor 633-transferrin. Clathrin-mediated endocytosis was investigated by confocal microscopy doing time kinetics. In order to inhibit CME, PMN were pre-incubated with chlorpromazine for 30 minutes at 37°C. As shown in **Figure 40 A**, human PMN already start to engulf FITC-nucleosomes after 14 minutes at room temperature, whereas clathrin-coated pits were not detectable. FITC-nucleosomes were still internalized in the presence of the CME inhibitor chlorpromazine after 14 minutes (**Figure 40 B**). These results indicated that FITC-nucleosomes were unlikely to be internalized via clathrin-mediated endocytosis. However, we cannot conclude from this result, since clathrin-coated pits were either too small-sized or not detectable. Consequently, we cannot be sure that chlorpromazine inhibited CME.

A B

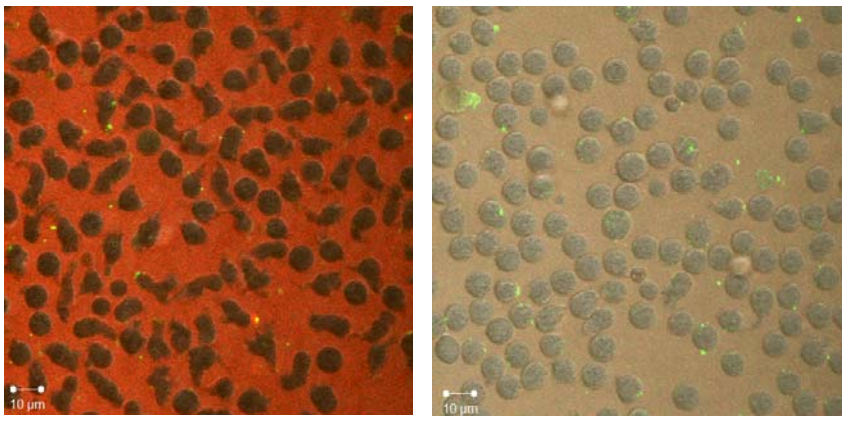


***Figure 40**. Endocytosis of nucleosomes by human PMN. Freshly isolated human PMN were cultured in medium containing 10 % autologous serum in a chambered cover glass previously coated with 0.1 % BSA. (**A**) PMN were analyzed by confocal microscopy in the presence of 30 µM FITC-nucleosomes (Green) and Alexa Fluor 633-labelled transferrin (25 µg/ml) (Red). (**B**) PMN were pre-treated with chlorpromazine (10 µg/ml) for 30 minutes at 37°C, then 30 µM FITC-nucleosomes (Green) and Alexa Fluor 633-labelled transferrin (25 µg/ml) (Red) were added. Shown is the superposition of fluorescein and Alexa Fluor 633 fluorescence channels. Size is indicated.*

Macropinosomes are large endocytic vesicles that sample large volumes of extracellular milieu and originate from membrane ruffles (315). Fluorescent dextran conjugates can be used to monitor macropinocytosis. Here, freshly prepared human PMN were incubated in the presence of FITC-nucleosomes and Alexa Fluor 647-conjugated dextran and macropinocytosis was analyzed by confocal microscopy.

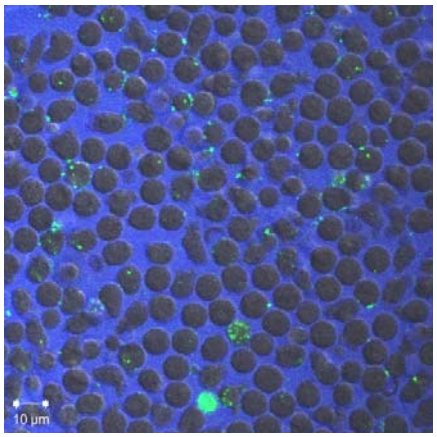


***Figure 41**. Endocytosis of nucleosomes by human PMN. Freshly isolated human PMN were cultured in medium containing 10 % autologous serum in a chambered cover glass previously coated with 0.1 % BSA. PMN were analyzed by confocal microscopy in the presence of 30 µM FITC-nucleosomes (Green) and Alexa Fluor 647-conjugated dextran (10 µM) (Blue). Shown is the superposition of fluorescein and Alexa Fluor 647 fluorescence channels. Size is indicated.*

In contrast to human monocyte-derived DCs (data not shown), human PMN did not endocytose dextran-conjugates via macropinocytosis, whereas FITC-nucleosomes were obviously taken up (**Figure 41**). This result indicated that human PMN did not form macropinosomes and were therefore unlikely to ingest FITC-nucleosomes via macropinocytosis.

Finally, caveolae/lipid rafts-dependent endocytosis of human PMN was examined. Caveolae are flask-shaped invaginations of the plasma membrane and characterized by the presence of caveolin-1 (317). Cholera toxin subunit B (CTB) (from *Vibrio cholerae*) can be used as marker for lipid rafts, which are membrane microdomains enriched in cholesterol and sphingolipids, and thereby for caveolae-mediated endocytosis. The cyclic heptasaccharide methyl-β-cyclodextrin (MβCD) can selectively block caveloae- and raft-mediated pathways by the extraction of cholesterol from the plasma membrane (318).
Enriched human PMN were cultured in medium containing 10 % autologous serum in the presence of FITC-nucleosomes and Alexa Fluor 555-conjugated Cholera toxin subunit B. Caveolae-mediated endocytosis was estimated by confocal microscopy employing time kinetics.

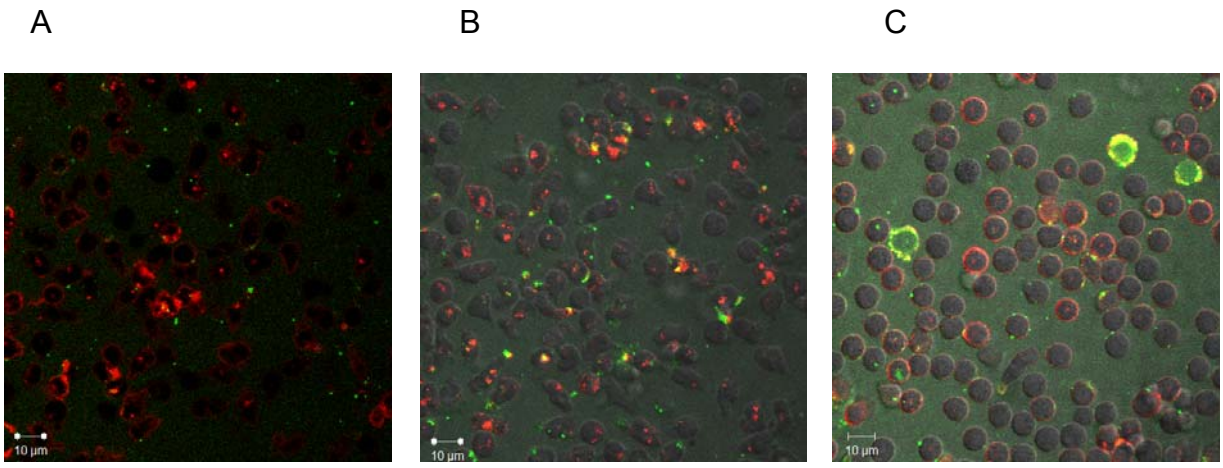


***Figure 42**. Endocytosis of nucleosomes by human PMN. Freshly isolated human PMN were cultured in medium containing 10 % autologous serum in a chambered cover glass previously coated with 0.1 % BSA. (**A**) PMN were analyzed by confocal microscopy in the presence of 30 µM FITC-nucleosomes (Green) and Alexa Fluor 555-labelled Cholera toxin subunit B (20 µg/ml) (Red) after 8 minutes. (**B**) After 48 minutes. (**C**) PMN were pre-treated with methyl-β-cyclodextrin (5 mM) for 30 minutes at 37°C, then caveolae-mediated endocytosis was examined in the presence of 30 µM FITC-nucleosomes (Green) and Cholera toxin subunit B (20 µg/ml) (Red) after 14 minutes. Shown is the superposition of fluorescein and Alexa Fluor 555 fluorescence channels. Size is indicated.*

As shown in **Figure 42 A**, human PMN strongly internalize Alexa Fluor 555-conjugated CTB into lipid rafts after 8 minutes at room temperature. FITC-nucleosomes co-localized to some extent with CTB at the cell surface, but also inside the cells (**Figure 42 A** after 8 minutes and **Figure 42 B** after 48 minutes). However, the raft-mediated pathways inhibitor MβCD did not prevent the endocytosis of FITC-nucleosomes after 14 minutes (**Figure 42 C**), but inhibited the uptake of CTB into lipid rafts. These results implied that FITC-nucleosomes may be partially taken up via caveolae-mediated endocytosis. Since the engulfment of FITC-nucleosomes was not inhibited by MβCD, it is very likely that some additional pathways are involved. Possibly, FITC-nucleosomes were also taken up via clathrin- and caveolae-independent endocytosis. However, this pathway is poorly understood and has to be further investigated.

In addition to the endocytic pathways, the probable destination of FITC-nucleosomes within the cell was examined. Monomeric cyanine nucleic acid stains, such as TO-PRO-3, specifically bind double-stranded nucleic acids, which are concentrated in the nucleus. Thus, freshly prepared human PMN were cultured with FITC-nucleosomes in PBS containing 10 % decomplemented FCS for 30 minutes at 37°C. Cells were then fixed, permeabelized and incubated with TO-PRO-3 for 15 minutes at room temperature. The staining of the nucleus was analyzed by confocal microscopy. As shown in **Figure 43**, TO-PRO-3 specifically stained the multilobular nuclei of human PMN. FITC-nucleosomes did not co-localize with TO-PRO-3, but were obviously taken up into the cytoplasm. The same results were obtained using 7-AAD or Propidium iodide (PI) (data not shown). (PI and 7-AAD do stain DNAs, but also RNAs. Therefore a pre-treatment with RNase A (200 µg/ml, 20 minutes, 37°C) was done.)

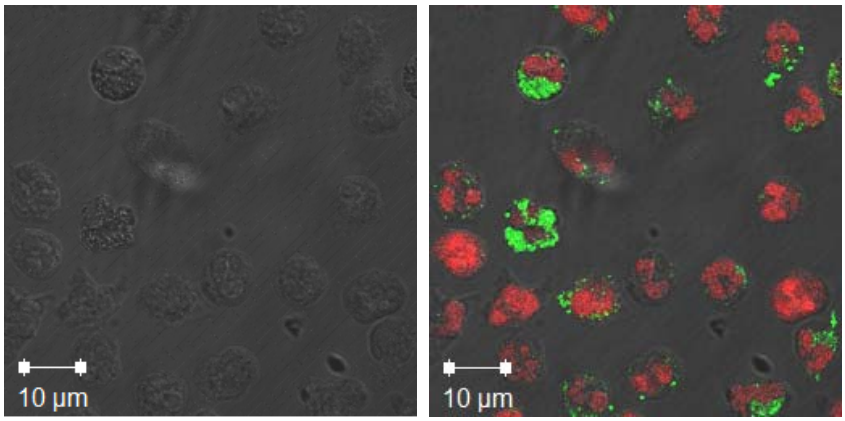


***Figure 43**. Endocytosis of nucleosomes by human PMN. Freshly isolated human PMN were cultured in PBS containing 10 % FCS in a 96 well-V-bottom plate with 10 µM FITC-nucleosomes (Green). Cells were then fixed, permeabelized and incubated with TO-PRO-3 (1/1,000) (Red). PMN were analyzed in a chambered cover glass by confocal microscopy. Transmission image is shown in the left panel and fluorescence image is shown as superposition of fluorescein and Alexa Fluor 633 fluorescence channels in the right panel. Size is indicated.*

Having shown that nucleosomes were internalized by human PMN, we next addressed a potential involvement of lysosomes in the cellular trafficking of nucleosomes. LysoTrackers are cell-permeant, weakly basic amines, which accumulate in cellular compartments with low internal pH and can therefore be used for labelling lysosomes in live cells. Thus, freshly isolated human PMN were incubated in medium containing 10 % autologous serum in the presence of either FITC-nucleosomes or FITC-conjugated CpG 2006 ODN and LysoTracker Red DND-99. Endocytosis by human PMN was analyzed using live cell confocal microscopy. FITC-conjugated CpG 2006 ODNs were expected to co-localize with LysoTracker, since CpG motifs are supposed to be recognized by the endosomally expressed TLR9.

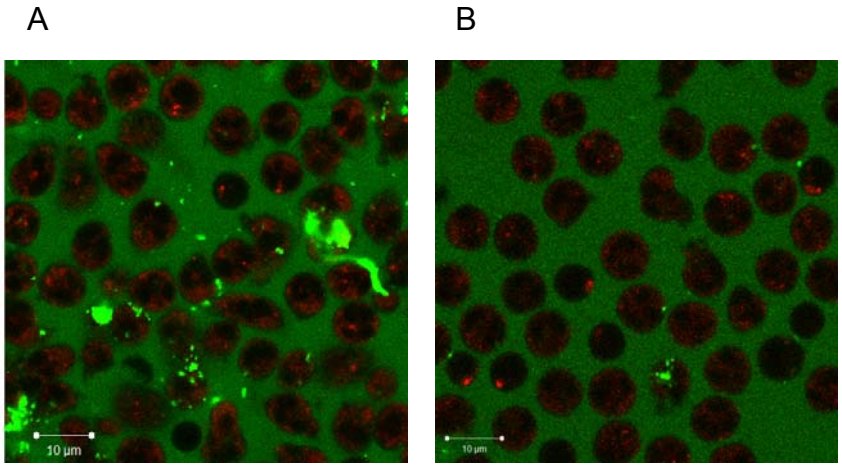


***Figure 44**. Endocytosis of nucleosomes or CpG 2006 ODN by human PMN. Freshly isolated human PMN were cultured in medium containing 10 % autologous serum in a chambered cover glass previously coated with 0.1 % BSA. (**A**) PMN were analyzed by confocal microscopy in the presence of 30 µM FITC-nucleosomes (Green) and LysoTracker Red DND-99 (100 nM; Red) after 34 minutes. (**B**) PMN were examined in the presence of 2 µM FITC-CpG 2006 (Green) and LysoTracker Red DND-99 (100 nM; Red). Shown is the superposition of fluorescein and Red DND-99 fluorescence channels. Size is indicated.*

FITC-nucleosomes (**Figure 44 A**) as well as FITC-CpG 2006 ODN (**Figure 44 B**) were endocytosed by human PMN, however, neither FITC-CpG 2006 ODN nor FITC-nucleosomes co-localized with LysoTracker. In order to find an explanation for this result, we had to investigate if the low pH in early (pH 5.5 – 6.3) and late endosomes (pH 5.5) or lysosomes (pH 4.6) interfered with the pH-sensitive fluorescein.

Thus, endosomally disruption of fluorescein could be the reason why co-localization of FITC and LysoTracker was not detected. Therefore, human PMN were pre-treated with ammonium chloride to inhibit endosomal acidification and then incubated with either FITC-nucleosomes or FITC-CpG 2006 ODN. As shown in **Figure 45 A**, endocytosis of FITC-nucleosomes by human PMN did not interfere with ammonium chloride, whereas FITC-CpG 2006 ODN obviously bound more to the cell surface than rather being taken up (**Figure 45 B**).

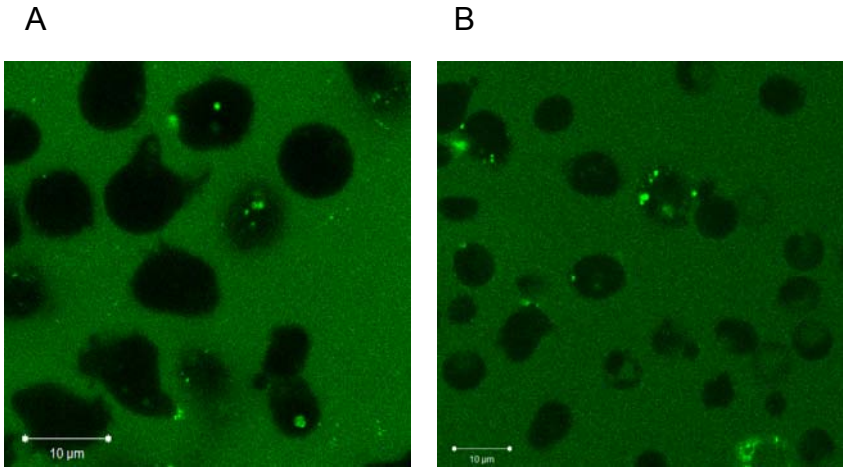


Figure 45*. Endocytosis of nucleosomes or CpG 2006 by human PMN. Freshly isolated human PMN were cultured in medium containing 10 % autologous serum in a chambered cover glass previously coated with 0.1 % BSA. PMN were analyzed by confocal microscopy in the presence of ammonium chloride (7.5 mM) with 30 µM FITC-nucleosomes (Green) (**A**) or 2 µM FITC-CpG 2006 (Green) (**B**). Shown are the fluorescein fluorescence channels. Size is indicated.*

In conclusion, FITC-nucleosomes were partially taken up via caveolae-mediated endocytosis by human PMN. However, it is very likely that other endocytic pathways are involved. The uptake of FITC-nucleosomes was not prevented in the presence of any inhibitors of endosomal acidification or endocytosis. The fate of FITC-nucleosomes inside the cell needs further investigation. If technical reasons were not responsible, then FITC-nucleosomes might be partly endocytosed via a novel pathway or by direct translocation across cell membranes. Finally, it may be possible that nucleosome uptake is low and not necessary for the PMN activation due to direct signalling via a cell surface receptor. Furthermore, it seems like that the final destination of internalized nucleosomes is the cytosplasm. Then, analysis of the role of a recently described cytosolic DNA sensor might be of interest.

4 Discussion

In the present study, it was shown for the first time that purified nucleosomes directly induce activation and recruitment of neutrophils *in vitro* and *in vivo*. Importantly, free nucleosomes induced activation without requiring immune complex formation. These results support the finding that autoantigens may play an active role in the pathogenesis of some autoimmune diseases through the activation of innate immunity. Nucleosome-induced PMN activation was determined by CD11b/CD66b cell surface upregulation, IL-8 secretion, increased phagocytosis and delayed apoptosis. Indeed, the nucleosome core structure was crucial for PMN activation, because only nucleosomes and histone H1-depleted nucleosomes were able to induce PMN activation, whereas DNA, histones or mixtures of both were not.

Nucleosome assembly is a complicated molecular process. By simply mixing purified histones and nucleosomal DNA under physiological conditions, some nucleosomes will form, but there will also be many non-nucleosomal histone-DNA complexes and aggregates (319). *In vitro* nucleosome assembly methods using salt-dialysis circumvent these non-nucleosomal products. The PMN activation induced by nucleosomes (with and without histone H1) prepared from calf thymus, *in vitro* reconstituted nucleosomes or histone/DNA mixture was compared. We found that only stimulation with nucleosomes and H1-depleted nucleosomes resulted in PMN activation. However, it was not possible to draw a conclusion regarding the ability of reconstituted nucleosomes to activate human PMN, because they were likely to contain contaminating LPS. (The Rec Nuc buffer, which was used as negative control for the reconstituted nucleosomes, was prepared under sterile conditions according to the protocol and did not activate the cells).

PMN from healthy donors and SLE patients were sensitive to nucleosome-induced activation, supporting such a mechanism *in vivo* in patients with SLE. It is noteworthy that nucleosome-induced PMN activation was observed in the presence of the LPS inhibitor, polymyxin B, and independently of TLR2 and TLR4. This convincingly demonstrates that the nucleosome-induced PMN stimulation was not due to endotoxin contamination.

Likewise, nucleosome-induced PMN activation was not caused by HMGB-1 contamination. Interestingly, it was recently shown that HMGB-1 protein purified from calf thymus, containing minimal contaminating DNA or RNA, was inactive (320). In contrast, recombinant HMGB-1 from E.coli was reported to induce activation of human neutrophils (308) and monocytes (321). However, the E.coli-derived HMGB-1 activity was reduced by DNase treatment, suggesting that at least a part of the activity was mediated by HMGB-1 associated with DNA (320). Indeed, HMGB-1 (HMGBiotech) used in the present study was produced in E.coli from an expression plasmid coding for the rat protein, which is 99% identical to the human HMGB-1. However, no IL-8 was induced by this HMGB-1.

The fact that nucleosomes induced human neutrophil activation *in vitro* and in mice *in vivo*, further provides evidence for such a mechanism *in vivo* in lupus patients, supporting the key role of nucleosomes in the development and pathogenesis of SLE.

Recently, several new functions have been attributed to autoantigens, such as the activation of pDCs or chemoattraction of immune cells. In agreement, Decker and co-workers have demonstrated previously that purified nucleosomes induce DC activation *in vitro* and *in vivo* in a MyD88-independent manner (281). In contrast to other studies, it was shown here that free nucleosomes and not nucleosome-containing immune complexes, stimulated neutrophil activation. In addition, we demonstrated that the whole nucleosome complex, and not the DNA moiety, was involved in neutrophil activation. Although it has been shown that mammalian DNA is able to activate some immune cells under particular circumstances, it is generally accepted that DNA is only present in the form of nucleosomes in the serum of SLE patients (173). However, extracellular mammalian DNA in the form of immune complexes can induce activation in pDCs (140). Accordingly, self DNA is only able to activate immune cells, when DNA is artificially introduced into cells by transfection (54,280) or in lipidic vesicles enhancing the endosomal translocation (322) or when endogenous DNA from apoptotic cells escapes lysosomal degradation (323). Furthermore, it was shown that endosomally translocated self DNA activates TLR9 independently of CpG motifs (324).

Recently, a novel cytosolic innate immune response to DNA linked to type I interferon production was described (44,55).

We thus identified a new activation mechanism of innate immunity driven by free nucleosomes.

Likewise, we found that DNA purified from the nucleosome preparations did not induce PMN activation, suggesting that the nucleosome structure is important for the signalling and that unmethylated CpG motifs *per se* were not responsible for the activation. In agreement with this finding, Decker et al. showed that nucleosomal DNA did not induce DC maturation (281). Thus, these results clearly demonstrate that nucleosome-induced PMN activation does not occur via the DNA moiety of the nucleosome, but implies the involvement of a different signalling pathway. Indeed, facilitation of the delivery of DNA to the cytoplasm by different transfection methods is sufficient to activate TLR9. This hypothesis is further supported by the demonstration of a mutant TLR9 that translocates to the cell surface and confers responsiveness to normally non-stimulatory vertebrate DNA (325). Thus, the intracellular localization of TLR9 partly prevents the recognition of self DNA. Similarly, it is conceivable that the recognition of nucleosomes by PMN requires the native nucleosome structure, but upon uptake and degradation in endocytic vesicles, PMN activation occurs in the cytoplasm via the DNA moiety.

In line with our results is the recent finding that bacterial DNA, but not mammalian DNA, induced neutrophil activation in a CpG-independent manner (314). Furthermore, it was shown that bacterial DNA stimulated human neutrophils through a TLR9-independent, but MyD88-dependent pathway (313). This supports our results that the whole nucleosome complex is necessary for PMN activation, whereas the free DNA moiety is unlikely to be involved. Of note, nucleosomes employed in all experiments were prepared from calf thymus. Accordingly, it was demonstrated that calf thymus DNA did not activate human neutrophils (312). Additionally, it was found that unlike DNA only DNA-anti-DNA complexes were taken up by human neutrophils (326). This is consistent with our assumption that free (self) DNA is not recognized by neutrophils.

Altogether, the present results clearly demonstrate that free nucleosomes are able to activate neutrophils, highlighting a yet unknown pathway of innate immunity.

One of the most potent neutrophil chemoattractants is the chemokine IL-8 (327,328). Many cell types, including neutrophils, produce IL-8 during inflammatory states.

Serum IL-8 levels were found to correlate with the disease activity in SLE patients (329). Thus, significantly higher levels of IL-8 were detected in patients with renal involvement and urinary IL-8 levels were higher in patients with glomerular leukocyte infiltration (330).

Since SLE is an inflammatory disease and neutrophils are the first cells recruited to infection and inflammation sites, nucleosome-induced IL-8 secretion may lead to an increased neutrophil recruitment and activation, subsequently resulting in inflammation via an amplification loop. In agreement with our results is the demonstration that synovial fluids from inflamed joints of rheumatoid arthritis patients contained increased nucleosome concentrations (mean 14 µg/ml), which correlated with the concentration of infiltrating neutrophils (331). Thus, there is evidence to suggest that the presence of free nucleosomes characterizes an inflammatory state.

Interestingly, we have shown that nucleosomes bind to the cell surface of human PMN and are subsequently endocytosed, suggesting a signalling pathway, which might involve a nucleosome-specific cell surface receptor and/or might take place in the cytoplasm of human PMN. However, it cannot be excluded that the internalization of nucleosomes is not necessary.

Our results suggest that nucleosomes enter human PMN at least partly via caveolae-mediated endocytosis. Moreover, the endocytosis of nucleosomes was increased in the presence of medium containing autologous serum and strongly increased with C1q. Hence, our group has recently found that free nucleosomes directly interact with C1q (Erbacher et al., manuscript in preparation). However, the interaction of C1q and nucleosomes, as well as its resulting effects are currently under investigation and will not be further discussed in this work.

In our experiments, cholera toxin subunit B was used as marker for lipid rafts. However, it was recently shown that CTB can be endocytosed via three distinct pathways: clathrin coated pits, caveolae and by a major clathrin- and caveolin-independent pathway (332). In general, the intracellular distribution of fluorescent tracers is based on their physico-chemical characteristics. LysoTrackers, for example, are weak bases, which are able to cross membranes in the deprotonated form, and are trapped inside acidic organelles by protonation. For this reason, the specificity of these compounds is also limited. Thus, the discriminatory ability of the compounds does not reflect the complexity of vesicular trafficking (316).

Considering these facts, further approaches have to be undertaken to be able to identify the entry portal and the destination of nucleosomes within the cell. Interestingly, it was demonstrated that histones are able to directly transverse the cell plasma membrane and mediate the penetration of macromolecules covalently attached to them (333). The authors came to this conclusion, because several known endocytosis inhibitors (alone or in combination) did not have any inhibitory effect on the internalization of histones.

Human neutrophils express almost all TLRs (TLR1-10, except TLR3) (334). The ubiquitous expression profile may reflect their important role as first-line effector cells in host defence. Importantly, the inappropriate recognition of self nucleic acids in particular by TLR7 and TLR9, can lead the development of autoimmune disorders, such as SLE (335). Based on sequence homology, TLR7, TLR8 and TLR9 are believed to form an evolutionarily conserved cluster within the TLR family (336,337). Furthermore, they share functional dependency on endosomal maturation for the initiation of cellular activation and are thus specialized to sense pathogen-derived DNA and RNA nucleic acid motifs in endosomal-lysosomal compartments (42,338). The synthetic antiviral compound, the imidazoquinoline resiquimod (R848), is known to activate immune cells through human TLR7/8 and mouse TLR8 (339,340). Although TLR7 expression is reported to be low in human PMN (334), R848 induced the activation of human PMN. Considering the reported facts that human PMN do not express TLR3 (334) and bacterial DNA induces neutrophil activation through a TLR-9 independent pathway (313), our data suggest that endosomally located TLRs are not involved in nucleosome-induced PMN activation. Hence, alkalization of endosomal/lysosomal pH had no blocking effect on nucleosome-induced PMN stimulation.

Interestingly, treatment of human neutrophils with GM-CSF was shown to increase the TLR9 expression levels and enhance the response to CpG DNA (334). The inducible expression of TLRs was also found in human monocytes, where IFNγ augmented the expression of TLR4, MyD88 and IRAK, whereas IL-10 had an inhibitory effect (341-343). Furthermore, CpG ODN was shown to induce the up-regulation of TLR9 expression in B lymphocytes (344,345).

In contrast to previous reports, it was recently demonstrated that human monocytes infected with *Yersinia pestis* express TLR9 at the cell surface (346). The authors claimed that the cell surface expression of TLR9 was also increased on B cells treated with CpG ODN. Furthermore, a weak expression of TLR9 was found at the cell surface of CD41-positive platelets (347).

Consistent with these findings, we found that part of human PMN express TLR9 at the cell surface. While the majority of neutrophils showed a considerable intracellular TLR9 expression, a small CD66b-positive subpopulation also expressed TLR9 at the cell surface. CpG 2006 ODN and nucleosomes induced a slight increase in TLR9 cell surface expression on human PMN after 18 hours of cell culture.

The fact that a typically intracellular TLR is localized on the cell surface was already described for TLR3. The expression of human TLR3 is limited to the intracellular compartment in DCs and mast cells (43,348), whereas in fibroblasts (349) and NK cells (350) TLR3 is expressed at the cell surface.

In view of the fact that TLR9 is also expressed at the cell surface, the inhibition of endosomal acidification may, consequently, not prevent the interaction of ligand and receptor on the cell membrane. Thus, the presence of TLR9 at the cell surface of human PMN may explain why CpG ODN-induced activation was not inhibited by ammonium chloride. Unless Alvarez et al. are right and bacterial DNA-stimulated neutrophil activation does not involve TLR9 (313).

It is, however, important to remind that PMN are short-lived cells, which start to die after several hours in cell culture. For this reason, it might be difficult to further describe or purify this subpopulation of human PMN expressing TLR9 at the cell surface. Certainly, it has to be examined in future experiments, if TLR9 at the cell surface of human PMN is involved in nucleosome-induced activation. If cell surface TLR9 is involved in nucleosome-induced PMN activation, it does not explain why nucleosomal DNA does not stimulate PMN. Hence, it seems unlikely in this case, that surface TLR9 only recognizes whole nucleosomes and not free DNA. Nevertheless, PMN may be more sensitive to DNA/nucleosomes once they are pre-sensitized and up-regulate TLR9 at the cell surface. In this context, it may be interesting to investigate, whether some serum factors associated with SLE development might increase the surface expression of TLR9 on PMN.

Much evidence suggests that nucleosomes bind selectively to cells or tissues and are in some instances taken up by appropriate target cells. Thus, diverse cell types, such as fibroblasts (297), monocytes (294) and splenocytes (299) are capable to bind nucleosomes.

Koutouzov and co-workers (298) provided evidence for the presence of a ubiquitous cell surface receptor for nucleosomes in fibroblasts. They claimed that the binding of nucleosomes to its putative receptor mediates further binding of anti-dsDNA or anti-histone antibodies and subsequent internalization of these immune complexes. Consistent with this is the finding that nucleosome-restricted antibodies complexed to nucleosomes bind to rat GBM *in vivo* (287).

However, our data support the basic concept of cell surface binding and subsequent endocytosis of nucleosomes without requiring immune complex formation. In contrast to PMN activation, which involved the whole nucleosome complex, cell surface binding and eventually internalization may be mediated either through the DNA or histone moiety of the whole nucleosome complex. Consequently, nucleosomes may bind via their DNA moiety to TLR9 located on the cell surface of human PMN. Since histones are able to cross the plasma membrane, nucleosomes could also penetrate human PMN via direct translocation.

TLR9 localization has been studied extensively, leading to the observation that this receptor resides in the endoplasmic reticulum and is recruited to endosomal and lysosomal compartments upon stimulation with CpG DNA (351,352). Notably, the activation of TLR9 by unmethlyated CpG motifs requires endosomal acidification, as compounds that alkalize endosomal pH usually block CpG DNA-driven TLR9 activation (353). Recently, Latz and co-workers presented a revised model of TLR9 activation in which only stimulatory CpG DNA led to the activation of TLR9 in endosomes by inducing a receptor conformation, which enabled the signalling through the recruitment of MyD88 (354). Overall, the data provided by Latz et al. explains how different DNA sequences can elicit distinct TLR9 responses.

Recent findings demonstrate that HMGB-1 binds to CpG ODNs and enhances their immunostimulatory potential in a TLR9-dependent manner (320,355). Thus, HMGB-1 contributes to the redistribution of TLR9 from the endoplasmic reticulum to early endosomes in response to CpG ODN (355).

HMGB-1 is an abundant highly conserved nuclear protein that modulates chromatin structure, facilitates interaction of proteins with DNA, regulates transcription and assists in V(D)J recombination (356,357). HMGB-1 is released from cells undergoing necrosis (but not apoptosis) (358) or actively secreted after inflammatory cytokine stimulation (359). Nevertheless, HMGB-1 was also shown to suppress pDC cytokine secretion and maturation in response to TLR9 agonists (360).

Thus, CpG DNA binds to the cell surface, enters the cell by means of clathrin-dependent pathways and is recognized by TLR9 located in the ER and endosomal compartments (351,361). However, TLR9 requires distinct species-specific CpG motifs for signal initiation. While GTCGTT was the optimal CpG motif for human TLR9, the optimal mouse sequence was GACGTT (361).

Furthermore, TLR9 responds to different types of ODN depending on the sequence motif and secondary structures. Class A CpG ODNs (CpG-A) (e.g. CpG 2216) mainly elicit IFNα/β production and contain a single CpG motif and a poly-G tail at the 3' end on a mixed phosphorothioate-phosphodiester backbone. In contrast, CpG-B type ODNs (e.g. CpG 2006) display a phosphorothioate backbone, but lack poly-G tails (362,363).

Our data revealed that, both CpG-A and CpG-B ODNs activated human PMN as well as mouse BMDCs, whereas CpG 2006 ODN, in contrast to the mouse specific CpG 1826 ODN, only weakly activated mouse BMN. Moreover, the CpG ODN-induced activation of mouse BMDCs was blocked by the inhibition of endosomal acidification. In contrast, neither chloroquine nor ammonium chloride prevented CpG-stimulated activation of human PMN. Nucleosomes activated PMN and BMDC, but in both cases, the inhibition of endosomal acidification did not prevent nucleosome-induced stimulation.

Finally, we found that nucleosomes might induce the activation of mouse BMN in a TLR9-independent manner, because nucleosomes slightly stimulated the up-regulation of Ly-6G and CD11b in BMN from TLR9-deficient mice. However, the latter result has to be confirmed in further experiments, since this system was not highly sensitive to nucleosome- and CpG ODN-induced activation. Altogether, these results suggest that there are different endosomal and TLR requirements for nucleosome- and CpG ODN-induced activation of human PMN and mouse BMDC.

Our data suggest that these aforementioned immune cells behave differently in their sensitivity and specificity to synthetic TLR9 ligands. For this reason, is has to be verified first that they all express TLR9, either intracellularly or at the cell surface, before drawing a conclusion.

It might be possible, that human PMN are unique in their inducible expression of TLR9 at the cell surface. Certainly, the intracellular localization of TLR9 was found to control the access of the receptor to different sources of DNA.

Thus, the intracellular localization of TLR9 might contribute to the avoidance of self-DNA recognition (325). The expression of TLR9 at the cell surface would also enable the recognition of DNA that does not normally activate TLR9. However, nucleosomal DNA, in contrast to nucleosomes, was not able to induce human PMN activation.

Consequently, it has to be proven in further experiments, whether or not this minor population expressing TLR9 at the cell surface mediates nucleosome-induced PMN activation.

In the present study, it was shown that free nucleosomes induce neutrophil activation in a TLR2/TLR4-independent manner. Moreover, the nucleosome-triggered PMN activation probably did not involve endosomal/lysosomal TLRs.

It was also hypothesized that nucleosomes only stimulate PMN via the DNA moiety, because their endosomal translocation is more effective than that of free mammalian DNA (which only activates cells upon enforced endosomal translocation, as explained above), favouring the interaction of nucleosomes and TLR9. Nevertheless, our results with human PMN suggest a TLR9-independent mechanism, since endosomal acidification was not required. In some cases, maybe, TLR9-mediated activation does not necessarily need endosomal acidification. However, this seems to be unlikely in our experiments, as an acidic pH is probably required for nucleosome denaturation and degradation into single-stranded (ss) DNA. Thus, only ssDNA, in contrast to double-stranded (ds) DNA, binds to TLR9. Moreover, this implies that PMN behave differently from mBMN, since a slight nucleosome-induced activation was detectable in TLR9-/- cells.

However, whether or not the internalization of nucleosomes involves a cell surface receptor (TLR9?), occurs via the DNA (CpG motif?) or histone moiety (direct translocation?) of the whole nucleosome, recruits acidic vesicles as well as the trafficking of nucleosomes within the cell is still not resolved.

Nevertheless, the present study contributes to a more complete understanding of the pathogenesis of SLE. It is noteworthy to mention that neutrophils are key players of the innate immune system that provide a first line of defence against invading pathogens. In addition, they have been suggested to link innate and adaptive immunity. Indeed, nucleosomes trigger a previously unknown pathway of innate immunity, which may provide some evidence of how self-tolerance is overcome in genetically predisposed patients with SLE.

5 Summary

Nucleosome, the basic structure of chromatin and normal product of cell apoptosis, plays a pivotal role in both the induction and pathogenesis of systemic lupus erythematosus (SLE). Nucleosomes are found to circulate at high levels in patients with SLE and are thought to be one of the major autoantigens in SLE.

In the present work, it is shown for the first time that physiological concentrations of purified nucleosomes trigger the activation and recruitment of neutrophils *in vitro* and *in vivo* in a Toll-like receptor (TLR) 2- and TLR4-independent manner. Nucleosomes directly induce the activation of human polymorphonuclear neutrophils (PMN) upon internalization, as determined by up-regulation of the cell surface molecules, CD11b and CD66b, IL-8 secretion, increased phagocytic activity and delayed apoptosis. The chemokine IL-8 is one of the most potent neutrophil chemoattractants. Serum IL-8 levels strongly correlate with the disease activity in SLE patients. Since SLE is an inflammatory disease and neutrophils are the first cells recruited to infection and inflammation sites, nucleosome-induced IL-8 secretion may thus result in enhanced inflammation via an amplification loop.

Nucleosomes induce PMN activation without the requirement of immune complex formation and independently of CpG motifs in free nucleosomal DNA, endotoxin or high-mobility group box-1 (HMGB-1). PMN from both normal healthy donors and patients with SLE show responsiveness to nucleosome-induced activation.

Moreover, nucleosomes probably trigger the activation of human PMN without engagement of endosomal nucleic acid-sensing TLRs. However, we identified a previously unknown subpopulation of human PMN, which do express TLR9 at the cell surface. Although nucleosomes slightly induce the up-regulation of Ly-6G and CD11b on the cell surface of bone marrow-derived neutrophils from TLR9-deficient mice, the role of TLR9 in nucleosome-induced innate immune cell activation needs further detailed investigation.

Indeed, nucleosomes trigger a previously unknown pathway of innate immunity, which provides an essential piece of the puzzle in understanding how self-tolerance is overcome in patients with SLE.

6 Zusammenfassung

Nucleosomen sind die Grundbausteine der Chromatinstruktur und entstehen als Abbauprodukt beim programmierten Zelltod (Apoptose). Darüber hinaus spielen Nucleosomen eine entscheidende Rolle bei der Entstehung und dem Krankheitsverlauf des systemischen Lupus erythematodes (SLE). Hohe Konzentrationen zirkulierender Nucleosomen werden bei SLE-Patienten während eines Schubs festgestellt und deshalb als wichtiges Autoantigen angesehen.

In der vorliegenden Arbeit wird erstmals gezeigt, dass physiologische Konzentrationen aufgereinigter Nucleosomen die Aktivierung und Rekrutierung von Neutrophilen *in vitro* und *in vivo* induzieren; und das unabhängig von Toll-like Rezeptor (TLR)2- und TLR4. Nucleosomen induzieren die Aktivierung dieser humanen polymorphkernigen Leukocyten durch Internalisierung, wie mittels Hochregulierung von Zelloberflächenmolekülen (CD11b und CD66b), Sekretion von Interleukin (IL)-8, erhöhter Phagocytoseaktivität und verzögerter Apoptose festgestellt werden konnte. Das Chemokin IL-8 lockt vor allem Neutrophile aus dem Blut zum Infektionsherd. Der IL-8-Gehalt im Serum von SLE-Patienten korreliert stark mit dem Krankheitsverlauf. Da der SLE eine entzündliche Erkrankung ist und Neutrophile die ersten Zellen an Infektions- und Entzündungsherden sind, bewirkt die Nucleosomen-induzierte Freisetzung von IL-8 das Aufrechterhalten der Entzündung über eine Verstärkerschleife.

Die Nucleosomen induzieren hier die Aktivierung von Neutrophilen ohne Bildung von Immunkomplexen mit Autoantikörpern, außerdem unabhängig von CpG-Motiven (in der nucleosomalen DNA) sowie Endotoxinen oder HMGB-1. Neutrophile von gesunden Kontrollpersonen und SLE-Patienten reagierten sensitiv auf die Nucleosomen-induzierte Aktivierung. Es spricht vieles dafür, dass die Nucleosomen die Neutrophilaktivierung ohne Mitwirkung intrazellulärer TLR triggern.

Zudem haben wir eine bis dato unbekannte Subpopulation humaner Neutrophiler entdeckt, die TLR9 auf der Zelloberfläche exprimieren.

Obwohl die Nucleosomen eine schwache Hochregulierung der Zelloberflächenmoleküle Ly-6G und CD11b bei (Vorläufer-) Neutrophilen aus dem Knochenmark TLR9-defizienter Mäuse induziert haben, müssen weitere Experimente durchgeführt werden, um zu einem schlüssigen Ergebnis zu gelangen.

In der vorliegenden Arbeit wird aufgezeigt wie Nucleosomen einen bisher unbekannten Signalweg der angeborenen Immunantwort triggern. Diese neuen Erkenntnisse liefern somit einen wichtigen Beitrag zum Verständnis wie die Toleranz gegenüber Selbstantigenen bei SLE-Patienten verloren geht.

7 Reference list

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8 Supplement

8.1 List of figures

8.2 List of tables

8.3 Acknowledgement

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8.4 Publication list and curriculum vitae

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- Erbacher, A.I., **Rönnefarth, V.M.** and Decker, P. 2007. Circulating nucleosomes due to a defective clearance of dying cells: consequences in systemic lupus erythematosus. Autoimmunity; 40 (4): 311-4
- **Rönnefarth, V.M.**, Erbacher, A.I. and Decker, P. Circulating nucleosomes in systemic lupus erythematosus: not only a passive target for autoantibodies. (manuscript in preparation)
- Erbacher, A.I., **Rönnefarth, V.M.**, Botto, M., Rammensee, H.-G. and Decker, P. C1q is involved in an autoantigen clearance backup mechanism leading to complement consumption in systemic lupus erythematosus. (manuscript in preparation)
- **Rönnefarth, V.M.**, Erbacher, A.I., Rammensee, H.-G. and Decker, P. Different endosomal and TLR requirements for nucleosome- and CpG ODN-induced activation of PMN and BMDC: consequences in lupus. (manuscript in preparation)

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