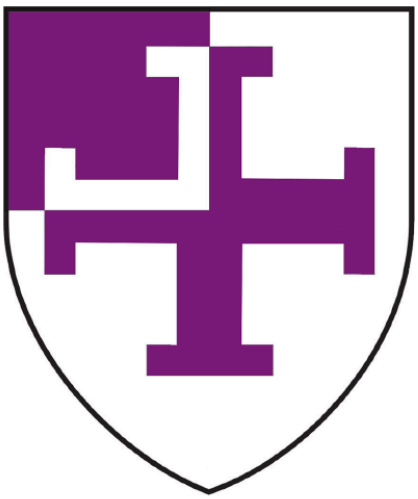
The role of IRAK3 in regulating immune-mediated inflammatory arthritis



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Thesis submitted for D.Phil in Musculoskeletal Science

Michaelmas Term 2018

Abstract

A repeated or prolonged stimulation with LPS induces in macrophage-lineage cells a refractory state known as endotoxin tolerance (ET). ET curtails inflammation and prevents tissue damage caused by sustained immune activation in sepsis. TNFα is required for ET induction and it is hypothesized that TNFα exerts its immuno-modulatory action via ET-associated molecules. In models of TNFα-driven disease, ET-associated molecules were investigated to determine their role in the progression from acute, self-limiting inflammation to chronic inflammatory disease. An *in vitro* model of ET was developed to evaluate the gene expression profile of ET macrophages. The ET-associated genes *IRAK3*, *TNFAIP3* and *PTPN6* were further investigated in collagen-induced arthritis (CIA). Although arthritic paws had increased macrophage-lineage cell infiltration and high expression of TLRs and TNFα, the expression of ET-associated genes was not uniformly increased. *Tnfaip3* and *Ptpn6* were up-regulated in arthritic paws but *Irak3*, a key molecule in ET which inhibits the MyD88 pathway, was expressed at a lower level than the naïve control throughout the inflammatory process. CIA was performed in IRAK3$^{-/-}$ mice and WT controls. In IRAK3$^{-/-}$ mice the disease was significantly accelerated, inflammatory gene expression was higher in paws, plasma IL1β concentration was higher and there was a decrease in the prevalence of regulatory T cells in spleen and lymph-nodes. We hypothesize that an alteration in *IRAK3* gene expression or activity potentiates the development and chronicity of inflammatory disease.

Declaration

This is to certify that

(i). the thesis comprises only my original work towards the D.Phil,

(ii). due acknowledgement has been made in the text to all other material used,

The work was carried out at the Kennedy Institute of Rheumatology, NDORMS, University of Oxford.

The research was funded by a BBSRC CASE Studentship.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisors Prof Richard O. Williams and Dr Felix I. Clanchy. Prof Williams has always been a calming reassuring presence, even in difficult moments and I really thank him for all his support. Dr Clanchy has taught me all I know in the laboratory and I really thank him for his patience, motivation, and immense knowledge. Their guidance helped me all the time of research and writing of this thesis.

I would also like to extend my gratitude to Dr John Silva, and UCB Celltech (Slough, UK) for facilitating and supporting this studentship.

I would like to thank the whole of my group as well. This has been a very long journey and it would not have been possible without all of you.

Dr Ida Parisi provided me with her huge histology experience and I really thank her for her motivation and for the emotional support.

Dr Wen Yi Tseng and Dr I-Shu Huang worked with me often side by side and helped me with encouragement and valuable comments.

Dr Joanna Kawalkowska was, and still is, always available for thoughtful insights and emotional support. She has always been there for me and I am grateful of her friendship.

Dr Kay McNamee, who provided me with all her experience and patience, helping me with valuable comments and suggestions.

Dr Joy Ogbechi, we met at the very end but nonetheless she helped with all her experience and insightful comments.

I would like also thank the Biotechnology and Biological Sciences Research Council (BBSRC) for providing the funding who allowed me to undertake this research.

My husband Andrea Guerra, who has probably done what most husbands would not. He provided me with the time to fully dedicate myself to such a demanding task like completing a DPhil in Oxford. I am so grateful for that.

My daughter Gaia who made it slightly more challenging but who also kept me grounded and taught me to not give up to anxiety.

Last but not least, my parents, who raised me to endure. It was a tough school but it was worth it.

Publications

The research presented here will be published or presented in the following forms.

Journal articles

Characterisation of endotoxin tolerance-associated molecules in rheumatoid arthritis.

In preparation.

Interleukin 1 Receptor Associated Kinase 3: a key molecule in inflammatory disease.

In preparation.

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Figures and Tables

List of abbreviations

AHR	aryl hydrocarbon receptor
AP-1	Activator protein 1-transcription factor
APC	Antigen presenting cell
BCL2	B-cell lymphoma 2
BCL-X	B-cell leukemia x
bp	Base pair
CCL-	CC chemokine
CCR-	CC chemokine receptor
CD-	Cluster of differentiation
cDNA	complimentary DNA
CEBPA	CCAAT/enhancer-binding protein alpha
CEBPD	CCAAT/enhancer-binding protein delta
COPD	Chronic obstructive pulmonary disease
CREB	cAMP response element-binding protein
CSF1	see M-CSF
CSF2	see GM-CSF
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
CXCL-	CXC chemokine
CXCR-	CXC chemokine receptor
DAMPs	Disease associated molecular patterns
DAS28	Disease activity score 28
DC	Dendritic cells
DD	death domain

DMARDs	Disease modifying anti-rheumatic drugs
DNA	Deoxyribonucleic acid
EC50	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ET	Endotoxin tolerance
EULAR	European league against rheumatism
FBG	Fibrinogen
Foxp3	Fork-head box P3- Regulatory T cell transcription factor
GATA3	Th2 transcription factor
GM-CSF	Granulocyte macrophage colony stimulating factor
Het	heterozygous
HIF1	Hypoxia inducible factor
HLA	Human Leukocyte Antigen
HMGB1	High mobility group box 1 protein
HSP	Heat shock protein
ICC	Circulating immuno-complex
IDO1	Indoleamine-pyrrole 2,3-dioxygenase
IFNγ	Interferon g
IL-	Interleukin
INPP5D	Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1
IRAK	Interleukin 1 receptor associated kinase
IRF	Interferon regulatory factor
KC/GRO	CXC chemokine also known as CXCL1
KO	Knocked out

LPS	Lipopolysaccharide
LRP2	Low density lipoprotein-related protein 2
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
M-CSF	macrophage colony stimulating factor
MD2	Lymphocyte antigen 96
MHCII	Major histocompatibility complex
miRNA146a	microRNA
MMPs	Metallo-proteinases
MyD88	Myeloid differentiation factor 88
NEMO	NF-kappa-B essential modulator also known as inhibitor of nuclear factor kappa-B kinase subunit gamma (IKK-γ)
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NOS2A	Nitric oxide synthase 2A
Pam3CSK4	Synthetic triacylated lipoprotein - TLR1/2 ligand
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PMNs	Polymorphonucleates
PSORS1C1	psoriasis susceptibility 1 candidate 1
PTPN2	protein tyrosine phosphatase non receptor type 2
PTPN6	Tyrosine-protein phosphatase non-receptor type 6
PU-1	tissue-specific transcription factor expressed in cells of the hematopoietic lineage
qPCR	quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa-B

RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acid
RORgt	RAR related orphan receptor gamma
SIGIRR	Single Ig IL-1-related receptor
SMAD	Family of signal transducers for TGFβ receptors superfamily
SNPs in	Single nucleotide polymorphism
STAT4	Signal transducer and activator of transcription 4
TAB2	TAK1-Binding Protein 2
TAK1	Transforming growth factor β-activated kinase 1
TAMs	Tumour associated macrophages
TBET	transcription factor specific of Th1 cells encoded by TBX21
TBX21	T box transcription factor TBX21
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th1	T helper cell 1
Th2	T helper cell 2
THP1	human acute monocytic leukaemia cell line
TIP	Thioglycolate induced peritonitis
TIR domain	Toll interleukin 1 receptor domain
TIRAP	TIR Domain Containing Adaptor Protein
TLR	Toll like receptor
TNFAIP3	Tumour necrosis factor α induced protein 3
TNFRSF1A	Tumour necrosis factor receptor superfamily member 1A
TNFRSF2B	Tumour necrosis factor receptor superfamily member 1B
TRAF6	Tumour necrosis factor receptor-associated factor 6

TRAM	TRIF-related adaptor molecule
Tregs	Regulatory T cells
TREM1	Triggering receptor expressed on myeloid cells 1
TRIF	TIR domain-containing adaptor protein inducing interferon
VEGF	Vascular endothelial growth factor

Chapter 1: Introduction

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1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease that without adequate treatment is characterized by progressive destruction of the joints [1]. RA affects 0.5-1% of adults in developed countries and is 3 times more frequent in women than men [2]. Incidence rises with age and is highest in women older than 65 years [3]. Considerable variation exists between ethnicities with a prevalence of 0.3-1.1% in populations of European ancestry and increased prevalence reported in native American populations (5-7%)[4].

1.1.1 Genetic factors

Although the precise aetiopathogenesis of the disease remains unknown, there is evidence to suggest that a genetically susceptible individual becomes immunological aware of an arthritogenic antigen, resulting in a breakdown of immunologically self-tolerance and a chronic inflammatory reaction. Several genes associated with increased susceptibility to RA have been identified. In particular, HLA-DR alleles (HLA-DRB1*0401, *0404, *0405, *0408, *0101, *0102, *1001 and *1402) have been strongly associated with higher risk of developing the disease especially in the European population [5-8]. HLA-DR, together with HLA-DP and HLA-DQ, are highly polymorphic genes located on the chromosome 6 in humans and chromosome 17 in mice, which encode the α and β chains of MHC class II molecules. The MHCII are trans-membrane glycoproteins expressed on the surface of APCs and present antigens to $CD4^+$ cells, directly interacting with their T cell receptor (TCR). Although, the different branches of the immune system participate in the development and perpetuation of the disease, RA is mainly a T cell-driven disease, hence the major role played by MHCII in its pathogenesis [10].

These genes alone are not sufficient in inducing the disease as indicated by the low level of concordance (ranging between 12 and 15% in different studies) on monozygotic twins [9,11].

1.1.2 Role of SNPs

While the allele is a variant form of a gene, a single-nucleotide polymorphism (SNP) represent a single base variation in a sequence of DNA [9]. In a recent study, Ciccaci C. et al. demonstrated the different role of SNPs in STAT4, IL10, PSORS1C1, PTPN2 and microRNA146a in the development of the disease [12]. Patients bearing specific SNPs in STAT4 showed an increased risk in developing RA, while a specific SNP in IL10 gene had a protective effect.

SNPs in PSORS1C1 (psoriasis susceptibility 1 candidate 1) and PTPN2 (protein tyrosine phosphatase, non-receptor type 2) were associated with an increased severity of the disease with high number of bone erosions, while a SNP in microRNA146a correlated with the presence of rheumatoid factor in the serum. SNPs in different members of the toll-like receptor (TLR) family of proteins have been associated with severity of disease and the response to anti-TNFα [13-16]. The role of tumour necrosis factor alpha induced protein 3 (TNFAIP3) in RA development appears to be more complex as some SNPs in this gene have been associated with an increased risk of developing RA [17] while others have a protective action[18].

1.1.3 Environmental factors

Smoking, hormonal factors, microbiota composition, and the presence of periodontitis are factors that play a role in the development of the disease. Smoking is one of the main environmental factors that increases the risk of developing RA, and the putative mechanism is the relative increase in the

oxidative stress and circulating levels of pro-inflammatory mediator [19] compared to non-smokers. The risk of developing RA is more strongly associated with the duration of the smoking habit rather than with its intensity [20]; smoking is associated with an increase frequency of periodontitis that often precedes the development of RA [21].

An etiological agent of periodontitis is *Porphyromonas gingivalis*, a gram-negative anaerobic bacterium producing peptidyl-arginine deiminase (PAD) that catalyses the citrullination of arginine residues. This could trigger the production of anti-citrullinated protein/peptide antibodies (ACPA) able to cross-react with citrullinated peptides present in the inflamed joint [27].

The gut microbiota is an emerging area of research for environmental triggers for RA development; differences between RA patients and controls in microbial diversity have been observed and are associated with the development of autoimmunity [23]. Sex hormones influence antigen presentation, homing of lymphocytes to various organs and the Th1/Th2 balance [24]. Female hormones levels after menopause increase the risk of developing RA while oral contraceptives and breast-feeding represent protective factors [25, 26].

1.1.4 Development of RA

The arthritic joint develops a non-suppurative proliferative and inflammatory synovitis with cellular infiltrate in the intra-articular space, degradation of the articular cartilage, and ankyloses. The development and persistence of inflammation requires the involvement of different cell lineages: resident cells (fibroblasts-like and macrophage-like synoviocytes), cells of the innate immune

system (dendritic cells, macrophages, polymorphonuclear cells, natural killer (NK) cells, mast cells) and adaptive immune cells (B and T lymphocytes).

Macrophages and macrophage-like synoviocytes produce pro-inflammatory cytokines (IL1β, TNFα, IL6, IL23, IL12, PGE2, GM-CSF and TGFβ1) that, upon activating the endothelial cells, facilitate further transmigration of inflammatory cells. This accumulation of cells is associated with a characteristic imbalance between proliferation and apoptosis due in part to increased expression of anti-apoptotic genes (e.g. BCL2 and Bcl-X) which favour the formation of pannus [28].

The hyperplastic synovia, progressively engulf the articular ends destroying the cartilage evolving eventually into the pannus.

Pannus is a granular tissue containing proliferating synovial cells and infiltrating inflammatory cells [29] that release pro-inflammatory cytokines and destructive enzymes responsible of the cartilage damage, chiefly matrix metallo-proteinases (MMPs) [31]. Among the pro-inflammatory cytokines, IL6 acting in synergisms with IL1β and TNFα, induce the pro-angiogenic mediator, vascular endothelial growth factor (VEGF-A), also up-regulated by the hypoxic conditions of the inflamed paws [30]. The same cytokines are also involved in the up-regulation of RANK-L gene expression [113] and the activation of the RANK-RANKL pathway in osteoclasts potentiates the differentiation of osteoclast precursors and increases the bone resorption of mature, multinucleated osteoclasts [32].

After the cartilage has been destroyed, the pannus bridges the opposing bone, ossifies progressively, inducing joint deformation and increasing limiting the motion of the joint [29, 33].

It has been proposed that joint damage represents a source of new antigens and inflammatory mediators that perpetuate the inflammatory process through activation of antigen receptors and TLRs and the promotion of the production of

autoantibodies (by B cells) with the formation of circulating immune-complexes (ICC) that activate complement [34].

The clinical course of RA is extremely variable. The disease begins slowly and insidiously for more than a half of the patients and the pattern of joint involvement varies but is generally symmetrical with the small joints being affected before the large ones [35]. Symptoms usually develop in the hand (metacarpophalangeal and interphalangeal joints) and feet followed by the wrists, ankles, elbows and knees. The involved joints show the classical signs of inflammation: *dolor, calor, rubor* and *functio lesa* being swollen, painful, warm and particularly stiff on arising or following activity. The disease course may be slow or rapid with fluctuations over time, with the greatest damage occurring in the first 5 years from diagnosis [36]. The clinical condition is usually followed up using the DAS28 (Disease activity score in 28 joints) used as reference [37].

The therapeutic regime for RA is well codified by the EULAR (European League Against Rheumatism) guidelines which state that therapy with DMARDs (Disease Modifying Anti-Rheumatic Drugs) should be started as soon as the diagnosis of RA is confirmed [38]. DMARDs are a group of otherwise unrelated medications that are used to slow down the disease progression. They are separated in two main groups – the synthetics (methotrexate, leflunomide, sulfasalazine, hydroxychloroquine) and the biologics (of which the TNFα-inhibitors adalimumab, certolizumab, etanercept and infliximab represent a large part).

1.2 Key molecules in inflammatory disease

While the specific cause of RA in genetically susceptible individuals is an ongoing area of research, recent discoveries in immune system biology have shed light on the processes that initiate inflammation, and the progression of acute, short-lived inflammation to chronic auto-immune disease. The most relevant molecules to RA pathogenesis are those that sense danger (such as TLRs), mediators that signal to other cells (cytokines and chemokines) and key intracellular signalling molecules (e.g. the NFκB pathway).

1.2.1 Toll-like Receptors (TLRs)

The innate immune system appears to be involved from the early stage of the disease, probably through the TLR pathway [39-41]. TLRs are a family of evolutionary conserved single span trans-membrane receptors which recognise pathogen associated molecular pattern (PAMPs) from viruses, bacteria, protozoa and fungi. They can also bind endogenous molecules associated with inflammation and tissue damage such as cytosolic HSP [42] and HMGB1 [43], FBG [44], fibronectin [45] and tenascin-C [40] which are collectively known as damage associated molecular patterns (DAMPS); DAMPs are vital for the initiation of sterile inflammation and clearance of cellular debris[46].

TLRs1-11 are conserved between human and mouse with TLR10 being non-functional in mice while in humans it dimerizes with TLR2 [47]. Mice also express TLR12 and TLR13 genes which in humans are pseudo-genes. TLRs are expressed on myeloid cells, fibroblasts and endothelial cells [48-50].

TLR1, 2, 4, 5 and 6 are expressed on the cell surface while TLR3, 7, 8, and 9 at intracellular level. TLR4 can be expressed at both levels and its cellular

localization determines which signalling pathway will be activated (MyD88 when TLR4 is extracellular and TRIF when it is intracellular) [51]. Furthermore, Aksoy et al. demonstrated in 2012 that blocking TLR4 internalization is associated with a more severe response to LPS hypothesising that controlling TLR4 cellular localization could represents a mechanism of inflammation control [52].

TLR structure includes an extracellular binding domain for the ligand containing 18-28 copies of a leucine-rich repeat (LRR) and a C-terminal intracellular highly conserved TIR-signalling domain which interacts with the TIR domains located in other signalling molecules. After binding their specific ligand, TLRs homo/hetero-dimerize causing their cytoplasmic TIR domains to come into close proximity and interact with the TIR domain of one of four cytoplasmic adaptor molecules: MyD88, TRIF, TIRAP and TRAM. These adaptors are necessary for the signalling because the TLRs TIR domain has no intrinsic tyrosine kinase activity. TLRs signal through MyD88 activating NFκB and AP-1, with the exception of TLR3 which signals through TRIF activating IRF3 and IRF7 [53]

All the intracellular TLRs induce type I IFNs production but while TLR3 signals through TRIF, TLR7, 8 and 9 signal through MyD88 [54]. TLR4 is unique among the TLRs in that it can signal through both MyD88 and TRIF. TLR5, 7, 8 and 9 can directly recruit MyD88 while TLR1, 2, 4 and 6 require TIRAP as bridging adaptor. TLR4 needs also another bridging adaptor, TRAM, to activate the TRIF pathway.

Most TLRs appear to be involved in the development of RA [3, 41, 55-59]. TLR2, 3, 4, 5, 7 and 8 are highly expressed in RA by inflammatory cells and synovial fibroblasts. Overexpression of TLRs appears to be an early event in the

pathogenesis of RA and may be caused by a non-specific trigger, such as an endogenous ligand or an environmental factor like cigarette smoke. Roeflofs et al. (2005) described the observation that TLR3/7 signalling can then result in an up-regulation of TLR2 and TLR4 leading to a higher sensitivity toward endogenous DAMPs and starting a vicious inflammatory cycle; simultaneous activation of multiple TLRs could induce a synergistic effect with an increased inflammatory response leading to a breakdown of tolerance [60]. TLRs are over-expressed by the cells of the immune system in the early stage of the disease [3]. The release of DAMPs by the dying cells and degraded matrix may perpetuate inflammation by binding to TLRs and causing further stimulation.

According to this scenario, all TLRs could be involved in binding intracellular antigens released by the dying cells in established disease. Furthermore, TLR4 could play a pivotal role in the establishment and perpetuation of RA, being up-regulated both in early and late stage disease [3,61], reacting with multiple ligands [39] and playing different roles in controlling the activity of other inflammatory cells [62] and inducing bone erosions [63].

By extension, we could hypothesize that the different TLRs present in RA are activated following a hierarchy with TLR3, 7 and 8 (that recognize RNA and are up-regulated in the early stages of the disease [3, 60] involved at the beginning of the disease with the consequent breakdown in immune tolerance, while other TLRs, like TLR2 and TLR4, are involved in the perpetuation of the disease following activation by DAMPs that are released during joint damage [64].

1.2.2 Inflammatory mediators

A role for many inflammatory mediators has been demonstrated by *in vitro* and *in vivo* animal models [31]. In the clinic, therapeutic targeting of only CTLA-4, IL1β, IL6, and TNFα has regulatory approval for the treatment of RA [65].

For activation, T helper (Th) lymphocytes require two signals, one provided by the TCR-MHCII interaction, the other by the binding of the co-stimulatory receptor CD28 which is expressed on the surface of the Th cells. The cognate CD28 ligands of the B7 family (CD80/CD86) are expressed on the surface of the APCs [66]. CTLA-4 modulates this signalling by binding B7 and blocking the co-stimulus in this way [67]. IL6 is a pleiotropic cytokine produced by T and B cell, monocytes, fibroblasts and synoviocytes which is responsible for osteoclast activation [68] and pannus formation [69] and together with TGFβ1 can facilitate the differentiation of Th17 cells, skewing the balance of Tregs and Th17 [70]. IL1β acts locally in the joint, amplifying the production of inflammatory mediators and MMP release [31], increasing bone loss [71] and maintaining the phenotypic stability of Th17 cells [72].

1.2.3 TNFα is a key inflammatory mediator

The central role of TNFα in RA has been well established by Feldmann et al. in a series of in vitro experiments on RA synovial cells [73, 74] which lead to the development of anti-TNFα therapy [38, 75]. TNFα is a homo-trimeric cytokine well known for its pro-inflammatory role [76]. It is produced by different cell types (macrophages, DCs, B and T cells) and signals through two different receptors:

TNFRSF1A (p55) and TNFRSF1B (p75) [77]. It is synthesized as a membrane-bound protein which can be cleaved and released as a soluble form; the transmembrane and the soluble forms are both active.

The two different receptors for TNFα can also exist as soluble forms. TNFR1SF1A is expressed on almost all nucleated cells and it already exists in its trimeric form before the binding to TNFα. It binds both forms of TNFα (membrane bound and soluble) and initiates signalling via the classical NFκB pathway. The expression of TNFR2SF1B is more restricted and is found on endothelial cells, immune cells like monocytes/macrophages, T and B cells and NK cells, where binds mainly to the membrane-bound form of TNFα. The intracellular portion of TNFR2 lacks the death domain [78] and can activate the canonical and the non-canonical NFκB pathway.

Although anti-TNFα therapy represents a highly efficacious biological treatment of RA, not all patients treated with anti-TNFα achieve remission (defined as DAS28 score <2.6) and often suspension of treatment is associated with relapse [79]. Patients initially responding to anti-TNFα therapy can stop responding within one year [80]. In several clinical studies, TNFα blockade has been associated with an increase in Th17 cells prevalence in non-responders [81-85]. The increase in Th17 cells prevalence has also been measured in animal models of disease [86]. The association of elevated numbers of Th17 lymphocytes with a poor response to therapy [87] suggests that TNFα may not be the only mediator responsible for disease chronicity in non-responding patients, and that other mechanisms of

disease may take precedence in the establishment and perpetuation of inflammation.

1.2.4 TNFα has an immuno-modulatory function

In addition to its pro-inflammatory role, an immuno-modulatory effect has been described for TNFα. Different animal models of autoimmune disease indicate that TNFα can modify the course of the disease, delaying the development of complications [88] and reducing or blocking the establishment of disease [89, 90]. In addition, TNFα is also involved in the termination of inflammation [91] and in the establishment of endotoxin tolerance (ET) [92], in particular through up-regulation of IRAK-M and TNFAIP3 [93]. Blocking TNFα during LPS Cells stimulation prevents ET [92].

It has been demonstrated that blocking TNFα with anti-TNFα therapy increases the expression of IL12B, suggesting that TNFα can block the production of IL12B [86]. As the common subunit of IL12 and IL23, IL12B is plays an important role in the polarisation of T lymphocytes towards Th1 and Th17 phenotypes [59, 86, 94].

1.2.5 Inflammation-associated signalling molecules

Several molecules are required for the optimal detection of LPS, which is a canonical stimulator of TLR signalling. LPS interacts with LBP (LPS binding protein) an acute phase protein which is secreted by the liver and circulates in the blood [95]. At the cell surface, the LPS-LBP complex binds to CD14, a glycoprotein expressed on the surface of myelomonocytic cells, that acts as co-receptor for several TLRs (TLR1, 2, 3, 4 and 6) [96]. TLR4 is complexed with MD-

2, a glycoprotein that is necessary for TLR4 surface expression and activity [97, 98].

After binding with LPS, TLR4 dimerises and recruits through TIR-TIR interactions the bridging molecule TIRAP that activates MyD88. MyD88 has a C-terminal TIR domain that interacts with the TIR domain of recruiting TLRs, an intermediate domain and a N-terminal death domain (DD) that interacts with the DDs of IRAK family members. MyD88 recruits IRAK4 and IRAK1. The IRAK4-IRAK1 dimer becomes phosphorylated and dissociates from MyD88, activating TRAF6. TRAF6 interacts through its K63-ubiquitinated chains with the two adaptors proteins TAB1 and TAB2/3, inducing TAK1 phosphorylation, oligomerisation and activation. TAK1 is recruited to the plasma membrane where it interacts and activates the IKK complex. The IKK complex is composed by two kinase subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ/NEMO) [99].

The interaction with TAK1 triggers the K48-linked ubiquitination of the IKB proteins (IκBα, IκBβ and IκBε) enabling the nuclear translocation of the NFκB members (NFκB1/p50, its precursor p105, NFκB2/p52 and its precursor p100, RelA/p65, RelB and c-Rel) with consequent increased expression of pro-inflammatory cytokines (such as TNFα, IL1β and IL6)[100].

1.3 Macrophage-lineage cells in inflammatory disease

Macrophages play a significant role in the pathogenesis of chronic inflammatory diseases such as COPD [101], atherosclerosis [102], multiple sclerosis [103], Alzheimer's disease [104] and joint replacement failure [105] and the severity of

bone lesions has been observed to correlate with the macrophage numbers in RA [106]. Macrophages represent extremely plastic cells that can oscillate between two different phenotypes, M1 (classical activated) and M2 (alternative activated), according to the cytokines present in the micro-environment. While the M1 phenotype is mainly involved in the response against pathogens and is typified by prolonged production of pro-inflammatory cytokines, the M2 phenotype has an immuno-regulatory, pro-fibrotic action and sustained production of IL10 and TGFβ1 [107]. For that reason, macrophages appear to be important cells not just for the inflammatory process but also for its resolution. At the onset of inflammation, they begin as resident or infiltrating monocytes and progressively switch phenotypes, according to the cytokine micro-environment, becoming first pro-inflammatory M1-like macrophages, producing TNFα, IL12/23, IL6, CXCL9, CXCL10 [108]. After the phagocytosis of apoptotic PMNs they switch to M2-like macrophages which potentiates the resolution of the inflammation, wound-healing and pro-fibrotic/pro-angiogenic activity due to a change in mediators secreted (IL10, CCL2, CCL7, CCL22 and TGFβ1 [109]. Towards the end of the inflammatory process, macrophages undergo another phenotypic switch by becoming resolution–promoting macrophages (Mres) and migrate to remote sites [110-112]. The importance of macrophages in the resolution process is not limited to acute inflammation. Thus, macrophages are also involved in inflammatory conditions by self-limiting organ damage due to the immune response.

1.4 Endotoxin Tolerance

The initial stimulation with LPS induces production of key inflammatory mediators (e.g. TNFα, IL6, IL1β, IL12) but if the LPS stimulation persists the cell significantly

reduces the production of inflammatory mediators and up-regulates immuno-modulatory cytokines (IL-10, TGFβ) and intracellular molecules that promote macrophage polarization toward an M2-like phenotype (e.g. IRAK3, TNFAIP3, PTPN6 [113-117]. The prolonged stimulation with LPS induces a phenomenon known as endotoxin tolerance (ET). ET is normally defined as a refractory state of the cell of monocyte/macrophage lineage subsequent a prolonged stimulation with LPS. ET is not simply a refractory, non-responsive state of the cell but it is characterised by a complex re-programming of the global gene expression.

Under normal, physiological conditions ET prevents inflammation in tissues constantly exposed to a potential inflammatory stimulus (e.g. LPS from commensal bacterial flora in the gut [118, 119], exposure to airborne LPS in the respiratory tract [120] and exposure to LPS from *Porphyromonas gingivalis* in gingival epithelial cells [121]. In a pathological context, ET represents a mechanism to prevent further damage due to a prolonged immune response in the presence of a very strong or chronic inflammatory stimulus (i.e. sepsis or HCV infection [122]) and this positive effect is counterbalanced by the risk of opportunistic infections [123].

Characteristically, the down-regulation of pro-inflammatory mediators (TNFα, IL6, IL12, CXCL10, CCL5 and CCL3) is accompanied by a down-regulation of antigen presentation and co-stimulatory molecules (MHC, CD80 and CD86). TLRs on the cells surface are reduced, while there is an increase in the expression of immune-regulatory cytokines like TGFβ1 and IL10, and increase the expression of M2 markers and ET-associated genes; see Chapter 3 for expanded discussion.

ET is a reprogramming of gene expression rather than a down-regulation of gene expression or an exhausted phenotype. The altered response to LPS appears to be mediated by an alteration in intracellular signalling [92] as the only significant change on the cell surface is the down-regulation of MHCII expression [124]. As reported by several studies, TNFAIP3, PTPN6 and IRAK-M are essential for the establishment of ET [113, 125, 126].

TNFAIP3 (TNFα-induced protein 3) is an intracellular ubiquitin-editing protein with a fundamental role in the termination of the canonical NFκB signalling [127]. TNFAIP3 is expressed in physiological conditions by the enterocytes maintaining tolerance to the commensal flora [128]. In resting conditions TNFAIP3 is expressed in thymocytes and peripheral T cells but its transcription is rapidly up-regulated upon TNFα-induced NF-κB activation in all tissues [93, 129, 130]. TNFAIP3 inhibits TLR4-induced NFκB activation through de-ubiquitination of TRAF6, altering its ability to recruit TAK1 [131].

PTPN6 is an intracellular protein tyrosine phosphatase (PTP) with two SH2 (Src Homology 2) domains in its N-terminus and is alternatively known as SHP-1. PTPN6 is expressed by mature T cells, granulocytes, macrophages, mast-cells and platelets [132]. It is reported that PTPN6 is able to block the activity of the Src-related kinases [132] and JAK kinases [133]. PTPN6 prevents NFκB translocation by directly binding the kinase domain of IRAK-1 [134], representing another mechanism by which the TLR4-MyD88 pathway and NFκB activation can be attenuated [135].

IRAK3 (also known as IRAK-M) is a member of the IRAK family, together with IRAK-1, 2 and 4, and is expressed predominantly in macrophages. After LPS stimulation, the TLR4-MyD88 complex recruits IRAK4 which induces phosphorylation of IRAK-1. IRAK1 permits an association with TRAF6 that then leads to NFκB activation. IRAK3, which lacks the catalytic domain and kinase activity, acts as an inhibitor of the TLRs-MyD88 pathway by interacting with IRAK4 and altering the normal assembly of the MyD88-IRAK4-IRAK1 signalling complex. In particular, IRAK3 expression has been linked with a down-regulation in the production of the p40 subunit (the translated product of *IL12B*) of IL12 and IL23 [136], and has been shown to be necessary for ET in human and mouse cells [92, 113, 136]. IRAK-3 is discussed in greater detail in Chapter 5.

1.5 Hypotheses and aims.

Several studies have highlighted the important role played by the innate immune systems in the development and perpetuation of the inflammatory response which is responsible of the joint destruction in RA. Cells of the monocyte/macrophage lineage represent the link between innate and adaptive immune system. They are activated through TLR ligands derived from the damaged tissues, producing inflammatory cytokines like TNFα, IL6, IL1β and IL12/23 that initiate the recruitment of immune cells to which they then present the antigens.

For TNFα, in particular, a double role has been described. Studies propose TNFα at the top of a hierarchy among the different inflammatory cytokines [75] and the key role of TNFα in RA is confirmed by the therapeutic actions of anti-TNFα therapy [74]. TNFα also exerts an immuno-regulatory role by being able to modulate the immune response through different mechanisms such as ET. The establishment of a tolerant state in response to prolonged stimulation with TLRs ligands is a phenomenon characteristic of monocytes/macrophages and it is obtained through a global reprogramming of the cell gene expression profile.

ET mechanisms are required to finely regulate the inflammatory response and a perturbation of ET mechanisms is often involved in pathological conditions which result in a persistent inflammatory response. Persistent inflammation and defects in ET have been observed in transgenic mice lacking IRAK-3, TNFAIP3 [137] and PTPN6 [125]. Considering the role of TNFα in RA and in ET-associated gene expression, the principal hypothesis in this study was that the prolonged

inflammatory response responsible in chronic inflammatory disease, such as RA, could be contributed to by defective activity of ET mechanisms

The first aim was to investigate the immuno-regulatory role of TNFα in the context of ET mechanisms by developing an in vitro model to better characterise the gene expression phenotype in macrophages.

The second aim was then to characterise ET mechanisms in disease and how they can be modified by blocking TNFα with anti-TNFα therapy in an animal model of RA; for his purpose collagen-induced arthritis (CIA) was used.

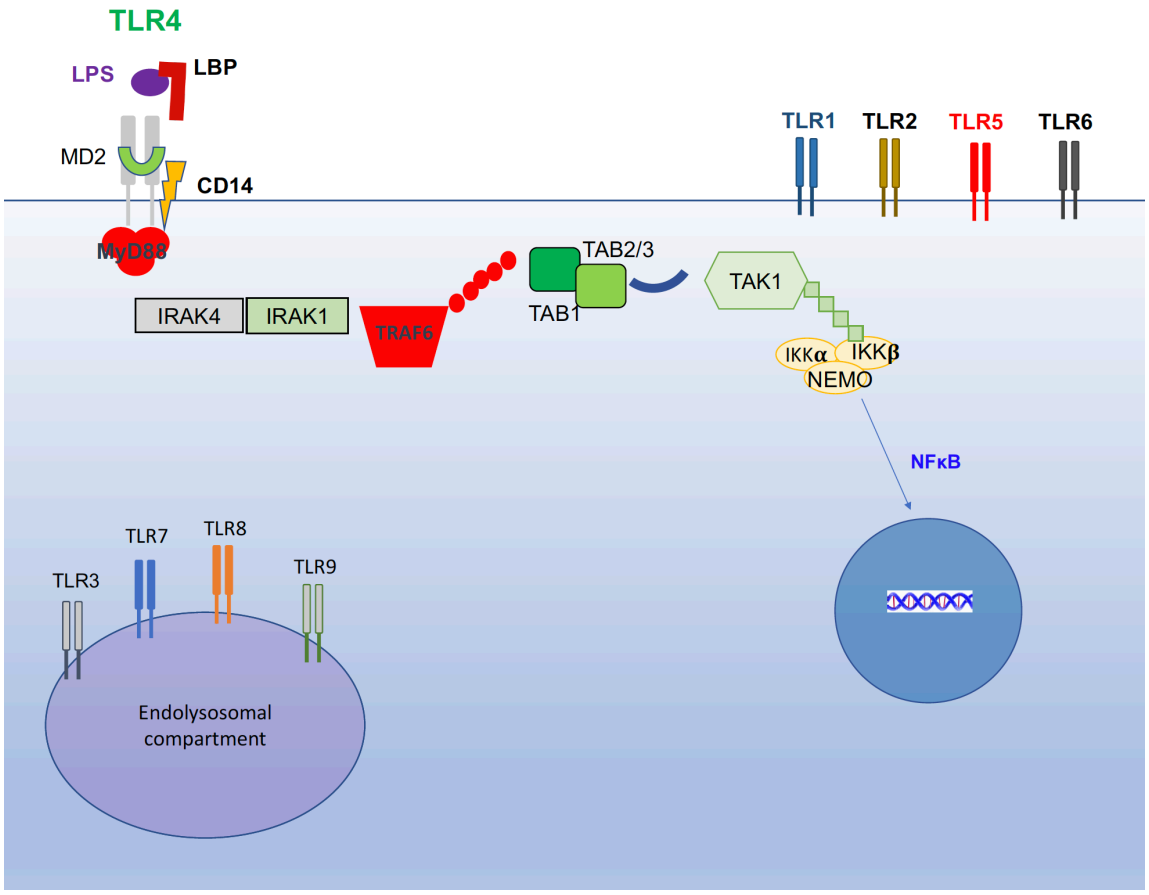

Figure 1.1 TLR4 signalling pathway.

TLR4 is expressed on the cells surface together with TL1, TLR2, TLR5 and TLR6, although its intracellular localization has been described. Together with CD14 and MD2 interacts with LPS. On the cell surface TLR4 activates a signalling pathway that will lead to the activation of NFκB and its nuclear translocation.

Chapter 2 : Materials and Methods

2.1 Ethical Approval

All animal procedures were undertaken pursuant to Project and Personal licences granted by the UK Home Office. Ethical approval was granted for the use of human aphaeresis cones.

2.1 Animal studies

2.1.1 Irak3 KO mice

Mice heterozygote/KO for IRAK-3 (B6.129S1-IRAK3 ^{tmlFlv}/J), were a kind gift from Richard Flavell, and bred on B6 background. All animals were maintained in the mouse facility at the Kennedy Institute of Rheumatology, NDORMS department, Oxford University. To genotype them, genomic DNA (gDNA) extraction was performed on the animal ear-clips using Genomic DNA Sigma Extraction Kit (Sigma). The genotype reaction was performed using the Jackson Laboratory Protocol, with the primers shown in Table 1.

The genotype PCR reaction was prepared mixing Mango MixTM (Bioline) 12.5 μL with dH_2O 6.5 μL adding 2 μL of each primer (100pmol/μL) and 2μl of gDNA. The thermo-cycler was then set as shown in Table 2. The PCR product was then run on 1.5% agarose gel at 120 V for 1 hour. For the mutant mouse a product of 500 base pairs (bp) was expected, while for the wild type of 600 bp. The heterozygote showed instead both the 500bp and 600bp bands. The bands size was checked against HyperLadder^{TM} 1kb (Bioline).

2.1.2 Breeding Irak3 transgenic mice onto the C57Bl/6.NQ background

Based on the discovery that C57Bl/6 mice, require MHC-II A^q (which is similar to the molecules encoded by RA susceptibility alleles, HLA-DRB1*0101 and *0401) to develop auto-reactive T cell responses in CIA [138], I then decided to cross the C57Bl/6 IRAKM$^{-/-}$ (expressing A^b) to congenic C57Bl/6N.Q (expressing A^q) to acquire a phenotype more susceptible to the development of arthritis, expressing the MHC-II A^q molecule.

2.1.3 Collagen-Induced Arthritis

For the CIAs described in Chapter 4 (paragraphs 4.3.1-4.3.4), DBA/1J mice were used while for the CIA described in chapter 5 mice C57Bl/6N.Q IRAK3$^{-/-}$ and wild type were immunized with bovine type II collagen in complete Freud's adjuvant as previously described [139].

2.1.4 Assessment of CIA disease and treatment

After a period of time ranging between two and four weeks the mice developed the disease.

Arthritis severity in the paws was scored as follows: 0, normal; 1, slight swelling and/or erythema; 2, clear swelling; 3, pronounced oedematous swelling/ankylosis. The clinical parameters were recorded for 10 days after the onset and then the animals were euthanized by CO_2 and blood, spleen, inguinal lymph-nodes and paws were harvested.

For the CIA described in Chapter 4, the animals were injected i.p. with 100 µl PBS or 100ug of Enbrel (Amgen/Pfizer) diluted in PBS every other day for a total of 5 treatments.

2.2 In vitro derivation of human and murine macrophages

2.2.1 Isolation of human CD14$^+$ monocytes

PBMCs were obtained from blood cones. The blood was first diluted to ~100mL with PBS. The diluted blood product was layered over Lympholyte®-H (Cedarlane, CL5010) for density gradient centrifugation at 2000rpm, at room temperature, for 30mins. The PBMC interface was collected and washed with PBS.

Isolation of CD14$^+$ cells was performed using the Miltenyi CD14 positive selection kit according to the manufacturer's instructions. PBMCs were incubated with CD14 micro-beads (Miltenyi Biotech) for 15mins at 4°C, washing and applying the cell suspension to Miltenyi LS column (Miltenyi) on a MACS (Magnetic Cell Separation) magnet (Miltenyi). The purity of the cells isolated was measured by flow cytometry. All isolations were greater than 95% purity.

2.2.2 Murine bone marrow isolation

C57Bl/6 mice were euthanized with CO_2 and death was then confirmed with cervical dislocation. Ethanol 70% was used to sterilise the animals before to proceed with the dissection. The femurs were collected and the flesh was completely removed using scissors and tissues. The femurs were cut at either end to open the bone marrow cavity for bone marrow cell extraction by centrifugation. The femurs were placed into a 0.5mL micro-centrifuge tube which had been pierced at the bottom with a 23 gauge needle (0.6x25mm, BD Microlance 3). The 0.5mL tube was placed inside a sterile 1.5mL micro-centrifuge tube and spun at

8000 rpm (6153.5g) at 4 C for 5 minutes to force the bone marrow cells from the femurs and into the 1.5mL tube. The cells were then counted and cultured.

2.2.3 Thioglycolate elicited murine macrophages

Murine peritoneal macrophages were collected from C57Bl/6 mice as previously described by Cohn and Benson in 1965 [116]. The mice were injected i.p. with 1ml thioglycolate (3% in RPMI 1640, Gibco). The mice were euthanized at day 7 post-injection and cells were harvested by peritoneal lavage through peritoneal injection of 1mL of cold RPMI. The lavage cells were centrifuged, washed, counted and then cultured as described under *Section 2.3 Experimental assays*.

2.2.4 Macrophage culture

Human $CD14^+$ monocytes and murine bone marrow cells were cultured following the same protocol to generate macrophages. Cells were re-suspended in medium (10% FCS, 1% penicillin/streptomycin RPMI 1640 with L-glutamine, Gibco 61870-010) and plated in culture dishes (10^7 per plate) in complete medium with the addition of 50ng/mL M-CSF. After 3 days, the cells were harvested using a cell lifter, centrifuged at 1500g for 5 mins at 4°C and seeded in a 6 well plate ($1.5\text{-}2x10^6$ per well) with 2ml of medium and 50ng/mL M-CSF, allowing them to differentiate for 4 more days. On day 7 the cells were stimulated (ET bioassay or LPS time course).

2.3 Experimental assays

2.3.1 In vitro ET bioassay

On day 7 the cells were cultured with four conditions (a) nil (no stimulation), (b) LPS 10ng/mL 4 hours, (c) LPS 1ng/mL for 20 hours then LPS 10ng/mL 4 hours

and (d) IFNγ 40ng/mL/LPS 1ng/mL overnight then LPS 10ng/mL for 4 hours. At the end of the stimulation period, the medium was collected, aliquoted and stored at -80ºC for measurement of cytokines. The cells were washed with ice-cold PBS and stored at -80ºC prior to RNA or protein extraction.

2.3.2 RNA extraction from cells

Cells were stored at -80°C as dry pellets after washing in PBS. Total cellular RNA was isolated from cell using a Qiagen RNeasy mini kit (Qiagen) or TRIzol™ Reagent (Ambion-RNA by life Technologies') according to the manufacturers' protocols. RNA that was not used immediately was stored in Nuclease free/endotoxin-free water at -80°C for up to 12 months. The extraction of RNA from the cells of spleens, lymph-nodes and blood was performed following the same protocol.

2.3.3 RNA extraction from tissue

The paws were then snap-frozen in liquid nitrogen and pulverized with the BioPulverizer™ (BioSpec). Paw powder was then homogenised in 500µL of TRIzol reagent (Invitrogen) using the Sample Grinding Kit (GE Healthcare). The aqueous phase of the phenol/chloroform extraction was mixed with an equal volume of Isopropanol and then added to an RNA extraction column (microRNeasy Mini Kit, Qiagen) and mRNA extraction completed according to manufacturer's instructions.

2.3.4 cDNA synthesis

cDNA was reverse transcribed from RNA using a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific), according to the manufacturer's

protocol, in a 40 µL reaction which was then diluted to a total of 120 µL with nuclease free water.

2.3.5 Quantitative Real-Time Polymerase Chain Reaction (qPCR)

The qPCR was performed using standard 384-well reaction plates (ABI PRISM® 384-Well Clear Optical Reaction Plate with Barcode) and the Applied Biosystems™ ViiA™7 system. The reactions were set up using 10ng of cDNA (in a volume of 2.4 µL), 3µL of Mastermix Takyon (Low ROX Probe 2X dTTP blue), 0.3µL of gene specific TaqMan probes (Applied Biosystem) and 0.3µL of nuclease free water.

The expression of my target genes is showed as relative to HPRT gene expression using the ΔΔCT approximation method [247].

2.3.6 Phenotyping T cell subsets by flow cytometry

Cells were isolated from LN and Spleen using cell strainer. Cells from the spleen were then incubated for 3 minutes with Red cells lysis buffer. The cells were washed, counted and seeded into 96 well round-bottom plates (10^5cells/well) in 10% FCS RPMI. To assess the production of cytokines, the cells were stimulated with PMA 20ng/mL (Calbiochem), Ionomycin 1mM (Calbiochem) and BrefeldinA 12.5ng/mL (Sigma) added at the same time.

After stimulation the cells were washed prior to extracellular staining with antibodies for CD4 (PeCy7 BD Pharmingen 552775) and CD8 (FITC Biolegend 100706) for 30 min at 4° C, washed, and then fixed in Cytofix (eBiosciences). Cells were permeabilised using Permealization Buffer (eBiosciences) and stained with anti-mouse IFNγ (AF647 Biolegend 505814) and anti-mouse IL-17 (PE BD

Pharmingen 559502) and analysed on FACS Canto II using FACSDIVA software (BD Biosciences).

To analyse Th cell-associated transcription factors, cells from the spleen and LN were directly stained with antibodies for CD4 (BV510 Biolegend 100559), CD25 (PerCp Cy5.5 Biolegend 102030), then fix/permeabilised and stained intra-cellular with antibodies for FoxP3 (PeCy7 Ebioscience 25477742), Gata3 (efluor660 Ebioscience 50996642), RORγt (PE BD Pharmingen 562607), Tbet (BV711 Biolegend 644819), Helios (Pacific blue Biolegend 137220).

The cells were then run on the flow cytometer using calibration beads to assist with the compensation set up. Fluorescence minus one controls (FMO) were prepared for each stain. FACSDIVA software (BD Biosciences) and FlowJo (TreeStar) was used to analyse the results.

2.3.7 Extraction of cells from arthritic joints of mice

The skin was cut around the ankle and vertically towards the toes with a scalpel and peeled off. The joint was removed, snipped and incubated for 90 minutes in RPMI 1/100 for DNase (10 mg/ml stock, Roche) and 1/40 Liberase (5mg/ml stock).The digested tissue was washed then passed through a cell strainer. The single cell suspension was then centrifuged at 1500 rpm for 5 min and stained for flow cytometry.

2.3.8 Measurement of cytokines in plasma and cell culture medium

Inflammatory mediators in murine plasma and cell culture supernatant were measured using the Mouse Cytokine Assay Multi Array Assay Ultra-sensitive kit

(MesoScale Discovery, catalogue number N45022B-1) according to the manufacturer's instructions.

2.3.9 Measurement of anti-collagen antibody response

On day 10 post-onset, mice were sacrificed and blood was collected through cardiac puncture in 1.5 mL microcentrifuge tubes containing heparin. The samples were then centrifuged at 13,000 rpm for 10 minutes at 4ºC. Plasma was then collected in clean 0.5 mL microcentrifuge tubes and stored at -80ºC. The plate was coated with 50 µL of type II bovine collagen, and incubated overnight at 4ºC. The day after, the plate was washed 3 times with washing buffer (1XPBS 0.05% Tween 20 (Sigma-Aldrich P1379)) and then blocked with 200 µL blocking buffer (1XPBS 2%BSA (Sigma-Aldrich A 7906-500G)) and incubated for 1 hour at room temperature.

The plate was washed again and 100 µL of sample diluted 1:100 in washing buffer.

Washing buffer was added to the first row while 7 serial 3-fold dilutions were added to the remaining wells. CIA wild type non-treated mice (n=8) sera was pooled and used as a standard. Samples were incubated for 2 hours at room temperature. After washing, 100 µL HRP-conjugated anti-mouse IgG1 or IgG2a (diluted 1:1000 in washing buffer) were added and incubated for 1 hour at room temperature. The plate was washed again and then 50 µL of TMB (3,3', 5,5"-tetramethylbenzidine) substrate were added followed by 25 µL of 4.5 N H_2SO_4 (sulfuric acid) to stop the reaction. The Optical densities (OD) were then measured at 450 nm and 540 nm using a Thermo Labsystems, Multiskan Ascent software.

EC50 values were calculated from the range of values for each sample according to a 4-parameter titration curve.

2.3.10 Histological assessment of arthritic joints

Paws were harvested and fixed in 10% neutral buffered formalin for at least 48 hours, followed by decalcification with 5.5% EDTA in buffered formalin for 4 weeks. Fixed paws were embedded in paraffin, and sections cut and stained with haematoxylin and eosin.

2.4 Statistical analyses

Data were collected and analysed using Excel (Microsoft Ltd), Graphpad Prism (Graphpad Software Ltd) and MultiExperiment Viewer (TM4 software). Student's t-test or one 1-way ANOVA with post hoc analysis was used to test statistical significance. A p-value of < 0.05 was considered statistically significant.

Wild type Forward	GCC AGA AGA ATA CAT CAG ACA GGG
Wild type Reverse	TGT TTC GGG TCA TCC AGC AC
Mutant Forward	CGT TCC ATA ACA CAC CTC TCT GC
Mutant Reverse	TTC TAT CGC CTT CTT GAC GAG TTC

Table1. IRA3 KO genotype primers

Reaction set up
1.94°C 3mins
2.94°C 30 sec
3. 57°C 1min
4. 72°C 1min
5. Go to 2 for 35 cycles
6. 72°C 2mins
7. 10°C for ever

Table2. Thermo-cycler setting for IRAK3KO genotype

Chapter 3 : In vitro characterisation of Endotoxin Tolerant macrophages

3.1 Introduction

Endotoxin tolerance (ET) is a refractory state in macrophage-lineage cells in response to prolonged stimulation with LPS (or other TLR ligands). In comparison to cells without prior prolonged stimulation, tolerant cells are characterized by the down-regulation of pro-inflammatory mediators (TNFα, IL6, IL1β, IL12/23, CCL3, CCL5, CXCL10) upon second TLR ligand stimulation, [140]. The intracellular signalling molecules associated with the ET phenotype *(*e.g. IRAK3, PTPN6, TNFAIP3), act at different cellular levels [106, 125, 126, 128, 141, 142]. The ET phenotype is distinct from the alternatively or M2-like macrophage phenotype [117]. The establishment of ET and M2 macrophage polarization are dissociable processes which are the result of different signalling pathways [117, 143].

3.2 Cytokines modulate ET induction

3.2.1 Immuno-modulatory cytokines

Several cytokines are relevant for the induction and prevention of ET. IL10 and TGFβ, two immuno-regulatory cytokines, have been both shown to be involved in ET. TGFβ1 up-regulates IRAK3 gene expression [144, 145]. IL10 has an immuno-regulatory effect that facilitates the transition from M1-like to M2-like macrophages after TLR-ligand stimulation [146]. While these cytokines are associated with the ET phenotype, their role does not appear fundamental.

ET is still possible in IL10$^{-/-}$ animals and IL10 expression levels are not increased in all the ET animal models [147]. Several studies have reported a reduction in IL10 gene expression level after a prolonged stimulation with LPS. The role of IL10 may be more crucial in balancing the immune response to the acute stimulation with LPS rather than a necessary condition for ET.

3.2.2 Inflammatory cytokines

IFNγ is a cytokine necessary for innate and adaptive immunity. It is produced in response to viral, bacterial or protozoan-associated antigens by the cells of the innate and the adaptive immune systems [148] and induces polarization toward the M1-macrohage phenotype[149]. GM-CSF is a white blood cell growth factor that stimulates stem-cells to produce granulocytes and monocytes but has also an inflammatory action inducing differentiation, proliferation and activation of macrophages and dendritic cells [150]. These two cytokines are able to prevent or revert, the establishment of ET [151].

IFNγ is able to induce the expression of a class of specific transcription factors (Interferon-regulatory factors or IRFs [108]) and through the induction of IRF5 and IRF8 exerts a strong M1-polarizing action that is able to prevent or revert, the development of ET [152, 153]. GM-CSF can induce the production of pro-inflammatory cytokines in both in vitro stimulated human septic macrophages and in an animal model of sepsis [151, 154].

A pivotal role in the development of ET is played by TNFα.

In addition to the well-described pro-inflammatory effects, TNFα also has regulatory activity. The effect of TNFα in several animal models of autoimmunity indicate that it can modify the course of the disease, delaying the development of complications [88] and reducing or blocking the establishment of disease [89, 90]. TNFα is also involved in the termination of the inflammation [91] and in the establishment of ET [92] in particular through up-regulation of IRAK3 and

TNFAIP3 [93]; blocking TNFα when stimulating cells with LPS reduces IRAK3 expression [142]. In vitro studies using pro-monocyte THP-1 cells demonstrate a resistance to acquiring the ET phenotype, retaining their inflammatory response to LPS, in the presence of anti-TNFα[112], suggesting that the ET phenotype is restricted to macrophage-lineage cells that have developed beyond the monocyte precursor stage.

TNFα stimulation alone is able to induce a tolerant state in macrophages that relies more on TNFAIP3 than IRAK3, which is induced later in the process [156] and pre-treatment with TNFα before LPS can induce a condition similar to ET. This effect appears to be mediated mainly by TNFR1 [156] and by blocking TNFR1 it is possible to inhibit the LPS-induced ET [117]. The role of TNFR1 in the immuno-regulatory role of TNFα appears more evident with the observation that TNFR1$^{-/-}$ animals develop a more severe CIA with constantly high level of IL12 [157].

3.3 Rationale

TNFα may exert its immuno-regulatory action through ET-associated mechanisms in vivo. Defective or altered functions of ET mechanisms may be responsible for susceptibility to chronic inflammatory conditions such as RA. The aim of this study was to investigate the possible altered functions of ET-associated mechanisms in pathological conditions. To do so it was pivotal to better understand which molecules are modulated by ET and the conditions that induce ET by developing an *in vitro* assay.

3.4 In vitro ET bioassay

Using macrophages, ET was induced *in vitro* with a double stimulation of LPS. The doses of LPS used for the double stimulation were selected on the basis of the results obtained from preliminary experiments using various LPS concentrations and stimulation time. The macrophages in an ET state are cells polarized toward a phenotype similar to the M2, but these cells maintain their plasticity and can revert to a different phenotype depending on the milieu. Chen J. et al. demonstrated[153] that IFNγ was able to prevent the establishment of the ET status. The experiments described in this paper were taken as initial reference to select the best dose of IFNγ to block the establishment of ET. Preliminary experiments using different doses and stimulation time on LPS-stimulated cells were performed, allowing the selection of the dose of IFNγ to be used in the experiments described in this chapter. On the basis of the literature and preliminary experiments, the tolerant state was obtained by stimulating the cells overnight with LPS 1ng/mL and then a second stimulation for 4 hours with LPS 10ng/mL was used to demonstrate the ET status of the macrophages, compared to macrophages stimulated for 4 hours alone.

Human macrophages were derived from monocytes as describe in Methods and Materials. On day 7, the cells received no treatment, treatment with LPS 1ng/mL 20 hours or treatment with IFNγ 40ng/mL and LPS 1ng/mL 20 hours. The day after, the cells were washed twice with PBS and then stimulated again with LPS10ng/mL for 4 hours (see fig. 3.1 A). The cell culture medium was collected to assess the levels of cytokines. RNA extraction was performed on the cell pellet and then the RNA was reverse-transcribed into cDNA to assess gene expression using qPCR.

3.4.1 ET bioassay gene expression profiles

The different cell treatments are indicated as follows:

- Nil (**N**);
- No pre-treatment + LPS 4 hours (**L4**);
- LPS 20 hours + LPS 4 hours (**LL**);
- IFNγ/LPS 20 hours + LPS 4 hours (**ILL**).

The heat map in Fig 3.1(B) shows the gene expression profiles induced under the different conditions, with the N and the LL conditions showing some similarities. The L4 condition clearly shows a profile with up-regulated inflammatory genes and the ILL condition shows a pro-inflammatory phenotype, due to the contemporary 20 hours treatment with LPS and IFNγ; as anticipated the pre-treatment with IFNγ in the ILL condition is able to revert/prevent the development of the ET phenotype (LL) [151, 153].

3.4.2 ET bioassay: Key mediators

In Figure 3.1(C) the gene expression of the main pro-inflammatory cytokines is shown alongside the relative cytokines levels in the cell culture medium (Fig. 3.1D). TNFα and IL6 are reduced both at the gene and protein level in the LL group, compared with the L4. This demonstrates that these pro-inflammatory cytokines are fully tolerisable by the double treatment with LPS. In comparison, this was not the case for IL1β where gene expression was marginally higher after LL stimulation. IL1β levels in the supernatant were similar in L4 and LL conditions and showed a sharp increase in the ILL condition presumably due to the strong pro-inflammatory and M1-polarising action exerted by IFNγ pre-treatment.

The role played by IL10 did not appear to be fundamental to the establishment of ET, but more a response to the acute inflammation induced by LPS. Consistent with the acute induction of IL10 gene and protein expression was lower expression in the LL condition than in the L4. Furthermore, the pre-treatment of cells with IFNγ reduced the levels of IL10 in both gene expression and supernatant.

3.4.3 ET bioassay: M1/M2-associated genes

Analysing in more detail the differences in gene expression induced by the different conditions it appeared evident that the LL and N showed some similarities and so did L4 and ILL. Most of the similarities between the N and the LL conditions were in the expression of M1 specific markers like *CCR2, CSF2*, *CXCL10* and *CXCR3*, *CD40* (characteristic of APC and activated macrophages) and the two subunits of IL12, *IL12A* and *IL12B*; all were expressed at low levels in both conditions. *HLA-DRA* and *HLA-DRB1*, genes that encode for MHC-II molecules, and *CD86*, a co-stimulatory molecule for T cells activation, are expressed at lower levels.

Conversely, the main difference in expression between the N and LL conditions were non-tolerisable genes (*IFNG, IL1B* and *IL1*A), some putative M2 markers (*CSF1, IL10, CCL2, LRP2, SMAD7*) and also other M1 markers that are up-regulated in the LL condition (*CCL5, CCR5, CCL3*). The most interesting difference between the ILL and the L4 conditions was a lower expression of some pro-inflammatory mediators (*IL12A, CXCL10, NOS2A and CXCR3*) in the ILL condition. In addition to the up-regulation of M1 markers (*CCR7, CD86*, *CCL5, CCR5*) it indicates that the pre-treatment with IFNγ does not revert all the change induced by ET induction.

To further characterize ET gene expression, a comparison was made of L4 and LL macrophages, focussing on a panel of TLR receptors, inflammatory mediators and transcription factors.

3.4.4 ET bioassay: TLR profiles

A reduction of TLR4 gene expression has been described in some models of ET [158, 159]. Furthermore, cross-tolerance between different TLRs has been demonstrated [156, 160]. With the aim to better characterise the receptors that initiate the induction of ET, gene expression levels of the different TLRs were assessed using qPCR. As shown in Figure 3.2, while the expression of TLR1, 2, 3 and 6 was higher in the LL condition, the difference with the L4 condition was not statistically significant for TLR3 and 6. TLR4, 5, 7, 8, 9 and 10 were instead expressed at lower levels in the LL condition even if the difference was not always statistically significant. It is interesting to note that structurally similar TLR1, 2 and 6 follow the same trend, and that TLR4, 7 and 9 that are all down-regulated in the LL condition can induce tolerance to each other [196].

3.4.5 ET Bioassay: M1/M2 markers

ET macrophages express lower levels of M1 markers; however, they lack a clear polarization toward the M2 phenotype. As shown in Figure 3.3, *IL12B*, *CCL5*, *CXCL10*, *CCL3*, *CXCR3* and *CCR7*, which are genes characteristic of the M1 polarized macrophages and controlled by the transcription factor IRF5 [161], were reduced in LL macrophages in comparison to macrophages stimulated with LPS for just 4 hours.

While M2 markers (such as *CCL2* and *IDO1* [162]and *VEGF* [146] were expressed at higher level in the ET macrophages, the difference was not always statistically significant, despite the clear trend (Fig. 3.4). IL10 and IL4R were significantly decreased in LL macrophages.

3.4.6 ET bioassay: Transcription factors

The expression of specific transcription factors involved in the polarization toward the M1 or M2 macrophages phenotypes was investigated. As shown in Figure 3.5, the gene expression of *IRF4*, *IRF5* and *IRF8* was reduced in the LL condition, while *MAFB* was expressed at significant higher levels. While the *IRF5* and *IRF8* higher expression in the L4 conditions could be anticipated by their known association with M1-like macrophages [108], lower level of IRF4 in the LL condition is somehow unexpected, considering that it represents an M2-associated transcription factor [163]; however, the statistically significant expression of *MAFB* in the LL group is consistent with the polarization of the cells toward a M2 phenotype. CEBPA is a transcription factor that has been found involved in both M1 and M2 polarization [164] and while CEBPD is M2 transcription factor involved in the production of IL10 and TGFβ [165], the much lower expression levels in the LL condition were not statistically significant.

3.4.7 ET bioassay: ET-associated genes

When analysing molecules specific for ET, the LL condition showed many but not all the hallmarks of the ET state, at the gene expression level. As shown in Figure 3.6, LL macrophages expressed higher levels of some typical ET markers such as *IRAK3*, *PTPN6* and *AHR* [166, 167] while other genes usually described

associated with ET (*TNFAIP3*, *INPP5D*, *SIGIRR*) were expressed at levels lower than the L4 condition. *IRAK4*, which is one of the targets of IRAK3 action [140], was somewhat increased in the LL cultures. TREM1 is a pro-inflammatory mediator associated with a M1-polarization [168] but previous studies have described the induction of TREM1 by TLR4 interacting with LPS [169] and an increase of *TREM1* gene expression levels in ET [170]. TREM1 expression can be induced by hypoxia which has been demonstrated to be a M2 polarizing factor [171] although in the ET model, the gene expression levels of *HIF1A* were significantly higher in the L4 condition. *TREM1* was also expressed at higher levels in the LL stimulated macrophages. The expression of TNF receptors was very similar in both L4 and LL stimulation.

3.5 Murine ET bioassay

Having developed the ET bioassay in human macrophages, the same experimental model was then applied on bone marrow-derived murine M-CSF macrophages to check the reproducibility of the results on cells from a different species. To establish that the murine macrophages represent a valid alternative to human cells was necessary as it would allow a deeper understanding of the underlying ET mechanisms due to the availability of transgenic mice that lack molecules associated with ET (e.g. TNFR1 and TNFR2). Additionally, a murine ET bioassay would be the first step to further investigate the ET-associated mechanisms in an animal model of RA. Bone marrow-derived macrophages were differentiated with murine M-CSF 25ng/mL for 7 days and then stimulated with single, or double dose of LPS with or without IFNγ 20ng/mL. The gene expression and protein levels of TNFα were measured to confirm the acquisition of the ET phenotype (Fig. 3.7A).

3.5.1 Murine ET-associated gene expression

When ET-associated gene expression (*Irak3*, *Tnfaip3* and *Ptpn6*) was measured by qPCR, a statistically significant increase in *Irak3* gene expression in LL macrophages was observed compared with all the other conditions (Fig. 3.7B). *Tnfaip3* showed relatively lower expression in LL macrophages, while *Ptpn6* was expressed at higher level, although the difference was not statistically significant (p=0.1) (Fig. 3.7B).

3.5.2 TNFα signals through TNFRSF1A to control Irak3 gene expression

As previously detailed, TNFα exerts an important role in IRAK3 induction [142]. To determine whether TNFα receptors differ in their ability to induce IRAK3 expression, a time-course of LPS stimulation was performed using bone marrow-derived macrophages from TNFR1$^{-/-}$, TNFR2$^{-/-}$ and WT mice.

The aim of this experiment was to define if the action of TNFα signalling was different when conveyed by signalling originating from TNFR1 or TNFR2. Among the ET genes considered (*Irak3*, *Tnfaip3*, *Ptpn6*), *Irak3* was the one to show the better correlation between the human and murine samples. There is a degree of overlap in the signalling pathways of each TNFR, which are not well described, so a time-course of LPS stimulation was used in preference to the previously described *ET bioassay* to mitigate and measure any differences in the kinetics of gene expression.

Irak3 gene expression levels were reduced in both TNFR1$^{-/-}$ and TNFR2$^{-/-}$ macrophages comparing with the WT cells, as shown in Figure 3.8. While the difference in *Irak3* between TNFR1$^{-/-}$ and WT cells, after 12 hours of stimulation with LPS, was statistically significant, the difference between TNFR2$^{-/-}$ and WT cells and TNFR2$^{-/-}$ and TNFR1$^{-/-}$ cells did not reach statistical significance (Fig. 3.8).

3.5.3 Ex vivo macrophages express Irak3 after ET induction

A prolonged in vitro culture may push the phenotype of macrophages towards one not found *in vivo* [172]. To obtain a more reliable model of what happens *in vivo*, an *ex vivo* model was set up, using thioglycolate induced peritonitis (TIP) derived cells. Since TNFR1$^{-/-}$ cells had showed *in vitro* a reduced expression of IRAK3 in response to LPS, it was decided to induce ET in TIP derived macrophages from TNFR1$^{-/-}$ and TNFR2$^{-/-}$mice.

TNFR1$^{-/-}$, TNFR2$^{-/-}$ and WT mice were injected with thioglycolate as described in Materials and Methods. The cells were then stimulated *in vitro* with a single or double dose of LPS. As shown in Figure 3.9, while there was no significant difference between the TNFα levels in the supernatant of the different strains (Fig. 3.9A), *Irak3* gene expression was again reduced in the TNFR1$^{-/-}$ cells (Fig. 3.9B). Together these data show that despite the different receptors of TNFα having a different effect on in the induction of *Irak3*, the deficiency of one TNFR is not enough to significantly alter the establishment of ET in TIP macrophages.

3.6 Discussion

ET is usually described as a refractory state of the monocyte/macrophages cells after a prolonged stimulation with LPS. As demonstrated by the results described in this chapter, it represents a state of altered expression rather than the down-regulation of all genes. An in vitro ET bioassay was developed to determine the molecules involved in ET induction. The stimulatory conditions were selected based on the relevant literature and after preliminary experiments (data not shown); TNFα supernatant levels were selected as the readout of ET status.

In the first experiment, the cells received 4 possible stimulations: N (nil), L4 (LPS4h), LL (LPS20h+LPS4h) and ILL (IFNγ/LPS 20h+LPS4h) (see Figure 3.1A). LL macrophages showed a gene expression profile more similar to the N condition than to the L4, with a reduction in the gene expression of the pro-inflammatory mediators (e.g. TNFα and IL6), the protein levels of which were also reduced in the cell culture medium. IL1β did not appear tolerisable, while IL10 (a cytokine that has already been demonstrated as dispensable in the development of ET), was expressed at higher levels by the L4 cells. A general reduction in the expression of M1 markers was observed in the LL stimulated cells which, broadly speaking, acquired a phenotype similar to M2-polarised macrophages. In the ILL treatment, the tolerising action of 20 hours pre-treatment with LPS was abolished by the presence of IFNγ, a cytokine able to prevent the establishment of ET, as already described in literature [153].

When comparing the gene expression of the L4-stimulated macrophages and the LL-stimulated macrophages (Figures 3.2-3.5) it appeared clear that the double stimulation with LPS induces a co-ordinated change in the transcriptome.

With the exception of TLR1, 2 and 6 (that form together heterodimers upon activation) all of the TLRs appeared to be expressed at a lower level in the LL macrophages compared with the L4 macrophages. In particular, TLR4 expression was down-regulated in the LL cells as previously described in other models of ET [158, 159]. The M1 chemokines (CCL3, CCL5 and CXCL10) and their receptors (CXCR3 and CCR7) were expressed at lower levels in the LL macrophage. The LL-stimulated cells expressed at higher levels some of the characteristic M2 markers (e.g. CCL2, CD86, IDO1, VEGFA) although the differences were not always statistically significant.

When analysing the gene expression of transcription factors which are differentially expressed in polarized macrophages, there was relatively lower expression of *IRF4, 5* and *8* in LL stimulation, and an increase in the gene expression of *MAFB*. *IRF4* has been described as a M2 macrophage-associated transcription factor [120] but it was expressed in human LL macrophages at lower levels compared with the L4 stimulation while *MAFB,* again a transcription factor for the M2 macrophages was expressed at higher level in the LL stimulation [173]. *IRF5*, which is promotes the expression of classical M1 genes such *IL12B*, *IL23A*, *IL1β*, *TNFα*, *CCL3*, *CCL5*, *CD40*, *CD86* and *CCR7* [161] was expressed at higher levels in the L4 stimulated cells as was *IRF8*, which is also involved in M1 polarization[174].

Finally, LL stimulated macrophages showed an increased expression of some typical ET markers like *IRAK3*, *PTPN6* and *AHR* [166, 167] while other genes usually described associated with ET (*TNFAIP3*, *INPP5D*, *SIGIRR*) were expressed at higher levels in L4 stimulated macrophages. A higher gene

expression of *TREM1* was found in LL stimulated macrophages comparing with the L4 stimulated. TREM1 is a pro-inflammatory mediator associated with a M1 polarization [168] but previous studies have described the induction of TREM1 by LPS stimulation [169] and an increase of *TREM1* gene expression levels in ET [170].

The same in vitro model of ET was then applied to murine bone marrow-derived macrophages (Fig 3.7) and the acquisition of the ET phenotype was determined using TNFα in the culture medium. Consistently with results already demonstrated in human macrophages, *Irak3* gene expression appeared increased after a double stimulation with LPS and so *Ptpn6* gene expression (though the difference was not statistically significant), while *Tnfaip3* appeared expressed at higher levels in the L4 stimulated cells, as it was in the human macrophages.

With the aim to understand which of the two TNFα receptors played the most important role in inducing *Irak3* gene expression WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ bone marrow derived MCSF macrophages were stimulated with LPS 10ng/mL for different time span and qPCR for *Irak3* was then performed. As shown in Figure 3.8, the levels of *Irak3* were lower in both the TNFR1$^{-/-}$ and TNFR2$^{-/-}$ macrophages even though there was a statistically significant difference for only TNFR1$^{-/-}$ macrophages after 12h of LPS stimulation.

To investigate *Irak3* gene expression in a macrophage population more similar to the one that occurs naturally in vivo, instead of that derived in vitro, gene expression was measured in macrophages from the exudate of thioglycolate-induced peritonitis. In the transgenic strains used (TNFR1$^{-/-}$, TNFR2$^{-/-}$ and WT)

there was a substantial reduction of TNFα detected in the culture medium after the induction of ET, but no differences between strains however, the expression of *Irak3* was lower in the TNFR1$^{-/-}$ cells after stimulation with LPS.

3.6.1 Conclusion

In conclusion, a double stimulation with LPS is able to polarise human monocyte-derived macrophages some way toward a M2 phenotype with an increase the expression of ET-associated genes and reduced production/secretion of pro-inflammatory cytokines. The acquisition of the ET phenotype in these cells is inhibited by pre-treatment with IFNγ. This ET bioassay appeared to work equally well on murine M-CSF bone marrow-derived macrophages. Of the two TNFα receptors, TNFR1 appeared to be the one more able to induce expression of *Irak3*, although the lower *Irak3* gene expression in TNFR1$^{-/-}$ macrophages did not induce any relevant modification in terms of ET establishment.

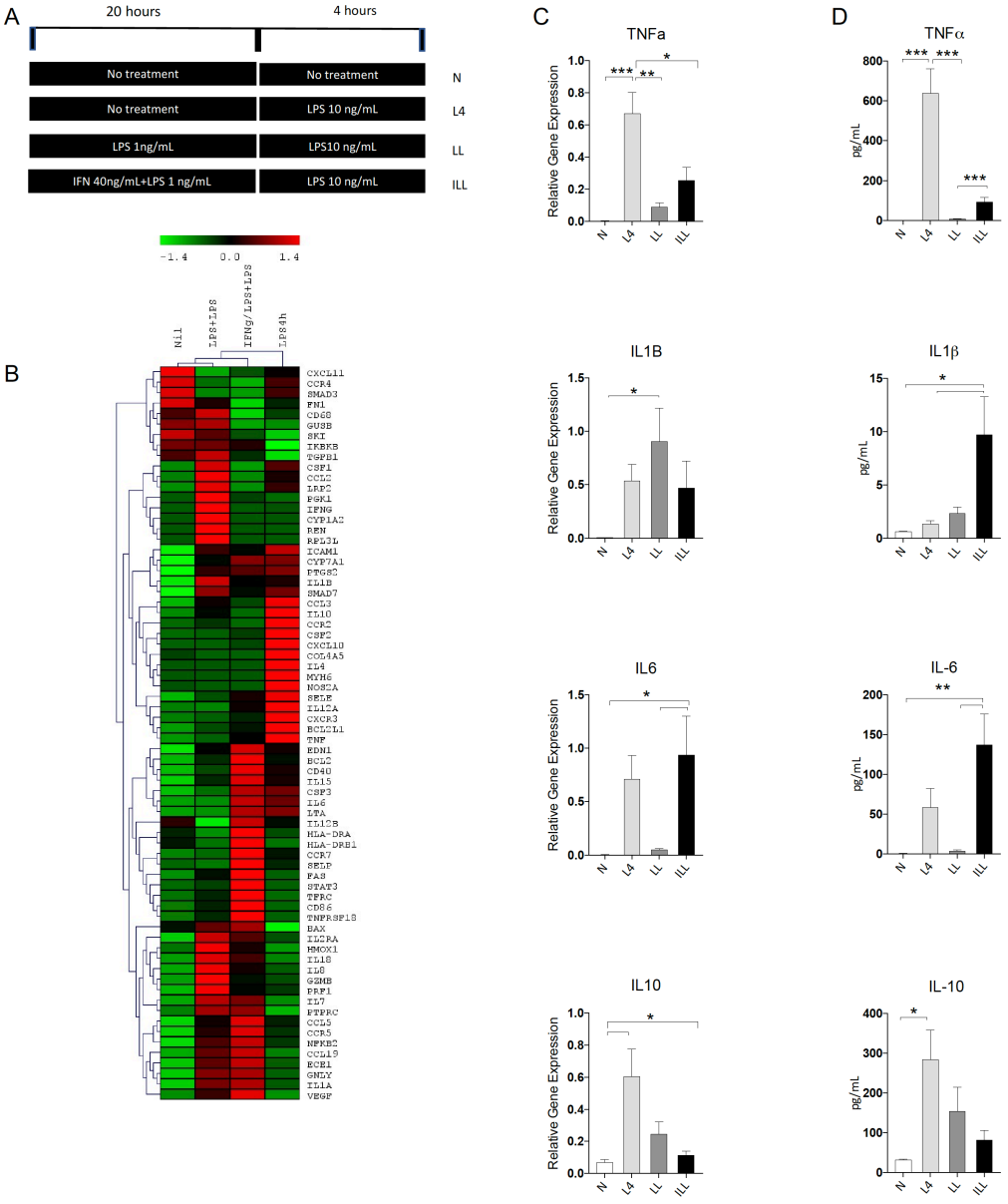

Figure 3.1 Gene expression and cytokine secretion in endotoxin tolerant cells.

Human monocytes were cultured *in vitro* for 7 days in presence of human MCSF at 50ng/mL. (A) The cells were left un-stimulated (N), challenged with a single 4 h LPS stimulation (L4), a 4 h LPS stimulation preceded by a 20 h dose of LPS alone(LL) or in the presence IFNγ (ILL). (B) The gene expression of pro-inflammatory and immuno-regulatory mediators is shown as a heat map; values are row normalised means of n=4 donors. (C) Gene expression of the principal inflammatory mediators, with the cell culture medium levels of the same cytokines assessed through Mesoscale Discovery analysis show in (D); values are mean±SEM, ordinary one-way ANOVA with Tukey's multiple comparison test, * $p<0.05$, **$p<0.005$, *** $p<0.0005$.

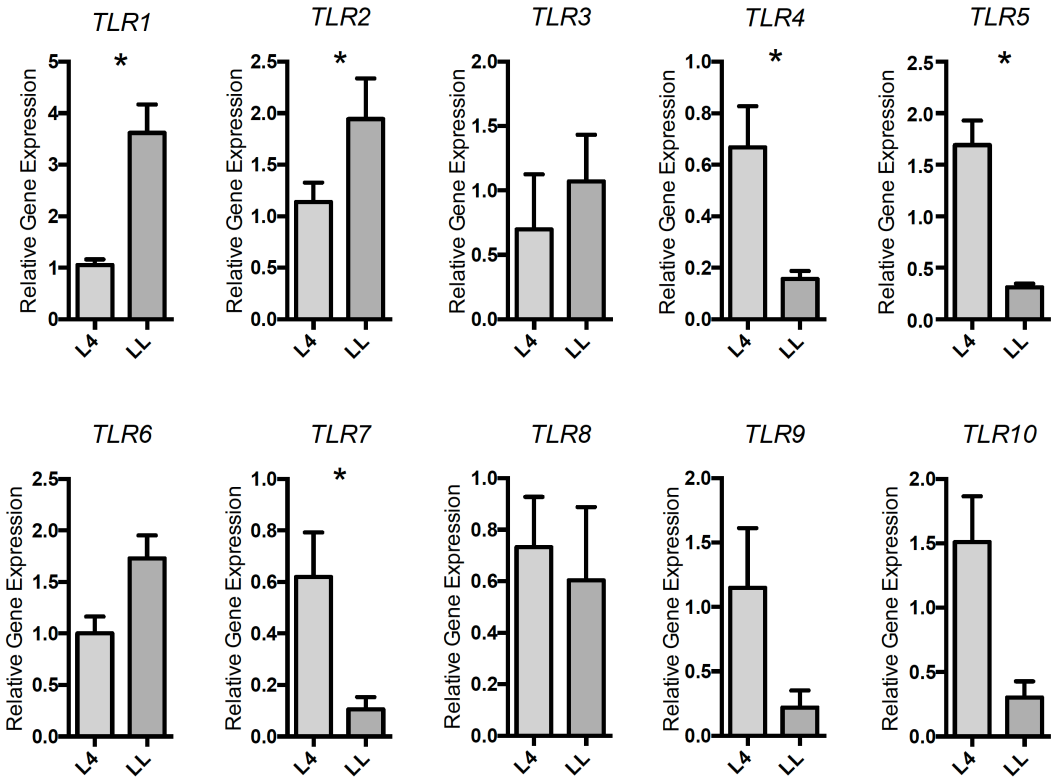

Figure 3.2 ET modifies TLR gene expression. Macrophages were stimulated with a single dose of LPS 10ng/mL for 4 h (L4) or double stimulated (LL) with two different doses of LPS (1ng/mL for 20 h followed by 10ng/mL for further 4 hours); values are mean±SEM, Student's t-test, * $p<0.01$ (n=4).

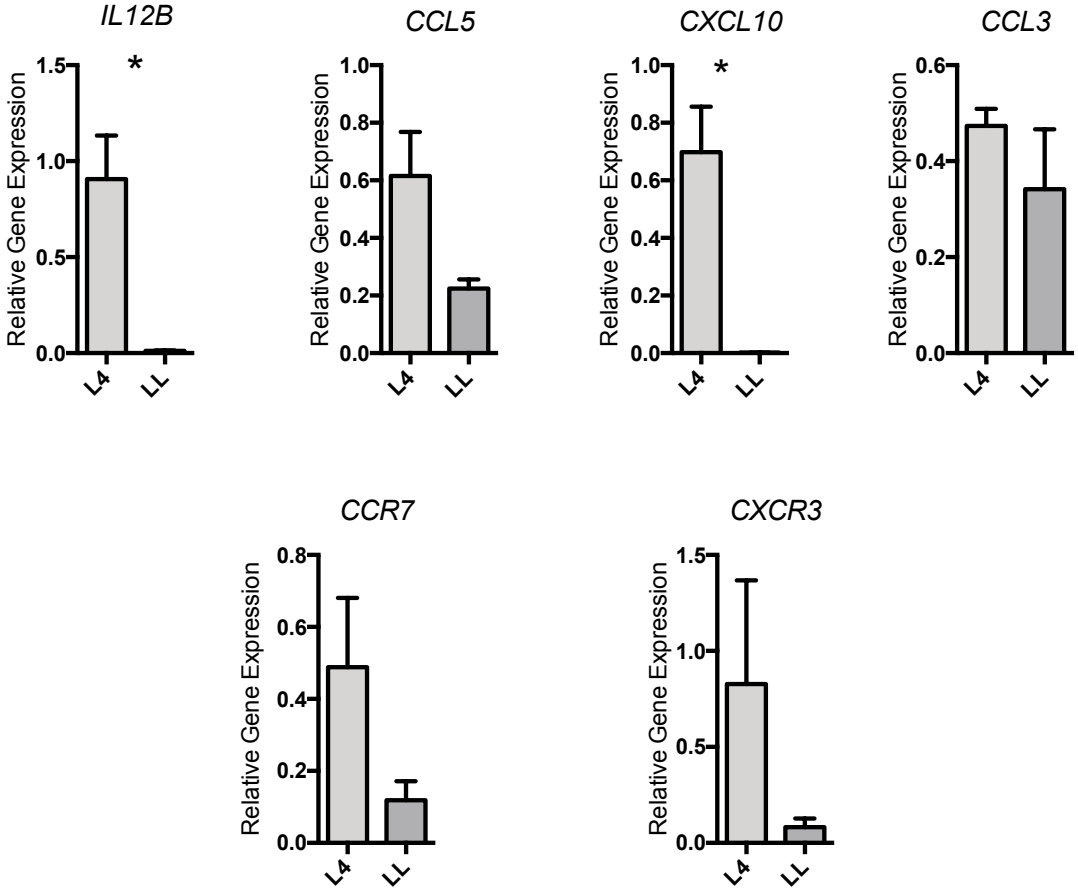

Figure 3.3 ET bioassay expression of M1 markers.
Macrophages were stimulated with LPS for 4 h alone (L4) or for 4 h after a 20 h pre-treatment with LPS (LL); values are mean±SEM, Student's t-test, * $p<0.01$ (n=4).

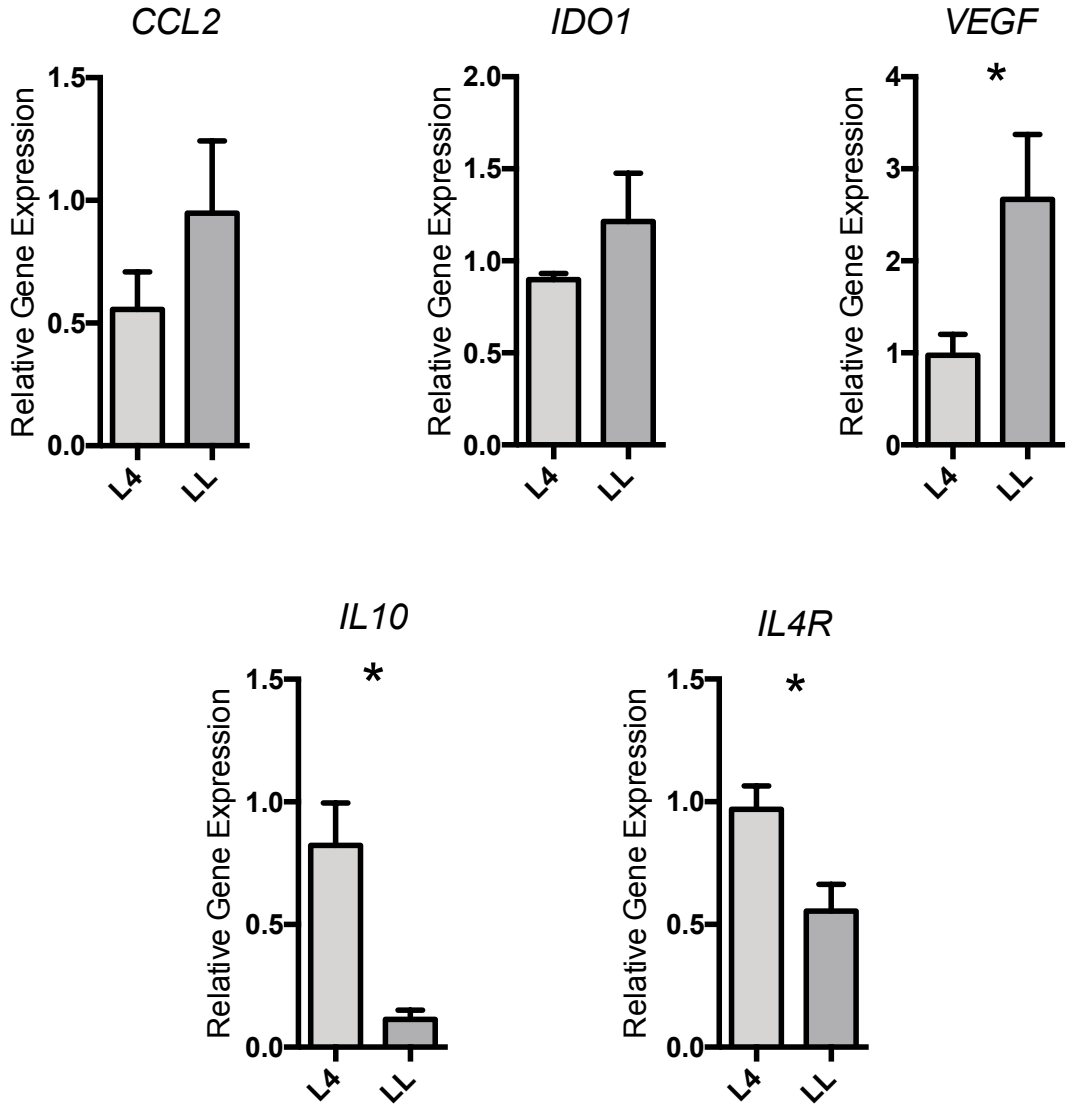

Figure 3.4 ET bioassay expression of putative M2 markers.
Macrophages were stimulated with LPS for 4 h alone (L4) or for 4 h after a 20 h pre-treatment with LPS (LL); values are mean±SEM, Student's t-test, * $p<0.01$ (n=4).

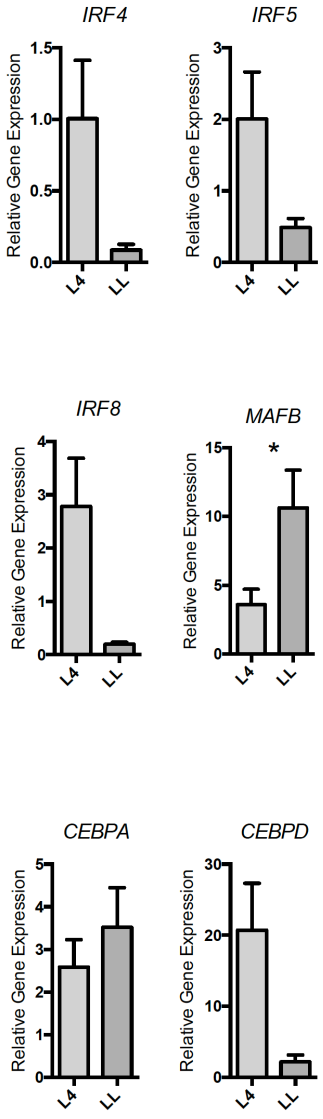

Figure 3.5. ET bioassay expression of M1/M2-associated transcription factors.
Macrophages were stimulated with LPS for 4 h alone (L4) or for 4 h after a 20 h pre-treatment with LPS (LL); values are mean±SEM, Student's t-test, * $p<0.01$ (n=4).

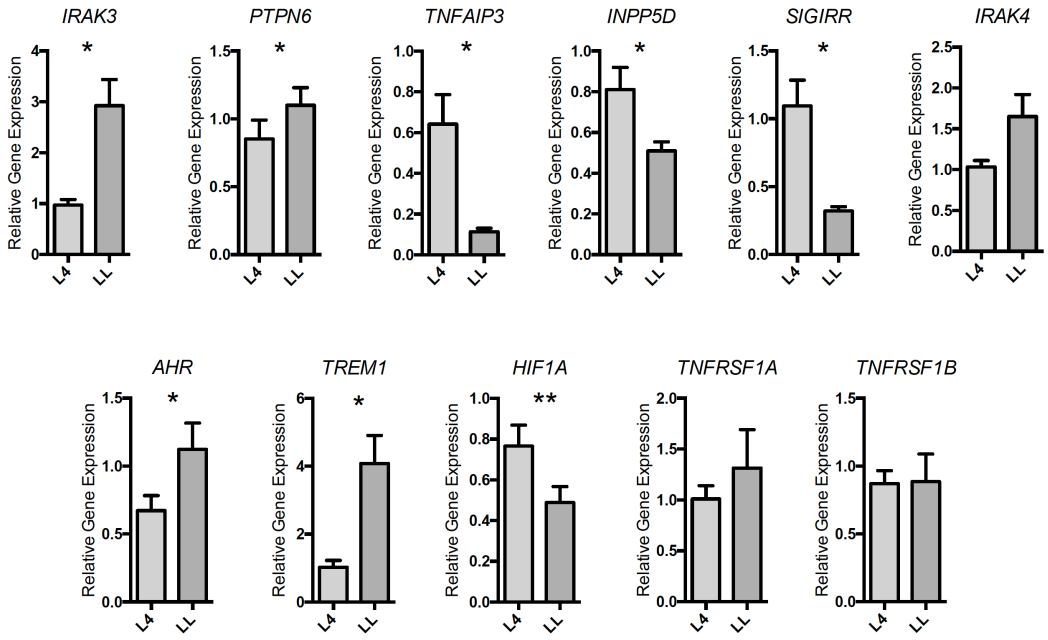

Figure 3.6 ET bioassay expression of ET-associated genes.
Macrophages were stimulated with LPS for 4 h alone (L4) or for 4 h after a 20 h pre-treatment with LPS (LL); values are mean±SEM, Student's t-test, * p<0.01, **p<0.005, (n=4).

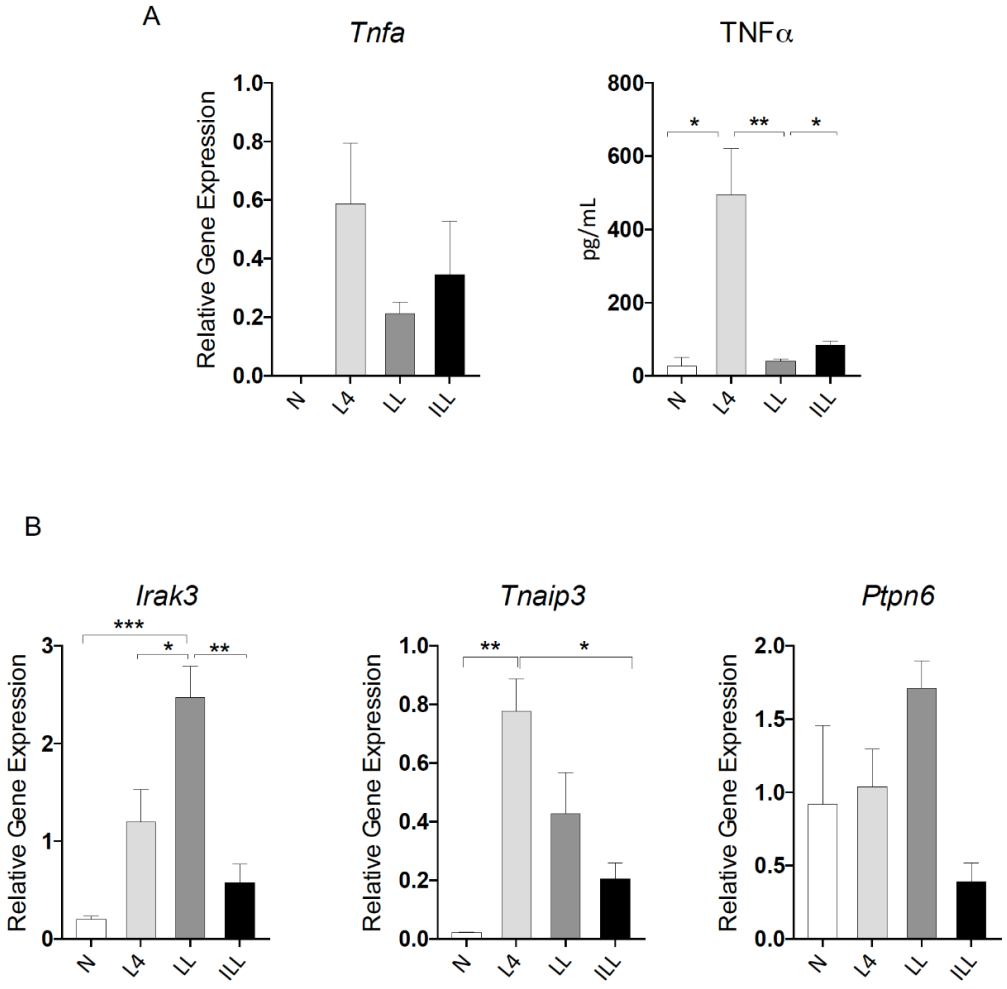

Figure 3.7. ET model in BMM derived MCSF murine macrophages.
Murine bone marrow-derive macrophages were differentiated in vitro for 7 days in presence of MCSF. The cells were left un-stimulated (Nil, N), challenged with a single 4 h LPS stimulation (L4) or a 4 h LPS stimulation preceded by an 20 h dose of LPS alone (LL) or in the presence IFNγ (ILL).(A) *Tnfa* gene expression and TNFα levels in the cell culture medium. (B) Differential expression of ET-associated genes, *Irak3*, *Tnfaip3* and *Ptpn6*); values are mean±SEM, ordinary one-way ANOVA multiple comparison test, * p<0.05, **p<0.005, *** p<0.001.

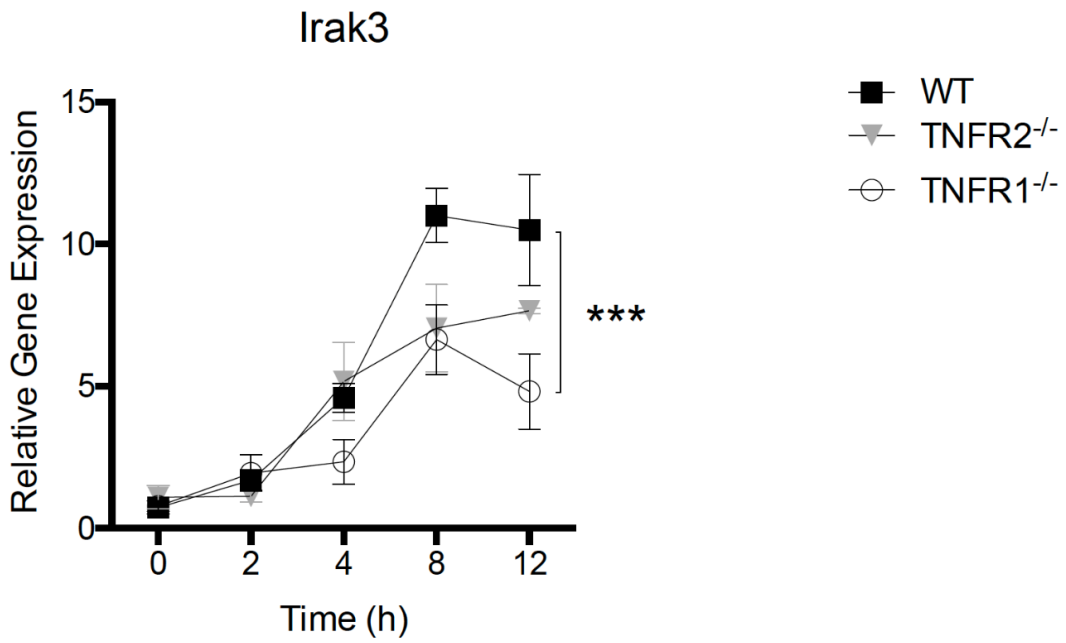

Figure 3.8. TNFα signals predominantly through TNFR1 to induce Irak3 expression
The gene expression of IRAK3 was assessed by qPCR on bone marrow derived MCSF macrophages from TNFR1 $(p55)^{-/-}$, TNFR2 $(p75)^{-/-}$ and WT mice stimulated for different with LPS 10ng/mL; values are mean±SEM, ordinary one-way ANOVA with Tukey's multiple comparison test, ***p<0.001, (p55 vs WT). N=3 independent experiments.

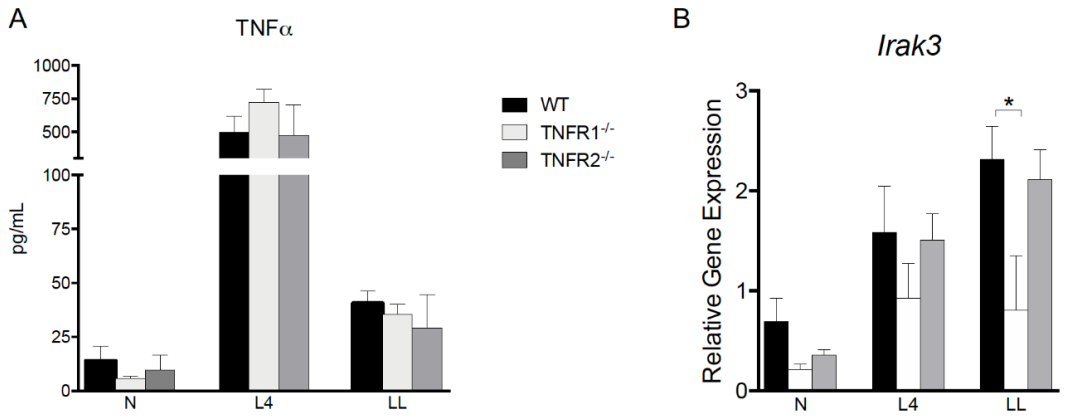

Figure 3.9. *Irak3* gene and protein expression in peritoneal macrophages
Thioglycolate-induced peritonitis macrophages were stimulated in vitro with a single or double dose of LPS. (A) TNFα levels were assessed by ELISA of the cell culture medium. (B) *Irak3* gene expression was assessed by qPCR; Ordinary one-way ANOVA with multiple comparison test, *p<0.05.

Chapter 4 Endotoxin Tolerance-associated mechanisms in Experimental Arthritis

4.1 Introduction

The therapeutical management of RA is based on the use of two different classes of disease-modifying anti-rheumatic drugs (DMARDs): the synthetics (like methotrexate, sulfasalazine, leflunomide and tofacitinib) and the biologics [38].

Among the latter, anti-TNFα agents (adalimumab, certolizumab, etanercept, golimumab and infliximab) are widely used but not all the patients respond to them achieving remission.

Bunch et al. demonstrated in 2007 [80] that up to 25% of patients failed to achieve any response to anti-TNFα within 12 weeks of starting therapy and among the responding group 55% of patients stop responding within the first year of therapy. Furthermore, among responding patients the use of anti-TNFα is associated with an increase in Th17 cell number [81-85]. This could be just the consequence of a redistribution of inflammatory cells from the inflamed joint, as anti-TNFα can induce a change in chemokines expression [84], or an altered balance Tregs/Th17 induced by anti-TNFα [85].

On the basis of these observations it appears evident that TNFα is not the only mediator responsible for the inflammatory process in RA but other factors are capable of driving the disease, as confirmed by the efficacy of biologics targeting other inflammatory molecules (for example tocilizumab to block IL6 [175, 176] and anakinra to target IL1β [177]).

The increase in Th17 cells prevalence consequent to anti-TNFα therapy, suggests an immuno-regulatory role for TNFα. As there is an increase in the number of Th17 cells, the mediators that promote their differentiation, survival and phenotypic stability (such as IL23, IL6 and IL1β [178]) must also be increased. Evidence supporting this hypothesis was published by Notley et al who showed that the

increase in Th17 cells in arthritic mice treated with anti-TNFα had higher levels of IL12B (the common subunit of IL12 and IL23) [86]; subsequent studies have confirmed this hypothesis [179].

TNFα is important for the establishment of ET and is probably responsible for much of its immuno-regulatory action in myeloid cells. TNFAIP3 gene expression is induced by TNFα [180] while PTPN6 is expressed at constant levels and translocates to different cellular compartments in response to inflammatory stimulation [141]; these molecules have both been demonstrated to be involved in inhibiting macrophage activation in response to LPS [114, 141, 181]. The relationship between IRAK3 and TNFα has been previously investigated and TNFα appears to be a potent inducer of IRAK3 expression [142]. Alteration of IRAK3 expression has been found to be associated with cancer [144], infections [182] and autoimmunity, in particular diabetes [183] and lupus [184].

4.2 Experimental Rationale

To investigate if an alteration in ET-associated molecules was associated with the development and status of disease, an in vivo model of chronic inflammatory disease was used. In the first experiment, to understand if the expression of ET-associated molecules was different in association with the stage of the disease, the animals were euthanized at different time points with the objective to map ET-associated gene expression at different stages of disease. In a subsequent CIA, the animals were treated with anti-TNFα, to determine if the blockade of TNFα modified ET-associated gene expression.

DBA/1J mice (8 weeks old) were immunized as previously described [139]. After roughly 14 days from the immunisation, the animals started to develop the disease and a clinical score ranging from 0 to 3 was assigned to each paw according to the presence of signs of inflammation. The samples were harvested as shown by the experiment layout in Figure 4.1.

Naïve mice were used as a control to compare to ET-associated genes expressed in the physiological steady state. The T cell expansion phase, during which T cells encounter their antigen and accordingly expand [185], has an average length of 14 days [9, 186]; the time points PI-5 (Post-Immunisation day 5) and PI-10 (Post-Immunisation day 10) were chosen to assess the targeted gene expression at the beginning and toward the end of the T cell expansion phase. The paws were harvested at the beginning (O-1) and at the end of the inflammatory process (CIA 0 and 3). Unaffected paws (CIA 0) were collected and investigated because despite the absence of clinical signs they are still affected by inflammatory mediators derived from arthritic lesions via the blood.

The clinical score of each paw was recorded every day for 10 days after the onset of the disease and the average global clinical score (resulting from the sum of each paw clinical score) is showed in Figure 4.2A.

4.3 Characterisation of innate inflammatory pathways in CIA

4.3.1 TNFα up-regulation in affected CIA paws

To confirm the pathological relevance of TNFα, gene expression was measured through qPCR in unaffected (clinical score 0) and affected (clinical score 1-3)

paws and then compared with naïve paws. Figure 4.2B shows the difference between the naive group and, in arthritic mice, the affected and unaffected paws. Naive and arthritic but unaffected paws had similar levels of TNFα gene expression but there was a statistically significant difference compared to affected paws in arthritic mice.

4.3.2 Macrophage-lineage cells accumulate in arthritic paws

As ET is a phenotype of macrophage-lineage cells, the proportion of these cells in the joints of mice with differing disease status was measured. Cells extracted from CIA paws were stained with antibody for CD115 and analysed by flow cytometry; CD115 is the receptor for M-CSF and is expressed on monocytes and macrophages. As anticipated, affected paws from arthritic mice had a higher proportion of $CD115^+$ cells after 10 days of disease, comparing with the unaffected paws from the same mice (Figure 4.3); affected and unaffected paws harvested on day 1 of disease onset showed similar levels of $CD115^+$ cells.

4.3.3 TLR expression is dependent on disease status in CIA

As already discussed, the different TLRs play a role in the initiation and in the perpetuation of the disease [187]. They are also responsible of the establishment of the tolerant state discussed in Chapter 3. The gene expression levels of the different TLRs were assessed by qPCR (Figure 4.4). All the TLRs were expressed at significantly different levels among the different time points considered. TLR1, 2, 4 and 7 were expressed at higher levels in the CIA affected paws group than in the naïve control, although the difference was not always statistically significant. TLR5, 6, 8 and 9 were expressed at lower levels. TLR3 was the only one expressed at similar levels in both CIA affected paws and naïve paws. With the exception of TLR1 and 2 which showed an increased expression up to the first day

of onset, (O-1), all the other TLRs followed the same trend of expression, with a progressive reduction that reached its lowest in the CIA 0 group.

To summarise, a global increased in TLR gene expression appeared associated with the later stage of the disease in affected paws while in the absence of clinical signs (in CIA 0) was associated with a reduced expression of all TLRs.

4.3.4 ET-associated gene expression in CIA

Having mapped the kinetics of the cells required for ET, the TLRs required for the initiation of ET and the putative mediator required for the induction of ET-associated intracellular molecules (TNFα), the expression of ET-associated molecules was then measured at different stages of disease by qPCR. As shown in Figure 4.5, the kinetics of *Tnfaip3* and *Ptpn6* expression differed from that of *Irak3*. While *Tnfaip3* and *Ptpn6* levels at the beginning and at the end of the inflammatory process were similar, *Irak3* was reduced soon after immunization, remaining stably lower than the naïve group up to at least 10 days post-onset (Figure 4.5). These results were unexpected since they show lower levels of *Irak3* in an environment enriched with $CD115^{+}$ cells and with relatively higher levels of TNFα.

4.4 Anti-TNFα modulates the expression of ET associated genes in CIA

Blocking TNFα in vitro in the presence of TLR ligands prevents the development of ET and reduces IRAK3 [188] and selectively blocking TNFR signalling in macrophages lacking either TNFR1 or TNFR2 led to lower expression of *Irak3* (Chapter 3, Figure 3.8) compared to WT macrophages. To further delineate the

relationship between inflammation and ET-associated mechanisms of immunomodulation, arthritic mice were treated with anti-TNFα.

At the onset of the disease the animals were treated with an intra-peritoneal injection of 3 mg/Kg of etanercept (Enbrel™, Amgen/Pfizer) or vehicle every other day for a total of 5 treatments. The animals treated with etanercept showed a less severe disease with a lower global clinical score than the PBS treated animals (Figure 4.6). While the number of paws affected was similar between the two groups, the paws from treated mice had reduced swelling as indicated by a reduced paw width compared with the PBS-treated paws (Fig. 4.6C). The treated group had a reduced width in the first affected paws although the difference was not statistically significant (Fig. 4.6D).

To confirm the previously described effect of anti-TNFα on Th17 cells [86], lymphocytes from the inguinal lymph nodes were analyzed for intracellular cytokine expression by flow cytometry (Figure 4.7); see Materials and Methods.
The proportion of $CD4^+IFN\gamma^+$ and $CD4^+IL17^+$ cells (Figure 4.7B) in lymph nodes from Etanercept-treated mice was higher than in PBS-treated mice, although statistically significant only for $CD4^+IL17^+$ cells; the number of $CD4^+Foxp3^+$ cells was similar in the two groups. The percentage of $CD8^+IFN\gamma^+$ in lymph-nodes from Etanercept-treated mice was 5 times greater than in PBS-treated group. This data was consistent with previous observations that anti-TNFα therapy improves the disease clinically but is associated with an increase in Th17 cells [179].

The expression of ET-associated genes was then assessed by qPCR in the paws, spleens, lymph nodes and blood. The only significant difference in *Irak3* gene expression was an increase in Etanercept-treated lymph nodes (Figure 4.8). *Tnfaip3* was expressed at higher levels in the Etanercept group in the paws, spleen and blood while *Ptpn6* was expressed at higher levels in the Etanercept group in the lymph nodes.

4.5 Discussion

On the basis on the literature cited in Chapter 1 and the experiments shown in Chapter 3, it appears clear that there is a mechanistic relationship between IRAK3 and TNFα; the immuno-regulatory action of TNFα is likely to be due in part to its induction of IRAK3 expression. While in vitro experiments generally support this hypothesis, the heterogeneous milieu of inflamed synovial joints and lymphoid organs contain many confounding factors which may modulate the effect of TNFα.

To investigate the association between TNFα and ET-associated gene expression in an in vivo model that relies heavily on TNFα, *Tnfaip3*, *Ptpn6* and *Irak3* were measured after confirming in the joints of arthritic mice the kinetics of macrophage-lineage cells, the expression of TLRs and expression of TNFα.

4.5.1 Kinetics of ET-associated gene expression in CIA

With the aim to map the expression of ET-associated genes to a specific time point during the establishment of the disease the first CIA was performed, by sacrificing the animals 5 and 10 days post immunisation and then on day 1 and day 10 post-onset.

In the un-treated CIA, the gene expression levels of *Tnfaip3* and *Ptpn6* followed a very similar trend, with a low expression in all conditions except for affected paws at 10 days post-onset and in the naive paws (Figure 4.5). In contrast, *Irak3* gene

expression levels remained lower than the naive control through the whole experiment. This finding was unexpected for the higher levels of TNFα gene expression and prevalence of $CD115^+$ in the arthritic paws.

CIA is a TNFα driven disease but other pro-inflammatory cytokines, such as IFNγ and GM-CSF which can attenuate the establishment of ET, are also produced [151]. The low *Irak3* gene expression level could then be either a consequence of the strong M1 polarization state of the macrophages which in turn may be the reason responsible of the perpetuation of the disease, despite the up-regulation of *Tnfaip3* and *Ptpn6*, which do not appear able to control the inflammatory process.

This un-expected finding prompted to further investigate the association between IRAK3 and TNFα to understand if the link between TNFα levels and IRAK3 gene expression was preserved even during the pathological condition. This was investigated firstly by TNFα blockade in arthritic mice.

4.5.2 ET-associated gene expression in TNFα blockade

CIA was induced in DBA/1J mice and the animals were treated at the onset of disease with PBS or Etanercept. As expected on the basis of the literature and previous observations [75], the group treated with etanercept showed an improvement in the disease symptoms with reduced clinical severity comparing with the PBS treated group (as showed by the clinical data and by the histology) but such improvement was associated, as previously described by Williams et al[86], to increased levels of Th17 cells in the lymph-nodes of the etanercept-treated group.

ET-associated gene expression levels were measured by qPCR in paws, spleen, lymph-nodes and blood of PBS and etanercept-treated animals. No significant difference was found between the two groups in term of *Irak3* gene expression with the exclusion of a higher gene expression in etanercept treated lymph-nodes. Broadly speaking all of the genes measured (*Irak3*, *Tnfaip3* and *Ptpn6*) were expressed at higher levels in the etanercept treated group, though not all reached statistical significance (Figure 4.8).

4.5.3 Conclusion

In the light of the experiments described in this chapter it appears evident that an inadequate activation of ET mechanisms may play role in the pathogenesis of CIA due to the modulation of gene expression of ET-associated genes. The fact that *Irak3* remained permanently low in the CIA tissues, toward the end of the post-onset period when *Tnfaip3* and *Ptpn6* were up-regulated, is of particular interest considering the already demonstrated involvement of this molecule in several different inflammatory conditions. The fact that anti-TNFα therapy did not modify *Irak3* gene expression in the paws may be ascribable to the fact that CIA is associated with low *Irak3* gene levels at an early stage of disease and that, thus, the very low *Irak3* levels cannot be further reduced by anti-TNFα therapy.

There are indications of a possible role of IRAK3 in RA [189] therefore with all its limitations the animal model still represents a valid opportunity to further investigate the role of IRAK3 using knock-out animals to allow us to dissect its action.

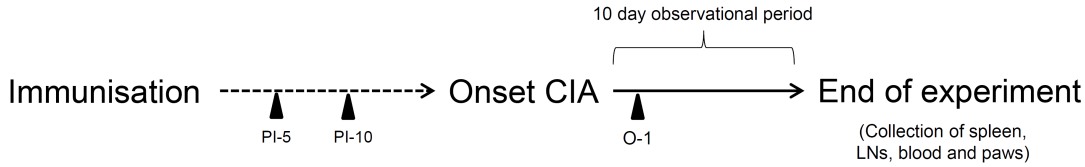

Figure 4.1. Experiment layout. 8 weeks old DBA mice were immunised with type II bovine collagen and **s**pleen, Lymph-nodes and paws were collected at different time-points.

(PI-5:post-immunisation day5; PI-10:post-immunisation day 10; O-1: onset day; end of experiment: collection of samples from all the remaining CIA animals)

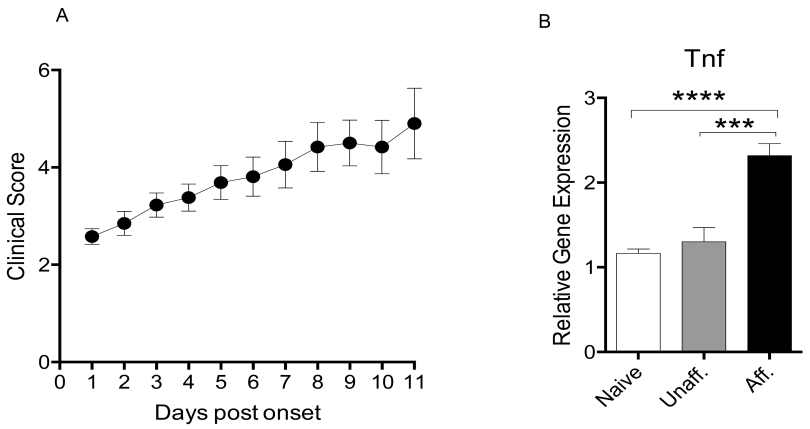

Figure 4.2. Collagen-induced arthritis: clinical progression and TNFα levels.

(A) Average of the global clinical score (sum of all paws) recorded during the observation period. (B)*Tnf* gene expression was assessed by qPCR in unaffected (clinical score 0) and affected (clinical score 1-3) paws from arthritic mice 10 days after onset and compared with paws from unimmunised, naïve mice; one way ANOVA with Kruskal-Wallis multiple comparison test. Values are mean±SEM; ** p<0.01, *** p<0.005, ****p<0.0001

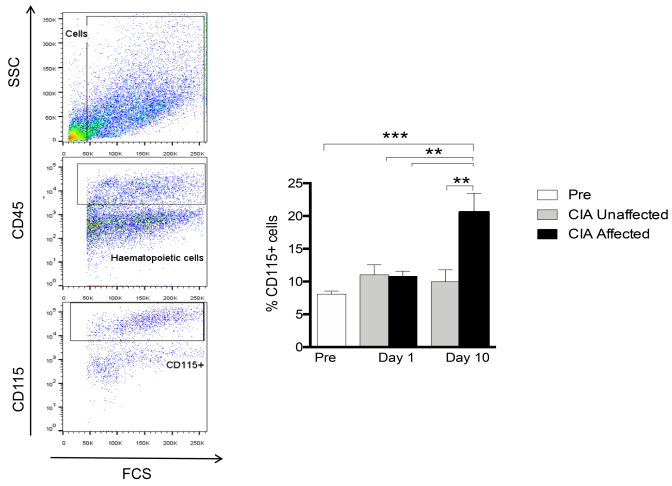

Figure 4.3. Collagen-induced arthritis: macrophage infiltration.

The proportion of macrophage-lineage cells ($CD115^+$) was measured in the paws by flow cytometry in mice prior disease onset (Pre), on the first day of arthritis (Day 1) and 10 days after disease onset (Day 10); one-way ANOVA with Tukey's multiple comparison post-test. . Values are mean±SEM; ** p<0.01, *** p<0.005, ****p<0.0001

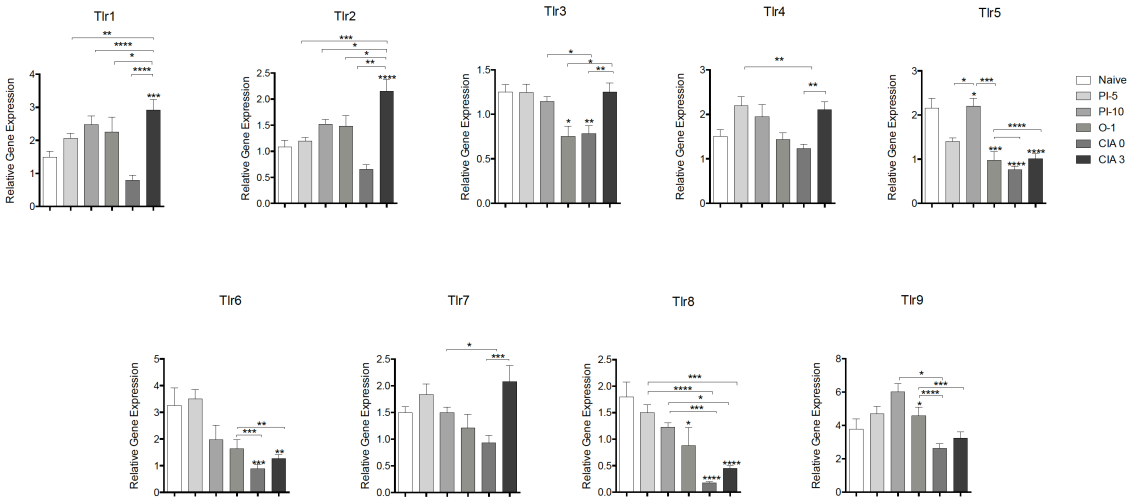

Figure 4.4. TLR gene expression is dependent on disease status in CIA.
The expression of *Tlr1-9* was measured in unimmunised mice (Naïve) and mice at 5 and 10 days post immunisation (PI-5 and PI-10, respectively). In arthritic mice, the arthritic paw from mice euthanized on the day of arthritis onset (O-1) was assessed, as were as the affected (CIA 3) and unaffected paws (CIA 0) 10 days after onset. Values are means±SEM, n≥12; one-way ANOVA with multiple comparison, * p<0.05, ** p<0.01, *** p<0.005, ****p<0.0001. Stars located on top of the columns are relative to the Naïve control.

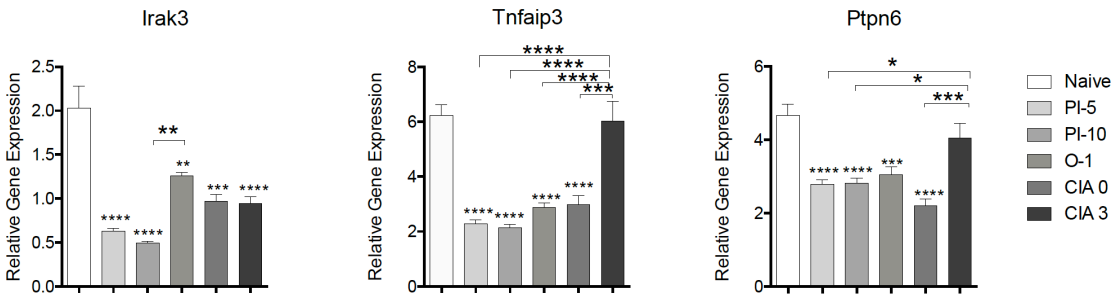

Figure 4.5. Gene expression of Irak3, Ptpn6 and Tnfaip3 in CIA.
The expression of Irak3, Ptpn6 and Tnfaip3 was measured in unimmunised mice (Naïve) and mice at 5 and 10 days post immunisation (PI-5 and PI-10, respectively). In arthritic mice, the arthritic paw from mice euthanized on the day of arthritis onset (O-1) was assessed, as were as the affected (CIA 3) and unaffected paws (CIA 0) 10 days after onset. Values are means±SEM, n□12; one-way ANOVA with multiple comparison, * p<0.05, ** p<0.01, *** p<0.005, ****p<0.0001. Stars located on top of the columns are relative to the Naïve control.

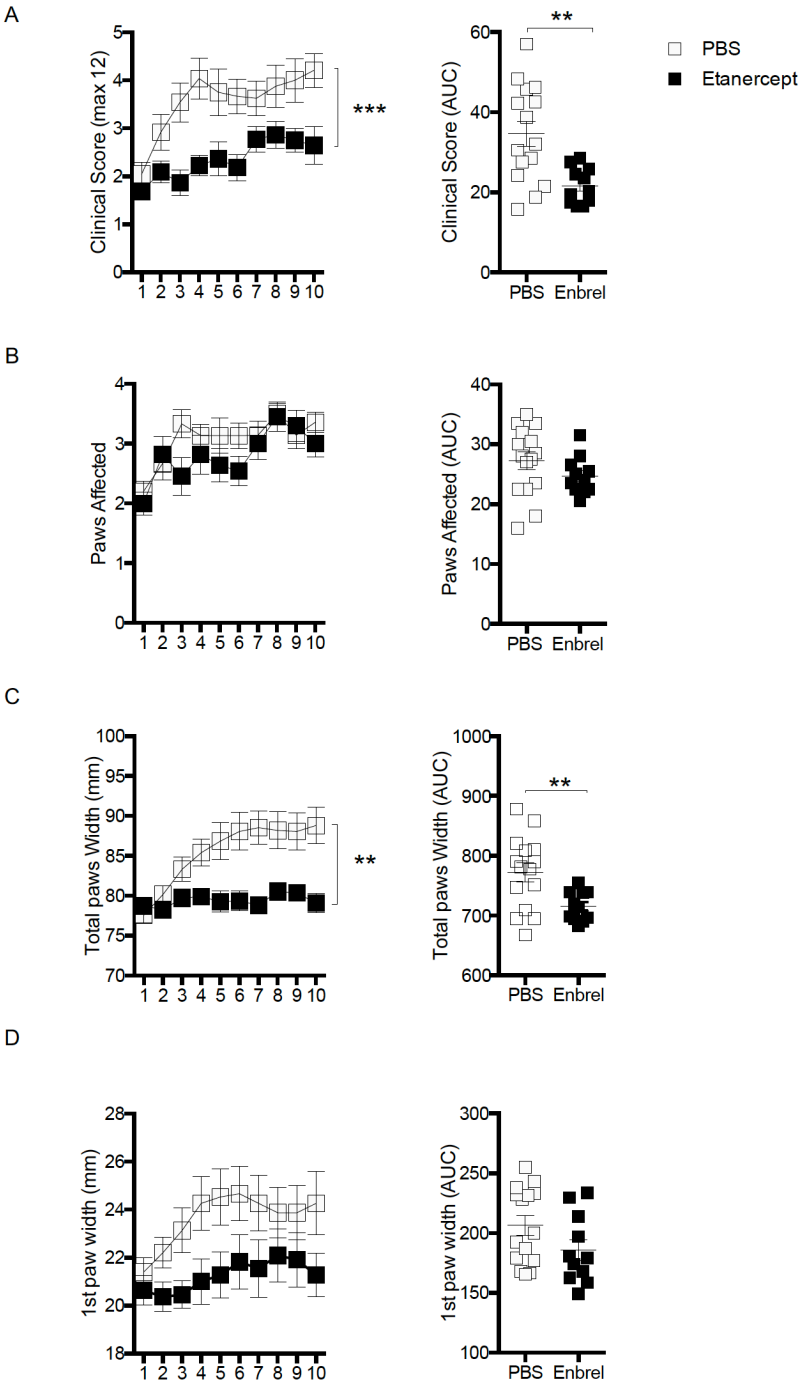

Figure 4.6. Etanercept-treated CIA DBA/1J mice.
Daily clinical data (left side) and calculated Area Under the Curve (AUC, right side) for individual mice. All the clinical data was collected during the post-onset period (10 days). (A) Total clinical score of Etanercept-treated versus PBS treated mice. (B) Number of affected paws. (C) Total combined width of all paws in mm. (D) Width of the first-affected paws in mm. Values are means±SEM. Daily clinical data 2-way ANOVA, ***p<0.0001, **p=0.0018. AUC, Student's t-test ** p=0.008.

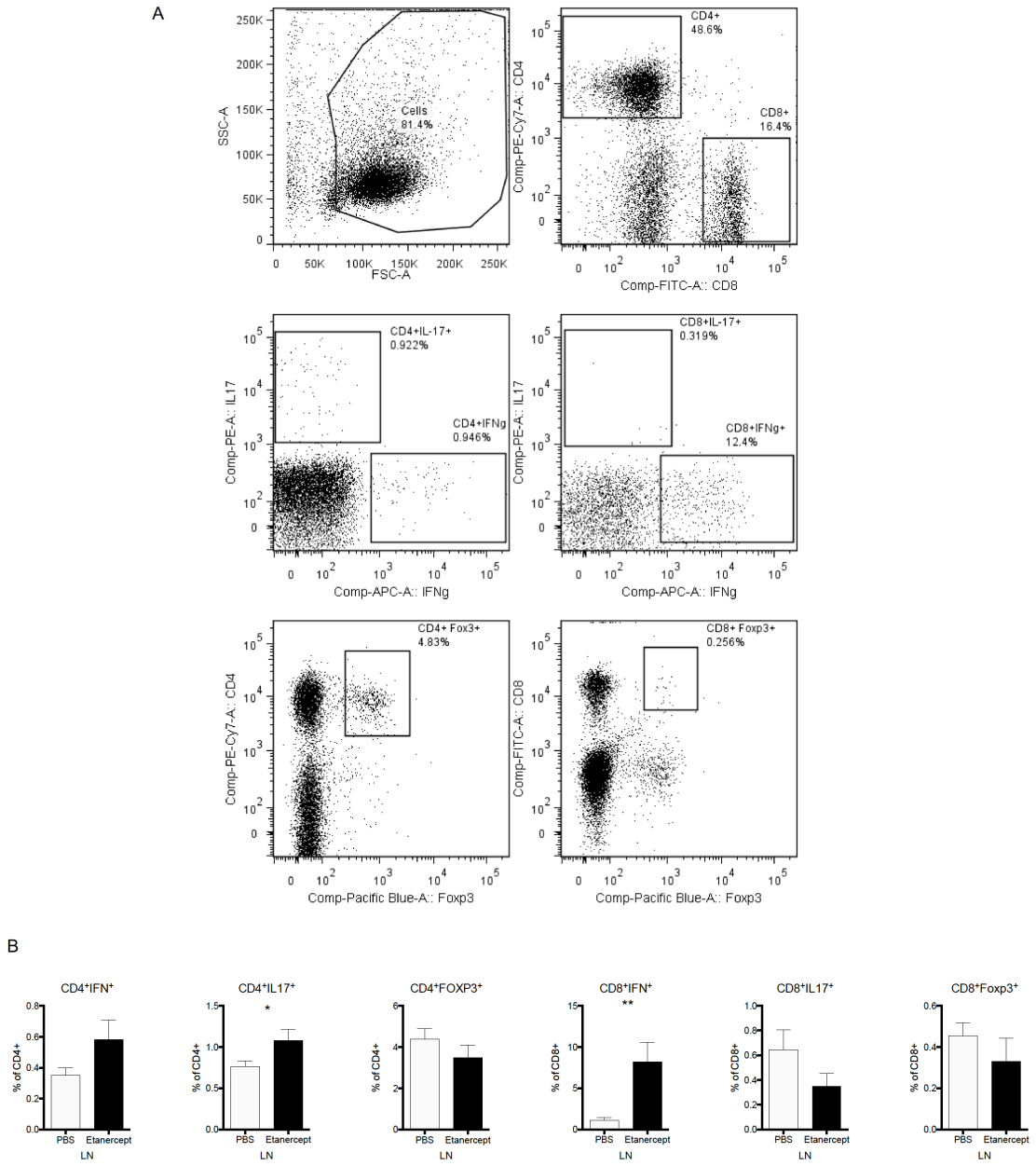

Figure 4.7. Lymph-nodes from Etanercept-treated mice have an increased proportion of Th17.

(A) The gating strategy for functionally-defined lymphocytes. (B) The percentage of subsets of $CD4^+$ and $CD8^+$ cells, defined by expression of IFNγ, IL-17 or FoxP3; values are means±SEM, Student's t-test, *p<0.05,

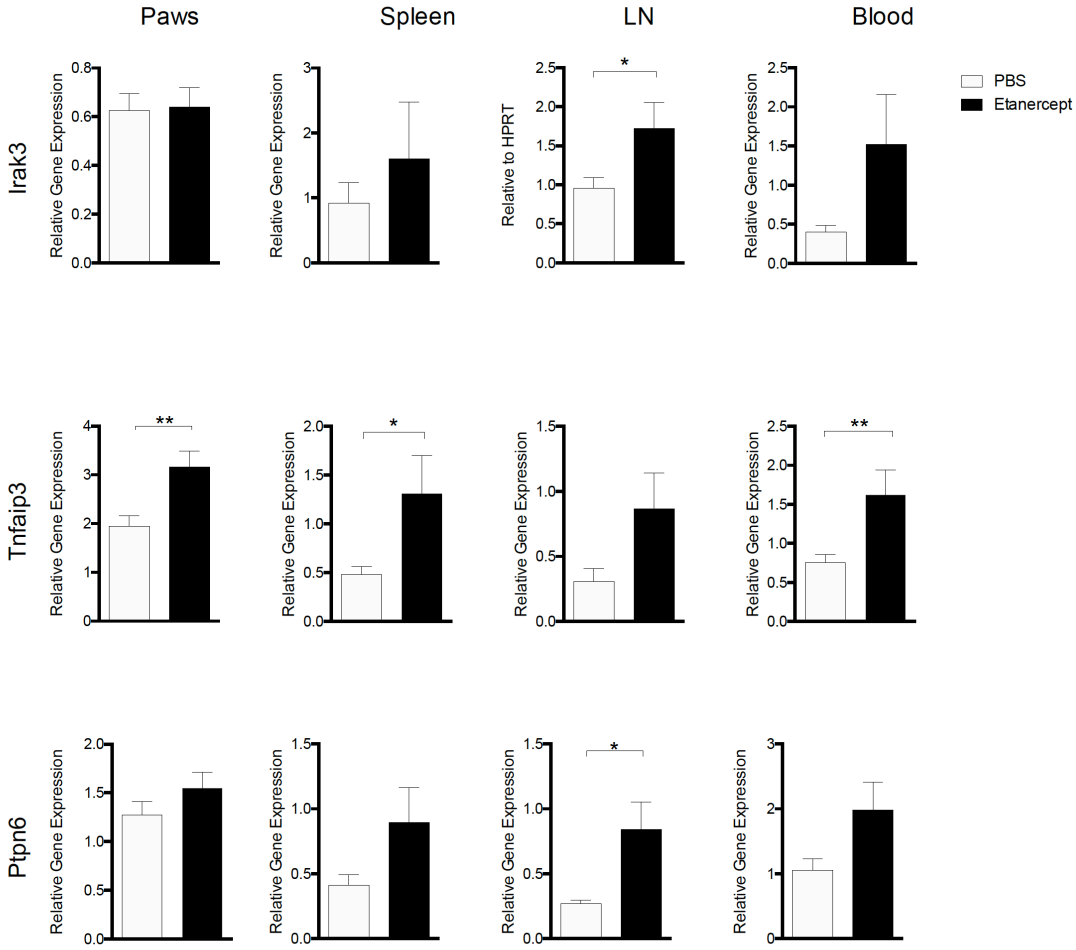

Figure 4.8. Modulation of ET-associated gene expression by Etanercept in CIA. Gene expression of Irak3, Ptpn6 and Tnfaip3 was assessed in paws, spleen, lymph-node (LN) and blood of arthritic mice treated with Etanercept or vehicle; values are means±SEM, Student's t-test, *p<0.05, ** p<0.01.

Chapter 5 : IRAK3 deficiency accelerates experimental arthritis

5.1 Introduction

IRAK3 is a member of the Interleukin 1 receptor-associated kinase family. The gene is located on human chromosome 12 at position 12q14.1-12q15, while on the mouse is on chromosome 10. Both mouse and human genes encode for a protein of 68kDa. Murine IRAK3 shows 71% sequence identity with human IRAK3 at the protein level. Different transcription factors are reported to be involved in inducing IRAK3 expression (including NFκB, AP-1 and CREB [190], PU-1 [191] and C/EBPβ [192]) and are recruited for transcription at different stages of the inflammatory process [192]. While human IRAK3 expression is mainly confined to macrophage-lineage cells, murine IRAK3 has a broader expression pattern and has also been reported in neutrophils, B cells, epithelial intestinal cells, alveolar macrophage and Kupffer cells [193, 194].

5.1.1 IRAK3 modulates experimental signalling

IRAK3 structure includes an N-terminal death domain (DD), which is required to bind with other IRAK family members (and MyD88), a central serine/threonine kinase domain (with 12 subdomains) and a C-terminal domain with a TRAF6 binding motif [195]. Although IRAK3 shows between 30-40% of similarity with IRAK4 (and IRAK2) and is able to interact with MyD88 and TRAF6, it is unable to undergo auto-phosphorylation [35, 63]. IRAK3 has no kinase activity and it exerts its action by binding the IRAK1-IRAK4 complex, which prevents dissociation of IRAK1 from the complex and subsequent interaction with TRAF6 [195]. In this way, it exerts a negative regulatory role in TLR/IL1R signalling. IRAK3 is ubiquitous throughout the cell and becomes exclusively cytoplasmic upon TLR stimulation [196].

IRAK3 expression has been most comprehensively studied during LPS-TLR4 stimulation in the context of ET but IRAK3 expression can be induced by different TLR ligands [196]. As different TLRs (notably TLR2, 4, 7 and 9) share common signalling pathways, the induction of cross-tolerance by one ligand for another is possible. Thus, IRAK3 has been described to be involved in cross-tolerance to TLR4 and TLR9 ligands [160]. Furthermore, its up-regulation after TLR7 stimulation has been associated with increased levels of other ET molecules (e.g. SOCS-1; the phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1 encoded by gene INPP5D and also known as SHIP-1; TNFAIP3) [197]. IRAK3 expression in DCs is reported to be TLR dependent and its expression limits their pro-inflammatory activity and their maturation process [198].

Ligand binding to TLRs causes receptor dimerization and recruitment of MyD88, IRAK1 and IRAK4 with phosphorylation of the IRAK1/4 complex [99]. Phosphorylation of IRAK1 and IRAK4 results in a conformational change that causes a loss of affinity for binding to the TLR signalling complex thereby allowing association with TRAF6 [195]. Once IRAK1 is in an activated open conformational status, after the phosphorylation induced by IRAK4, IRAK3 appears able to bind to IRAK1 through the DD domain [199] and inhibit the dissociation of IRAK1 and IRAK4, therefore blocking TRAF6 activation [33]. In this way, IRAK3 inhibits the classical NFκB pathway [68, 196, 200]. In addition, Su J. et al. [201] demonstrated in 2007 that IRAK3 can attenuate p38 activation in response to Pam_3CSK_4, a TLR2 ligand, and, thus, down-regulate the non-canonical NF-κB pathway.

Although the list of IRAK3 functions keeps growing as new studies are published, the consensus is that it prevents inflammatory signalling, and that deficiencies in IRAK3 activity are associated with an increased activation of inflammatory pathways [202, 203]. Furthermore, some IRAK3 gene variants have been associated with specific pathological conditions [182, 217, 218 and 222]. The importance of IRAK3 in regulating the inflammatory response is highlighted by the fact that IRAK3$^{-/-}$ mice have a pro-inflammatory phenotype with increased inflammatory responses *in vitro* and *in vivo,* increased production of TNFα, IL1β, IL6 and IL12p40 and enhanced NFκB and MAP kinase signalling in response to TLR ligand engagement [202]. Importantly, IRAK3$^{-/-}$ macrophages are unable to develop ET as demonstrated by persistently high production of pro-inflammatory cytokines after a prolonged stimulation with LPS [33].

5.1.2 Physiology of IRAK3

In physiological, steady-state conditions IRAK3 prevents inflammation in regions of the body constantly exposed to pro-inflammatory stimuli such as the respiratory tract, where IRAK3 expression is induced in alveolar macrophages by the surfactant maintaining them in a “tolerant” state [204]. Similarly, in the gut Biswas et al. demonstrated in 2011 that IRAK3 gene expression is modulated by the intestinal commensal flora and that knocking down IRAK3 in a colitis model in IL10$^{-/-}$ animals increased the susceptibility to the disease [205]. IRAK3 is also diffusely expressed in the intrahepatic biliary tract preventing inflammation in response to the PAMPs physiologically present in the bile [41].

IRAK3 plays an important role in modulating the phenotype of professional APCs, such macrophages and dendritic cells. IRAK3 expression in APC is TLR

dependent and controls their pro-inflammatory activity and maturation process [198]. By fine-tuning the TLR signalling pathway, IRAK3 can modulate APC function and have significant effect on the immune response, such as the induction of an optimal vaccine response [206]. APC also play a major role in determining T cell phenotype through expression of co-stimulatory molecules and cytokines, which are in turn influenced by intracellular signalling that is modulated by IRAK3 [207].

5.1.3 Pathophysiology of IRAK3

In pathological conditions, several studies have demonstrated how IRAK3 can be induced directly by a number of pathogens to create a pathogen-friendly environment in which host defences are impaired as a consequence of ET-like attenuation of TLR/IL-1 signalling [136, 198, 208-211]. Naturally occurring genetic polymorphisms within the IRAK3 locus have also been reported to be associated with a higher risk of developing serious infectious diseases [182]. IRAK3 has also been demonstrated to be involved in the functional reprogramming toward the M2-like phenotype that monocytes/macrophages undergo during sepsis mediated which is by HIF-1α [212] and indeed, IRAK3 involvement in macrophage polarization towards a pro-healing M2 phenotype has been demonstrated in a number of diseases, including influenza-induced pneumonia, cardiovascular disease and bleomycin-induced lung injury [9, 213-216]. IRAK3 has also been reported to be involved in the inflammatory process associated with metabolic syndrome and obesity [203] and alcohol-induced liver disease [24, 42]. Hayashi et al. demonstrated that repeated low dose stimulation with a synthetic TLR7 agonist could inhibit the onset and attenuate the severity of the disease in two different

animal models of autoimmune disease (namely EAE and antibody induced arthritis) through up-regulation of IRAK3 expression [43].

APCs deficient for IRAK3 have superior antigen presenting function and induce a stronger immune response [207]. The absence of IRAK3 alone is not enough to induce autoimmunity but animals already carrying mutations that predispose them to develop autoimmune disease, show a more severe phenotype when IRAK3 is silenced [15, 40, 184, 217, 218].

IRAK3 up-regulation could be a mechanism of cancer escape from the immune response. The M2 polarized Tumour-associated macrophages (TAM) [246] express higher levels of IRAK3 and TGFβ1-induced IRAK3 expression is hypothesized be one of the mechanisms through which tumour cells subvert an anti-tumour response [144, 219]. This is consistent with the report that IRAK3$^{-/-}$ mice appear to be resistant to tumour growth after inoculation with tumour cells by developing a stronger immune response against them compared to WT controls [220]. Conversely, it has been reported that the methylation of *IRAK3* represents a negative prognostic marker in hepatocellular carcinoma [87]. IRAK3 deficiency has been associated with aggressive tumour development in an animal model of colon cancer with increased level of Tregs in the tumour site putatively responsible for the failure of the immune response [221]. Genetic polymorphisms of IRAK3 are also reported to be associated with a higher risk of developing breast cancer in a population of Korean women [222]. Further research on the role of IRAK3 in inflammation-dependent tumour development and the effect of IRAK3 expression in tumour cells, as opposed to the APC within the tumour, will further clarify the role of IRAK3 in cancer.

Currently, there are conflicting data concerning the effect of IRAK3 in regulatory T cell (Tregs) populations. Some papers describe an increased prevalence of Tregs in IRAK3$^{-/-}$ mice [221] whereas other papers report a reduction in Tregs in the absence of IRAK3 [220] that may be related to an increased production of pro-inflammatory cytokines (e.g. IL6) or pro-inflammatory mechanisms that would induce a skew in the production of Tregs cells toward Th17 cells [70, 223, 224].

5.1.4 IRAK3 and anti-TNFα therapy

To gather further information about the possible effect of anti-TNFα therapy on IRAK3 gene expression in vivo, the Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) was searched for relevant studies [225]. Studies that used microarrays to identify markers predicting response to infliximab were searched for and analysed, and among them two that contained data on IRAK3 were selected [226-228]. The samples analysed in these studies were obtained from patients affected by pathological conditions for which infliximab use is well codified by specific guide-lines [229, 230] (e.g. RA, Crohn disease (CD), ulcerative colitis (UC), psoriasis and psoriatic arthritis). These studies were performed on peripheral blood and on biopsies from synovial tissue and intestinal mucosa.

As shown in Figures 5.1 and 5.2, some of the samples analysed showed a reduction in IRAK3 expression after treatment with Infliximab. The cells from the PBMC of RA and CD patients showed a very significant reduction in IRAK3 gene expression [227], but this finding was not confirmed on CD14^{+} blood cells from RA, psoriasis and psoriatic arthritis patients [226].

Similarly, while the reduction in IRAK3 was particularly notable in mucosal biopsies of patients that responded well to infliximab therapy [226], in the synovial biopsies of 3 patients who underwent a synovial biopsy before and after 10 weeks of infliximab therapy, did not display any reduction in IRAK3 expression [227]. For samples of intestinal inflammatory diseases, a reduction of IRAK3 expression after anti-TNFα therapy in human patients would be consistent with the important role of TNFα in IRAK3 expression and ET establishment described in literature.

5.2 Experimental Rationale

The main effect of IRAK3 is to reduce the inflammatory response in both pathological and physiological conditions. Being expressed mainly in macrophage-lineage cells, IRAK3 is able to influence antigen presentation to the other cells of the immune system. Previous studies have demonstrated that IRAK3 deficient antigen-presenting cells are more efficient in initiating an immune response compared with the normal cells [207]. IRAK3$^{-/-}$ animals show a pro-inflammatory phenotype [202]. They are prone to develop autoimmunity [184, 217, 218], are unable to develop ET and produce higher levels of pro-inflammatory cytokines (TNFα, IL6 and IL12) in response to inflammatory stimuli. Due to the role of IRAK3 in controlling osteoclast activation, IRAK3$^{-/-}$ develop early osteoporosis [231]. IRAK3 polymorphisms in humans are associated with an increased risk of developing sepsis [182] and the pathogenesis of early-onset asthma [217].

Having measured reduced IRAK3 expression throughout the course of CIA (Chapter 4, Figure 4.3), the aim then was to investigate if the complete absence of

IRAK3 was associated with a change in the characteristics of disease. CIA was induced in transgenic mice lacking IRAK3 and in wild type.

Both groups of mice were on a C57Bl/6 background expressing MHC-II A^b ($H\text{-}2^b$), a strain able to develop CIA but with variable efficiency. Bäcklund et al. described in their paper published in 2013 [138] the generation of a mouse with the C57Bl/6 background expressing the MHC $H\text{-}2^q$ haplotype, the C57Bl/6N.Q. It is hypothesized that these mice respond to the collagen in the CFA, rather than the residual pepsin remaining in the collagen from its purification [138].

The $IRAK3^{-/-}$ mice were bred with C57Bl/6N.Q mice to generate $IRAK3^{-/-}$.NQ mice and WT.NQ controls (Figure 5.3 shows an example of genotyping). CIA was then induced by immunising the animals with bovine collagen in CFA as detailed in Chapter 2 Materials and Methods. After the onset of the disease, the clinical parameters were assessed for 10 days post-onset and the animals were then euthanized. Blood was removed and plasma separated to measure the levels of inflammatory cytokines and anti-collagen antibodies. Flow cytometry analysis was performed on spleen and lymph-node cells to define the T cell phenotype; gene expression was assessed on arthritic paws by qPCR.

To better characterise the disease induction period prior to onset, animals were also euthanized 14 days after immunisation, before the onset of the disease. The T cell phenotype was assessed by flow cytometry and the levels of anti-collagen antibodies were measured by ELISA.

5.3 Characterisation of experimental arthritis on IRAK3KO background

After immunisation, WT and IRAK3$^{-/-}$ mice were observed for signs of disease (joint swelling and tenderness). The two groups developed symptoms of disease at different times, with the IRAK3$^{-/-}$ group having a shorter pre-onset period and a slightly higher incidence (Figure 5.4).

5.3.1 Disease onset is accelerated but differences in clinical severity are nominal

Although the development of CIA was accelerated and more efficient in IRAK3$^{-/-}$ mice, the clinical severity between the two strains was similar with no significant differences in the clinical score, number of affected paws, paw width or first affected paw width (Figure 5.5). Histological sections of WT and IRAK3$^{-/-}$ CIA joints with a clinical score of 3 were prepared (Materials and Methods) and showed similar inflammatory infiltrate, bone and cartilage erosions and destruction of joint architecture (Figure 5.6).

5.3.2 IRAK3 deficient arthritic mice have increased plasma IL1β

Cytokines were measured in the plasma of the arthritic mice. While TNFα and IL12 were present at similar levels in the peripheral blood, IL1β was present at considerably higher levels in the IRAK3$^{-/-}$ group. KC/GRO (also known as CXCL1) showed a similar but non-significant trend. IL5 was present at higher levels in the WT group, (Figure 5.7).

5.3.3 IRAK3 deficiency alters antibody class switching

An ELISA for anti-collagen auto-antibodies was performed using the plasma of arthritic animals as described in Chapter 2, Methods and Materials. While the levels of IgG1 antibodies were virtually the same in the WT and IRAK3$^{-/-}$ animals, the level of IgG2a antibodies was lower in the IRAK3$^{-/-}$ group (Figure 5.8). When the ratio of IgG2a to IgG1 was calculated the statistical significance of the relatively lower IgG2a titres was reduced, however, a clear trend remained.

5.3.4 IRAK3 deficiency does not alter Th1/Th17 composition in lymphoid organs

Cells from lymph nodes and spleen were analyzed for CD4 and CD8 lymphocytes that expressed IFNγ and IL17 (Figure 5.9). There was no significant difference in CD4^{+}IFNγ^{+} and CD4^{+}IL17^{+} cells populations in both spleen and lymph-nodes, although the average of CD4^{+}IL17^{+} cells was lower in the lymph-nodes of IRAK3$^{-/-}$ mice. Statistically significant differences were observed in CD8^{+} lymphocytes with CD8^{+}IFNγ^{+} cells present at a higher proportion in IRAK3$^{-/-}$ lymph-nodes, while the CD8^{+}IL17^{+} population was higher in the WT lymph-nodes.

5.3.5 Arthritic IRAK$^{-/-}$ mice have reduced Treg populations

To further characterized the cells population in spleen and lymph nodes, the CD4^{+} and CD8^{+} cells were stained for transcription factors associated with different T lymphocyte lineages. Tbet is a transcription factor associated with Th1 cell phenotype; it is encoded by the gene TBX21 and it regulates the transcription of IFNγ [232]. GATA3 is the transcription factor for the Th2 cells and it is encoded by the homonymous gene; it regulates the expression of IL4 [233]; RORγt is the RAR-related orphan receptor gamma and is encoded by the gene RORC; it promotes

the differentiation toward the Th17 phenotype [234]. Foxp3 is the transcription factor for promoting the phenotype of Treg cells [235]. While in the lymph nodes of both groups, $CD4^+Tbet^+$, $CD4^+ROR\gamma t^+$ and $CD4^+GATA3^+$ cells were present at similar levels, the $CD4^+FoxP3^+$ population was considerably lower in $IRAK3^{-/-}$ animals (Figure 5.10). A similar profile was observed in spleen samples, with the only difference being that the $CD4^+GATA3^+$ cells were present at lower levels in the $IRAK3^{-/-}$ group.

Although Foxp3 can be expressed by different cell populations, its expression is characteristically associated with Tregs [236]. There are 3 different subsets of Tregs expressing Foxp3 (nTregs, iTregs, Th3) [237], and the expression levels of Foxp3 can be modulated by the cell activation state [238]. To investigate the prevalence of inducible Tregs (iTregs), the cells were gated on the $CD4^+CD25^+Foxp3^+$ cells. This population appeared reduced in both spleen and lymph-nodes of the $IRAK3^{-/-}$ group comparing with the WT (Figure 5.11).

5.3.6 IRAK$^{-/-}$ paws have increased pro-inflammatory gene expression

The paws of arthritic mice were harvested, grouped on the basis of the clinical score and then processed individually to perform RNA extraction and qPCR (see Materials and Methods). Surprisingly, the $IRAK3^{-/-}$ group showed higher expression of inflammatory genes (Figure 5.12 (A)). In particular, *Tnf* and *Tgfb1* gene expression levels were significantly higher in the $IRAK3^{-/-}$ group. Higher levels of *Il12b*, *Ilb*, *Il6*, *Il17* and *Il10* were also measured although the difference did not reach statistical significance (Figure 5.12 (B)).

5.3.7 Th17 and Treg cell-associated gene expression is increased in IRAK3$^{-/-}$ joints

Despite the IRAK3$^{-/-}$ and the WT CIA paws not showing any clinical significant difference, a clear difference between the groups was observed when a panel of genes specific for the Th17 and Tregs sub-populations was assessed. As shown in Figure 5.13, the IRAK3$^{-/-}$ CIA paws expressed higher levels of *Cd3e* and *Cd4* indicating a general increase of CD4^{+} lymphocytes in the paws of IRAK3$^{-/-}$ mice compared with WT. *Ctla4*, *Cd25*, *Foxp3* and *Smad3* levels were higher compared with the WT group although the difference in *Foxp3* gene expression was not statistically significant. At the same time the IRAK3$^{-/-}$ paws expressed higher levels of *Stat3* and *Il23r*, two markers of Th17 cells.

5.4 Characterisation of IRAK3 deficiency during disease induction

5.4.1 IRAK3 deficiency reduces splenic Th1 cells prior to disease onset

To determine if the differences observed in the regulatory T lymphocyte population in IRAK3$^{-/-}$ mice were associated with the differences in the time from the immunisation to onset and thus secondary to the development of the disease, another CIA was initiated and 14 days from the immunisation, before the onset of the disease, the mice were euthanized.

In the lymph-nodes, the proportions of CD4^{+} cells were the same in the WT and IRAK3 $^{-/-}$ mice, with similar levels of CD4^{+}IFNγ^{+} and CD4^{+}IL17^{+} cells (Fig.5.14A). In the spleen, the CD4^{+} cell fraction was reduced for in IRAK3$^{-/-}$ mice with statistically significant lower levels of CD4^{+}IFNγ^{+} cells, while the CD4^{+}IL17^{+} cells remained at levels similar to the WT group (Figure 5.14B). For CD8^{+} lymphocytes, spleen and lymph-nodes showed a similar trend, although the differences were not

statistically significant in the spleen. The $CD8^+$ cells were increased in the $IRAK3^{-/-}$ group, with a higher proportion of $CD8^+IL17^+$ cells while the proportion of $CD8^+IFN\gamma^+$ cells was greater in the WT group (Figure 5.14B).

5.4.2 IRAK3 deficiency reduces Treg in pre-onset lymphoid organs

The cells of lymph-nodes and spleen were analysed by flow cytometry for the expression of different Th cell-associated transcription factors. While the proportion of $CD4^+Tbet^+$ cells was not statistically different in both groups in the lymph nodes, the levels of $CD4^+ROR\gamma t^+$, $CD4^+FoxP3^+$ and $CD4^+Gata3^+$ cells were lower in the $IRAK3^{-/-}$ group although the difference was not statistically significant for the latter.

No significant differences between the two groups was found in the spleen for $CD4^+ROR\gamma t^+$ and $CD4^+FoxP3^+$ cells. The $CD4^+Gata3^+$ cells were lower also in the spleen of the $IRAK3^{-/-}$ group even if the difference, once again, was not statistically significant, (Figure 5.15).

A reduction in the proportion of $CD4^+CD25^+Foxp3^+$ cells was measured in $IRAK3^{-/-}$ pre-onset lymph nodes (Figure 5.16); no difference was found in the spleen (Figure 5.16) although there was greater variation in Treg numbers in this organ.

5.4.3 Pre-onset anti-collagen antibody composition is IRAK3-independent

An ELISA for anti-collagen antibodies was performed on plasma samples that were collected on the day 14 post immunisation. The results (Figure 5.17) are presented as relative EC_{50} (the EC value for each plasma sample relative to the EC50 of the standard curve). Virtually no difference was found between the

IRAK3$^{-/-}$ and WT groups in the levels of anti-collagen IgG1 and IgG2a antibodies or their ratio (IgG2a/IgG1).

5.5 Discussion

The absence of IRAK3 is associated with a pro-inflammatory phenotype in response to pathogenic stimuli both in vitro and in vivo. As described in the Chapter 4, a reduction in IRAK3 gene expression is associated with the development of the CIA. To understand through which mechanisms IRAK3 might be involved in the pathogenesis of the disease IRAK3$^{-/-}$ and WT mice were first backcrossed on C57Bl/6N.Q background, to obtain a more efficient CIA model, and then immunised with bovine collagen in CFA [139].

The first difference observed between the IRAK3$^{-/-}$ and the WT group was in the incidence of the disease and in a shorter pre-onset phase. In the absence of IRAK3, the induction of the disease was more efficient than in WT mice with roughly 20% more mice developing the disease. The severity of the disease in the two groups was comparable, with a similar clinical score, arthritic paw width and a similar level of inflammatory infiltrate observed histologically. As further confirmation of the similar clinical severity, the level of pro-inflammatory cytokines detected in the plasma of peripheral blood were similar with the exception of IL1β and KC/GRO that were present at higher levels in the IRAK3$^{-/-}$ group, although for the latter the difference was not statistically significant. The WT group showed higher level of IL5, IL4 and IFNγ although the difference was statistically significant just for IL5. The levels of anti-collagen antibodies were similar for IgG1 while the IRAK3$^{-/-}$ group showed significant lower levels of IgG2a, probably indicating a reduced Ig class switching in the IRAK3$^{-/-}$ group during the CIA. When the cells

from lymph-nodes and spleen were analysed, no significant difference was observed for Tbet (Th1) and RORγt (Th17)-expressing cells and the two groups showed similar percentages of $CD4^+IFN^+$ and $CD4^+IL17^+$ in both spleen and lymph-nodes.

A difference was observed in the proportion of the $CD4^+Foxp3^+$ population in both lymph-nodes and spleen, where lower levels of $CD4^+Gata3^+$ cells were also observed. Foxp3 is phenotype-defining transcription factor for the regulator T cell populations but its expression is not just limited to Tregs [236].

As CIA is an inflammatory process localized principally in the joints, particular focus was placed on a specific population of Tregs, the inducible Tregs (iTregs) which maintain immunological tolerance in peripheral organs and tissues [237]; This $CD4^+$ sub-population is characterised by high expression of CD25 (IL2Ra) and Foxp3 [235]. The prevalence of $CD4^+CD25^+Foxp3^+$ was reduced in both spleen and lymph-nodes of the $IRAK3^{-/-}$ mice. Other studies have already demonstrated that IRAK3 can modulate Treg levels in animal models of cancer [221] and autoimmunity [184]. Conversely, while some papers describe an increased presence of Tregs in $IRAK3^{-/-}$ mice [221], other papers report a reduction in Tregs in the absence of IRAK3 [220] which may be due to increased production of pro-inflammatory cytokines (e.g. IL6) or pro-inflammatory mechanisms that would skew the differentiation of naïve T cells toward Th17 cells [50, 70, 223, 224]. To investigate if this was the case, the gene expression of a panel of inflammatory mediators was assessed in the $IRAK3^{-/-}$ and WT CIA paws. A significant difference in the production of pro-inflammatory mediators in the paws of the $IRAK3^{-/-}$ was measured. In particular pro-inflammatory cytokines

genes like *Tnf*, *Il12b*, *Il1b*, *Il6* and *Il17* were all expressed at higher levels in the IRAK3$^{-/-}$, even though the difference was not always statistically significant. The expression of *Tgfb1* and *Il10* was also increased.

When genes specific for different T cell subsets were assessed in the arthritic paws, an increased expression of both Th17 and Treg markers was observed. IRAK3$^{-/-}$ arthritic paws expressed higher levels of pan-lymphocytic markers *Cd3* and *Cd4*. *Smad3* and *Foxp3,* which are Treg-associated transcription factors, were higher in IRAK3$^{-/-}$ paws as were *Stat3* and *Il23r*, which are more specific for the Th17 subset. Interestingly, *Ctla4* appeared to be expressed in the IRAK3$^{-/-}$ joints at higher levels. CTLA-4 binding the B7 family members blocks the co-stimulatory signal represented by CD28-B7. This mechanism is used by nTregs to control T cells activity and some evidence suggests that it represents a marker for nTregs [239].

An increased gene expression can suggest an increase in the expression of a protein but is not directly indicative of such. Nevertheless, on the basis of these results, we could hypothesise that Th17 and Tregs are increased in the IRAK3$^{-/-}$ paws. Fujimoto, M., et al. demonstrated in a paper published in 2011 [70] that the generation of Foxp3^{+} iTregs is inhibited under conditions of excessive IL6 production. The IRAK3$^{-/-}$ paws expressed considerably higher levels of *Tgfb1* and *Il6* comparing with the WT group, and increased Treg and Th17 associated genes.

5.5.1 Conclusion

It could be hypothesized that the levels of iTregs observed in the IRAK3$^{-/-}$ lymph-nodes and spleen are reduced due to the pro-inflammatory milieu containing high

levels of IL6 and TGFβ1, which then switch cells toward the Th17 phenotype. Increased levels of CTLA-4 suggest an increase in the nTregs population, possibly as compensatory mechanisms in a pro-inflammatory phenotype. Alternatively, the difference observed in spleen and lymph-nodes could be due to a cellular redistribution to the main site of the inflammatory response, the joint. The pre-onset samples showed a similar trend to the CIA lymph-nodes with regard to the $CD4^+CD25^+Foxp3^+$ distribution, while in the pre-onset spleen there was not a significant difference between the WT and the $IRAK3^{-/-}$ mice. This indicates a cellular re-distribution of Th sub-population between the different sites of the inflammatory cells during the pre and post onset periods. The paws as the primary site of the inflammation would contain more pathological cells, like Th17, and lymphocytes driven to adopt a Th17 phenotype due to the higher levels of IL6 and TGFβ1.

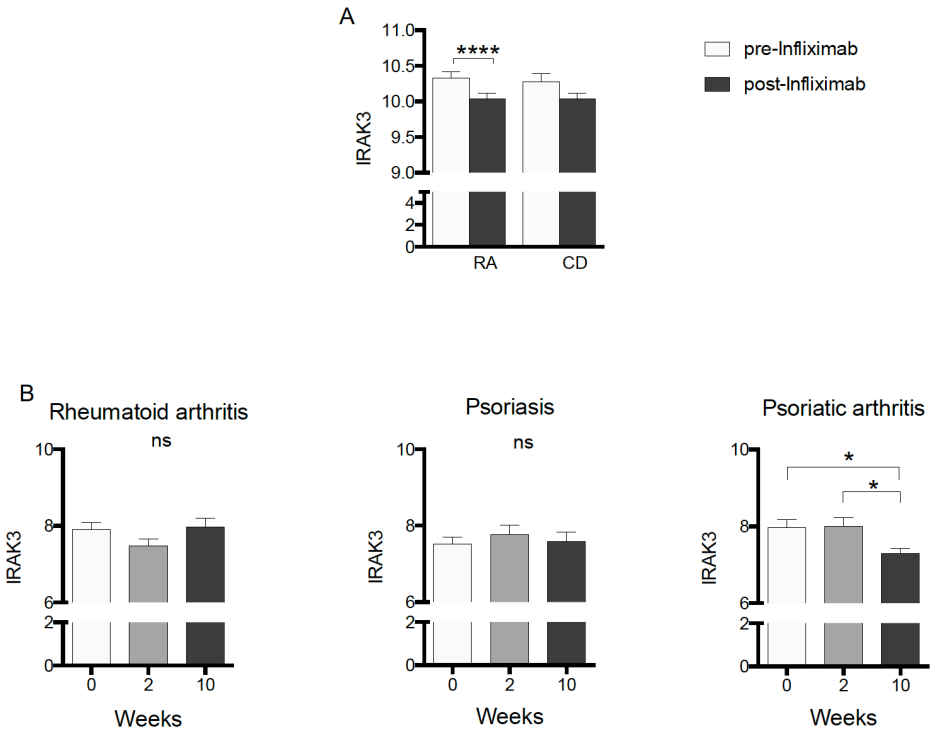

Figure 5.1. *IRAK3* expression in peripheral blood was modified by anti-TNFα therapy. (A) *IRAK3* expression in peripheral blood samples from 19 RA and 20 Crohn's disease patients before and after 2 weeks of therapy with Infliximab. (B) *IRAK3* expression was analysed in $CD14^+$ cells from the peripheral blood from 9 RA, 12 Psoriatic arthritis and 10 Psoriasis patients before therapy and after 2 and 10 weeks of infliximab treatment. Student's t-test, ****p< 0.0001; *p<0,05.

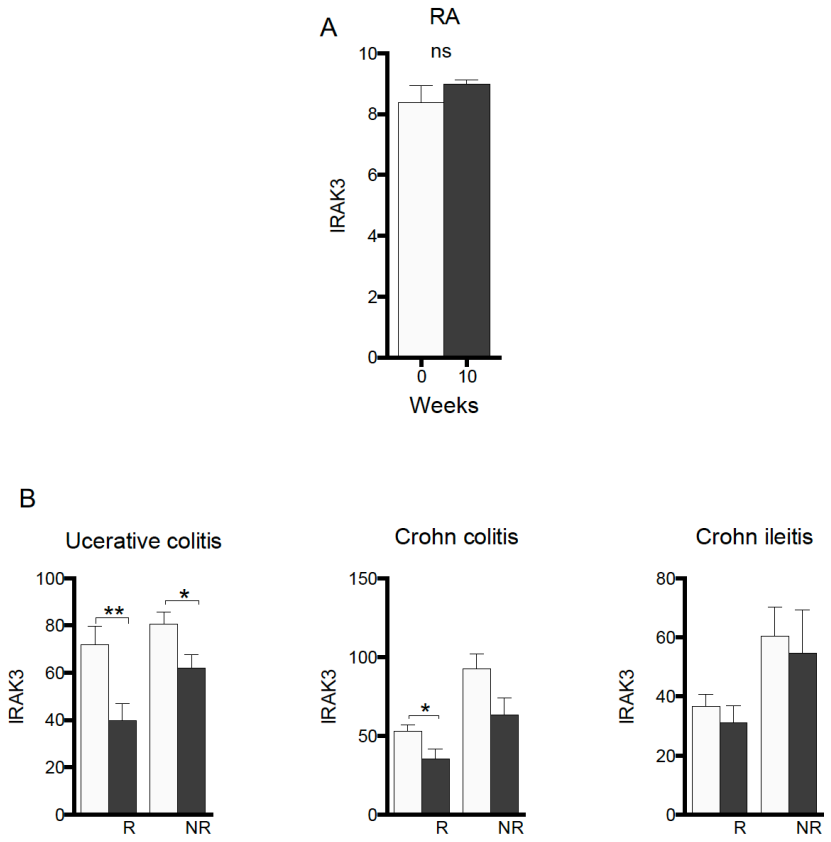

Figure 5.2. Anti-TNFα therapy does not modify *IRAK3* expression in RA synovial cells.
(A) Synovial biopsies were performed on the knee of 3 RA patients under ultrasound guidance at baseline and repeated on the same knee at week 10. (B) Mucosal biopsies were obtained in endoscopy in actively inflamed mucosa of 24 ulcerative colitis, 19 Crohn's colitis and 18 Crohn's ileitis patients. The patients were classified for response to infliximab in responders (R) and not responders (NR) based on endoscopic and histologic findings at 4-6 weeks after first infliximab treatment; Student's t-test **p<0.005; *p<0.05.

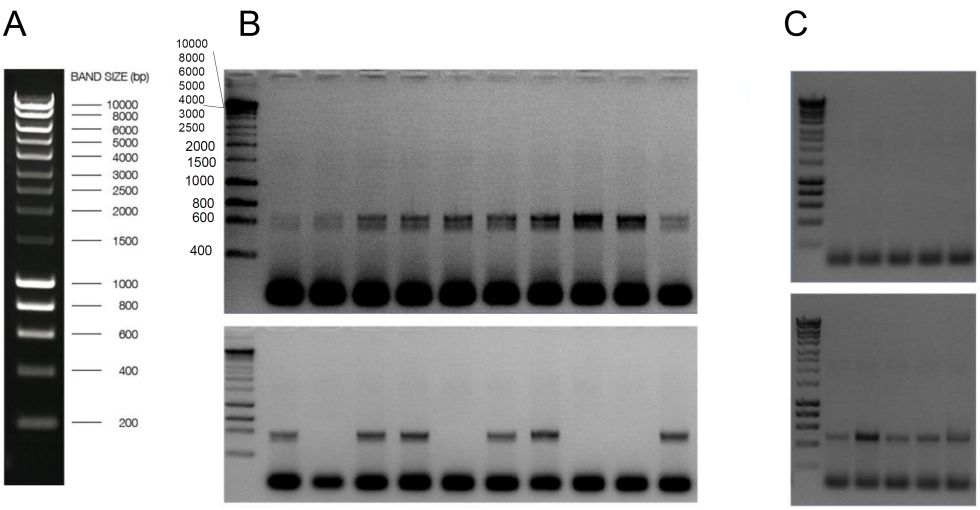

Figure 5.3 Genotyping of IRAK3$^{-/-}$ mice. Bands size was checked against HyperLadder 1^{TM} 1Kb (A). Wild type primers were used for the upper pictures and IRAK3-specific primers for the lower pictures. (B)Wild type animals present just one band in the upper picture (600bp) while the heterozygous for IRAK3 present a band in the upper and one in the lower picture (500bp). (C) IRAK3-/- mice with no band in the upper picture.

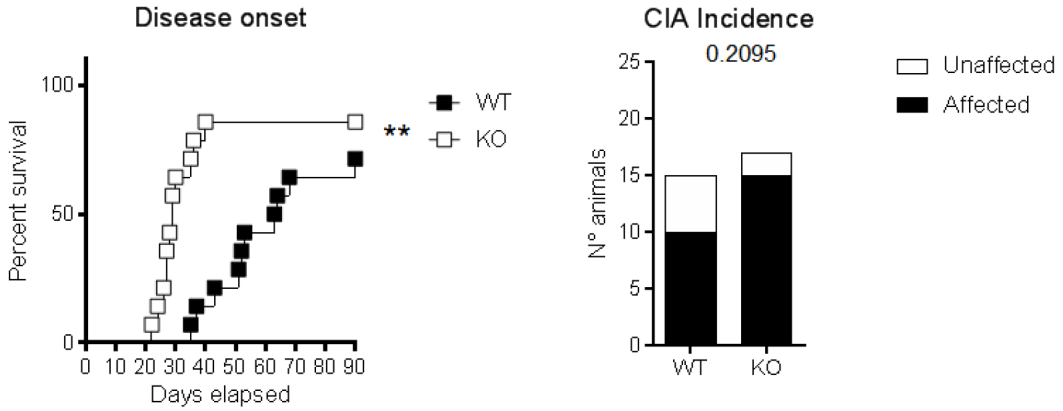

Figure 5.4. Experimental arthritis onset is accelerated and more efficient in IRAK3$^{-/-}$ mice. IRAK3$^{-/-}$ mice (KO) develop symptoms of arthritis significantly earlier than WT mice; Mantel Cox test, **p=0.0074. The number of mice affected within 90 days of immunisation was slightly higher in the KO group,

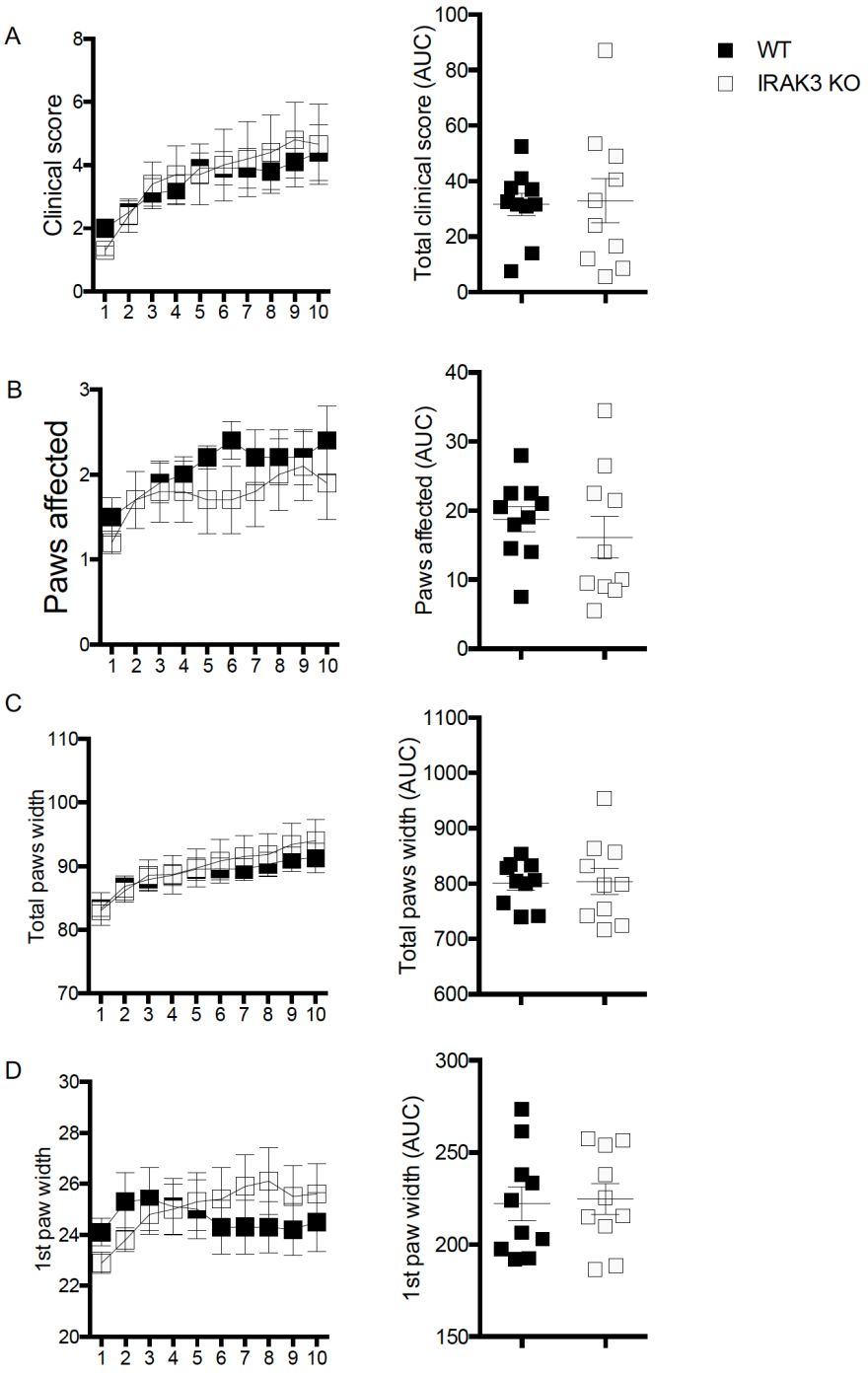

Figure 5.5 Clinical data of IRAK3$^{-/-}$ versus WT CIA.
Daily clinical data (left side), and calculated Area under the Curve (AUC, right side) for individual mice. All the clinical data was collected during the post-onset period (10 days). (A) Total clinical score of IRAK3$^{-/-}$ treated versus PBS treated mice. (B) Number of affected paws. (C) Total combined width of all paws in mm. (D) Width of the first-affected paws in mm. Values are means±SEM.

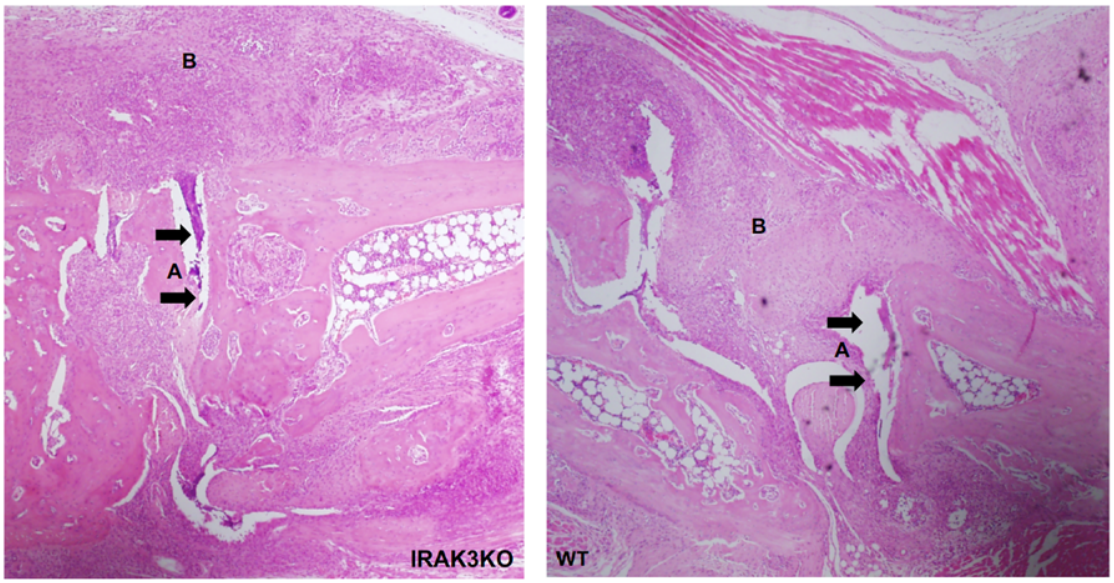

Figure 5.6. Arthritic IRAK3$^{-/-}$ and WT histology. IRAK3 KO mice and WT CIA mice showed both loss of the normal joint architecture with obliteration of the intra-articular space (A) and similar levels of inflammatory infiltrate (B).

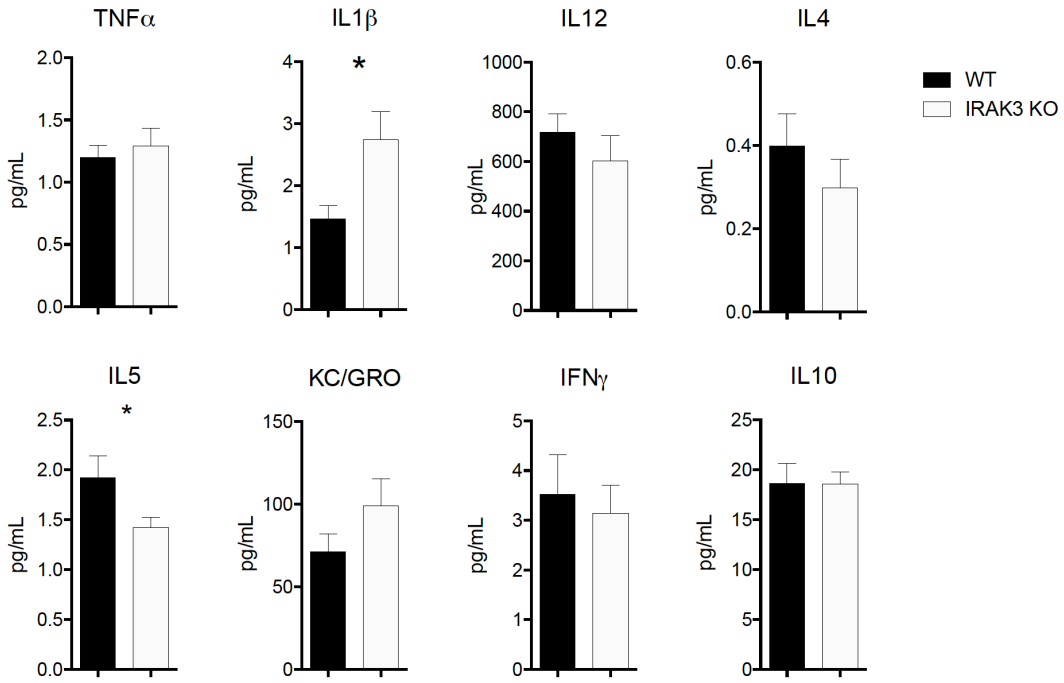

Figure 5.7. CIA IRAK3$^{-/-}$ mice have higher concentration of IL1β in peripheral blood.
From blood obtained from peripheral blood of arthritic IRAK3$^{-/-}$ and WT mice, the plasma concentration of a panel of cytokines was measured by Mesoscale Discovery Assay; values are means±SEM, Student's t-test, *p<0.05.

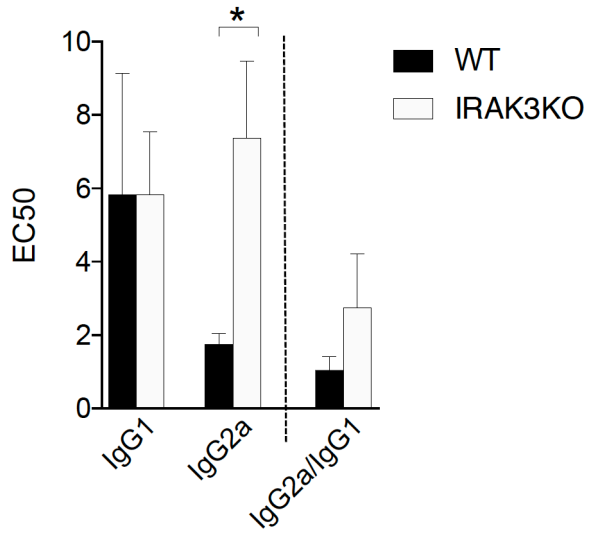

Figure5.8. CIA IRAK3KO mice showed lower levels of IgG2a antibodies. The graph shows the EC_{50} (half maximal effective dose) that represents the concentration of antibody that gives half-maximal binding. Since IRAK3KO mice have lower levels of IgG2a in the serum they need a higher amount to reach EC50 therefore the higher bars comparing with the WT.
Student's t-test, *p<0.05.

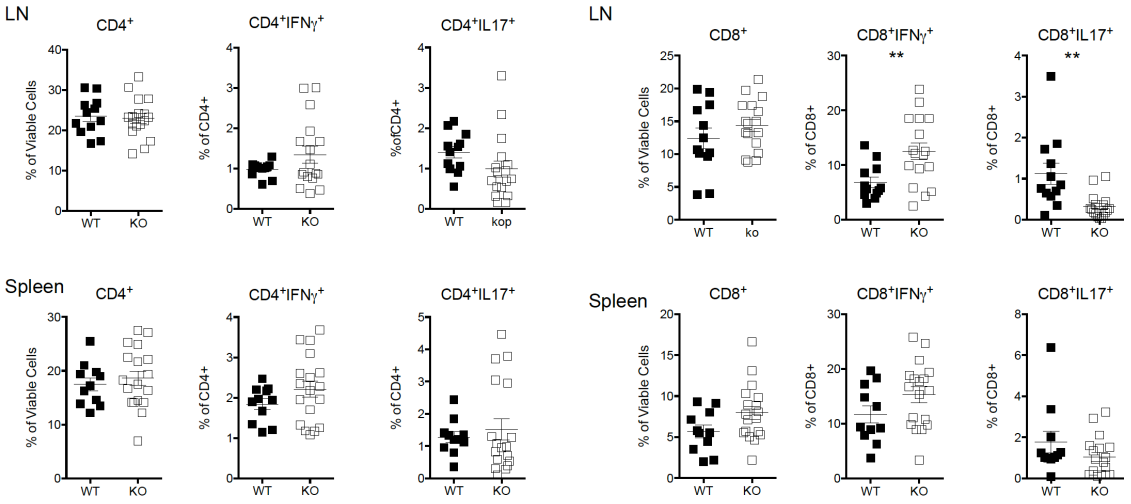

Figure 5.9. Th cells in the lymph-nodes and spleens of arthritic IRAK3$^{-/-}$ and WT mice.
The percentage of subsets of CD4^{+} and CD8^{+} cells, defined by expression of IFNγ or IL-17; values are means±SEM, Student's t-test, *p<0.05, ** p<0.01.

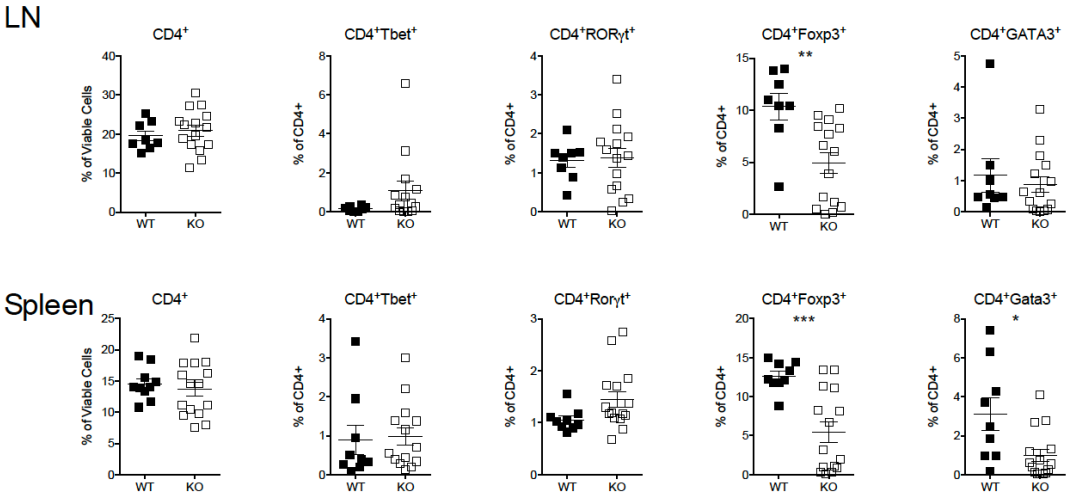

Figure 5.10. CD4$^+$FoxP3$^+$ cells are reduced in lymph-nodes and spleens of IRAK3$^{-/-}$ mice. The percentages of subsets of CD4$^+$ lymphocytes, defined by the expression of the transcription factors Tbet, Rorγt, FoxP3 or Gata3 in the lymph-nodes and spleens of arthritic of IRAK3$^{-/-}$ and WT mice; values are means±SEM, Student's t-test, *p<0.05, ** p<0.01, *** p<0.005.

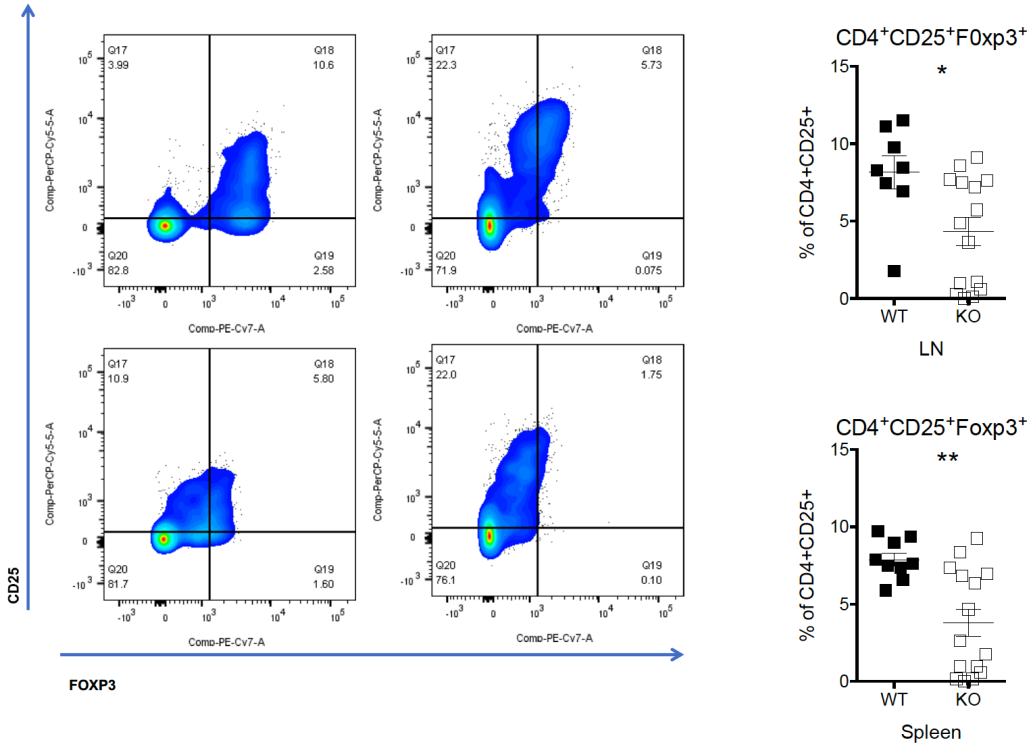

Figure 5.11. Arthritic IRAK3$^{-/-}$ mice have reduced CD4^{+}CD25^{+}Foxp3^{+} cells in lymph-nodes and spleen.
(A) Representative density plots, gated on CD4^{+} cells. (B) The percentage of CD4^{+}CD25^{+}Foxp3^{+} lymphocytes in lymph-nodes (LN) and spleen; values are means±SEM, Student's t-test, *p<0.05, ** p<0.005.

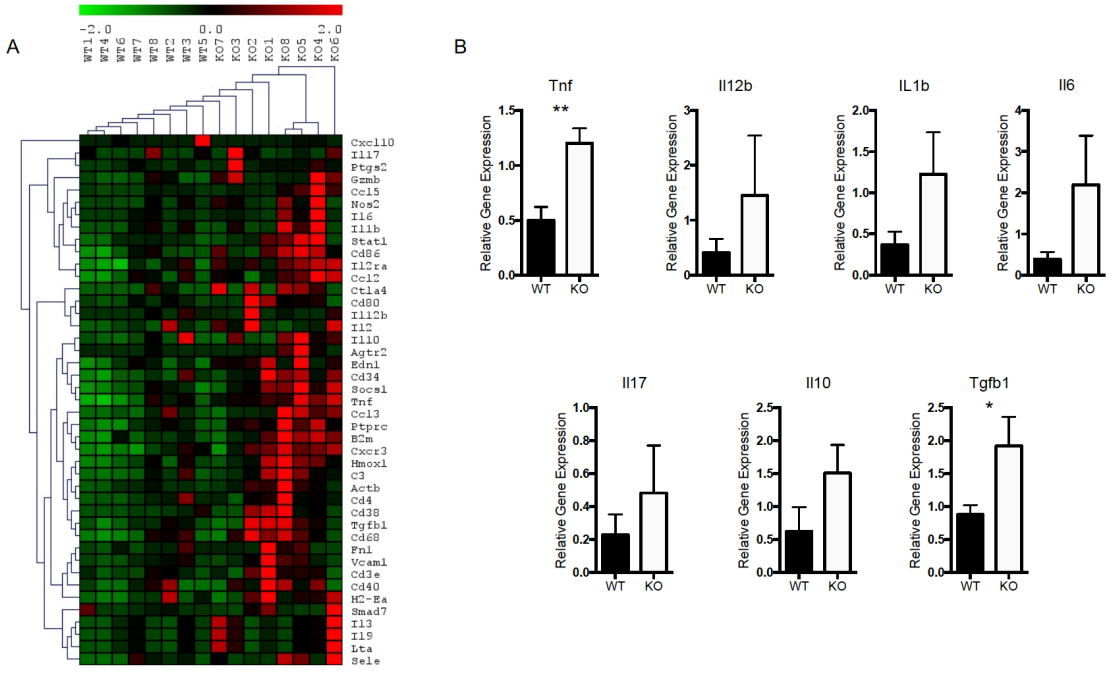

Figure 5.12. Expression of pro-inflammatory genes is increased in arthritic IRAK3$^{-/-}$ paws. Differential gene expression for a panel of pro-inflammatory genes expressed in IRAK3$^{-/-}$ arthritic paws; values are row normalised. (B) Gene expression of pro-inflammatory cytokines; values are mean±SEM, Student's t-test, n=8, * $p<0.05$, ** $p<0.005$.

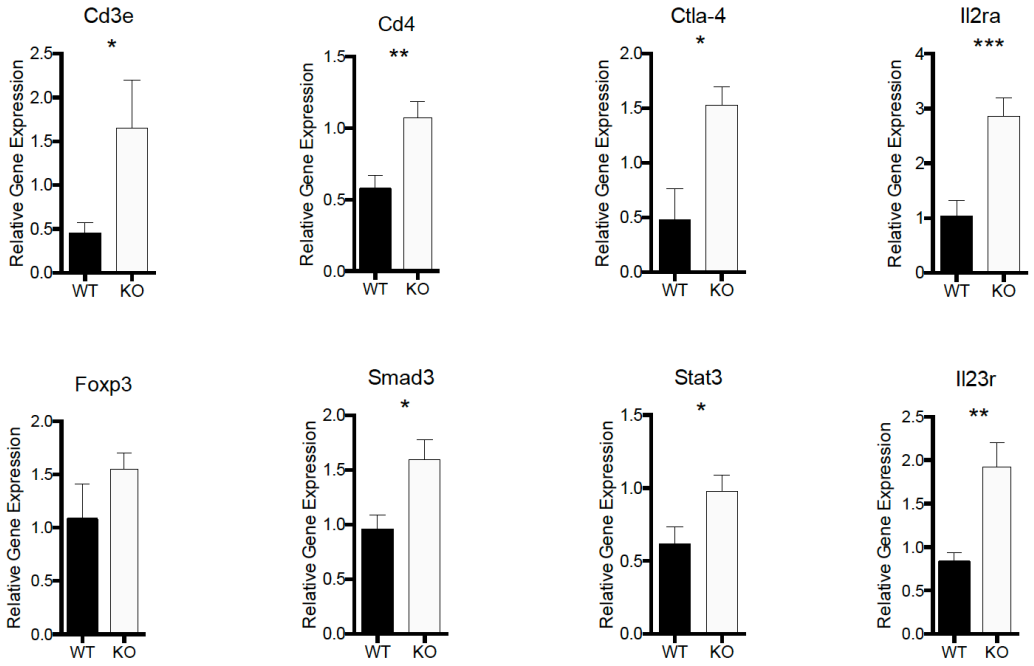

Figure 5.13.Th17/Treg-associated genes are increased in arthritic IRAK3$^{-/-}$ paws.
Gene expression of Th17/Treg-associated genes in arthritic paws from WT and IRAK3$^{-/-}$; values are mean±SEM, Student's t-test, n=8, * $p<0.05$, ** $p<0.005$.

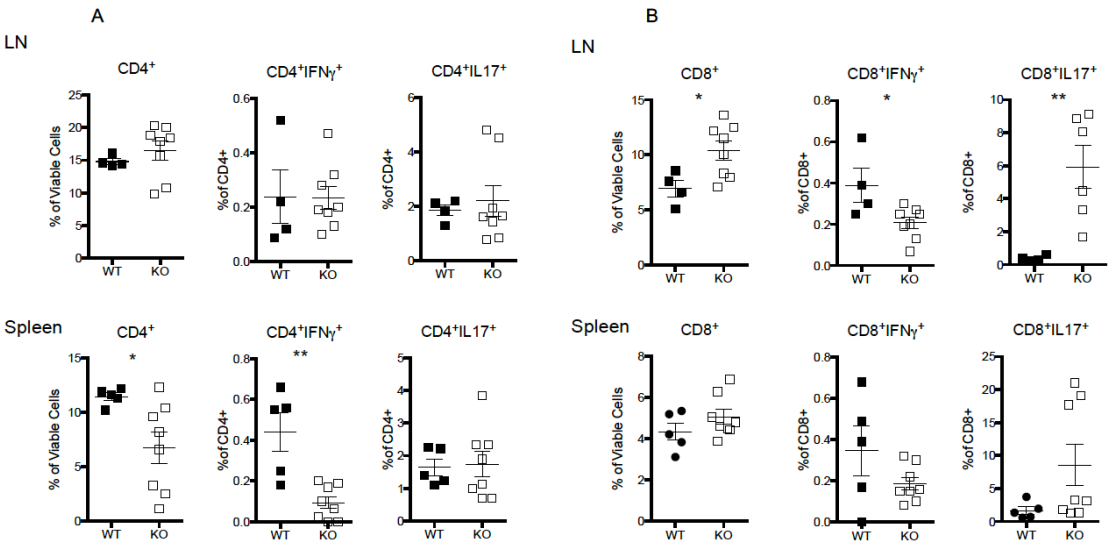

Figure 5.14. The prevalence of CD4$^+$INFγ$^+$ cells was reduced of IRAK3$^{-/-}$ mice prior to disease onset.
The percentage of subsets of CD4$^+$ and CD8$^+$ cells, defined by expression of IFNγ or IL-17; values are means±SEM, Student's t-test, *p<0.05, ** p<0.01..

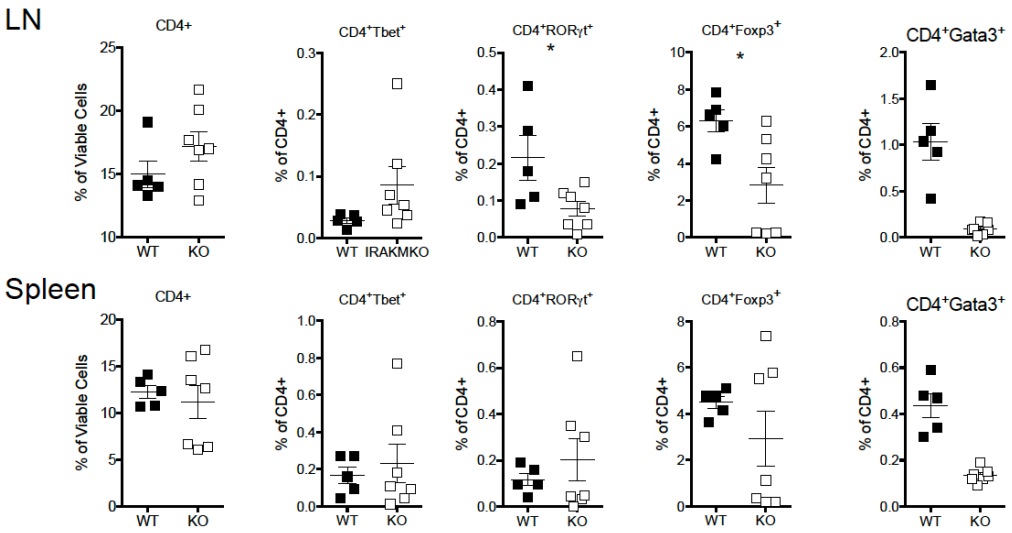

Figure 5.15. CD4$^+$RORγ$^+$ and CD4$^+$Foxp3$^+$ cells are reduced in IRAK3$^{-/-}$ lymph-nodes prior to disease onset.
The percentages of subsets of CD4$^+$ lymphocytes, defined by the expression of the transcription factors Tbet, Rorγt, FoxP3 or Gata3 in the lymph-nodes and spleens of arthritic of IRAK3$^{-/-}$ and WT mice; values are means±SEM, Student's t-test, *p<0.05.

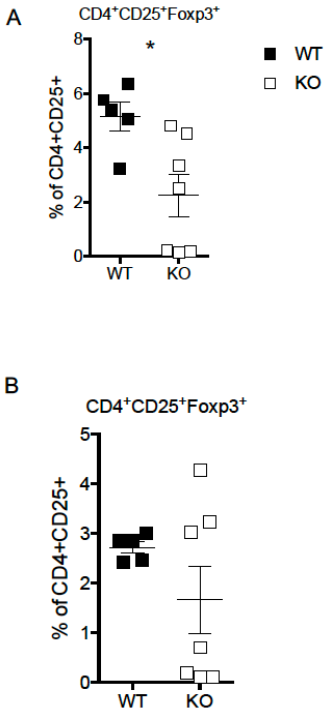

Figure 5.16. IRAK3$^{-/-}$ mice have reduced Treg in lymph-nodes prior to disease onset.
The percentage of CD4+CD25+Foxp3+ lymphocytes in (A) lymph-nodes and (B) spleen; values are means±SEM, Student's t-test, *p<0.05.

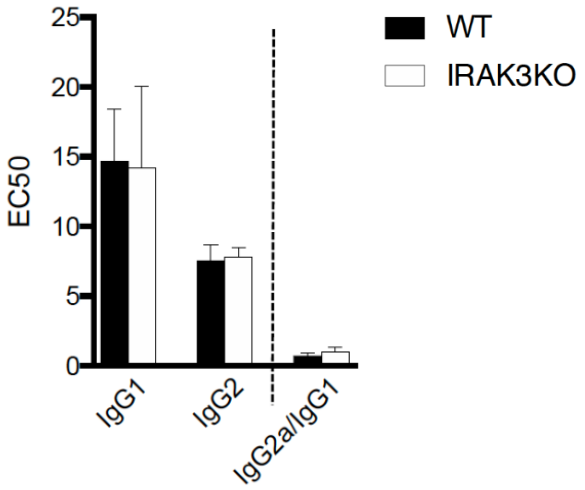

Figure 5.17. IRAK3$^{-/-}$ and WT mice had similar levels of anti-collagen antibodies prior to disease.
Plasma from pre-onset mice was titrated and assayed to determine the EC50 for anti-collagen IgG1 and IgG2a antibodies. For each mouse the ratio of IgG2a to IgG1 anti-collagen antibodies was calculated; No difference was found in the levels of anti-collagen antibodies in KO mice and WT before the onset of the disease

Chapter 6 : Summary and Discussion

6.1 Introduction

As demonstrated by several studies, the innate immune system is involved in the initiation and perpetuation of RA, with the involvement of TLRs. The TLRs are involved also in the establishment of a phenomenon of tolerisation to further inflammatory stimuli known as endotoxin tolerance (ET). ET represents a gene expression re-programming of macrophage-lineage cells and as different TLRs share the same signalling pathways, a ligand can induce a state of ET such that subsequent stimulation with another TLR ligand will result in a reduced inflammatory response.

LPS stimulation of TLR4 activates the NFκβ pathway leading to production of inflammatory mediators, such as TNFα which exerts an inflammatory effect. At the same time, in a negative, self-limiting feed-back effect, the expression of IRAK3 is induced.

IRAK3 is a member of *Interleukin 1 receptor associated kinases* family, and blocks intracellular signalling that induces the expression of inflammatory mediators (Figure 6.1). TNFα has been demonstrated to have a fundamental role in the up-regulation of molecules involved in the ET process [188] and ET establishment can be disrupted by blocking TNFα during LPS stimulation [92]. ET represents one of the mechanisms through which TNFα exerts its immuno-regulatory action.

The main hypothesis of this project was that in a TNFα-driven disease such as RA (and CIA), ET mechanisms, in particular IRAK3, that normally controls the

inflammatory process have failed to prevent the perpetuation of the inflammatory process (Figure 6.2).

Although ET is associated with pathological conditions [240], the underlying mechanism is the same involved in a physiological inflammatory response. The same molecules are involved in ET also participate in the termination of the inflammatory response. To further investigate these ET-associated mechanisms, as first step before moving to animal models of chronic inflammatory diseases, a fully in vitro ET model was established.

6.2 ET bioassay

Macrophages are phenotypically plastic cells depending on the stimuli present in their micro-environment for their polarization. They are usually classified as M1 (classical activated) or M2 (alternative activated) macrophages [107, 149]. M1 macrophages are induced by pro-inflammatory stimuli like LPS and IFNγ. They propagate and support the inflammatory process secreting TNF-α, IL1β, IL6 and IL-12, but their prolonged activation induces tissue damage. M2 macrophages instead, are induced by IL4, IL13, TGFβ1 and IL-10, and produce anti-inflammatory cytokines (TGF-β1 and IL-10) to control the immune response [107]. A M1/M2 balance is sufficient to avoid damage related to a prolonged inflammatory response [108, 110-112, 241, 242]. Therefore, in vivo the two phenotypes (which are further divided into subtypes [172]) represent two phenotypic nodes between which the cells oscillate, depending on the environment [242].

In Chapter 3 a long, low-dose stimulation with LPS was used to induce ET in monocyte-derived human M-CSF macrophages. When stimulated a subsequent time with LPS, the ET macrophage phenotype differed from the pro-inflammatory one induced by a single, short stimulation with LPS, and assumed a phenotype more similar to the M2 polarised macrophage. Expression of ET-associated genes (up-regulation of *IRAK3*, *PTPN6*, *AHR* and *TREM1*), and reduced levels of pro-inflammatory cytokines (in particular TNFα) were found in the supernatant of ET macrophages; IFNγ pre-treatment partially blocked such modifications in the ET phenotype. The same in vitro model of ET was able to induce similar modifications on bone marrow-derived murine M-CSF macrophages, and using the transgenic mice that lacked TNFRSF1A or TNFRSF1B, it appeared then that TNFα was able to induce an immuno-regulatory action (with sub-sequent down-regulation of TNFα gene expression as well) through the induction of ET genes, in particular IRAK3.

TNFα-driven immuno-regulatory mechanisms were then investigated in an animal model of a TNFα-driven disease, collagen induced arthritis (CIA).

6.3 ET-associated molecules in experimental arthritis

Despite higher levels of TLR and TNFα gene expression and an increased percentage of monocytes/macrophage cells in the joints of CIA mice, the levels of ET-associated genes expressed by the CIA samples was lower than the naïve controls. Among the ET-associated genes there was a further difference. While *Tnfaip3* and *Ptpn6* were expressed in the CIA paws at similar levels than the naïve controls indicating at least an attempt to down-regulate the inflammatory process, *Irak3* gene expression levels remained lower than the naive control through every phase of the disease. That *Tnfaip3* and *Ptpn6* were not able to modify the

inflammatory process in the absence of *Irak3* is persuasive evidence of its importance in the control of the inflammatory process.

This constant down-regulation of *Irak3* in a TNFα-driven disease with an increased number of macrophages at the site of the inflammation prompted a further investigation to understand if the normal relationship between TNFα and IRAK3 gene expression was preserved in the pathological model. For this reason the effect of TNFα blockade on ET-associated molecule expression was measured.

Anti-TNFα therapy represents the gold standard for the treatment of RA and its high position in the cytokine hierarchy in RA has been demonstrated by several studies [38, 73-75]. Despite its well-proven efficacy in the treatment of the disease, not all patients respond to anti-TNFα therapy [80]. Blocking TNFα by therapy is associated with an increase in Th17 cell numbers as demonstrated in animal models of the RA [81-86]. Anti-TNFα therapy may not prevent just its pathological action, but also its immune-regulatory activities. In arthritic mice, anti-TNFα treatment has been demonstrated able to increase the prevalence of Th1 and Th17 cells in lymph nodes even when it improved the symptoms of the disease [23]. Previous studies [59, 86, 94] demonstrated that TNFα can block IL12B expression [24].

In the anti-TNFα-treated CIA mice, despite the clinical improvement, the group treated with Etanercept showed an increased in the levels of Th17 cells. There was no significant difference compared with the PBS-treated group in the expression of ET-associated genes (with the exclusion of a higher gene expression in Etanercept-treated lymph-nodes). It must be considered that in human studies IRAK3 reduction after anti-TNFα therapy has been demonstrated in

peripheral blood cells while the analysis of gene expression of synovial biopsies (which are analogous to the paws studied here) before and after infliximab did not show any significant difference in IRAK3 gene expression levels. Furthermore, the levels of IRAK3 gene expression were already extremely low in the un-treated CIA which probably limits the further reduction induced by anti-TNFα therapy. Although TNFα blockade did not significantly affect the expression of ET-associated gene expression (*Tnfaip3*, *Ptpn6*, *Irak3*) in paws with established arthritis, the kinetics of *Irak3* expression throughout CIA and differences in *IRAK3* expression in human inflammatory disease prompted further investigation using mice lacking this gene.

6.4 IRAK3 deficiency accelerates experimental arthritis via reduced Treg

As previously described IRAK3 is a member of the IRAK family with no kinase activity and exerts its action by blocking the dissociation between IRAK4 and IRAK1 and in this way preventing the propagation of the activation signal of the NFκB pathway. IRAK3$^{-/-}$ mice are characterised by an extremely pro-inflammatory phenotype with increased production of TNFα, IL6 and IL12 in response to pro-inflammatory stimuli and inability to develop ET. A higher propensity to develop autoimmunity, cancer and other pathological conditions has been previously described in absence of IRAK3 [184, 243]. IRAK3$^{-/-}$ mice developed symptoms of arthritis more rapidly than WT controls and showed an increased incidence, although not an increased severity from a clinical and histological point of view.

The lower levels of IgG2a antibodies present in IRAK3$^{-/-}$ CIA mice could indicate a defect in Ig class switching which would be partly confirmed by the lower level of CD4^{+}Gata3^{+} cells in the spleen of IRAK3$^{-/-}$ CIA mice and by the reduced level of IL4 in the serum of the same group [245].

On the other side, IRAK3$^{-/-}$ mice showed an increased proportion of CD8^{+}IFNγ^{+} in the spleen and IFNγ act upon B cells to switch the Ig class to one more able to fix complement [244].

IRAK3$^{-/-}$ and WT mice did not show relevant differences in the Th1 and Th17 cells prevalence in spleen and lymph-nodes and a further analysis of the different Th cells subtypes revealed that the IRAK3$^{-/-}$ and WT mice showed similar level of Tbet^{+}, RORγt^{+} and Gata3^{+} positive cells in lymph-nodes (although, as already mentioned, in the spleen the CD4^{+}Gata3^{+} population was reduced in the IRAK3$^{-/-}$ mice).

An important difference between the two groups was found in the prevalence of CD4^{+}Foxp3^{+} cells that were reduced in both spleen and lymph-nodes of IRAK3$^{-/-}$ mice. The transcription factor Foxp3 is characteristic of the Tregs and interferes with AP-1 and NFAT at the IL2 gene promoter, preventing the production of IL2. The main function of Tregs is to tune down the inflammatory response, preventing any damage associated with a hyper-activation of the adaptive immune system. They are fundamental in the prevention of autoimmunity. There are two main groups of Tregs: natural and induced. The natural Tregs (nTregs) represent less than 15% of the CD4+ cells in the human circulation. They are CD4^{+}CD25^{+}Foxp3^{+} T cells that develop in the thymus. They can produce TGFβ1 and IL10 but they exert their action mainly through CTLA-4, expressed on their surface. The T cells during their differentiation in fact need two signals: a stimulatory one provided by the interaction of their TCR receptor with the antigen presented in the context of the MHCII molecules and a co-stimulatory one, provided by the interaction of their co-receptor CD28 with two molecules, CD80 and CD86, expressed on the surface

of the APCs. CTLA-4 expressed on the surface of nTregs can then interact with CD28, preventing the co-stimulatory signal [67].

The inducible Tregs (iTregs) differentiate from naïve CD4+ T cells in the periphery in presence of TGFβ1. The iTregs are $CD4^+CD25^+Foxp3^+$ and exert their function producing TGFβ1 and IL10. These cells are extremely plastic and in presence of strong inflammatory stimuli, that determine increased levels of IL6, can switch toward the Th17 phenotype.

In the CIA $IRAK3^{-/-}$, the observed reduction in the $CD4^+Foxp3^+$ population was mainly due to a reduction in the prevalence of the $CD4^+CD25^+Foxp3^+$ sub-population in both spleen and lymph-nodes. Previous studies have demonstrated a variable association between Tregs and the $IRAK3^{-/-}$ status. In an animal model of colitis, knocking down IRAK3 in $IL10^{-/-}$ mice induced an increased in the percentage of $CD4^+Foxp3^+$ cells in the lamina propria of the intestine, probably as a physiological mechanism to maintain a tolerant state in an organ constantly exposed to antigens [205] and in an animal model of colon cancer IRAK3 deficiency was associated with a more severe colon tumour development probably for the increased percentage of $CD4^+CD25^+Foxp3^+$ at the tumour site that could favour the tumour immunological escape [221].

Xie et al. demonstrated in 2007 that $IRAK3^{-/-}$ mice were resistant to tumour growth upon inoculation with transplantable tumour cells and that such resistance was probably associated with the reduction in the $CD4^+CD25^+Foxp3^+$ population observed in the spleen [220]. $IRAK3^{-/-}$ mice appeared to be more susceptible to the inflammation associated with alcohol consumption and such increased

susceptibility was associated with a reduction in the percentage of $CD4^{+}Foxp3^{+}$ cells in $IRAKM^{-/-}$ the liver.

This variable association with IRAK3 expression and Tregs could be due to a redistribution of the Tregs that could be present at higher percentage in the organ site of the pathological process (like the gut lamina propria in the experimental colitis and in the colon cancer model and the joint in my CIA) and reduced in the peripheral lymphoid organs like spleen and lymph-nodes. Another hypothesis is that an increased presence of the pro-inflammatory cytokine IL-6 associated with a high presence of TGFβ1 could induce a shift of the iTregs toward the Th17 phenotype as previously already described [70].

Analysing the gene expression in the joint of the CIA animals, an increase in the expression of pro-inflammatory cytokines in the $IRAK3^{-/-}$ group was observed, despite the animals showing similar clinical severity to the WT control group, and despite similar plasma levels of inflammatory cytokines in the plasma of the two groups of animals. Considering that the inflammatory process in the CIA is mainly located in the joint and that at the same time one animal could have joints with different clinical scores, it is possible the levels of pro-inflammatory cytokines in the peripheral blood, does not reflect adequately their level in the joint micro-environment.

When specific Th genes were assessed through qPCR in the joints of CIA animals an increase expression of Th17 and Tregs cellular markers was found in the $IRAK3^{-/-}$ group. The $IRAK3^{-/-}$ paws expressed considerably higher levels of TGFβ and IL6 comparing with the WT group. It would be possible to hypothesise that the

level of inducible Tregs in IRAK3$^{-/-}$ lymph-nodes and spleen are reduced because these cells are located in a highly pro-inflammatory micro-environment with high levels of IL6 and TGFβ1 capable of switching toward the Th17 phenotype.

Alternatively, the difference observed in spleen and lymph-nodes could be due to cellular redistribution in the main site of the inflammatory response, the joint. The pre-onset samples showed in the CD4^{+}CD25^{+}Foxp3^{+} in the lymph-nodes had a similar trend with the CIA samples, while in the pre-onset spleen a non-significant difference between the WT and the IRAK3$^{-/-}$ samples was observed. This could be simply explained with the beginning of cellular re-distribution of Th cells sub-population between the different sites of the inflammatory cells.

6.5 Conclusion

In conclusion, ET mechanisms appear to be involved in the control of the inflammatory process and are among the mechanisms caused by TNFα to exert an immuno-regulatory action (Figure 6.2). The in vitro model of ET, therefore, represents a valid model to characterise these mechanisms and in particular the use of different genetic strains of murine cells will allow a deeper knowledge of ET mechanism.

Among ET related genes, IRAK3 appear to be absent in chronic disease and the absence of IRAK3 appeared to be associated with the reduced prevalence of CD4^{+}CD25^{+}Foxp3^{+} cells. Further studies are necessary to fully characterise the mechanisms through which IRAK3 is related to the described effects.

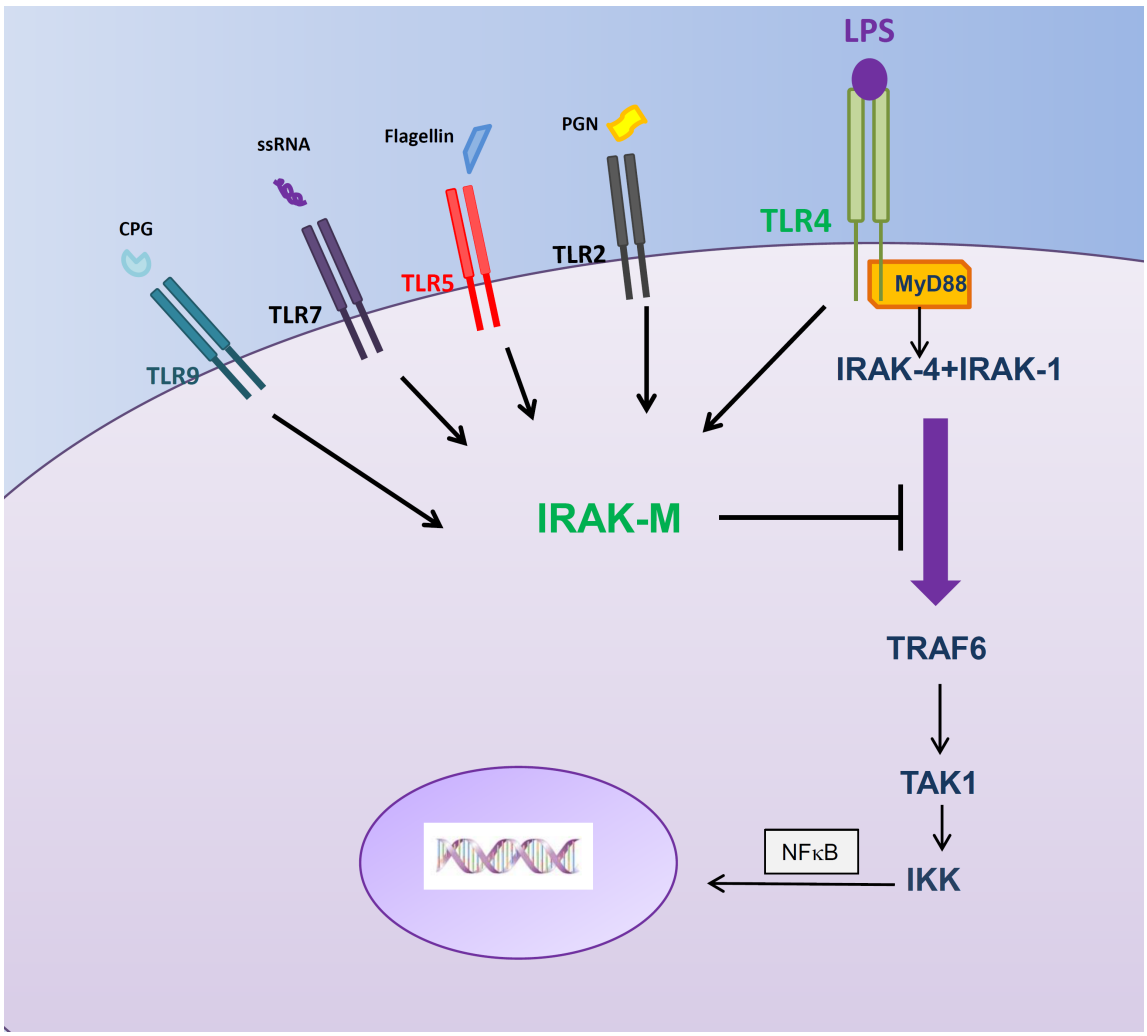

Figure 6.1. IRAK3 action on NFκB pathway. Several immuno-regulatory molecules are involved in the establishment of Endotoxin tolerance but IRAK3 is the only one that appears to be constantly up-regulated after stimulation with different TLRs ligands. IRAK3 acts binding the IRAK4-IRAK1 complex preventing their dissociation and activation of TRAF6.

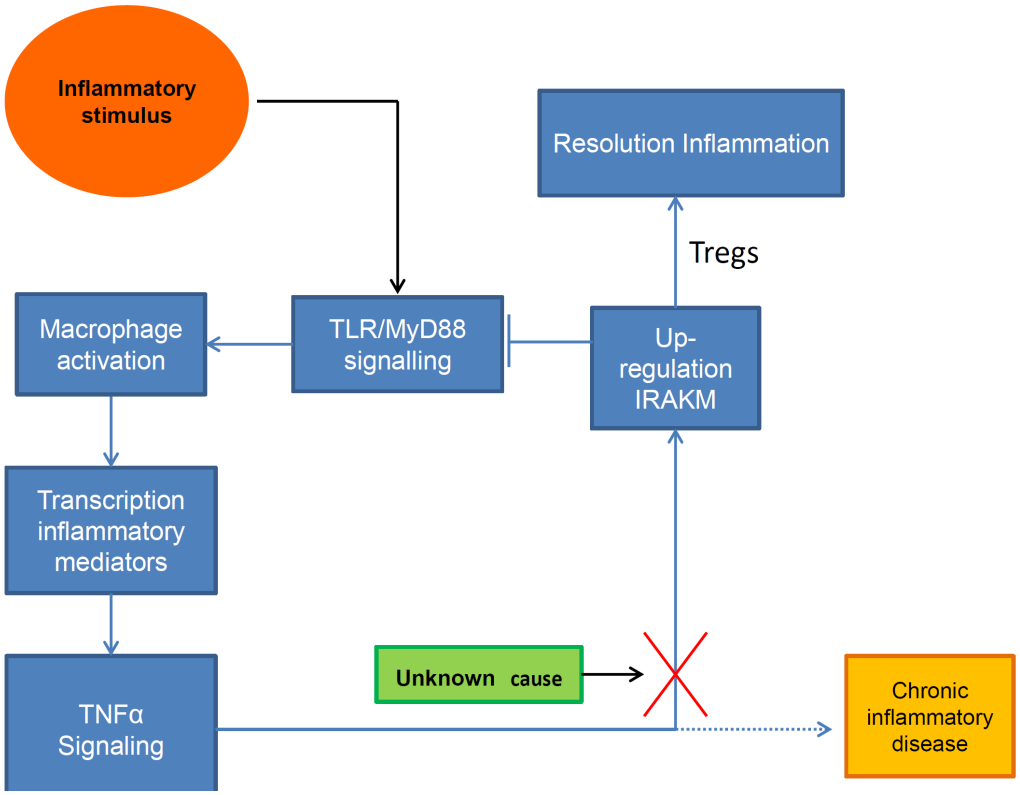

Figure 6.2. Endotoxin tolerance represents a physiological mechanism of inflammation control. TLRs are activated at the very beginning of the inflammatory response inducing the transcription of pro-inflammatory mediators among which TNFα. The up-regulated TNFα induces the immuno-regulatory molecule IRAK3 in a sort of negative feedback to guide the resolution of the inflammatory process. If this pathway is disrupted by unknown cause the inflammation perpetuates and the inflammatory process becomes chro

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