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Homologous association of the mouse TNFα locus

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ABSTRACT

Lipopolysaccharide (LPS) stimulation of murine macrophages triggers the activation of signalling pathways that lead to the nuclear translocation of Nf-κB and the subsequent expression of TNFα., among other pro-inflammatory cytokines.

In the present project we have studied the events that take place in the cell nucleus before and during the expression of TNFα and, specifically, we aimed in identifying the protein factors that regulate the inter-chromosomal interactions between the two murine TNF alleles.

We found that the homologous association of the two TNFα alleles takes place 30min upon the stimulation of macrophages with LPS. The highest level of TNF expression followed this homologous interaction, at 1h upon LPS stimulation and accordingly the simultaneous transition from mono- to by-allelic expression was detected at the same time point.

In correspondence to the model of transvection and the GAGA factor that mediates homologous pairing in Drosophila, we used a series of molecular and genetic approaches to identify the protein complexes that regulate these long-range interactions in mammals.

ΠΕΡΙΛΗΨΗ

Η ενεργοποίηση από λιποπολυσακχαρίτη (LPS) των μακροφάγων ποντικού ενεργοποιεί σηματοδοτικά μονοπάτια που οδηγούν στην πυρηνική μετατόπιση του μεταγραφικού παράγοντα Nf-κB και την έκφραση του TNFα, μεταξύ και άλλων προ-φλεγμονοδών κυτοκινών.

Στη παρούσα εργασία μελετήσαμε τις υπο-πυρηνικές αλληλεπιδράσεις που λαμβάνουν χώρα πριν και κατά τη διάρκεια της έκφρασης του TNFα και, συγκεκριμένα, προσπαθήσαμε να ταυτοποιήσουμε τους πρωτεϊνικούς παράγοντες που ρυθμίζουν τις δια-χρωμοσωμικές αλληλεπιδράσεις μεταξύ των δύο TNF αλληλίων.

Βρήκαμε ότι η ομόλογη αλληλεπίδραση των δύο αλληλίων του TNFα λαμβάνει χώρα 30 λεπτά μετά την ενεργοποίηση από LPS, ενώ τα μέγιστα επίπεδα μεταγραφής του TNFα έπονται του φαινομένου αυτού, μια ώρα μετά την ενεργοποίηση, όπως και η ταυτόχρονη μετάβαση από μονο-αλληλική έκφραση σε έκφραση και από τα δύο αλλήλια του TNFα.

Σε αντιστοιχία με το μοντέλο του ‘transvection’ και τον παράγοντα GAGA που ελέγχει την ομόλογη σύνδεση στη Δροσόφιλα, χρησιμοποιήσαμε μια σειρά μοριακών κι γενετικών προσεγγίσεων για να ταυτοποιήσουμε τα πρωτεϊνικά σύμπλοκα που ρυθμίζουν αυτές τις αλληλεπιδράσεις στα θηλαστικά.

It is a capital mistake to theorize before you have all the evidence. It biases the judgment.

Sherlock Holmes

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1. INTRODUCTION

Gene expression is mediated by regulatory elements which can be located far away from the genes they control. The mechanism that allows transcriptional regulation over large genomic distances involves the formation of direct associations between these elements and their gene targets via the combined action of multiple trans-acting factors[1-3].

So far, evidence has suggested that these elements, capable of transcriptional regulation, act *in cis* on genes physically linked to them, on the same chromosome. Recently, however, *trans*-interactions have been shown to occur between regulatory elements located on a chromosome different than the one carrying the gene to be regulated.

In diploid organisms, the two alleles existing for each locus do not always function independently, as is generally believed. There are circumstances in which one allele can "sense" the presence of the other allele *in trans* and initiate certain processes and epigenetic changes that alter the expression pattern[4].

1.1. Trans-allelic phenomena

Trans-allelic phenomena have been studied recently in several systems, pointing out that such a mechanism regulating gene expression *in trans* is a general phenomenon. Some of these phenomena are directional, in that the paired alleles are different from one another, whereas in other phenomena, identical alleles act reciprocally. The interactions can either be weakened by disruption of the association or not and they can either activate or suppress transcription. The term "trans-sensing effects" was introduced to deal with this diversity and describe every allelic-pairing dependent phenomenon, irrespective of the underlying mechanism[4].

1.1.1. RIP, MIP and quelling

In the filamentous ascomycete *Neurospora crassa* duplicated DNA sequences are subjected to mutations at high frequency during the sexual cycle. A silencing mechanism called "Repeat-Induced Point mutation" (RIP) identifies duplications that are greater than

~400bp (or ~1kb for unlinked duplications) and introduces G:C to A:T mutations into both copies of the sequence[5, 6]. It is also believed that sometimes a specific DNA rearrangement involving loss of one copy of the duplicated element and the intervening sequences takes place[5, 7]. In any case, the process results in a high probability of nonsense codons and in methylation of the mutated sequences, leading to gene inactivation (Fig.1). Evidence shows that the presence of repeats is detected by pairing of homologous DNA sequences[8-10]. Although the DNA pairing processes used to pair and recombine chromosomes in meiosis would seem likely to be used by RIP to detect mutations, experiments affecting pairing at meiosis and cases of unlinked duplications that do not undergo recombination, suggest that homologous pairing involved in RIP is a distinct process[5, 6]. In any case, RIP serves as a protection mechanism against repetitive DNA and transposable elements and preserves genome integrity by suppressing or eliminating chromosomal rearrangements[5, 11].

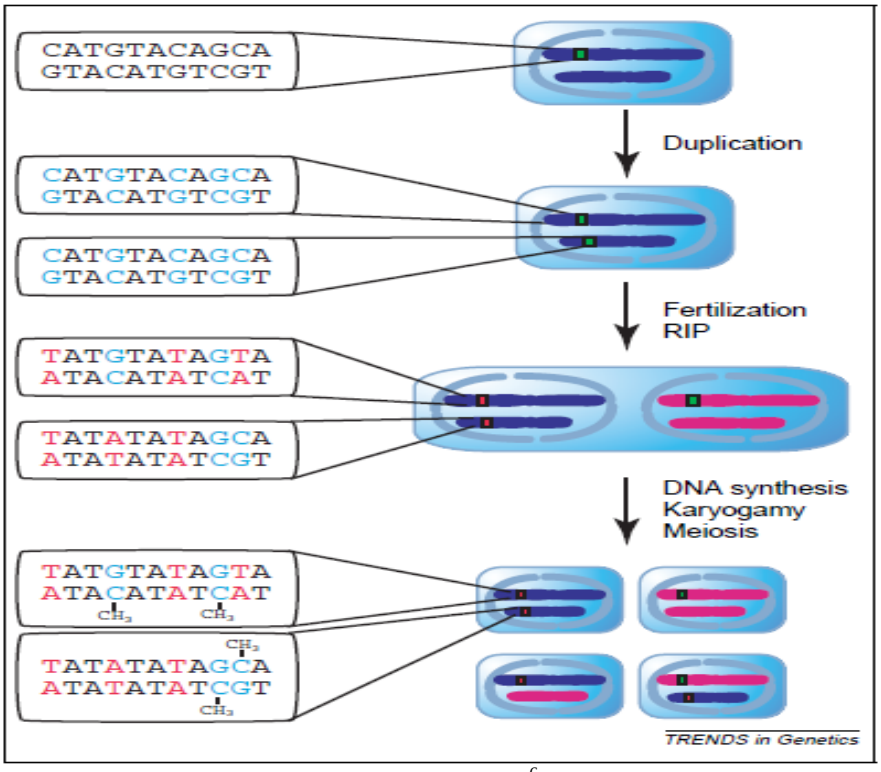


Figure 1. Repeat-induced point mutation process[6].

Although RIP is unique to *N. crassa*, epigenetic gene-silencing mechanisms similar to RIP have been identified in other fungi. In the filamentous ascomycete *Ascobolus*

immersus and in the basidiomycete *Coprinus cinereus*, MIP (Methylation Induced Premeiotically) detects duplications and results in methylated cytosines prior to meiosis[5]. The mechanism also involves a homologous pairing process during which duplications, with sizes similar to those needed for RIP, are identified and methylated at cytosine residues which blocks transcription elongation and results in gene silencing[5, 12].

Analogous to MIP, but acting during vegetative growth, quelling in *N. crassa* results in gene silencing of DNA sequences introduced by transformation. Transgene silencing, although believed to involve methylation events, occurs by a post-transcriptional or co-transcriptional mechanism, during which homologous transgene RNA-RNA interactions accelerate degradation of the native mRNA[5, 13].

1.1.2. Paramutation

Paramutation was discovered in *Zea mays* (maize) by Alexander Brink in the 1950s[14, 15]. It was described as an interaction between alleles of genes that leads to heritable changes in gene expression. It is now known that it is an epigenetic phenomenon involving changes in gene expression that are stably transmitted through mitosis as well as meiosis. These heritable changes are mediated by *in trans* interactions between homologous DNA sequences on different chromosomes. During these *in trans* interactions, epigenetic information is transferred from one allele of a gene to another allele of the same gene, resulting in a change of gene expression[15]. At a locus subject to paramutation, an inducing (paramutagenic) allele silences a sensitive (paramutable) one.

One classic example of paramutation is the *b1* locus in maize (Fig.2) where the *B-I* allele is converted (paramutated) to *B′* and, importantly, this new *B′* allele (designated *B′**) is equally capable as the parental *B′* allele of paramutating *B-I* to *B′* in subsequent generations[16]. It has been found that the key sequences required for paramutation are tandem repeats of non-coding DNA located ~100 kb upstream of the *b1* transcription start site[17] and that the heritability of paramutation is RNA-dependent[18-22].

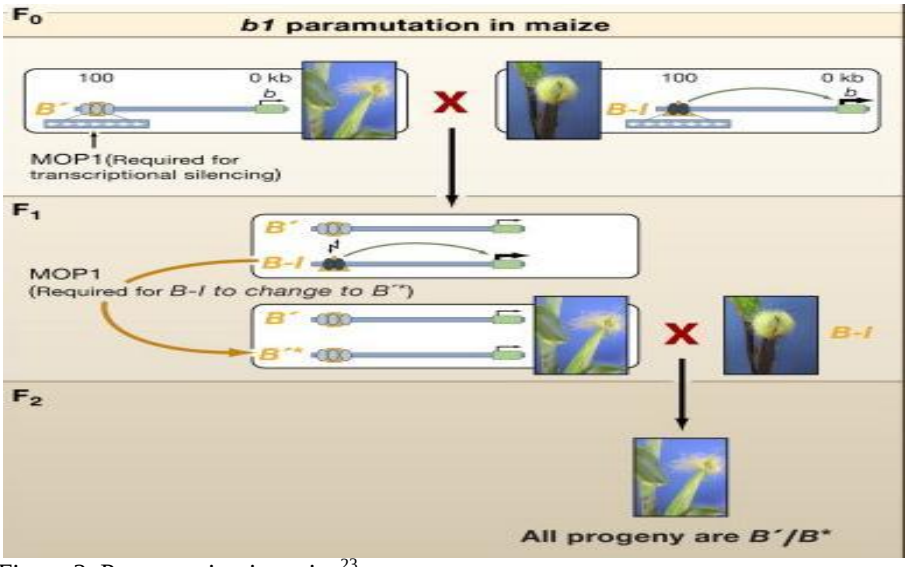


Figure 2. Paramutation in maize[23].

Although paramutation was initially discovered in plants, it has recently been observed in mammals as well, suggesting that the mechanisms underlying paramutation might be evolutionarily conserved. Remarkably, during studies on the Kit locus, a tyrosine kinase receptor that functions in melanogenesis, germ cell differentiation, and hematopoiesis, it was observed that when heterozygotes were crossed with wild type animals, the phenotypic result of the genetically wild-type progeny was similar to that of heterozygotes (Fig.3)[24].

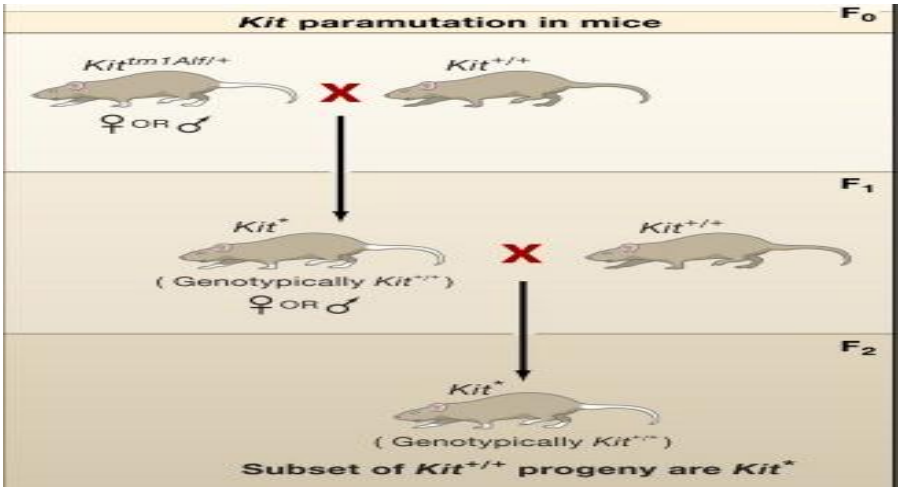


Figure 3. Paramutation in mice[23].

In this case, transmission of the paramutagenic state seems to involve small RNAs, which, in contrast to maize, appear sufficient to induce paramutation[24].

1.1.3. Transvection

The term transvection was introduced by E.B. Lewis in 1954 to describe phenomena in *Drosophila melanogaster* in which gene activity is influenced by homologous pairing. He illustrated transvection by showing how complementation between two alleles of the *bithorax* gene complex can be antagonized by disruptions of somatic pairing. Since then, transvection has been found at a rapidly growing number of Drosophila loci including *white, decapentaplegic, eyes absent, vestigial, and yellow*[25].

Transvection is an epigenetic phenomenon that results from an interaction between an allele on one chromosome and the corresponding allele on the homologous chromosome which can lead to either gene activation or repression. It is dependent upon chromosome pairing and it has been shown that a translocation leads to disruption of transvection and, thus, of complementation.

Transvection is believed to occur through a variety of mechanisms[25]. It may occur by the enhancers of one allele acting *in trans* on the promoter of a paired allele (Fig.4a) or by the enhancer bypassing an insulator to get in close proximity with the promoter (Fig.4b).

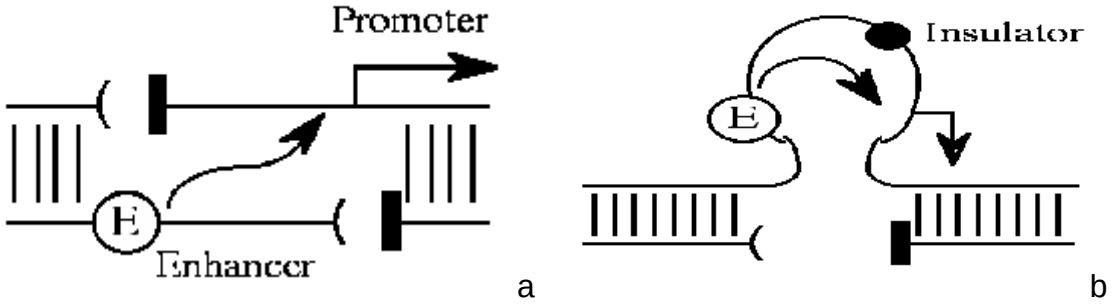


Figure 4. Mechanisms of transvection a) Enhancer action *in trans* b) Insulator bypass[26].

Several protein factors have been associated with these mechanisms, among which zeste protein, which acts generally holding DNA segments together either intra-molecularly during looping or inter-molecularly during transvection[25], the GAGA factor, which binds to GA-rich sequences, $(GA)_n$, and stimulates expression by opening chromatin and maintaining the promoter in a conformation that allows further binding of sequence-

specific factors[27-30] and members of the Polycomb group (PcG) that bind to Polycomb group response elements (PREs) and modify chromatin structure[25].

1.1.4. X chromosome dosage compensation

Sex chromosome dosage compensation is a chromosome-wide regulatory process which detects the number of X chromosomes in a cell and adjusts the levels of gene expression in males and females.

The strategies for dosage compensation are diverse in different organisms, but in all known cases, specialized dosage compensation complexes are targeted exclusively to the X chromosome to equalize the transcript levels in order to neutralize the difference between the sexes (Fig.5). Hermaphrodite worms keep both X chromosomes active, but repress transcript levels from each X chromosome by half [31, 32] while male flies double the transcription rate of their single X chromosome [33-35]. For mammals it is now known that X inactivation exists in two forms: *imprinted*, during which the paternal X chromosome is preferentially shut off and *random*, where inactivation takes place randomly in the soma so that either the paternally or maternally inherited X chromosome is silenced[36].

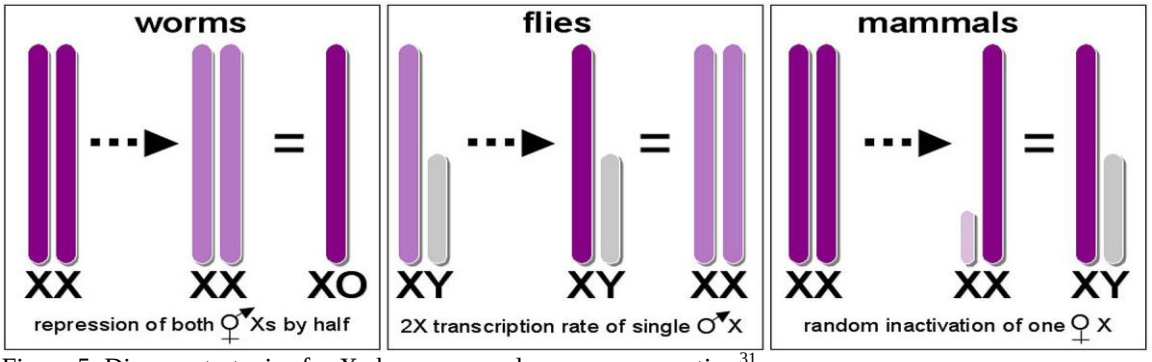


Figure 5. Diverse strategies for X chromosome dosage compensation[31].

In mammals, transcriptional silencing of a single female X chromosome is controlled *in cis* by Xist, whose RNA product coats the inactive X chromosome (Xi), and the X-inactivation centre (Xic). In the future inactive X *Xist* becomes upregulated and *Tsix* (its antisense unit) is down-regulated. The accumulation of the Xist transcript on the X chromosome chosen to be inactivated (Xi) triggers the initiation of X inactivation. The

chromosome-wide regulation is accompanied by the sex-specific modification of histones on the dosage compensated X chromosomes[37-45].

1.1.5. Inter-chromosomal interactions in mammals

Although somatic pairing is generally absent in mammals, the mechanism of X chromosome dosage compensation has been studied extensively. There are examples, however, of tissue and stage-specific pairing of particular loci, for which very little is known.

Besides paramutation in the Kit locus and the X inactivation mentioned above, inter-chromosomal interactions have been described between the alpha and beta globin loci, imprinted loci, the neuronal loci of olfactory receptors and the two mutually exclusive differentiation states of T helper cells.

The alpha and beta-globin loci have served as model systems for long-range gene regulation for many years. Besides the association of the Locus Control Region (LCR) of the beta-globin locus with distal regulatory elements and the long-range looping that activates the genes in the alpha locus, it was recently shown that the α and β globin alleles come into close proximity, sharing the same Polymerase II transcription factory. At the same time, the α-globin alleles have been shown to be colocalized when active, although no physical association has been detected so far[46-48].

Ling and colleagues have identified an unexpected co-association between two loci on separate chromosomes in mouse nuclei, the imprinting control region (ICR) in the *Igf2–H19* locus of chromosome 7 and the *Wsb1–Nf1* locus of chromosome 11. Strikingly, this interaction is CCCTC-binding factor (CTCF)-dependent, strictly allele specific and results in the regulation of expression of the *Wsb1–Nf1* genes[49-51].

In addition, it has recently been shown that the H enhancer, located on mouse chromosome 14, can associate with multiple odorant receptor (OR) gene promoters in olfactory sensory neurons. Specifically, this single enhancer element can trans-activate all different OR promoters and provides a mechanism for the stochastic choice of a single olfactory receptor gene in an individual odorant sensory neuron[52, 53].

Prior to all previous studies, in cells of the T-cell lineage it has been reported that inter-chromosomal interactions occur between the *Ifng* gene and the T_H2 cytokine gene loci.

Specifically, in naïve CD4+ T cells the *Ifng* locus was found to be in close proximity with the *Il5* and *Rad50* promoters and with the DNAse I hypersensitivity site RHS6 of the T_H2 LCR. Upon differentiation of naive T cells to T_H1 and T_H2 cell lineages the strong inter-chromosomal interactions between the Ifng gene and the TH2 cytokine locus were replaced by another TH1-specific intra-chromosomal interaction between the Ifng gene and Ifng CNS2 element, a tissue-specific transcriptional enhancer[54, 55].

1.2. Activation and transcriptional regulation in macrophages

Macrophages have long been considered to be important immune effector cells. They are present in virtually all tissues and differentiate from circulating peripheral blood mononuclear cells (PBMcs), which migrate into tissue in the steady state or in response to inflammation. These PBMcs develop from a common myeloid progenitor cell in the bone marrow that is the precursor of many different cell types, including neutrophils, eosinophils, basophils, macrophages, dendritic cells (DCs) and mast cells. During monocyte development, myeloid progenitor cells (termed granulocyte/macrophage colony-forming units) sequentially give rise to monoblasts, pro-monocytes and finally monocytes, which are released from the bone marrow into the bloodstream. Monocytes migrate from the blood into the tissue to replenish long-lived tissue-specific macrophages of the bone (osteoclasts), alveoli, central nervous system (microglial cells), connective tissue (histiocytes), gastrointestinal tract, liver (Kupffer cells), spleen and peritoneum[56, 57].

In the blood, monocytes are not a homogeneous population of cells, and there is substantial debate about whether specific monocyte populations give rise to specific tissue macrophages[58]. In mice, two populations of monocytes have been identified and termed as ‘inflammatory’ and ‘resident’ monocytes, based primarily on the amount of time they spend in the blood before migrating into tissues. These two mouse monocyte populations can be differentiated based on the expression of cell-surface markers[59]. Inflammatory monocytes in mice are defined as $CCR2^+$ (CC-chemokine receptor 2), $CX3CR1^{low}$ (CX3C-chemokine receptor 1) and $GR1^+$ (also known as Ly6), whereas resident monocytes are defined as $CCR2^-CX3CR1^{hi}GR1^-$. Monocytes that are not initially recruited to the tissue can further mature in the blood and eventually become part

of a blood-resident population of monocytes that is thought to contribute to the integrity of the endothelial cell lining of blood vessels and the maintenance of tissue-resident macrophage populations in the steady state[60].

Macrophages are phagocytic cells involved in the clearance of cellular debris generated during tissue remodeling, wound healing and necrosis after pathogenic infection. Due to this process they can respond to endogenous stimuli that are rapidly generated following injury or infection. These early stimuli are typically produced by innate immune cells and can exert a marked, though usually transient, effect on the physiology of macrophages. Macrophages can also respond to signals that are produced by antigen-specific immune cells. These signals are more focused and prolonged than innate immune stimuli and generally give rise to longer-term alterations in macrophages[61]. To complicate matters, macrophages themselves can produce several factors that influence their own physiology.

The combination of two signals, interferon-γ (IFNγ) and tumour necrosis factor (TNF) can result in a macrophage population that has enhanced microbicidal or tumoricidal capacity and secretes high levels of pro-inflammatory cytokines and mediators[62]. IFNγ can be produced by innate or adaptive immune cells: natural killer (NK) cells and T helper 1 (TH1) cells.

Typically, a Toll-like receptor (TLR) ligand acting in a MyD88-dependent manner will induce the transcription of TNF, which can then cooperate with IFNγ in an autocrine manner to activate this macrophage population. In addition to MyD88, some TLR ligands can also activate TIR-domain-containing adaptor protein inducing IFNβ (TRIF)-dependent pathways, which signal through IFN-regulatory factor 3 (IRF3) and result in IFNβ production which can replace IFNγ in activation[63].

The destructive potential of macrophages is generally stringently controlled. Recognition of conserved non-self molecules expressed by microorganisms is mediated by the so-called pattern recognition receptors, many of which belong to the Toll-like receptor family. The most studied of these receptors is Toll-like receptor 4 (TLR4), which mediates signals generated by lipopolysaccharide (LPS), a major component of the cell walls of gram-negative microorganisms. Stimulation of macrophages with exogenous TNFα or with a TLR ligand, such as lipopolysaccharide (LPS), can also result in the complete clearance of the parasite. In response to LPS, mouse macrophages undergo a

major change in gene expression, inducing the expression and release of numerous biologically active cytokines that orchestrate the inflammatory response. The LPS response in mouse macrophages has been analyzed on a number of different platforms and such profiling reveals a cascade of gene regulation and several LPS-induced genes[64]. However, it has not yet been possible to provide a reliable detailed map of the underlying regulatory transcriptional architecture.

1.3. Tumor necrosis factor-alpha

1.3.1. The LT/TNF locus

The tumour necrosis factor (*TNF*) locus comprises of the tandemly arranged genes coding for TNFα (cachectin), Lymphotoxin alpha (LTα) and Lymphotoxin beta (LTβ). The *TNFα* gene resides in a locus with the lymphotoxin (*LT*) α and β genes (Fig.6), their coding regions occupying ~12 kb of genomic DNA on human chromosome 6 and mouse chromosome 17. The *TNF* and *LTα* genes are in the same transcriptional orientation; *LTβ* is transcribed in the opposite orientation.

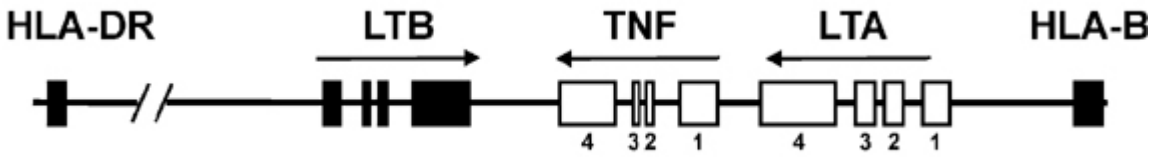


Figure 6. The LT/TNF locus.

The intergenic regions of the LT/TNF locus are generally poorly conserved, but they do contain short, highly conserved noncoding sequences. Among these is the *TNF* promoter itself, which shows almost complete conservation among primate species and complete conservation in humans in the proximal region critical for transcriptional regulation.

Transcription of the *TNFα* gene is regulated in a cell type- and stimulus-specific fashion through the recruitment of specific sets of transcription factors and coactivators to the promoter region (~200bp), forming distinct nucleoprotein complexes known as enhanceosomes. The TNFα gene is transcribed much more heavily than lymphotoxin-α in

T lymphocytes, even though the LTα mRNA is more abundant. Resting T cells and macrophages, which do not accumulate any TNF mRNA, nevertheless transcribe the TNFα gene actively. On the other hand, the LTα gene is transcriptionally silent in macrophages.

TNFα and the soluble lymphotoxin α are cytokines with similar biological activities. LTα when expressed by activated T cells forms a heterotrimer with LTβ which is a membrane-anchored ligand that binds specifically to the LT beta receptor (LT beta R). The LT beta R is implicated as a critical element in controlling lymph node development and cellular immune reactions. TNFα is a pleiotropic pro-inflammatory cytokine produced predominantly by monocytes and macrophages[65]. Proinflammatory cytokines act to increase their own production and the synthesis of small inflammatory mediators such as platelet-activating factor (PAF), eicosanoids, and oxidative radicals. Proinflammatory cytokines also recruit and stimulate cellular components of the immune system. TNF is an endogenous pyrogen that stimulates the production of other endogenous pyrogens, such as interleukin 1b (IL-1)[66].

TNFα is primarily produced as a 212-amino acid-long type II transmembrane protein arranged in stable homotrimers. From this membrane-integrated form the soluble homotrimeric cytokine (sTNF) is released via proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE, also called ADAM17). The soluble 51 kDa trimeric sTNF tends to dissociate at concentrations below the nanomolar range, thereby losing its biological activity.

1.3.2. Transcriptional activation of TNFα expression

The mechanisms leading to LPS-stimulated macrophage TNFα production appear multiple. LPS, released by invading bacteria, can trigger the expression of TNFα via two different receptors, the macrophage-specific surface receptor CD14 and the membrane protein Toll-like receptor 4 (TLR4) and through two respective signalling pathways.

In the first case, LPS-binding protein (LBP) facilitates LPS binding to the macrophage CD14. LPS-LBP-CD14 interaction provokes rapid activation of protein tyrosine kinase (PTK) causing tyrosine phosphorylation of several intracellular protein kinases. PTK activates a pathway involving Ras/Raf-1/mitogen-activated protein kinase (MEK)/

MAPKs/NFkB. Ras is an early target of the activated PTK and is able to interact directly with Raf-1. Raf-1/MEK appears to activate members of the MAPK family of protein kinases; of these the p38 MAPK appears to be a pivotal MAPK in the cascade leading to TNF gene induction. The protein kinase cascade leads to NFkB activation, its translocation to the nucleus and TNFα production[66-69] (Fig.7).

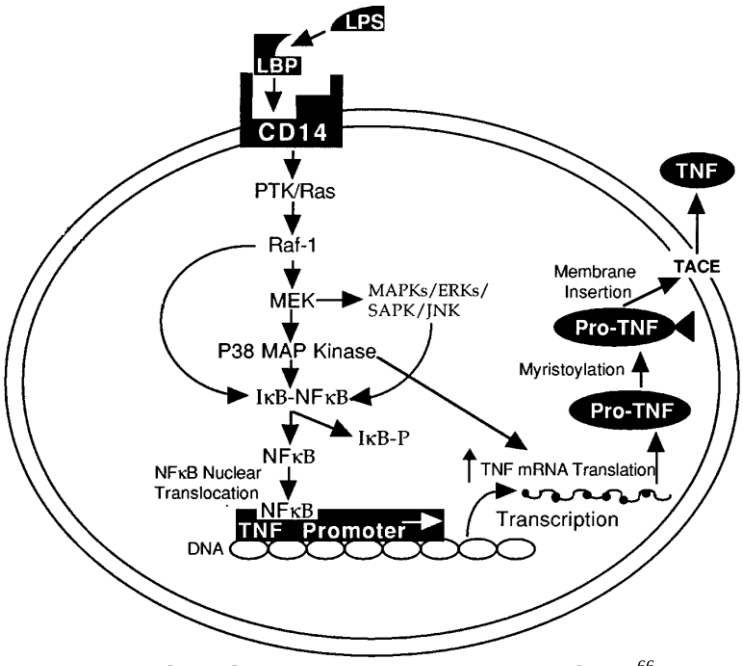


Figure 7. CD14-dependent TNFα expression in macrophages[66].

In the case of TLR4, signaling originates from its conserved cytoplasmic Toll/IL-1 receptor (TIR) domain. In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, is recruited. This MyD88-dependent pathway leads to the recruitment of IL-1 receptor-associated kinase (IRAK) to TLRs through the interaction of the death domains of both molecules. IRAK is activated by phosphorylation and then associates with TRAF6. The IRAK-1/TRAF6 complex dissociates from the receptor and associates with TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins, TAB1 and TAB2, at the membrane portion. TRAF6 is poly-ubiquitinated which leads to the activation of NF-κB and the production of inflammatory cytokines[70] (Fig.8).
There is also a MyD88-independent pathway in TLR signaling. In the absence of MyD88, LPS stimulation leads to the activation of the transcription factor IRF-3, and thereby

induces IFN-β. IFN-β, in turn, activates STAT1, leading to the induction of several IFN-inducible genes. The TRIF-TRAM module that is activated in this pathway can also recruit RIP1 and TRAF6, but the induction of NF-κB in this case only leads to the induction of IFN-inducible genes and not TNFα[70].

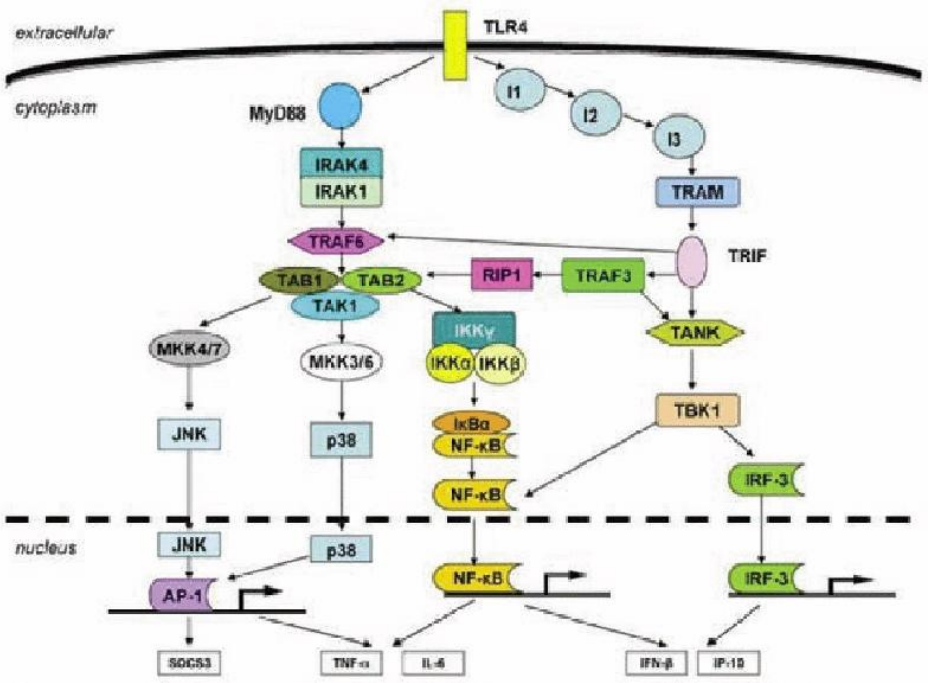


Figure 8. TLR4-dependent TNFα expression[70].

Lastly, TNFα can itself activate a pathway that leads to the induction of NF-κB and further pro-inflammatory cytokine expression. This is achieved through the binding of TNFα to its TNF receptor. Two receptors, TNF-R1 (TNF receptor type 1; CD120a; p55/60) and TNF-R2 (TNF receptor type 2; CD120b; p75/80), can bind TNFα. TNF-R1 is expressed in most tissues and can be fully activated by both the membrane-bound and soluble trimeric forms of TNFα, whereas TNF-R2 is found only in cells of the immune system, and respond to the membrane-bound form of the TNFα homotrimer[67, 71].

The TNF-TNF-R binding causes a conformational change in the receptor, leading to the dissociation of the inhibitory protein SODD from its intracellular death domain. This dissociation enables the adaptor protein TRADD to bind to the death domain, serving as a platform for subsequent protein binding. TRADD can either recruit TRAF2 and RIP and lead to the transcription of proteins involved in cell proliferation and survival, or TRAF2 can activate the JNK-inducing upstream kinases of MEKK1 and ASK1 (either directly or

through GCKs and Trx, respectively), and these two kinases phosphorylate MKK7, which then activates JNK. JNK translocates to the nucleus and activates transcription factors such as c-Jun and ATF2. The JNK pathway is involved in cell differentiation, proliferation, and is generally pro-apoptotic[72-75].

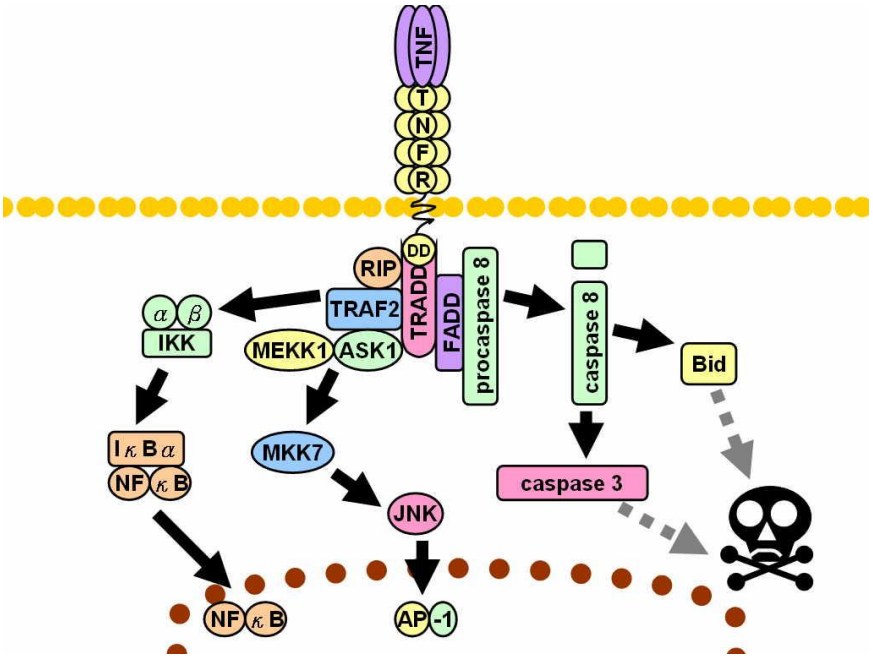


Figure 9. TNF-R signalling pathways[76].

As a third alternative TRADD binds FADD, which then recruits the cysteine protease caspase-8 to form the death-inducing signaling complex (DISC). A high concentration of caspase-8 in the DISC induces its autoproteolytic activation and subsequent cleaving of effector caspases, leading to cell apoptosis[75, 76] (Fig.9).

1.4. Objectives

Tumor necrosis factor (TNF), initially discovered as a result of its antitumor activity, has now been shown to mediate tumor initiation, promotion, and metastasis. In addition, deregulation of TNFα has been implicated in a wide variety of inflammatory diseases including rheumatoid arthritis, Crohn's disease, multiple sclerosis, psoriasis, scleroderma, atopic dermatitis, systemic lupus erythematosus, type II diabetes, atherosclerosis, myocardial infarction, osteoporosis, and autoimmune deficiency disease[66]. TNFα, however, is a critical component of effective immune surveillance and is required for

proper proliferation and function of NK cells, T cells, B cells, macrophages, and dendritic cells.

The goal of this work is to study the long range regulation of TNFα gene expression and further understand how nuclear organization and inter-chromosomal interactions affect its transcription. In addition, our main objective is to identify protein complexes that regulate these interactions in macrophages.

Work in the field of inter-chromosomal interactions is currently based on the identification of genomic interacting partners and merely on the protein complexes involved in organising the mammalian genome, regulating its subnuclear localisation and mediating the transcriptional activation or suppression. Our research efforts focused on identifying and characterising such factors and, ultimately, on proposing new mechanisms for the regulation of gene expression, that could be used in the future to study and possibly resolve the deregulation of gene expression in disease models. Identification of the proteins involved in inter-chromosomal interactions could lead to the development of small molecules aiming in the inactivation of such proteins and the future manipulation of the immune system and specifically TNFα.

2. MATERIALS AND METHODS

Cell lines

A mouse monocyte macrophage cell line, Raw 264.7, was cultured under 5% CO_2 at 37°C.

Cell culture medium

Dulbecco's Modified Eagle's Medium (ATCC, Cat No.30-2002) supplemented with 10% fetal calf serum (Sigma) and penicillin and streptomycin (GIBCO) at 5μg/mL each.

Macrophage stimulation

Cell line macrophages were stimulated with 50ng/ml LPS (Invivogen, O111:B4).

Total RNA isolation

A cell monolayer was rinsed with PBS and 1ml TRIzol Reagent (GibcoBRL) per 10cm^2 of cultured dish area was used to lyse them. The cell lysate was scraped from the dish and incubated at room temperature for 5min. Then, 0.2ml chloroform was added, the lysate was vortexed and centrifuged at 14000rpm for 15min at 4^oC. The RNA-containing supernatant was isolated and the RNA was precipitated with the use of 0.5 ml of isopropyl alcohol per 1 ml of TRIzol Reagent used for the initial homogenization. After centrifugation at 14000rpm for 20min at 4^oC, the RNA precipitate was dissolved in DEPC-treated water. Sample concentration and purity was determined with spectrophotometric analysis, measuring the OD at 260nm, taking into account that 1 OD at 260nm equals 40μg /ml RNA.

Reverse transcription - cDNA synthesis

cDNA was synthesized according to the following procedure:

2μg of total RNA and Oligo dT primers were used in a 20μl volume reaction. After 5min at 70^oC for the denaturation of secondary structures, 2μl 10x M-MuLV buffer, 1μl of

10mM dNTPs, 0.5μl M-MuLV Reverse Trascriptase (Finnzymes) and 1μl RNase inhibitor (HT biotechnology Ltd Human placental ribonuclease inhibitor) were added and left at 42^{o}C for 90min. The samples were then kept at -20^{o}C until use.

Real-Time PCR

An RT-PCR reaction were set up according to the iQ SUBR Green Supermix manual (BIO-RAD) with 5-10% of the synthesized cDNA, 10pmoles of each primer and 10μl 2x iQ SUBR Green Supermix, in a total volume of 20μl.

The PCR program was as follows:

50^{o}C for 2min, 95^{o}C for 10min, 40 cycles of 95^{o}C for 20sec and 60^{o}C for 1min, followed by plate read, 2min at 72^{o}C and melting curve formation from 72^{o}C to 92^{o}C with a plate read every 0.5^{o}C.

The data analysis was performed with the use of the Opticon Monitor software.

DNA FISH

Probe preparation

DNA FISH probes were constructed with the use of the Nick Translation kit (Roche Applied Science). The reaction was prepared with 2μg BAC DNA (LT/TNF locus, RP23-446-C22, BPRC, CHORI) in a final volume of 20μl according to the kit's manual.

The probe was then purified through a QIAquick PCR clean-up column following the manual procedure and stored at -20^{o}C.

Cell preparation

Glass coverslips were placed in a 24-well plate and Raw 264.7 macrophage cells were seeded on top (300-400x10^{3} cells/coverslip) and allowed to adhere for at least 2h. Cells were then stimulated with LPS (50ng/ml) for the desired time.

Cells were then washed with ice-cold 1x PBS and fixed with 4% PFA (paraformaldehyde 16% aqueous solution, EM Grade, Electron Microscopy Sciences) in 1x PBS for 12min. After 3x5min washes with 1x PBS, the cells were permeabilized with 0.5% Triton X-100 in 1x PBS for 10min and incubated in 20% glycerol in 1x PBS. Three freeze-thaws in liquid nitrogen follow and the cells were washed in 1xPBS and incubated in fresh 0,1N

HCl for 5min. The cells were finally rinsed in 2xSSC (for 20xSSC: 3M $NaCl_2$, 0,3M Sodium Pyruvate) and stored in 70% ethanol at 4^{o}C.

Hybridization

Probe: 100ng from each probe and 1μg mouse Cot-1 DNA (Invitrogen) were placed in a dark eppendorf tube and lyoplilized. The pellet was resuspended in 5μl prewarmed de-ionized formamide (Molecular biology grade, Promega) and reconstituted for 30min at 37^{o}C. DNA was then denatured for 10min at 95^{o}C and the probes were kept on ice until used for hybridization.

Cells: The cells were dehydrated with 4 consecutive washes of increasing ethanol concentration (70%, 80%, 95%, 100% - 3min each). The coverslips were dried on a heating block and then placed in a plate with fresh denaturation buffer (70% de-ionized formamide, 2x SSC pH 5.3, final pH7, pre-warmed at 73^{o}C) and incubated at 73^{o}C for 5min. The cells were then incubated in ice-cold 70% ethanol for 3min on ice and washed again in 80%, 95% and 100% ethanol for 3min.

5μl hybridization buffer (4xSSC, 20% Dextran sulfate, 50mM Sodium Phosphate) were added in the probe mix. The probe was then placed on a glass microscope slide (Marienfeld) and the coverslip with the cells was flipped on top and sealed with paper cement. When the paper cement dried (15-30min at room temperature) the slide was placed on a block heated at 73^{o}C for 5min and then incubated at 37^{o}C for 16h (overnight) in a humidified hybridization chamber.

The next day the coverslips were removed from the slide and placed in a 12-well plate where they were washed once with 2xSSC and once with 2xSSC pre-warmed at 37^{o}C, for 5min. When needed, the nucleus was counterstained with ToPro3 (1:8000 dilution from 1mM stock, in 2xSSC) for 1min at room temperature and the cells were washed with 2xSSC for 3min. The coverslips were rinsed in 1xPBS, allowed to air-dry and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen).

After at least 16h, the cells were visualized with the use of Confocal microscopy and the analysis and measurement of allele distances was performed with the use of the Volocity software (v.5.2.1 Build 0 by Improvision).

RNA FISH

Probe preparation

RNA FISH probes were constructed following the same procedure as for DNA FISH probes with the use of cDNA from the desired gene.

Cell preparation

For RNA FISH after the cells were washed with ice-cold 1x PBS, they were incubated in CSK buffer (Cytoskeletal buffer: 100mM NaCl, 300mM Sucrose, 3mM $MgCl_2$, 10mM PIPES, 0.5% Triton X-100, 1mM EGTA, 2mM Vanadyl Ribonucleoside) for 5min on ice. Then they were fixed with 4% PFA in 1x PBS for 10min, washed 3x5min with 70% ethanol and stored at -20^{o}C.

Hybridization

Probe: 100ng from each cDNA probe and 1μg mouse Cot-1 DNA (Invitrogen) along with 20μg ytRNA (Ribonoucleic acid, transfer, Backers, SIGMA) were placed in a dark eppendorf tube and lyoplilized. The pellet was resuspended in 5μl prewarmed de-ionized formamide and reconstituted for 20min at 37^{o}C. DNA was then denatured for 10min at 95^{o}C and the probes were kept on ice until used for hybridization.

Cells: The cells were dehydrated with 4 consecutive washes of increasing ethanol concentration (70%, 80%, 95%, 100% - 3min each).

5μl 2x fresh hybridization mix (40μl 20xSSC, 60μl DEPC-treated H2O, 20μl VRC, 80μl Dextran sulphate 50%) were added in the probe. The probe was then placed on a glass microscope slide (Marienfeld) and the coverslip with the cells was put on top and sealed with paper cement. When the paper cement dried (15-30min at room temperature) the slide was placed on a block heated at 73^{o}C for 5min and then incubated at 37^{o}C for 16h (overnight) in a humidified hybridization chamber.

The next day the coverslips were removed from the slide and placed in a 12-well plate where they were washed once with 2xSSC and once with 2xSSC pre-warmed at 37^{o}C, for 5min. When needed, the nucleus was counterstained with ToPro3 (1:8000 dilution from 1mM stock, in 2xSSC) for 1min at room temperature and the cells were washed with 2xSSC for 5min. The coverslips were allowed to air-dry and mounted in ProLong Gold antifade reagent with DAPI (Invitrogen).

Immuno:DNA FISH

The probes were prepared as for DNA FISH and cells as for RNA FISH. The hybridization followed the RNA FISH protocol.

The second day of the hybridization procedure, the coverslips were removed from the slide and placed in a 12-well plate where they were washed consecutively with 2x SSC for 5min, 2x SSC pre-warmed at 42^{o}C for 5min and 2x SSC for 5min at room temperature. The cells were then incubated with blocking buffer (200µl 20x SSC, 10µl 10% Tween, 200µl BSA) for 30min in a hybridization chamber pre-warmed at 37^{o}C. Then followed incubation with the primary antibody (1:500 in detection buffer: 200µl 20x SSC, 10µl 10% Tween, 50µl BSA) in a humidified hybridization chamber and after 1h the cells were washed 3x2min with 0.1% Tween-20 in 4x SSC, pre-warmed at 37^{o}C. After incubation with the secondary fluorescent antibody (1:500 in detection buffer) for 30min in a humidified hybridization chamber, the cells were washed again 2x2min with 0.1% Tween-20 in 4x SSC, pre-warmed at 37^{o}C, counterstained with ToPro3 in 2xSSC for 1min and finally washed in 2xSSC for 3min. The coverslips were rinsed in 1xPBS, allowed to air-dry and mounted in ProLong Gold antifade reagent with DAPI.

Nuclear protein extracts

Nuclear extracts were prepared from Raw 264.7 murine macrophage cells that had previously been stimulated with 50ng/ml LPS.

Cells were harvested by scraping, centrifuged at 1200rpm for 5min, washed twice in ice-cold 1xPBS and the pellet was resuspended in 5 vol. Buffer A (10mM Hepes pH7.9, 1.5mM $MgCl_2$, 10mM KCl, 5mM NaF, 1mM DTT, 1mM PMSF) and left on ice for 10min. 0.1% NP-40 was added and after <5min on ice, the cells were vortexed for no more than 10sec and centrifuged at 4000rpm, for 1,5min at 4^{o}C. The pellet (nuclei) was washed twice with Buffer A and after resuspension by hand Buffer C (20mM Hepes pH7.9, 25% Glycerol, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 1mM DTT, 1mM PMSF, 5mM NaF) was added while flicking. The extracted nuclei were left on ice for 30min and then centrifuged at 14000rpm for 15min at 4^{o}C.

For DNA affinity chromatography, the extracts were also dialyzed against at least 50 volumes of the DNA binding buffer with stirring for 2h at 4^{o}C.

The samples were kept at -80 ^{o}C until use.

Electromobility Shift Assay (EMSA)

The 5'-3' oligonucleotide sequences that were used are as follows:

GA repeat: 5'-GAGAGAGAGAGAGAGAGAGAGAGAG-3'

GA single: 5'- GGTGGTGCATGAGAGGCCCACAGTC-3'

GA mutant: 5'- GGTGGTGCATACACAGCCCACAGTC -3'

Double-stranded oligonucleotides (Microchemistry, IMBB, FORTH) were end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP for 45min at 37^{o}C and then purified with a G-50 column.

The binding reactions were carried out in a binding buffer containing (80mM KCl, 10mM Hepes pH7.9, 5mM $MgCl_2$, 10% Glycerol, 0.1% NP-40, 50μM $ZnCl_2$, 1mM DTT, 1mM PMSF) with 3μg nuclear extracts and 60-100x10^{3} cpm radio-labelled oligonucleotide. After 20min on ice the reactions were run in a 6% acrylamide gel (39:1) at 120-150V. The gel was dried for 1h at 72^{o}C and exposed either on a film or on a PhosphoImager screen.

SouthWestern blotting (SW)

Protein samples were separated on a 10% SDS-PAGE acrylamide gel and transferred on a nitrocellulose membrane (Protran, Whatman) overnight (~16h) at 50mA (21-25V) or 1.5h at 200-300mA at 4^{o}C. The membrane is placed in a glass box with 25ml Blocking/Renaturation buffer (25mM Hepes pH7.5, 50mM KCl, 6.25mM $MgCl_2$, 10% Glycerol, 0.1% NP-40, 1mM DTT, 1mM PMSF) with rocking for 10min at room temperature and then another 25ml Blocking/Renaturation buffer were added, this time with 3% non-fat milk and was left rocking (~8h) - the buffer was changed every 2h. The membrane was then washed with 25ml Binding/Washing buffer (12.5mM Hepes pH7.5, 50mM KCl, 6.25mM $MgCl_2$, 10% Glycerol, 0.05% NP-40, 1mM DTT, 1mM PMSF) for 5min and stored at 4^{o}C until hybridization.

The membrane was sealed in a plastic bag with hybridization buffer containing ~20x10^{6} cpm radio-labelled oligonucleotide and 60μg Salmon Sperm DNA in 3ml Binding/Washing buffer and left rotating ~16h at room temperature. The next day the

membrane was washed 3-4 times with Binding/Washing buffer for 15min each and it is exposed on a film.

DNA affinity chromatography

Binding of biotinylated oligo on beads:

A 30bp biotinylated oligo was bound on washed beads (200ng/sample biotinylated oligo with 20μl/sample beads in 1x Beads-Binding buffer) for >10min according to the manufacturer's protocol (Dynabeads M-280 Streptavidin, Invitrogen).

Preclearing of Nuclear Extracts (NEs) with beads:

100μg/sample of NEs were incubated with 100ng/μl poly(dI:dC) in 1x Biotin-Binding buffer (80mM KCl, 10mM Hepes pH7.9, 5mM $MgCl_2$, 10% Glycerol, 50μM $ZnCl_2$, 0.05% NP-40,1mM DTT, 1mM PMSF, 5mM NaF) for 10min on ice. 20μl/sample beads were added and left rotating for 1h at 4^oC.

Binding of NEs to biotinylated-oligo:

Either beads-bound to oligo or beads only were added to the extracts and incubated under rotation for 20min at room temperature. The beads were washed (Washing buffer: 80mM KCl, 20mM Hepes pH7.9, 5mM $MgCl_2$, 50μM $ZnCl_2$, 0.05% NP-40, 1mM DTT, 1mM PMSF, 5mM NaF) for 3x3min at room temperature, with the first wash containing 100ng/μl poly(dI:dC).

The samples were then denatured in 1x Protein loading buffer for 10min at 95^oC, loaded on a 10% SDS-PAGE poly-acrylamide gel and run at 100V. The gel was visualized with a silver staining method.

Silver Staining

The membrane was passed through the following buffers (200ml each):

Fixing: 50% Methanol, 5% Acetic acid, 20min, RT

Washing: 50% Methanol, 10min, RT

Rinsing: ddH_2O, 10min, RT

Sensitization: 0.02% Sodium thiosulfate, 1min, RT

Rinsing: ddH_2O, 2x1min, RT

Staining: 0.1% Silver nitrate (pre-chilled), 20min, 4^oC

Rinsing: ddH_2O, 2x1min, RT

Developing: 0.04% formalin, 2% Sodium carbonate with intensive shaking, when the solution turns yellow it is removed and fresh is added

Termination: 5% Acetic acid, >5min, RT

Storage: 1% Acetic acid, 4^oC

Yeast One Hybrid Screening Library

The Matchmaker One-Hybrid System (Clontech laboratories, Inc) was used according to the manufacturer's protocol.

3. RESULTS

3.1. Expression profile of TNFα in Raw 264.7 macrophages

Quantitative Real-Time PCR was used to detect the TNFα mRNA levels in Raw 264.7 murine macrophage cells, after 50ng/ml LPS stimulation. Total RNA was extracted after stimulation of the cells with LPS for 10min, 20min, 30min, 1h, 2h, 3h, 6h, 12h and 24h. The choice of 50ng/ml LPS for the stimulation was made after a dose-dependent experiment that enabled us to decide which concentration would resemble the normal inflammation levels and still have the same results.

Reverse transcription for every time-point provided the cDNA needed for RT-PCR. TNF transcripts were normalized to Hprt1 abundance.

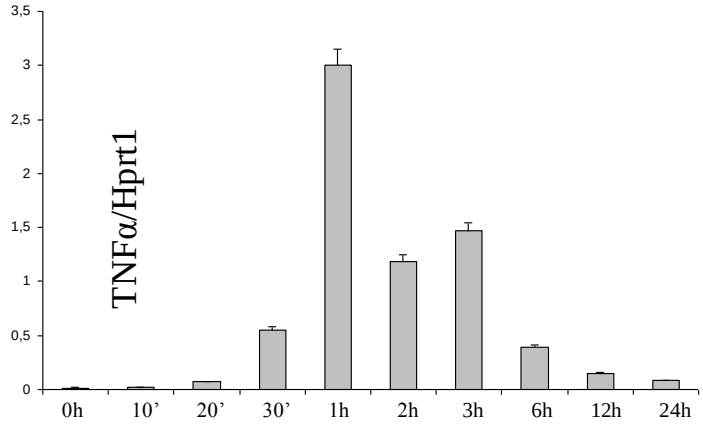


Figure 10. TNFα expression levels in LPS stimulated murine macrophages in relation to time.

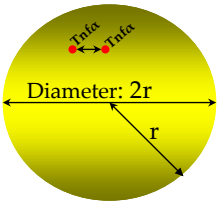
TNFα transcription initiates upon LPS stimulation, it is highly elevated after 1h and steadily declines until it reaches basal levels at 24h upon stimulation (Fig.10).

3.2. Homologous association of the TNFα alleles precedes transcriptional activation

In order to study the nuclear localization of the TNFα alleles in relation to space and to each other, DNA Fluorescence *in situ* hybridization (DNA FISH) experiments were performed. Hybridizations were performed using Raw 264.7 macrophages according to the protocol described in section 2 (Materials and Methods), which permits the

maintenance of the three-dimensional structure of the cells. The distance between the two TNFα alleles in every cell was calculated with the use of the Volocity software and all the distances were normalized to the diameter of the cell nucleus (ND: normalised distance). To avoid statistical errors, the experiments were repeated at least 10 times each and in every single experiment more than 100 cells were analysed. A distance of two signals shorter or equal to 0.6μm, in an average nucleus diameter of 6-8μm, is considered as co-localization (ND≤0.1) and it represents signals that are touching or are overlapping.

$$ND = \frac{Tnfa\text{-}Tnfa \text{ distance}}{2\sqrt{\frac{\text{nuclear area}}{\pi}}}$$



The following photographs depict an average representative image of the alleles' distance before stimulation (0h), after 30min of LPS stimulation, after 1h and 24h of stimulation (Fig.11).

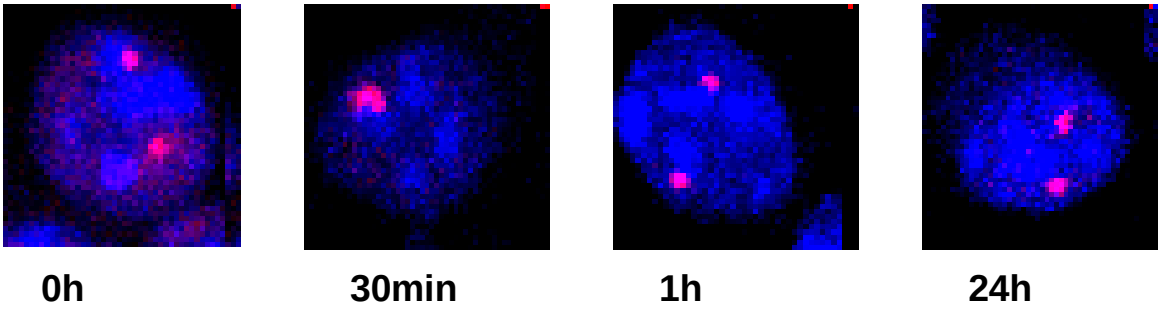


Figure 11. Confocal microscopy images with representative distances of the TNFα alleles in macrophage nuclei before, 30 min, 1h and 24h after LPS stimulation. DAPI staining of the nucleus (blue) represents the presence of DNA, LT/TNF probe labelled with Spectrum Orange is hybridized on the two TNFα alleles (red).

The distances between the two TNFα alleles were measured and normalized in more than 100 cells in each of 10 experiments and it was observed that the two alleles come in closer proximity 30min upon LPS stimulation and proximity declines again 1h following stimulation, when they were located the furthest away from each other (Fig.12). Average normalized distances (NDs) in relation to time of LPS stimulation are presented in the graph, with a representative image of a cell with the TNF alleles in the respective distance from each other. It is clear that homologous association of the alleles occured

30min upon LPS stimulation of macrophages. This co-localization is reversed 1h upon LPS stimulation and the alleles reached their furthest relative distance.

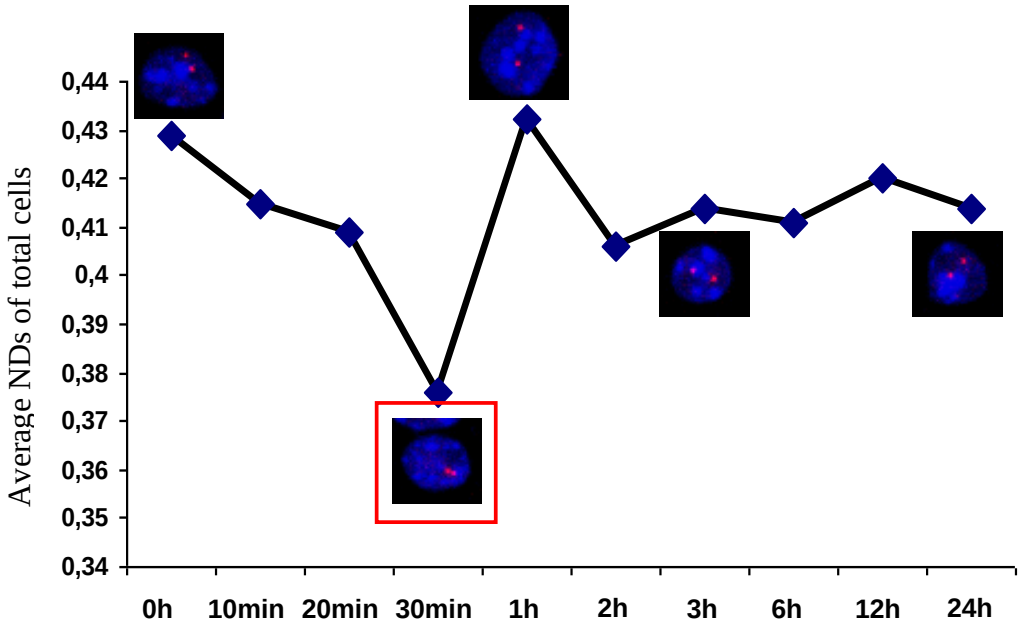


Figure 12. Time course of the homologous association of the TNFα alleles. Normalized Distances (NDs) of the total number of cells in relation to LPS stimulation time.

Again, as mentioned above the normalized distance equal or less than 0.1 (actual distance ≤0.6μm) was considered as co-localization. By separating and putting together all the measurements from the cells with an ND score ≤0.1, we plotted the graph that follows (Fig.13) which corroborates the observed phenomenon of the homologous pairing of the TNFα alleles 30min upon LPS stimulation of the macrophage cells.

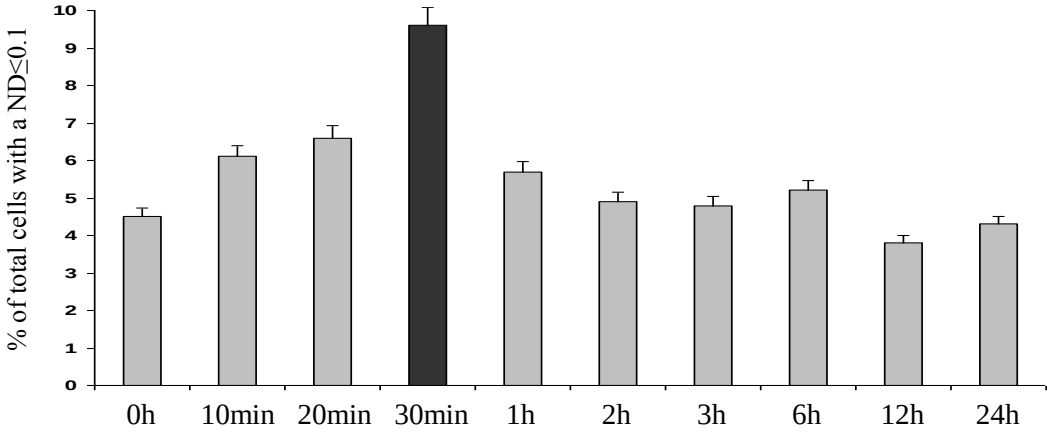


Figure 13. Percentages of co-localization of the TNFα alleles in respect to time in Raw 264.7 macrophages.

In order to examine if the homologous association of the TNFα alleles had any functional significance in the gene regulation of the locus, we analysed the pattern of expression at the single-cell level, with the simultaneous detection of both the newly-produced mRNA transcript and the DNA of the two alleles. To this end, we used RNA:DNA FISH experiments to visualize the nascent mRNA transcripts along with the two DNA loci, in relation to time, upon LPS stimulation of the cells. Figure 14 shows representative confocal microscopy photographs of the nascent mRNA transcript of TNFα and the two alleles, with the use of RNA:DNA FISH.

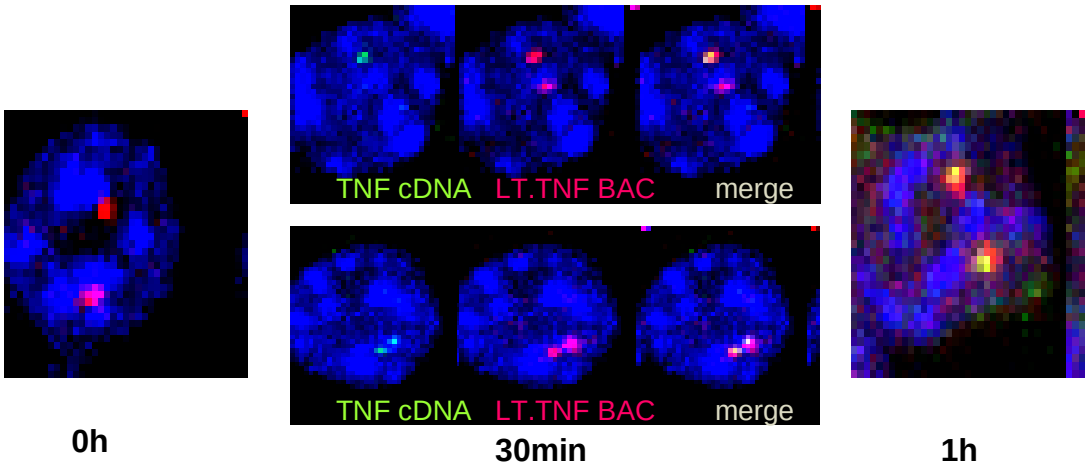


Figure 14. Confocal microscopy images with representative distances of the TNFα alleles, along with the nascent mRNA transcript in macrophage nuclei before, 30min and 1h upon LPS stimulation. DAPI staining of the nucleus (blue) represents the presence of DNA, LT/TNF probe labelled with Sp. Orange was hybridized on the two TNFα alleles (red) and TNFα mRNA transcripts were hybridized with Sp. Green - labelled probes (green).

The percentage of the cells expressing TNFα at the various LPS stimulation time-points (Fig.15), confirm the RT-PCR results and place the highest levels of TNF transcription at 1h upon LPS stimulation of the cells.

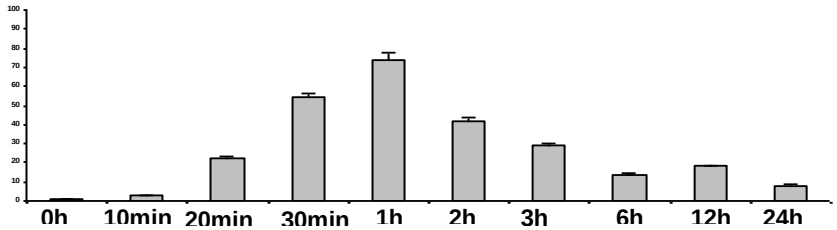


Figure 15. Percentage of total expressing cells in respect to time in Raw 264.7 macrophages upon LPS stimulation.

3.3. Allelic pattern of TNF expression

The advantage of the FISH technique, over the quantitative Real-Time PCR, which allows us to examine massively a population of cells, is that it permits the study of the allelic pattern of expression, that is, we are able to differentiate between the transcription from one or both alleles.

The examination of the allelic expression pattern showed a mono-allelic pattern of expression, during the first minutes of stimulation, a change to bi-allelic around 1h upon stimulation and the final turn to mono-allelic transcription of TNFα during the last hours of expression (Fig.16).

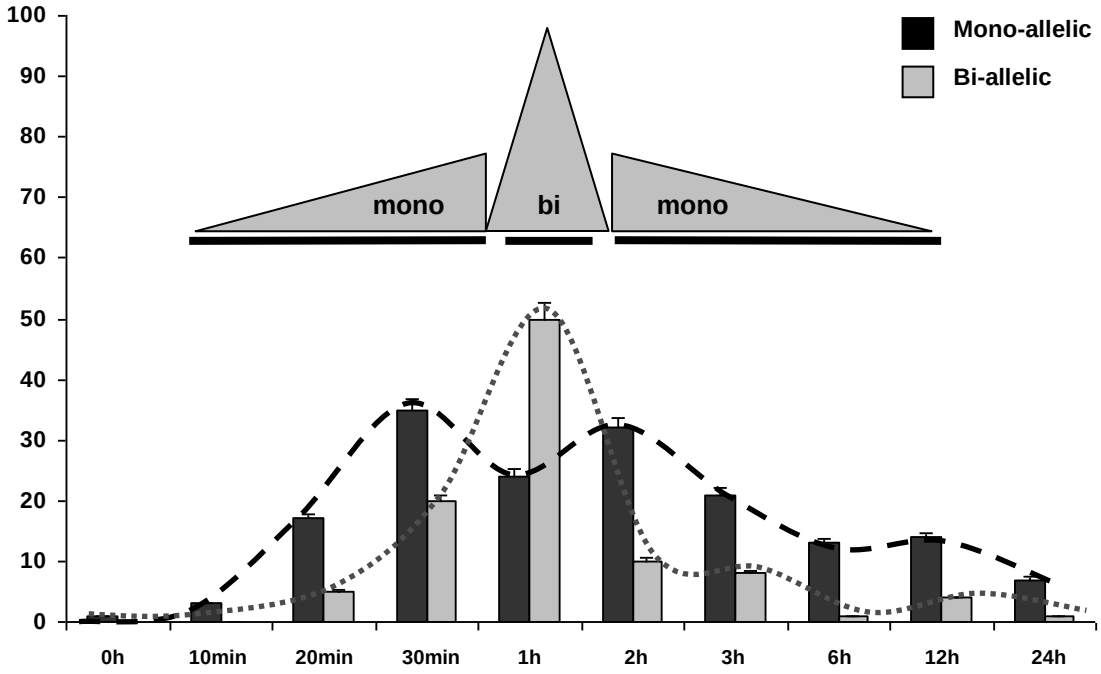


Figure 16. Allelic pattern of expression in Raw 264.7 macrophages after LPS stimulation.

Taking into account the results from all the FISH experiments we can conclude that somatic homologous pairing takes place between the two *TNFα* alleles and that this inter-chromosomal association precedes in time the transcriptional activation of the *TNFα* locus. In addition, the specific allelic pattern of TNFα expression changes exactly after the interaction. Specifically, the change from mono- to bi-allelic expression of TNFα

coincided with the highest expression levels of TNFα and followed in time the homologous association of the alleles.

3.4. A GA-specific binding factor as a putative mammalian GAGA factor

In order to fully understand the homologous pairing phenomenon so far identified between the TNFα alleles, we wanted to identify and characterize the protein complexes that mediate this inter-chromosomal interaction in macrophages.

Keeping in mind that the GAGA factor in Drosophila melanogaster regulates such inter-chromosomal interactions and that so far the mammalian homologue has not been identified, we checked if there were any $(GA)_n$ repeats within the LT/TNF locus. We selected these specific stretches to synthesize 25bp oligonucleotides which were P^{32} end-labelled with T4 polynucleotide kinase. Electromobility shift assays (Fig.17) with nuclear extracts from LPS-stimulated Raw 264.7 macrophages showed that there is indeed at least one GA-specific binding factor, which also seems to be LPS induced.

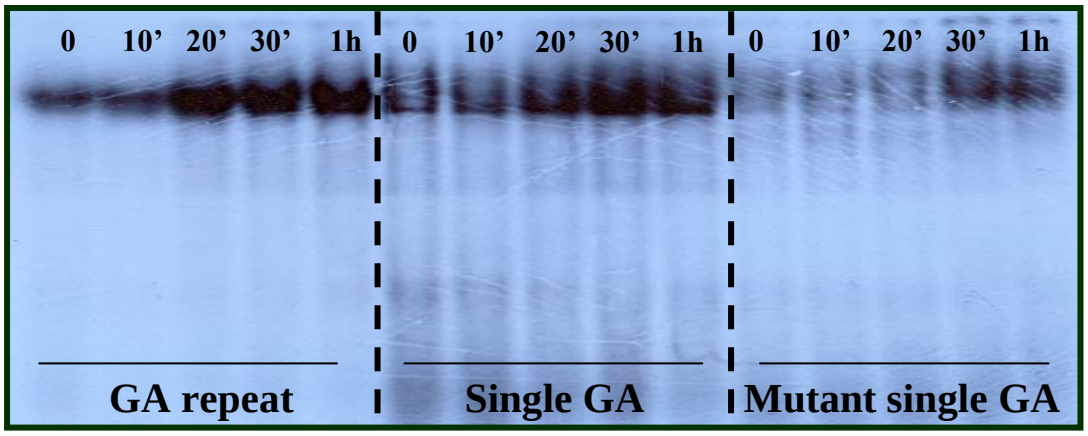


Figure 17. EMSA of P^{32}-labelled oligonucleotides and Raw 264.7 nuclear extracts before, 10min, 20min, 30min and 1h after LPS stimulation. First column: GA repeat 25bp oligonucleotide, second column: single GA binding site (GAGAG) chosen from within the TNFα promoter, third column: same sequence as "single GA" with the GAGAG site mutated to ACACA.

More specifically, we could observe a protein complex that was induced by LPS over time and the band shift was increased at the time when the homologous association occurs (20-30min). The gel shift was increased with the "GA-repeat" oligonucleotide, which contained multiple binding sites, it was sharper with the "single GA" binding site and disappeared with the mutated "mutant single GA" binding site.

Gel shifts can only inform us about the existence of a certain factor and only about its relative quantity between samples. The accurate molecular weight can be deduced with the use of a SouthWestern blot which separates the nuclear proteins by size in a poly-acrylamide gel and then the hybridization with the DNA probe on a nitrocellulose permits the visualization of the GA-binding proteins. The same LPS stimulation time-points were used for the preparation of nuclear extracts, as for the EMSA and the hybridization was performed with the multiple binding site "GA-repeat" 25bp oligonucleotide.

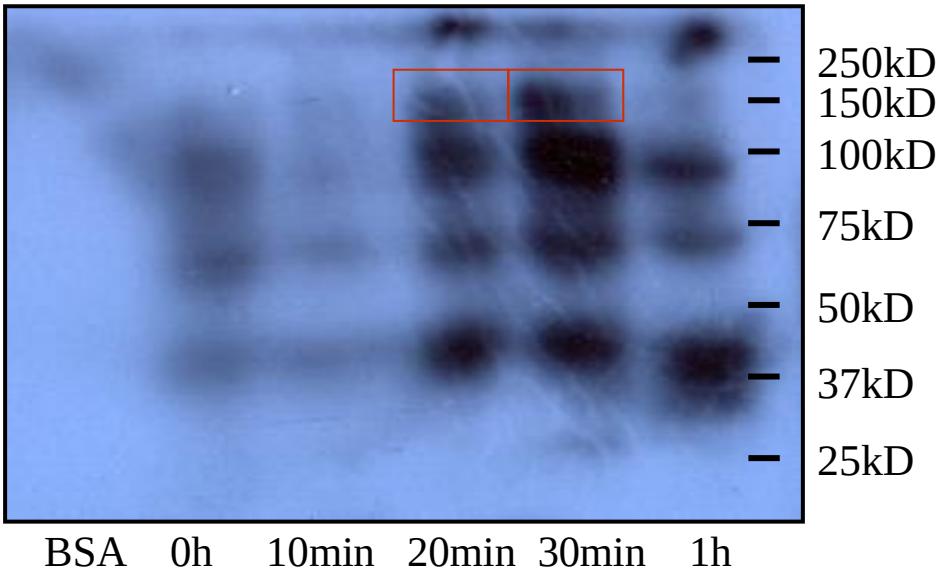


Figure 18. SouthWestern blot of Raw 264.7 nuclear protein extracts, hybridized with the "GA-repeat" 25bp oligonucleotide.

The multiple bands visible can be accounted for by either multiple GA binding proteins, or by slight degradation of the sample. In any case, we can distinguish 2 bands (square-marked in Fig.18) approximately 150kD, which were induced at 20-30min upon LPS stimulation and disappeared at 1h upon stimulation. This pattern would correspond to a factor that was induced to mediate the homologous association phenomenon (at 30min upon LPS stimulation) and would be removed/degraded after the completion of the pairing (1h upon LPS stimulation).

It is clear, however, that for the complete identification of any factor, we would need to incorporate mass spectrometric data in the analysis. To this end, we utilized DNA affinity chromatography for which 30bp synthetic DNA oligonucleotides were biotinylated and

immobilized on magnetic streptavidin beads and used to isolate proteins from LPS-stimulated macrophages.

More specifically, nuclear protein extracts from un-stimulated and 30min LPS-stimulated Raw 264.7 macrophage cells were prepared. We selected the 30min LPS stimulation of cells for the preparation of nuclear extracts, since it was the time when the homologous pairing appeared. The extracts were firstly pre-cleared with the use of a CA-repeat oligonucleotide which bound all the non-specific DNA binding proteins. Then, binding reactions were carried out between the extracts and the bead-immobilized biotinylated oligonucleotides (GA-repeat sequence). The bound proteins were denatured and separated on a 10% poly-acrylamide gel which was silver-stained for visualization (Fig.19).

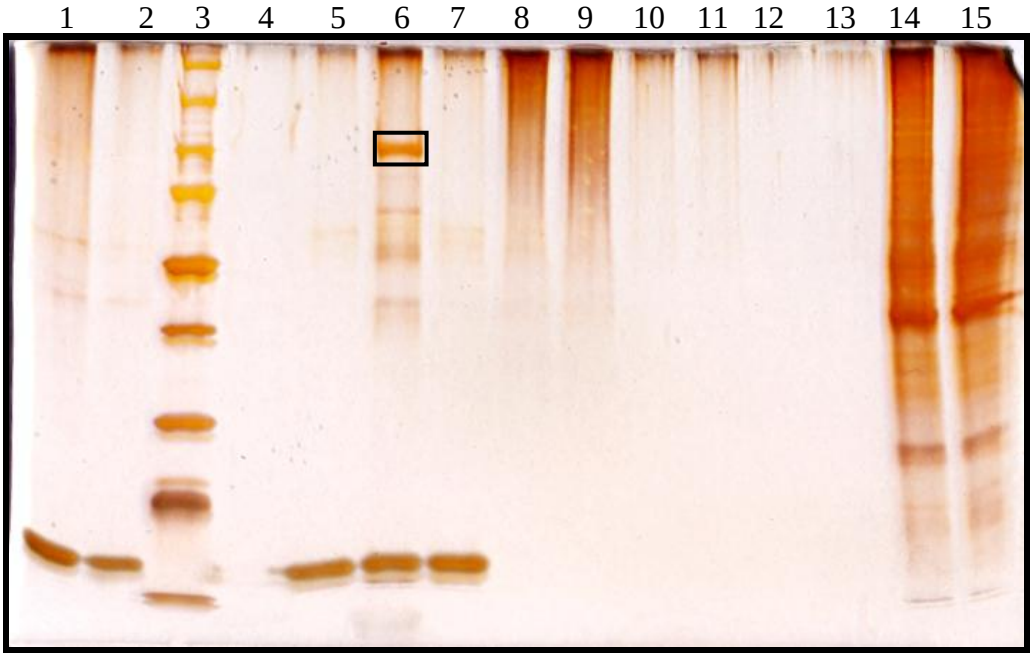


Figure 19. DNA affinity chromatography with a GA-repeat oligonucleotide. Lanes 1, 2: CA-bound beads used for pre-clearing, lane 3: protein marker, lane 4: proteins bound to GA-repeat (un-stimulated cells), lane 5: CA-control (un-stimulated cells), lane 6: proteins bound to GA-repeat (30min LPS stimulation), lane 7:CA-control (30min LPS stimulation), lanes 8, 10, 12: washes 1, 2, 3 from GA-bound beads (un-stimulated cells), lanes 9, 11, 13: washes 1, 2, 3 from GA-bound beads (30min LPS stimulation), lanes 14, 15: flow through from beads with un-stimulated and 30min LPS stimulation respectively.

The experiment was repeated several times and bands, such as the one square-marked in Figure 19, were excised and were analysed with Mass Spectrometry. However, the results were inconclusive since both the starting quantities of nuclear extracts were not enough for a definite identification and the protocol of isolation did not account for the fact that

many DNA-end/nick-binding proteins were selected in abundance and co-isolated with sequence-specific proteins. DNA-dependent protein kinase, Ku proteins, splicing factors, DNA helicase, and so on are known to bind non-specifically to free DNA ends, thus PARP-1 [Poly (ADP-ribose) polymerase 1] an enzyme associated with DNA repair that binds to single-strand DNA breaks, was identified as isolated with the specific protocol.

Another approach that was used in parallel as an *in vivo* assay to verify the results was a Yeast One Hybrid Screening. A 30bp GA-repeat DNA oligonucleotide was cloned in a plasmid vector which was used to transform a macrophage-derived cDNA expression library under stringent growth conditions. A clone was considered positive if it had the ability to express three selection markers, one of which comes from the successful interaction of an expressed protein with the DNA element cloned (Fig.20).

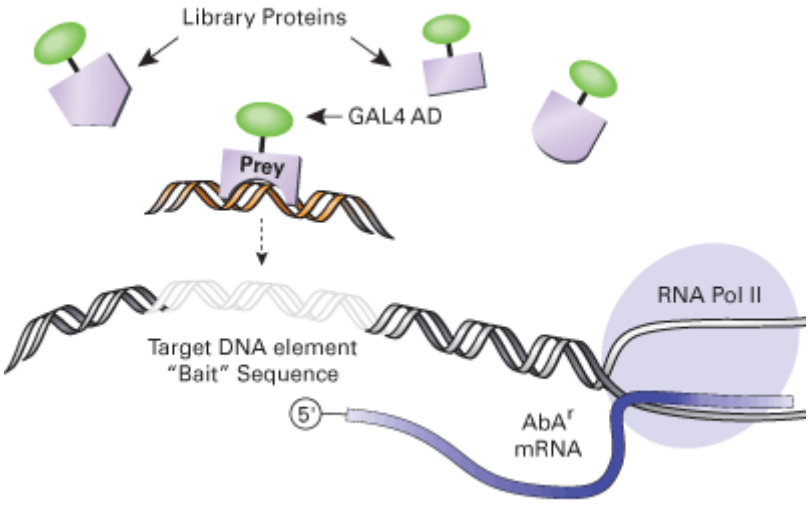


Figure 20. Yeast One Hybrid Screening library.

Most of the results were inconclusive as leaky expression of one of the selection markers (histidine) could not be completely avoided, even with the use of 3-AT (limits histidine biosynthesis to reduce leaky expression). The library screen was repeated twice and after rescue of the prey plasmid sequencing reactions were prepared.

A protein that was identified by both strategies was Pyruvate kinase isozyme M2, an enzyme involved mainly in glycolysis, but which was recently found to translocate into the nucleus along with STAT3-inhibitor, PIAS3[77-80] and to form homotetramers.

4. DISCUSSION

Inter-chromosomal interactions occur in many organisms and recent evidence has suggested an important role for the *in trans* regulation of gene expression. Among several examples of trans-interactions in mammals, homologous pairing of the two TNFα alleles has yet to be elucidated. The study of this phenomenon will shed light on the role of inter-chromosomal interactions in gene expression and more specifically in the mechanism of allele choice of expression and its relation to the innate immune response. Moreover, the identification of the protein complexes that mediate these interactions will provide information for a new mechanistic model of gene regulation and may lead to the development of molecules designed to assist in cases of deregulated gene expression in the immune system.

The main objective of this project was to apply a series of molecular, biochemical, imaging and genetic approaches in order to understand the homologous pairing phenomenon, identified between the TNFα alleles, and to try to characterise the protein complexes that mediate this inter-chromosomal interaction in macrophages.

Here we present a phenomenon of homologous association between the alleles of TNFα. In regard to time, the homologous pairing precedes the transcriptional activation and TNFα expression. In addition, LPS stimulation of macrophages changed the allelic expression of TNFα over time; from mono-allelic expression it turned to bi-allelic at the time-point of highest transcriptional activation.

There are several issues that still need to be addressed in the future, though. More specifically, specific genomic fragments that interact within the locus need to be identified and used to isolate binding proteins and other possible genomic partners. In addition, the protocols of protein isolation and DNA affinity chromatography need to be improved so that non-specific binding factors will be excluded from the isolation. The pathway of macrophage activation and TNFα expression needs to be studied in more detail so that certain aspects of the signalling pathway that may lead to the phenomenon of homologous association will be elucidated. For example, neutralizing antibodies for TNFα will be used to assess whether the early wave of TNF expression, upon LPS

stimulation, activates the homologous pairing, using a feedback loop of the secreted TNFα through the TNF receptor signalling pathway, or it is the LPS pathway itself that stimulates both phases of expression. Moreover, RNAi approaches will be used to evaluate the role of NF-κB in these interactions.

In addition, cohesin, a DNA-binding protein complex essential for the assembly and segregation of sister chromatids during meiosis and the repair of damaged DNA[81], has also been implicated in the regulation of gene expression[82-84]. The mechanistic basis of this function is still very poorly understood. Evidence, however, points to the cooperation of cohesin with CTCF[85-87], a DNA-binding factor that either activates or represses transcription in several studied loci. This link between CTCF and cohesin, although so far considered for its contribution of chromatin loops and gene regulation *in cis*, is tempting us to speculate that cohesin may also be involved in the long-range interactions between genomic loci on different chromosomes, observed during coordinated transcriptional events. The functional experiments that will be performed for the evaluation of the role of newly identified protein complexes could be used to study the role of cohesin proteins and CTCF in the regulation of gene expression in the TNFα-macrophage model.

Lastly, the same approaches will be followed in other macrophage cell-lines (e.g. human THP-1) to assess the cross-species conservation of the phenomenon.

We should keep in mind that DNA is not randomly distributed within the nuclear envelope. The nucleus includes several sub-compartments (nucleolus, speckles, transcription factories, PML bodies, chromosomal territories, Cajal bodies, etc), in any of which inter-chromosomal interactions could take place. Besides transcription, genomic organization is correlated with the coordination of replication and recombination of DNA, based on which it is widely accepted that the architectural organization of DNA in the nucleus is closely associated with genomic function. It is thus imperative to study the expression profile of TNFα in conjunction with the homologous pairing profile and the subnuclear localization of this interaction.

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