Trex1 and Nbs1 as Regulators of the Macrophage Inflammatory Response

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Моему мужу.

Без Тебя – невозможно!

Спасибо Тебе, с Тобой путь был более увлекательным и значимым!

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List of Abbreviations

AD, autosomal dominant

ADAR1, double-stranded RNA-specific adenosine deaminase 1

ADP-ribose, adenosine diphosphate ribose

AGS, Aicardi Goutieres Syndrome

AGT, O6-alkylguanine-DNA alkyltransferase

AIM2, absent in melanoma 2

ANA, anti-nuclear antibodies

ANA, anti-nuclear antibodies

APC, antigen presenting cell

AR, autosomal recessive

ASC, Apoptosis-associated speck-like protein containing a CARD

Asp, aspartate

AT, Ataxia-telangiectagia

ATLD, Ataxia-telangiectagia like disorder

ATM, ataxia telangiectasia mutated

ATRIP, ATR interacting protein

BER, base excision repair

Blm, Bloom syndrome protein

B-NHEJ, alternative non-homologous end joining

B-NHEJ, backup non-homologous end joining.

BRCA, breast cancer susceptibility protein

BRCT, BRCA1 C terminus domain

C/EBP alfa, CCAAT/enhancer-binding protein alpha

CA150, transcription elongation regulator 1

CD, cluster of differentiation

CDK, cyclin-dependent kinase

CDR, cytosolic DNA receptors

cGAMP, cyclic-GMP-AMP

cGAS, cyclic-GMP-AMP synthase

CpG, cytosine phosphodiester guanine

Csf1R, colony stimulating factor 1 receptor

CtIP, C-terminal-binding protein interacting protein

DAI, DNA-dependent activator of IFN-regulatory factor

DAMP, danger-associated molecular pattern molecule

DDB1, DNA damage-binding protein 1

DDX, DExD/H-BOX Helicases

DEDD, DnaQ-like

DExD/H, DEAH and the Ski families of proteins

DHX, probable ATP-dependent RNA helicase

DNA, deoxyribonucleic acid

DNase, deoxyribonuclease

D-NHEJ, DNA-PK-dependent non-homologous end joining

DR, direct repair

DSB, double-strand breaks

dsDNA, double-strand DNA

ERK, extracellular signal-regulated kinase

Exo1, exonuclease 1

FANCF, Fanconi anemia group F protein

Fas receptor, apoptosis antigen 1

Fc receptors, fragment crystallisable receptor

FCL, familial chilblain lupus

FEN, flap endonuclease

FHA domain, forkhead-associated domain

G1, first gap phase

G2, second gap phase

GAR, glycine-arginine rich

GAR, lycine-arginine rich

GAS, gamma-activated site

G-CSF, granulocyte colony-stimulating factor

Glu, glutamic acid

GPI, glycosylphosphatidylinositol

H2AX, H2A histone family member X

HIV, human immunodeficiency virus

HR, homologous recombination

IFI, interferon gamma-inducible protein

IFN, interferon

IFNGR, interferon-gamma receptor

IFNαR, interferon-α receptor

IKK alpha, inhibitor of nuclear factor kappa-B kinase subunit alpha

IKK complex, IκB kinase complex

IL, interleukin

IP-10, interferon gamma-induced protein 10

IRF, interferon regulatory factor

ISG, IFN-stimulated gene

ISGF3, ISG factor 3

ISRE, interferon stimulated response element

JAK1, janus-associated kinase 1

kDa, kilodalton

KO, knock-out

Ku70, X-ray repair complementing defective repair in Chinese hamster cells 6

LPS, lipopolysaccharide

LRR, leucine-rich repeat

LRRFIP1, leucine-rich repeat flightless-interacting protein 1

M, mitotic phase

MAPK, mitogen-activated protein kinases

MCP-1, monocyte chemotactic protein-1

M-CSF, macrophage colony-stimulating factor

MD2, myeloid differentiation 2

Mdc1, mediator of DNA damage checkpoint protein 1

MHC, histocompability complex

MIP, macrophage inflammatory protein

MMR, mismatch repair

Mre11, meiotic recombination 11

MRN, MRE11 complex or Mre11-Rad50-Nbs1 complex

mtDNA, mitochondrial DNA

mTOR, mammalian target of rapamycin

MyD88, myeloid differentiation primary response gene 88

NBS, Nijmegen breakage syndrome

NBSLD, Nijmegen breakage syndrome-like disorder

NER, nucleotide excision repair

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NHEJ, non-homologous end joining

NK, natural killers

NLR, nucleotide-binding oligomerisation domain receptor

NO, nitric oxide

O6MeG, O6-Methylguanine

OGG1, oxoguanine glycosylase

p53, protein 53

PAMP, pathogen-associated molecular pattern

PARP, procyclic acidic repetitive protein

PCNA, proliferating cell nuclear antigen

pDC, plasmacytoid dendritic cell

PIKK, phosphatidylinositol 3-kinase-related kinase

PKC, protein kinase C

Polβ, polymerase beta

PRR, pattern recognition receptor

PU.1, spleen focus forming virus proviral integration

PYHIN, pyrin and hin domain-containing protein

Rad50, DNA repair protein RAD50

RANKL, receptor activator of nuclear factor kappa-B ligand

RANTES, regulated upon activation normal T cell expressed and presumably secreted

RIG-I, retinoic acid-inducible gene 1

RIP1, receptor-interacting protein 1

RLR, RIG-I-like receptor

RNAse, ribonuclease

RNS, Reactive nitrogen species

ROS, reactive oxygen species

RPA, replication protein A

RVCL, retinal vasculopathy with cerebral leukodystrophy

S, DNA synthesis phase

SAM, Sterile alpha motif

SAMHD1, SAM domain and HD domain-containing protein 1

SH2, Src Homology 2

SLE, Systemic Lupus Erithematosus

SSB, single-strand break

ssRNA, single-stranded RNA

STAT, signal transducer and activator of transcription

STING, stimulator of interferon genes

TBK1, TANK-binding kinase 1

TFEB, mammalian transcription factor EB

TGF-β, transforming growth factor beta

Th1, T helper cell 1

Th2, T helper cell 2

TIR, toll/interleukin-1 receptor

TIRAP, toll/interleukin-1 receptor domain-containing adaptor protein

TLR, toll-like receptor

TNF, tumor necrosis factor

TRAF, TNF receptor associated factor

TRAM, translocation associated membrane protein

Trex1, three prime repair exonuclease 1

TRIF, TIR-domain-containing adapter-inducing interferon

TYK2, tyrosine kinase 2

Wrn, Werner syndrome

XLF, XRCC4-like factor

XRCC, X-ray repair cross-complementing protein

Introduction

1) Immune system

Immune system is a complex arrangement of biological structures and processes that protects an organism against diseases and is hence crucial for its survival. Pathogens have the ability to evolve and adapt, and thus tend to avoid being recognised by the immune system. In response to that, however, immune systems have built up multiple defense mechanisms to detect and eliminate pathogens. As a consequence and as a result of evolution, the human immune system is branched into innate and adaptive. The innate system is more ancient and provides for the first defense against pathogens. On the contrary, the adaptive immune system can recognise and memorise pathogens, which allows modern vaccination (Cooper and Alder, 2006). The Innate immune system includes the group of professional phagocytic cells, such as macrophages but also comprises anatomic barriers such as skin and mucosal surfaces. The adaptive immune system can be activated by interaction with the innate immune system and is comprised of T- and B-cells that possess antigen memory and can induce and produce antibodies (Hoebe et al., 2004).

Inflammation is a key characteristic of the immune system and is the first response to infection. This process is conducted by cytokines and chemokines produced by both immune and non-immune cells that recruit and activate immune effectors to the site of infection. A few centuries ago, John Hunter made the observation that "inflammation in itself is not to be considered as a disease but as a salutary operation consequent to some violence or some disease." This early finding stresses the importance of not only inflammation but also its resolution (Serhan and Savill, 2005). Resolution of inflammation includes elimination of all inflammatory immune cells, such as granulocytes and pro-inflammatory macrophages, cell debris and dead pathogens clearance and repair of damaged tissue, as a result of which, when correctly performed will not lead to scarring and the loss of organ function.

A healthy immune system possesses many different mechanisms, which are tightly regulated and can induce and down-regulate inflammation according to its needs. When exacerbated or not properly down-regulated inflammation is damaging and causes disease. atherosclerosis, autoimmunity, cancer and chronic

neurodegenerative diseases are among the long list of more than one hundred inflammatory associated diseases (Yu et al., 2012).

1.1) Monocytes and macrophages

Monocytes and macrophages are important effectors and regulators of the immune system. In the majority of cases, blood circulating monocytes are the predecessor of macrophages, but in some cases, macrophages are produced locally in different tissues, such as brain, dermis and spleen (Geissmann et al., 2010). These cells belong to the myeloid lineage of the innate immune system and have many different functions as illustrated in Figure 1. Macrophages functions are highly related to their environment which are dependent on a further differentiation of macrophages to tissue resident cells (Davies et al., 2013; Shi and Pamer, 2011).

In any of these conditions the failure to properly achieve their function leads to an imbalance in the immune response and in the extreme cases to a disease. Among these functions one should highlight, due to its importance, the initiation and resolution of inflammation. For this macrophages preform immune surveillance by continuously surveying their surrounding environment for signs of damage or infection. Together with pathogen elimination macrophages also help in the clearance of dust and allergens in lungs (alveolar macrophages), they clear toxins in the liver (kupffer cells), clear senescent red blood cells in spleen (splenic macrophage) and induce tolerance in intestine (intestinal macrophage) (Murray and Wynn, 2011).

These immune cells, although highly differentiated and diverse are characterised by expression of specific surface marker. Cluster of differentiation (CD)11b, EGF-like module containing (Emr1 or F4/80), CD68, colony stimulating factor 1 receptor (CSF1r), lymphocyte antigen 6 (Ly6)C and Ly6G are among these proteins (Murray and Wynn, 2011). When activate both in a pro-inflammatory and anti-inflammatory the expression of other markers is up- and down-regulated. The major histocompatibility complex (MHC) II and the mannose receptor are specific markers and, in themselves, represent examples, of pro-inflammatory and anti-inflammatory activation, respectively (Lawrence and Natoli, 2011).

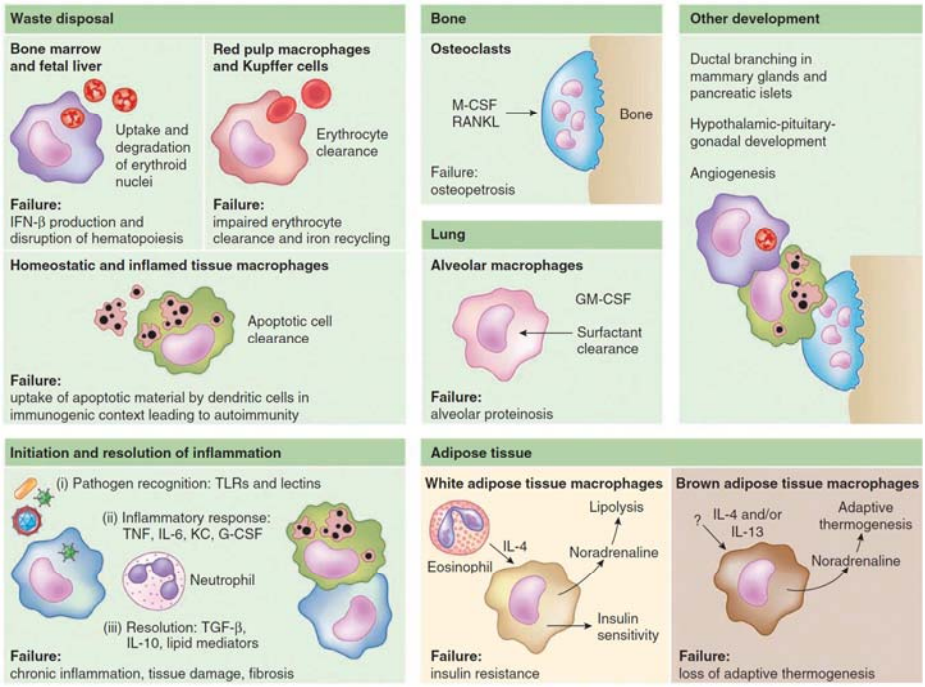

Figure 1: Different functions of tissue resident macrophages. This illustration depicts the roles of differentiated macrophages from diverse tissues. Failure of their function leads to indicated flaws or diseases (Davies et al., 2013).

1.2) Macrophages development

In recent years, our understanding about the origin of macrophages has evolved. Most of the tissue resident populations originate locally from stem cells that migrate from yolk sac prior to birth (Schulz et al., 2012). Another population of macrophages involved in the immune response is produced in the bone marrow. Their precursors, monocytes, migrate through the blood and under the effect of different cytokines and chemokines they are recruited in the tissues, where they subsequently differentiate into macrophages. Contrary to tissue macrophages, the functional activity of macrophages produced in the bone-marrow is mostly related to organism defense. They exercise patrol function in tissues and, whenever necessary, are activated at

inflammatory loci. During this activity, macrophages can act as antigen presenting cells.

Monocytes are originated in the bone marrow from hematopoietic stem cells through several steps of differentiation. PU.1 and C/EBPα transcription factors are crucial for the commitment of distinct differentiation steps (Valledor et al., 1998). Among cell fate decisions, throughout differentiation monocytes first become common myeloid progenitor cells and thereafter, are differentiated into granulocyte/macrophage progenitor cells and, prior to becoming monocytes, into macrophage/dendritic cell progenitors (Auffray et al., 2009). This differentiation process requires growth factors and cytokines. Macrophage colony-stimulating factor (M-CSF) is, however, critical for monocytes and macrophages differentiation (Mossadegh-Keller et al., 2013). In fact, PU.1 increases proliferation and differentiation through induction of M-CSF specific receptor (Celada et al., 1996). Although, M-CSF is the most powerful and specific growth factor for macrophages, its activity is also modulated by other factors, such as adenosine, present in the environment (Xaus et al., 1999a).

1.3) Macrophages proliferation

Leukocytes and lymphocytes proliferation is crucial for their development and for a fast and correct immune response. As referred above, macrophages proliferation is necessary in the process of monocyte generation and in the renewal of local resident macrophages. Macrophages' and monocytes' ability to respond to M-CSF is mediated through M-CSF receptor (CSF1r or also referred to as CD115).

M-CSF is the first hematopoietic growth factor to have been discovered. It was deemed to be important not only for macrophages proliferation, but also for macrophages development, recruitment and survival, by protecting macrophages from apoptosis (Hume and MacDonald, 2012).

M-CSF is expressed by endothelial cells, T-cells, fibroblast, cancer cells and macrophages. This growth factor has three different protein forms. Two of these proteins are soluble forms that circulate in the body. The third form is bound to the membrane and can be released by cleavage through local signaling. Active site is a

common feature of all M-CSF proteins and is composed of 149 amino acids. After secretion, M-CSF binds to its specific receptor that is expressed mainly in macrophages. Upon the interaction with M-CSF, CSF1r becomes tyrosine phosphorylated and dimerises. This, in turn, leads to further phosphorylation of other proteins and downstream signaling. Mitogen-activated protein kinase (MAPK) and extracellular-signal-regulated kinase (ERK) are one of the activated signaling pathways that are responsible for the induction of cell survival and proliferation (Comalada et al., 2004; Valledor et al., 2000).

1.4) Macrophages activation

To achieve and perform their function, macrophages need to be properly activated. Macrophages are multifunctional immune cells. During an inflammatory process they play two different opposite roles that contribute to tissue destruction and repair. On the one hand, they conduct a pro-inflammatory activity, M1 type, that is considered to occur due to classical activation. On the other hand, macrophages carry out an anti-inflammatory activity, M2 type, for tissue repair that is also designed as alternative activation. *In vitro*, macrophages can be activated by T helper 1 (Th1) type cytokines, such as interferon (IFN)-γ, or bacterial products, such as lipopolysaccharide (LPS), to become pro-inflammatory macrophages. Macrophages subsequently express molecules with a high degree of destruction, such as reactive oxygen species (ROS) and proteolytic enzymes. Once macrophages become activated by T helper 2 (Th2) cytokines, such as interleukin (IL)-4 or IL-10, they express an anti-inflammatory activity that results in a tissue repair (Biswas and Mantovani, 2010).

As other immune cells, macrophages possess a highly-diversified receptors network. These receptors can distinguish the surrounding signals and accordingly activate macrophages (Murray and Wynn, 2011). As mentioned above, macrophages can be activated by several cytokines, and these cells possess corresponding receptors for molecules, such as IFN-γ, IL-4 and IL-10.

In addition, macrophages can detect pathogens through pathogen-associated molecular patterns (PAMPs) and other harmful agents through danger-associated molecular pattern molecules (DAMPs) that induce activation. Both PAMPs and

DAMPs are relatively small highly-conserved molecules that are distinct from the host molecular features. Toll like receptors (TLRs), nucleotide-binding oligomerisation domain receptors, or NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and many others form part of pattern recognition receptors (PRRs). PRRs are able to bind to PAMPs and DAMPs triggering activation of the immune response (Kawai and Akira, 2010). Since macrophages, in addition to PAMPs and DAMPs, can also be activated by cytokines from other immune cells, this enables the immune system's communication and to act as a whole (Mosser and Edwards, 2008). Macrophages pro-inflammatory activators, including different PAMPs, cytokines and their corresponding receptors, are analysed below in further detail.

1.4.1) TLR4 and LPS

LPS is one of the outer membrane's components of gram-negative bacteria. It is divided into lipid A (endotoxin), core oligosaccharide and O-antigen. Lipid A is the only of these LPS regions that is detected by the immune system. TLR4 recognises LPS, which is a highly potent activator of the immune response, which, in high concentration, can cause septic shock. Lipid A recognition is not solely dependent on the presence of TLR4. Instead, Lipid A is bound by a circulating LPS-binding protein, which is able to transform Lipid A micelles into monomer. This complex is further concentrated by CD14 to a glycosylphosphatidylinositol (GPI)-anchored protein that enables it to bind to TLR4-myeloid differentiation 2 (MD2) complex. The abovementioned steps are needed for TLR4 triggering and demonstrate the importance of such a potent activator's regulation in the immune system (Miller et al., 2005) (Figure 2).

Like other TLRs, TLR4 consists of extracellular of leucine-rich repeats (LRRs) with a horseshoe-like shape. Intracellular part of TLR4 is composed of toll/interleukine-1 receptor (TIR) domains. These domains are highly conserved, and single point mutations can affect their function. To transduce signaling, TLR4 needs to interact with other TLR4-LPS bound elements by oligomerisation.

Although this process is to date not yet fully understood, TLR4 TIR domains bind different adaptor proteins, such as myeloid differentiation primary response gene 88

(MyD88), TIR domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF) and TRIF-related adaptor molecule (TRAM). Furthermore, MyD88 and TIR activate the so-called "MyD88-dependent pathway" that culminates with pro-inflammatory gene expression. On the other hand, binding of TRIF and TRAM to TLR4 TIR domains activates "MyD88-independent pathway" that culminates with type I IFN gene expression (Lu et al., 2008).

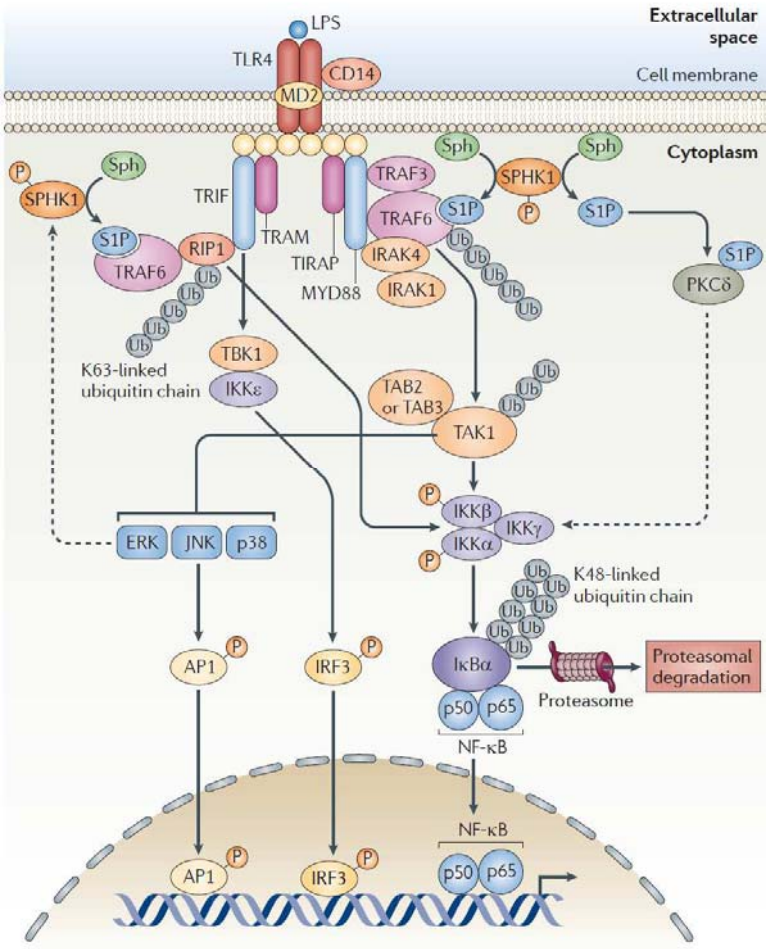

Figure 2. Signal transduction induced following activation of TLR4. MyD88 dependent and independent activation pathways are illustrated. (Spiegel and Milstien, 2011).

Since TLR4 and other TLR-triggering molecules are such powerful immune system's activators, they are tightly regulated. More precisely, TLR signaling pathway has many

negative regulators. A20 with double enzymatic activity of ubiquitin ligase and de-ubiquitinase is a regulator of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) activation through modulation of receptor-interacting protein 1 (RIP1) and TNF receptor associated factor (TRAF) 6 (Coornaert et al., 2009). TRAF family member-associated NF-kB activator (TANK) is also a negative regulator and TANK-deficient mice present autoimmune phenotype with uncontrolled production of IL-6 (Kawai and Akira, 2010).

1.4.2) DNA sensing and activation

DNA has been shown to be a potent activator of the immune system, and over the past few years, many of the DNA detectors have been discovered and characterized (Paludan and Bowie, 2013). DNA can be detected in cytoplasm, in endosome by TLR9 and in nucleus by interferon gamma-inducible protein 16 (IFI16). The latter contradicts with an earlier evidence that nucleus is an immune privileged cell compartment (Kerur et al., 2011).

DNA that triggers an immune response has different origins, including viral infections, microbial DNA originated from intracellular pathogens, DNA from apoptotic phagocytised cells, debris from DNA replication or endogenous retroviral products. For instance, when cells are infected by bacteria or by viruses their DNA is released to the cytosol. This DNA can be detected which hence trigger immune response. In the case of viral infections from herpes simplex virus and adenoviruses, proteins from capside are ubiquinated and degraded by proteasome and thereby expose viral DNA (Horan et al., 2013; Yan et al., 2002).

To prevent the accumulation of undesirable DNA, cells have nucleases that metabolise this DNA. Three prime repair exonuclease 1 (Trex1) and deoxyribonuclease (DNase) II are among these DNases. DNAse II, in particular, is crucial for apoptotic DNA's degradation in endosomes (Okabe et al., 2005).

Apart from cell specificity, reasons explaining the existence of a plethora of different nucleic acid detectors are to date not well understood (Unterholzner, 2013). In Figure 3, many of these detectors are illustrated together with signaling pathways they

activate. Several DNA sensors have been described to be present in macrophages, such as TLR9, TLR7 and IFI16. In contrast, the tissue location of more recently identified DNA sensors, such as DNA-dependent activator of IFN-regulatory factor (DAI) is not well determined.

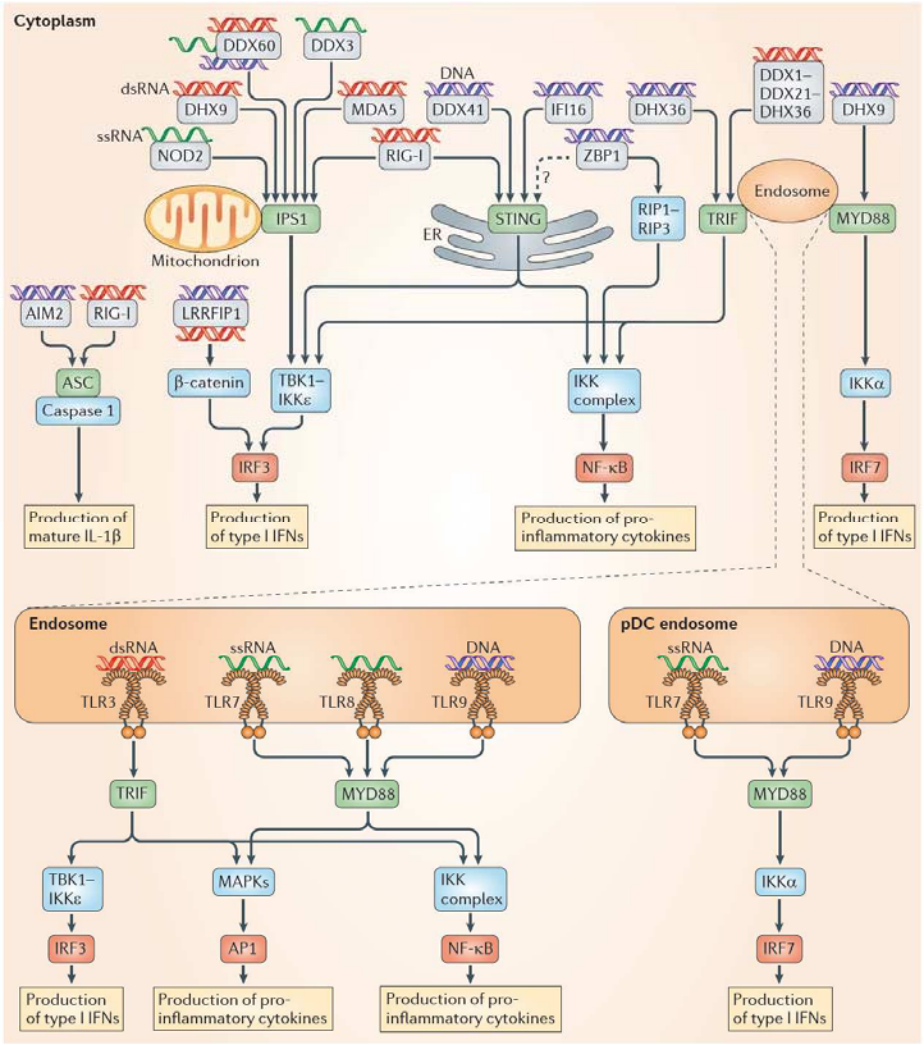

Figure 3. Overview of nucleic acid sensing pathways present in cytoplasm and in endosomes. Green boxes represent adaptor proteins, blue boxes refer to pathway-connecting proteins and red boxes represent transcription factors. The activation end products of the nucleic acid receptors are shown in beige boxes (Desmet and Ishii, 2012).

TLR9 is an endosomal TLR that is mostly expressed on antigen presenting cells, such as macrophages and dendritic cells. TLR9 presence in endosomes protects cells from detecting its own DNA. TLR9 ligand is CpG DNA found in bacteria and in mitochondrial DNA. Once bacterial DNA is in endosome it binds to TLR9 and triggers a signaling cascade dependent on MyD88 that activates interferon regulatory factors (IRF) 7 and NF-κB transcription factors in a cell (Kumagai et al., 2008). Recently, it also has been reported that TLR9 can recognise apoptotic DNA in the heart, and in the case of loss of DNAse II, cause autoimmunity (Oka et al., 2012).

Pyrin and hin domain-containing protein (PYHIN) proteins are characterised by pyrin domain in N-terminal and C-terminal HIN domain. These domains bind respectively proteins and DNA. Absent in melanoma 2 (AIM2) and IFI16 are two PYHIN proteins that are crucial for DNA-activated immune responses and have been described as DNA sensors. In response to double-strand DNA (dsDNA), AIM2 triggers the production of IL-1β in an inflammasome dependent way. IFI16 also binds dsDNA but activates IFN-β production through stimulator of interferon genes (STING). Both AIM2 and IFI16 are expressed in macrophages and are important for immune activation upon viral infection (Paludan and Bowie, 2013).

DExD/H-BOX Helicases (DDX) is a protein family of RNA and DNA helicases. These helicases are expressed in different cell types, including macrophages. Upon herpes simplex virus infection, DDX9 and DDX36 bind CpG DNA and trigger a MyD88 dependent response by producing IFN-α and TNF-α. DDX41, on the other hand, binds dsDNA through its DEAD domain and induces type I IFN production in a STING-dependent manner (Zhang et al., 2011). Recent evidence has shown that DNA-binding DDX can have a dual function, both as DNA sensor and as nuclease (Thompson et al., 2011).

DAI is a recently discovered cytosolic dsDNA receptor that signals through TANK-binding kinase 1 (TBK1) and IRF3. It has been shown to be expressed in fibroblasts, and to date, there is no evidence of its presence in immune cells. DAI is important for induction of IFN type I genes upon cell infection by cytomegalovirus (Paludan and Bowie, 2013).

c-GMP-AMP (cGAMP) synthase (cGAS) is a 60kDA protein. When cGAS encounters DNA it produces cGAMP (endogenous second messenger) activating STING (Civril et

al., 2013) The absence of cGAS is fibroblast, macrophages and other cells has been proven to abrogate the expression of IFN by these cells, indicating the importance of this DNA sensor is in the innate immune IFN production (Li et al., 2013).

DNA sensing in the nucleus is a recently observed phenomenon. IFI16 is expressed in both cytoplasm and nucleus. Besides detecting dsDNA in cytoplasm, as stated above, it also detects viral DNA in the nucleus. DNA damage response can also trigger IFN production. Recent publications report both Ku70 and meiotic recombination 11 (Mre11) as DNA detectors that are crucial in dsDNA break repair. These two molecules are also involved in triggering IFN production from nucleus and cytoplasm (Unterholzner, 2013).

1.4.3) IFNs and their receptors

IFNs are characterised by their role in antiviral and antimicrobial response that induce expression of IFN-stimulated genes (ISGs). The first data on these molecules dates back to more than 50 years ago, however, molecules pertaining to IFN type III sub family discovery dates back to just a decade ago (Borden et al., 2007). Figure 4 shows basic differences between the three subfamilies, their components and how they signal through their specific receptors.

Type I IFN sub-family is composed of 19 distinct molecules (Müller et al., 1994). Type I IFNs were extensively clinically studied and are applied in treatment of viral diseases, malignancies and autoimmune diseases. In an innate immune response, type I IFNs are expressed upon stimulation of PRRs, which detect viral and bacterial nucleic acids (Borden et al., 2007).

IFN I receptor is a heterodimer comprised of two chains, IFN alpha receptor (IFNαR)1 is constitutively associated to tyrosine kinase 2 (TYK2) and IFNαR2 to janus-associated kinase 1 (JAK1). When ligand and receptor interact, phosphorylation of kinases TYK2 and JAK1 occurs. Thereafter, signal transducer and activator of transcription (STAT) 1 and STAT2 interact with kinases. This binding leads to phosphorylation of tyrosine 701 and 690 in STAT1 and STAT2, respectively. Phosphorylated STAT1 and STAT2 hetero-dimerise and form a complex with IRF9

that is translocated to the nucleus. This complex is known as ISG factor 3 (ISGF3) and acts as a transcription factor that binds to promoter regions of genes containing sequence known as interferon stimulated response element (ISRE).

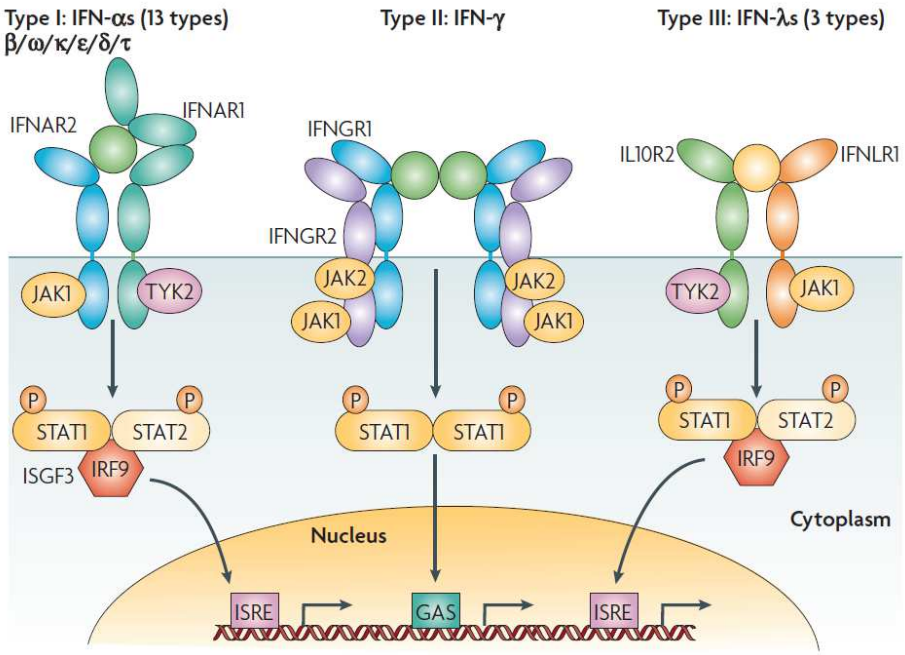

Figure 4. IFNs, their receptors and activated signaling pathways. The three types of interferons are depicted. All represented signalling pathaways depend on the phosphorylation and dimerization of STATs (Borden et al., 2007).

IFN-γ is the only **type II IFN** and has no structural resemblance to type I IFN. Although it also is relevant for the clearance of viral infections, IFN-γ has its own functions, including anti-proliferative effects on a range of different cell types (Valledor et al., 2008; Xaus et al., 1999b). IFN-γ has relevant function in elimination of intracellular bacterial infections and of tumor cells. It is also a stronger inducer of major histocompatibility complex (MHC) I and II molecules and leads to the activation of adaptive immune system by antigen presentation mechanism (Brucet et al., 2004; Cullell-Young et al., 2001). IFN-γ induces expression of type I IFN. This exemplifies the synergic functions of distinct IFNs for pathogen removal.

In humans, IFN-γ is expressed as non-covalent homo-dimer, which is, composed of two polypeptide chains with a molecular mass of each equal to 17-kDa. Upon dimerisation, however, in antiparallel form they are N-glycosylated and molecular mass of mature IFN-γ is 50kDa. Antiparallel structure allows IFN-γ to bind two IFN-γ receptors simultaneously. The main sources of IFN-γ are natural killers (NK), NK T-cells, CD4 and CD8 T-cells. NK and NK T-cells constitutively express IFN-γ mRNA. On the contrary, CD4 and CD8 T-cells require activation to express IFN-γ (Schoenborn and Wilson, 2007).

IFN-γ receptor is a heterodimer complex composed of two different sub-units IFNγR1 and IFNγR2, both of which are essential for signaling purposes (Bach et al., 1997). Both chains belong to class two cytokine receptor family. IFNγR1 chain is constitutively expressed and has a molecular mass of 90kDa. IFNγR2, on the other hand, is induced and has a variable molecular weight of approximately 65kDa.

Upon binding to IFN-γ in macrophages, that receptor is internalised with IFN-γ and thereafter are separated. More specifically, IFN-γ is transmitted to lysosome for degradation, while receptors' alpha chain is stored for subsequent re-use (Celada and Schreiber, 1987; Celada et al., 1984).

When IFN-γ binds to IFNγR1 connection between different receptors' chains becomes robust, and conformational change takes place. Induced changes in receptor allow auto-phosphorylation of JAKs that are constitutively expressed with IFN-γ receptor. JAKs phosphorylation activates them and phosphorylates tyrosine 440 in IFNγR1 chain. This phosphorylation event is followed by SH2 domain STAT1 docking. Thereafter, STAT1 is phosphorylated in tyrosine 701 and dimerisation of the two IFNγR1 bound STAT1s takes place. STAT1 homo-dimers are translocated to the nucleus, where they act as transcription factors binding to gamma-activated site (GAS) elements in the promoter regions of ISGs inducing their transcription (Kearney et al., 2013).

1.4.4) Macrophages pro-inflammatory activation response

As mentioned above, macrophages can be activated in many ways after triggering of proper receptors. Once activated, their different signaling pathways are induced, and a large number of genes is either up-regulated or down-regulated. As a result of inflammation, macrophages suffer modifications in their morphology and functional capacities. Through secretion of different cytokines, macrophages regulate both the activity of surrounding immune cells and of cells at long distance. Macrophages activation also induces antigen presentation process, which activates specific T cells. Activated macrophages also increase their ability to phagocyte pathogens and apoptotic cells produced upon pathogen destruction.

Cytokine and chemokine production are crucial for immune system. These molecules are secreted by different cells. Their expression and secretion occur upon activation of the abovementioned signaling cascades. Secretion of different pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12) and type I interferon are part of the pro-inflammatory response cytokines produced by macrophages. Besides the secretion of these cytokines, pro-inflammatory macrophages also produce chemokines that trigger migration of other immune cells to inflammatory loci. Macrophages are known to secrete IFN-gamma-inducible protein 10 (IP-10), macrophage inflammatory protein (MIP)-1α and monocyte chemoattractant protein (MCP)-1 that attract T-cells and other macrophages to inflammatory loci (Mosser, 2003).

Phagocytosis is a process used by myeloid cells to internalise and degrade target particulates larger than 0,5μM. These particles are usually micro-organisms, apoptotic cells, dead cells or cellular debris. Therefore, phagocytosis is crucial for proper immune response, including proper pathogen clearance and post-inflammatory tissue homeostasis (Underhill and Goodridge, 2012).

To be internalised, these particulates interact with specific receptors in macrophages' membranes, which include Fc receptors, mannose, scavenger and complement receptors. Binding to these receptors induces actin polymerisation on the site of internalisation and creates a vacuole called phagosome. When fully internalised, actin is shed and phagosome suffers a series of fusions and fissions episodes with endosomes and lysosomes. These steps allow phago-lysosome to achieve the

environment of necessary acidity that is required for proteolytic enzymes to digest and degrade phagocytosed particles (Aderem and Underhill, 1999).

Particles recognition process by phagocytes discriminates between pathogens (dead or alive) and apoptotic cells. Depending on the phagocytosed component, macrophages activate secretion of different cytokines. For example, upon phagocytosis of living bacteria macrophages secrete, among others, IL-6, IL-1β and TNF-α. If macrophages phagocyte dead bacteria, which does not have bacterial DNA, IL-1β is not secreted. Furthermore, phagocytosis of self-apoptotic cells does not induce secretion of pro-inflammatory cytokines, however, if these apoptotic cells are T-cells, MIP-2 has been shown to be secreted to the environment attacking other immune cells, such as neutrophils (Underhill and Goodridge, 2012).

Phagocytosis is a complex process that clears the organism from harmful particles, assesses the danger and communicates with other immune cells through cytokine production and release. Certain intracellular pathogens, however, have managed to circumvent this process. For instance, s*almonella typhimurium, legionella pheumophila* and *mycobacterium tuberculosis* have developed survival mechanisms and are even able to survive and continue growing within macrophages (Aderem and Underhill, 1999).

Antigen presentation by macrophages is a crucial link between innate and adaptive immunity. It is tightly related to macrophages phagocytic ability as peptides or antigens used for presentation arise from degraded proteins of phagocyte material. Proteins present in the endosomes are degraded and the antigens are loaded into MHC class II complex which can specifically bind a T-cell receptor and induce T-cell activation. Macrophages antigen presentation is increased upon stimulation, which induced MHC class II, CD86, CD80 and other co-stimulatory proteins expression levels. All these proteins are required in the process of antigen presentation. (Vyas et al., 2008).

2) DNA damage

Genomic integrity is vital for living organisms. Proper transmission of genetic information to descendants is crucial for ensuring continuation of species. Normal metabolic activities and environmental factors, such as ultraviolet (UV) light and radiation can cause DNA damage in human cells. This damage may amount to at least a few hundred lesions per cell per day (Allgayer et al., 2013). In particular, these lesions may produce structural alterations in DNA modifying genes or the capacity to transcribe such genes. This, in turn, results in either mutations or inhibition of the expression. In other cases, lesions in the genome, upon mitosis in cells, may affect daughter cells survival. For the above reasons, DNA repair processes are continuously repairing damaged DNA. If these processes fail, affected cells would either undergo senescence or apoptosis.

2.1) DNA damage lesions

DNA damage can be categorised as a) DNA base damages, such as O^6-methylguanine or mismatch that can be generated in the process of standard DNA replication, b) backbone DNA damage that includes single strand breaks and double-strand breaks (DSB) and c) crosslinks, which are also referred to as bulky lesions (Sancar et al., 2004) (Figure 5). These injuries can occur sporadically during DNA replication or exposure to endogenous damage, such as reactive oxygen species. Furthermore, they are also a result of exposure to UV light, ionizing radiation, impact of cigarette fumes or carcinogens present in the environment. “DNA damage sensors” detect damages in DNA and activate repair mechanisms.

The main DNA repair mechanism are base excision repair (BER), nucleotide excision repair (NER), direct repair (DR), mismatch repair (MMR), homologous recombination (HR) and non-homologous end joining (NHEJ) are the major repair pathways used by cells. The repair mechanisms together with the correspondent pathways involved are presented in Figure 5 (Postel-Vinay et al., 2012). Briefly, in BER, damaged bases are recognised by 8-Oxoguanine glycosylase (OGG1) with further recruitment of DNA

polymerase (i.e. Polymerase beta) and of DNA ligases (Robertson et al., 2009). NER is a form of repair that can be activated in two manners depending on the form of damage recognition. In both cases, 22-30 base excision occurs prior to repair. In MMR, single-strand break (SSB) is created to enable repair of damaged DNA. Finally, DR uses direct damage reversal and employs solely one protein, O^6-alkylguanine-DNA alkyltransferase (AGT) (Daniels et al., 2004).

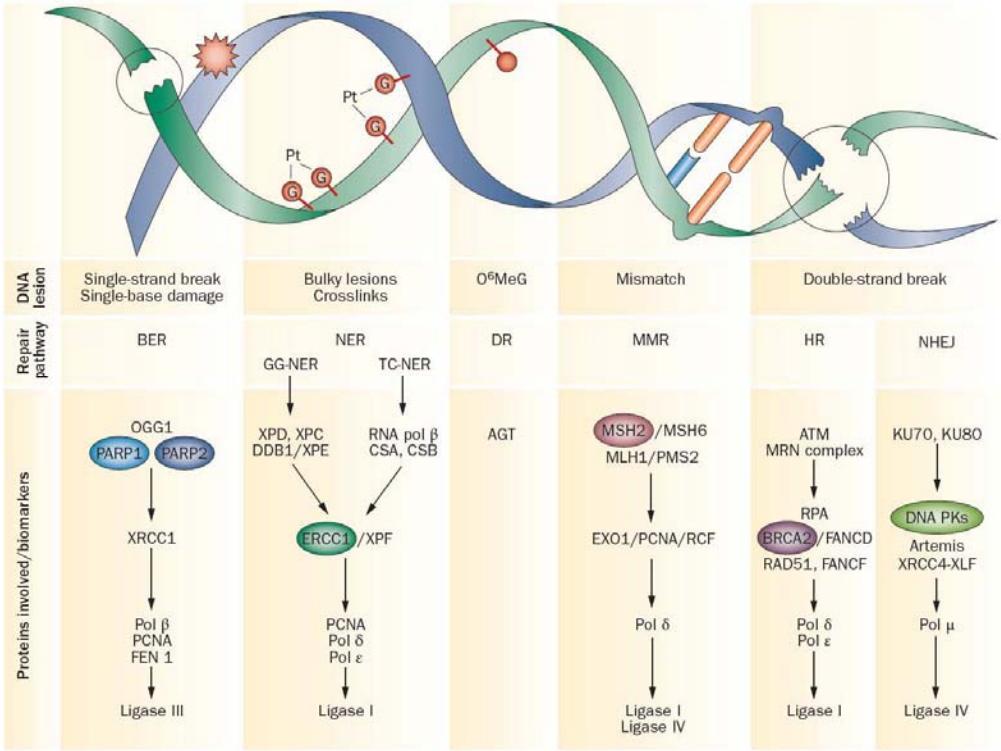

Figure 5. Types of DNA damage and repair pathways present in cells. (Postel-Vinay et al., 2012). BER, base excision repair; NER, nucleotide excision repair; DR, direct repair; MMR mismatch repair; HR, homologous recombination and NHEJ, non-homologous end joining.

2.2) DSB DNA damage response

DSB are deemed to be one of the most harmful DNA damage modifications as they cannot use the complementary strand sequence information for guided DNA repair (Kass and Jasin, 2010). Lack of control of these DNA lesions can generate chromosomal translocations, and result in an abnormal number of chromosomes causing disease. DSB can cause different maladies, such as cancer, immunodeficiency, neurodegenerative disorders, as well as ageing and infertility (Jackson and Bartek, 2009). Due to severity of DSB's impact, molecular response upon detection of damage is highly controlled by different molecular mechanisms, and if damage repair proves impossible, affected cells enters into senescence or apoptosis to avoid growth of precancerous cells (Bohgaki et al., 2010).

DSB repair is performed in three main ways: HR, DNA-PK-dependent non-homologous end joining (D-NHEJ) and backup non-homologous end joining (B-NHEJ). (Figure 6). Choice of the pathway is not well understood but is proven to depend on cell type, species and cell cycle when DSB is detected (Lamarche et al., 2010). HR is the prime choice when DSB is repaired in S and G2 phases of the cell cycle. This is due to the fact that sister chromatids are readily available and generate error-prone free repair. Since it does not depend on sister chromatid, NHEJ is normally chosen when DSB occurs throughout cell cycle (Delacôte and Lopez, 2008). B-NHEJ is slower and less efficient than D-NHEJ and is thus considered to be as a last resort to DSB repair. That is because in this case, the loss of DNA information is the highest and is the main origin of chromosomal translocation (Schipler and Iliakis, 2013).

HR starts with detection by MRE11 complex. DNA resection is initiated after commitment to HR. The first resection is accomplished by Mre11 with help of CtIP. Resection extension is done by Exonuclease 1 (Exo1) and Blm. Remaining ssDNA is stabilised by RPA and exchanged with RAD51 for search of matching sister chromatid. DNA polymerase extends 3' extremes that have sister chromatid as template. This process leads to error free DNA repair (Langerak and Russell, 2011).

D-NHEJ, on the other hand, does not involve DNA resection. Central proteins in this repair process are Ku70 and Ku80 that detect DSB and strongly bind to the break and protect it from resection. Artemis and DNA-PKCs are recruited to the site and prepare

DNA for polymerases and DNA ligase IV. This process is highly efficient and is commonly chosen for mammalian cells due to fact that they spend the longest time in G0/G1 phase of the cell cycle (Langerak and Russell, 2011).

B-NHEJ was first reported in 1996 in Ku70 deficient cells (Boulton and Jackson, 1996). This pathway uses micro-homologous sequences that do not normally exist in DSB. Due to this, resection occurs with great loss of nucleotide sequences. B-NHEJ uses part of HR machinery to accomplish this highly mutagenic repair, and was found to occur in many cancerous cells (Decottignies, 2013).

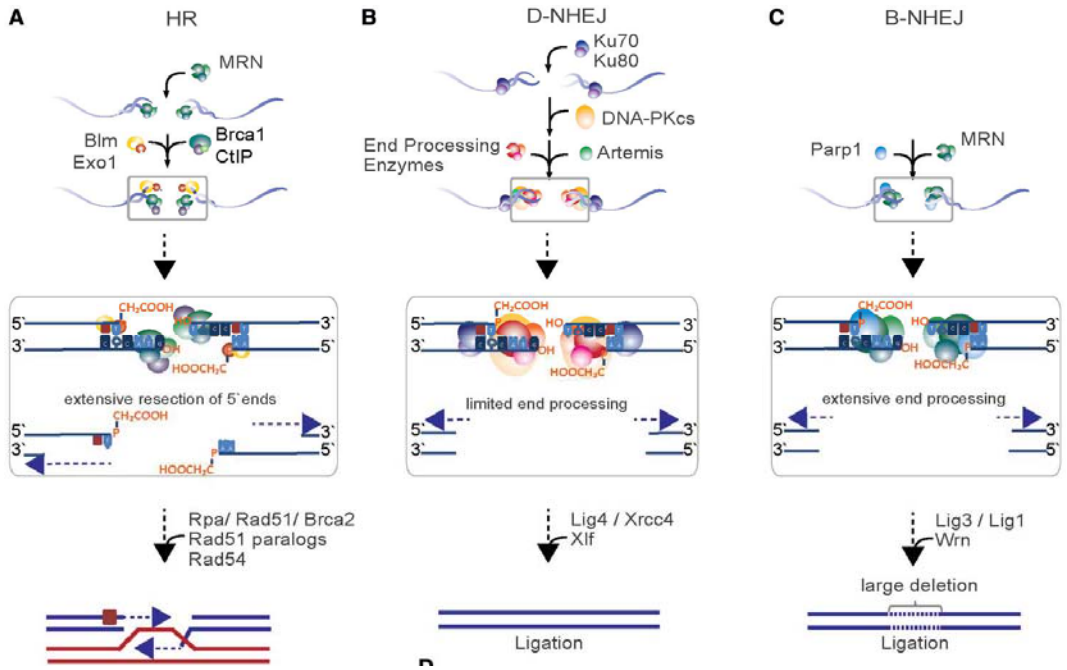

Figure 6. DSB repair mechanisms and machinery implicated in distinct processes (Schipler and Iliakis, 2013). HR, Homologous recombination; D-NHEJ, DNA-PK-dependent non-homologous end joining; B-NHEJ, backup non-homologous end joining.

2.3) Cell cycle checkpoints and DSB

Cell cycle is divided into four main phases: Mitotic phase (M), first Gap phase (G1), DNA synthesis phase (S) and second gap phase (G2). Progression in cell cycle is regulated by cyclin-dependent kinases (CDKs) which bind to cyclins originating a

complex that is inactivated by phosphorylation. When the cell is prone to continue in cell cycle the CDK-cyclin complex is dephosphorylated and the cell is allowed to progress (Santamaría et al., 2007).

If DNA is damaged cell cycle is stalled to enable the repair of the damage. This is enabled by three checkpoints in mammalian cells: G1/S, intra-S and G2/M. The proteins involved in checkpoints can be classified as DNA damage sensors, signal mediators, signal transducers and effectors. MRE11 complex is the main DNA damage sensor in DSB. This complex activates ataxia telangiectasia mutated (ATM) that serves as signal transducer. ATM belongs to PIKK kinase family; these proteins are responsible for activating effectors by phosphorylation. Effector proteins are, among others, histone2AX (H2AX) and protein 53 (p53) (Fragkos et al., 2009). These proteins are necessary for the decision of cell cycle progression, senescence or apoptosis.

2.4) Macrophages and DNA damage

Inflammation is generated in response to body damage, including infection. To clear pathogens, macrophages and other innate immune cells produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). However, in case of the overproduction of this reactive species surrounding tissue can be affected (Lonkar and Dedon, 2011). Macrophages produce nitric oxide (NO) and hydrogen peroxide (H_2O_2) (Figure 7) (Khansari et al., 2009). Depending on its concentration, NO exerts particular tasks. AKT phosphorylation, for instance, occurs when NO levels are approximately 30-100nM, while p53 is phosphorylated when the concentration is higher than 400nM. On the other hand, NO concentration exceeding 500nM bring about toxicity (Thomas et al., 2008). In normal conditions, production of these free radicals ends upon the removal of the pathogen. If this does not occur, as in the case of chronic inflammation, DNA is highly susceptible to free radical attacks causing DNA breaks and other damages, as discussed above. As a result, in the context of chronic inflammation, macrophages have a critical role in DNA damage as they are ROS producers.

Besides inducing DNA damage to cells in the environment, macrophages and monocytes can have their functional activity altered due to damaged DNA. Recent

studies have demonstrated that upon irradiation, the activation of macrophages can be altered and skewed to a more pro-inflammatory phenotype (Klug et al.; Mboko et al., 2012). Furthermore monocytes, precursors of macrophages, were shown to have ROS hyper-sensitivity, with increased apoptosis levels due to presence of DSB and activation of ATM and ATR DNA damage response pathways (Bauer et al., 2011).

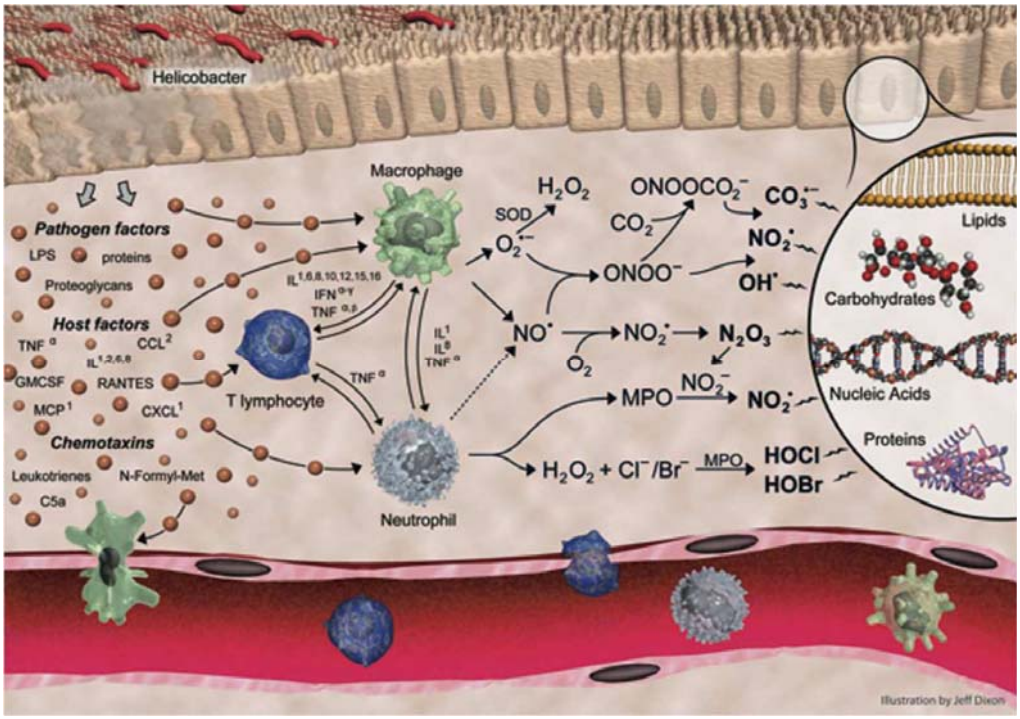

Figure 7. Reactive species produced in inflammation site. On the left side, molecules that activate neutrophils and macrophages are represented. In the center, ROS and RNS produced by macrophages and neutrophils and their interactions are represented. On the right side, biological molecules that are altered and damaged by interaction with ROS and RNS are depicted (Lonkar and Dedon, 2011).

3) Three-prime repair exonuclease 1 (Trex1)

3.1) Structure

Trex1 is characterised as an autonomous exonuclease since it is not associated to any polymerase (Mason and Cox, 2012). Trex1 belongs to DnaQ-like (DEDD) family, which displays low levels of sequence identity towards DNA that they degrade (Brucet et al., 2008). Structurally, Trex1 is homo-dimer composed of two globular domains. These domains consist of five beta sheets and nine alpha chains. Beta sheets are interior and two monomers bind through them in a perpendicular way (Brucet et al., 2007).

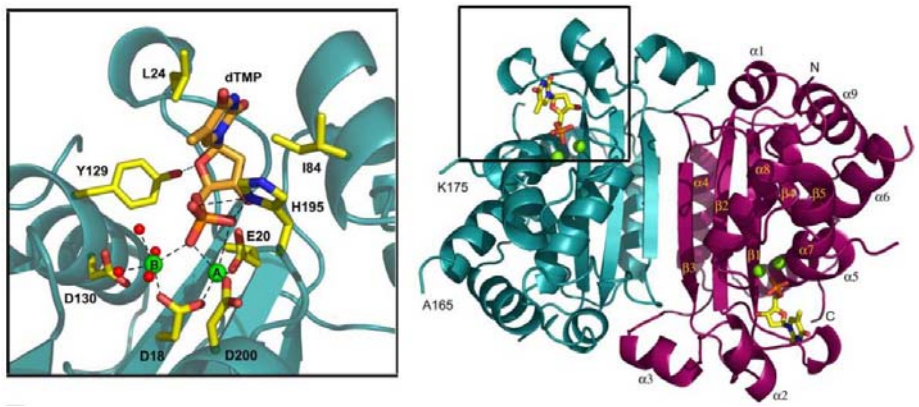

Figure 8. Trex1 structure and its active site. The left panel depicts the enlarged area of the active site of Trex1, where it binds DNA. In the right panel, the full structure of exonuclease is shown (Brucet et al., 2007).

In its catalytic site Trex1 has four highly conserved amino-acids. These are, Asp18, Glu20, Asp130 and Asp200 that bind manganese ions and create its active center, which cleaves DNA (Figure 8). It was observed that manganese is crucial for catalytic activity of Trex1, while lithium and sodium inhibit it. Trex1 is also composed of three leucine rich regions, two in N-terminal and a third one that is highly hydrophobic. C-

terminal forms a trans-membrane helix, this domain is important for Trex1 ability to stay in the cytoplasm, since the lack of this trans-membrane region leads to permanent translocation to the nucleus (Brucet et al., 2007). It is also important to note that Trex1 has a proline rich region that enables binding to other proteins, such as transcription elongation regulator 1 (CA150) (Brucet et al., 2007).

3.2) Function

Trex1 is an intracellular exonuclease that degrades excess DNA. The lack of function of Trex1 leads to accumulation of DNA in cells. This DNA is detected as a viral infection and generates an "anti-viral state" in the whole organism (Crow and Rehwinkel, 2009). The source of this DNA is still unclear although some studies suggest that its origin is diverse. Genomic retroelements, DNA damage and residual replication fork DNA have been demonstrated to be the endogenous sources of the Trex1degraded DNA. It has also been reported that some viruses use Trex1 as a way to avoid detection by the immune system. For instance, human immunodeficiency virus (HIV) uses Trex1 as a modulator of the quantity of retroviral DNA present in the cytoplasm, in this way, the levels of HIV DNA in the cell are maintained to a minimum avoiding immune detection but allowing viral replication (Yan et al., 2010).

Furthermore, cells lacking Trex1 from both human and mouse have been demonstrated to present chronic activation of cell cycle checkpoint in an ATM-dependent way. This checkpoint activation was related with an increase of 60 to 65 nucleotide length ssDNA originated in the lagging-strand DNA synthesis. Moreover, when Trex1 was absent in fibroblasts a blockage of cell cycle occurred in the G2/M phase (Yang et al., 2007). Trex1 has also been characterised as belonging to the endoplasmic reticulum-associated complex (SET complex). This complex is comprised of other endonucleases and together with Trex1 degrades DNA that has previously been nicked in granzyme A mediated apoptosis. To be activated, the SET complex has to undergo proteolysis, which induces its translocation to the nucleus. Furthermore, the SET complex is also involved in BER DNA repair and DNA replication. toguether, these evidences suggest that Trex1 has a function in DNA damage repair (Chowdhury et al., 2006).

More recently it was demonstrate that the oxidation of DNA, more specifically oxidized base-8-hydroxyguanosine (8-OHG) induced ISG transcription through cGAS/STING signaling. This DNA detection was due to DNA accumulation in the cytoplasm as Trex1 was unable to degrade the oxidized DNA (Gehrke et al., 2013). This oxidized DNA was generated from exposure to ROS, neutrophil extracellular trap (NET) from oxidative burst and UV exposure.

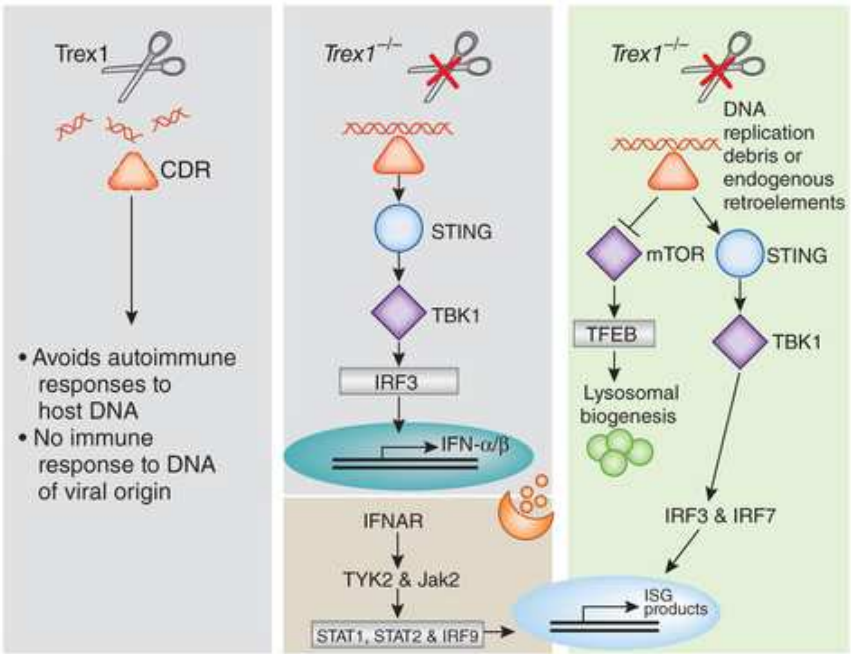

Figure 9. Effects of the lack of Trex1 on the cell. On the left, Trex1 digests cytosolic DNA avoiding autoimmunity. The middle panel shows what pathways are activated by accumulated DNA in the cytoplasm when Trex1 is absent in the cell. On the right panel, Trex1 role on lysosomal biogenesis is demonstrated (Simon and Ballabio, 2013). CDR, cytosolic DNA receptors.

By studying the deregulation at molecular and cellular levels of the lack of Trex1, more clarity has been added to its function. It has also been demonstrated that when Trex1 is absent in cells, lysosomes are deregulated with the change of gene expression profile (Hasan et al., 2013). Figure 9 provides for the illustration of a summary of Trex1 role in the cell. Although the detector of the accumulated cytosolic DNA is not yet discovered, STING (Stetson et al., 2008) is the first known signaling modulator which

is required for downstream activation of TBK1 and further IRF3 and IRF7 ISG induced transcription.

3.3) Associated diseases

In humans, mutations in the gene encoding Trex1 are associated to different diseases (Table 1). Aicardi goutieres syndrome (AGS), systemic lupus erithematosus (SLE) and familial chilblain lupus (FCL) are diseases that have been reported to be caused or aggravated by Trex1 mutations. The retinal vasculopathy with cerebral leukodystrophy (RVCL) is a rare disease also associated with mutated Trex1. Phenotypes in the four abovementioned diseases are different but the type I interferon is present in three out of the four diseases.

AGS was first reported in 1984 by Jean Aicardi and Francoise Goutieres. The disease was characterised by brain degeneration in children accompanied of chronic lymphocyte infiltration and brain calcifications. AGS phenotype mimics a congenital viral infection that is ruled out by serological tests (Kavanagh et al., 2008). There are six genes that have been shown to be mutated in this disease: Trex1, RNAseH2a, b, c, SAMHD1 and ADAR1 (Rice et al., 2013). These genes encode proteins with different DNA metabolic functions.

SLE is an autoimmune disease that seems to have been first characterised more than 2000 years ago by Hippocrates. "Lupus", as a term, was first introduced 1000 years ago by Herbernus due to the resemblance of the skin wounds in the face to wolf bites, hence the name that means "wolf" in latin (Smith and Cyr, 1988). SLE is a multisystem chronic disease that is present in 0.1% of the population, affecting women nine times more than men. This disease is depicted by high levels of anti-nuclear antibodies (ANA) and IFN production. In this disease, all immune cells are dysfunctional. Macrophages have a defect in apoptotic cell clearance and overproduction of pro-inflammatory cytokines, and an enhanced antigen presentation (Byrne et al., 2012). In 0,5% of patients mutations of *TREX1* have been reported (Namjou et al., 2011).

FCL is a rare form of cutaneous lupus erythematosus. Two missense mutations in *TREX1* were found in these patients. Twenty percent of individuals present later onset of SLE. Chilblains of these patients can be ameliorated upon treatment with steroids (Hedrich et al., 2008).

RVCL is caused by a mono-allelic mutation in Trex1. The symptomatology of this disease is quite different of other diseases associated to Trex1. Patients affected by RVCL suffer visual loss, dementia and stroke. The difference in the phenotype may be due to the specific mutation present in these patients. In RVCL, Trex1 is mutated in the transmembrane region and not in the catalytic domains. This leads to a translocation of Trex1 to the nucleus and not to an intrinsic loss of function (Kavanagh et al., 2008).

Table 1. Summary of recognised phenotypes associated with TREX1 mutations in humans. Autosomal recessive (AR) and Autosomal Dominant (AD). Adapted from Crow and Rehwinkel, 2009.

	AGS	RVCL	FCL	SLE
Inheritance	AR and rare AD cases	AD	AD	Rare monogenic forms
Genes	*TREX1*, *RNASEH2a,b, c*, *SAMHD1 and ADAR1*	*TREX1*	*TREX1*	Monogenic*:* *TREX1*, *DNASE1*, complement deficiency
Onset	Prenatal—usually <12 months	30–50 years	Childhood	Usually 15–40 years
Mortality	40% <10 years of age	5–20% 10 year mortality (from onset)	Non-lethal	5–20% 10 year mortality
Neurological involvement	Severe intellectual and physical disability	Strokes, seizures, migraine, cognitive decline	None	Neuro-lupus: strokes, seizures, psychosis, cognitive decline

3.4) Mouse model

Trex1$^{-/-}$ was generated by Morita et al., 2004 by eliminating the the only exon that codify the protein. Although initially the group was interested in this mouse model due to its possible role in DNA damage and expected an increased tumor incidence, *Trex1*$^{-/-}$ did not present these features. They did find that *Trex1*$^{-/-}$ showed a short half-life (10 to 20 weeks, depending on the group and mouse facility) (Morita et al., 2004; Stetson et al., 2008). As the majority of humans that have mutations in *TREX1*, mice present systemic inflammation with high levels of type I IFN. *Trex1*$^{-/-}$ is mainly affected in the heart and the production of type I interferon is initiated in this organ (Gall et al., 2012). Crossing *Trex1*$^{-/-}$ with IFN alpha receptor KO or with STING KO mice reverses the pathogenic phenotype. These experiments proved that the diseases' origin in the mouse is due to the type I IFN and that the signaling pathway used depends on STING (Stetson et al., 2008).

4) Nbs1 and MRE11 complex

4.1) Structure and function

The MRE11 complex is composed of Mre11, Rad50 and Nbs1, all these proteins are highly conserved over species and together they bind to DNA DSB to stabilise and brings support for DNA damage repair and signaling (Stracker and Petrini, 2011). The role of this complex was referred in connection with its crucial role in DSB repair. Apart from their enzymatic and structural functions in the DSB repair, MRE11 complex has an important role in telomere homeostasis. Below we will describe the characterisation of the structure and function of the components of MRE11 complex.

Mre11 is a 70 to 90 kDa nuclease protein that exists as a dimer. It is the connecting element of the complex binding Nbs1 and Rad50. The nuclease domain is located at the N-terminal, and is dependent on manganese and magnesium ions (Lamarche et al., 2010). The C-terminal of Mre11 has a DNA-binding domain and glycine-arginine rich (GAR) domain, that is methylated and is vital for the biochemical functions and for foci formations of this nuclease (Déry et al., 2008).

Rad50 is a 150kDa protein with N- and C-terminal Walker A nucleotide binding domains separated by two coiled-coil domains that are highly flexible and a middle zinc hook. The zinc domain mediates the assembly of the complex. RAD50 is the structure responsible for maintaining the constant distance between two sister chromatids and can be characterised as the scaffold of the complex (Stracker and Petrini, 2011).

Nbs1 (Nijmegen breakage syndrome 1), also known as Nibrin, is a ~90kDa protein with N-terminal forkhead-associated (FHA) domain and two BRCA1 C-terminus (BRCT) domains. In C-terminal, Nbs1 has a 24 amino-acid conserved motif which interacts with ATM and a Mre11 binding domain. FHA domain interacts with threonine phosphorylated residues from a Ser-X-Thr motif in other DNA damage proteins as mediator of DNA damage checkpoint protein 1 (Mdc1) and C-terminal-binding protein interacting protein (Ctp1). On the other hand, the BRCT domains bind serine

phosphorylated from the same motif (Kobayashi et al., 2002). Nbs1 is important also for the Mre11 and Rad50 translocation from the cytoplasm to the nucleus and the recruitment for the machinery of DSB repair. This protein can be phosphorylated and also has the ability to phosphorylate other genes. This demonstrates its central function in MRE11 complex (Lamarche et al., 2010).

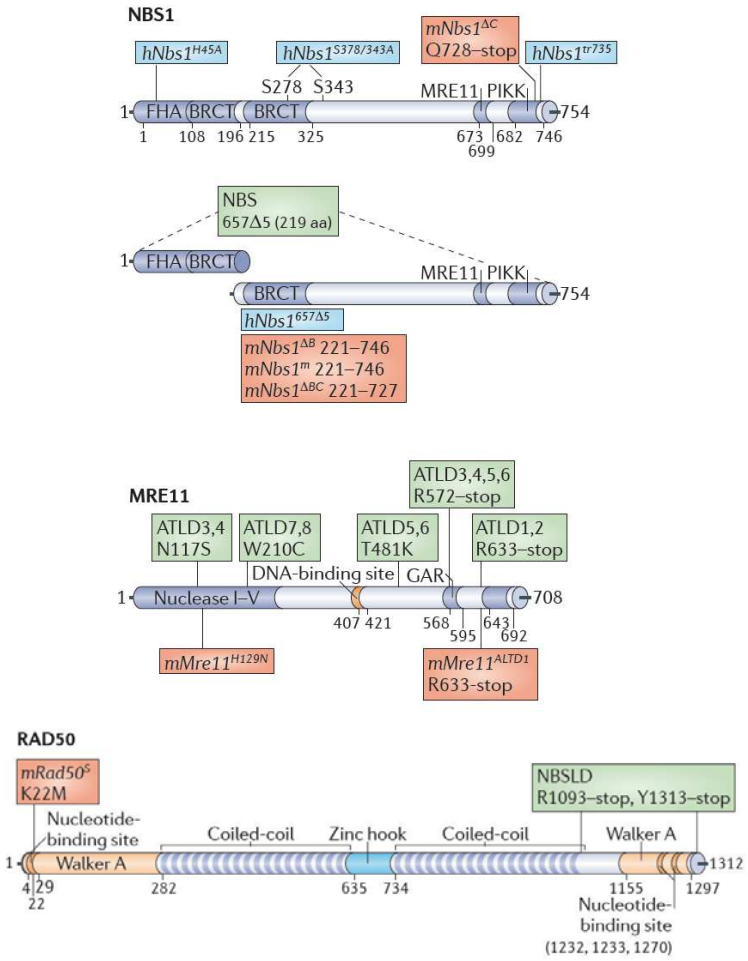

Figure 10. The domains of MRE11 complex in human disease. All functional domains of the component proteins of MRE11 complex are shown. The numbers indicate the number of amino acid. Green boxes indicate human mutations that cause different diseases. Proteins expressed in mouse models are represented in red boxes. Blue boxes show humanised mouse models. Adapted from Stracker and Petrini, 2011.

4.2) Associated diseases

It is important to refer that there are four syndromes with similar symptomatology that are caused by mutations in one of the genes encoding the proteins of MRE11 complex or ATM. The mutations in Nbs1 in humans are present in **Nijmegen breakage syndrome (NBS)** patients. This disease is defined by higher incidence in cancer, microcephaly, radio-sensitivity, growth delay and immunodeficiency. There are different Nbs1 mutations and the majority of them lead to Nbs1 truncation (Figure 10). The most common mutation is 657del5 which results in protein truncation. This leads to the expression of short N-terminal protein containing FHA/BRCT domain plus a 70kDa C-terminal protein. All Nbs1 mutations are likely able to retain part of Nbs1 functions, which would explain why the elimination of the full protein in mice is lethal (Bohgaki et al., 2010). Immunological defects in patients have been related to the modifications of the lymphocytes levels in blood. Reduced percentage of lymphocytes is present in NBS individuals and the differences between the NBS individuals and controls is reduced when the age increases (Piątosa et al., 2012)

Nijmegen breakage syndrome like disorder (NBSLD) has a similar phenotype to NBS. Patients present microcephaly, growth retardation and radio-sensitivity. However, they do not present immunodeficiency or cancer predisposition. This disorder is presented in humans with RAD50 hypo-morphic mutations (Lamarche et al., 2010).

Ataxia-telangiectagia (AT) is a disorder caused by a mutation in the gene encoding ATM. This disease is characterised, as it name indicates, by cerebral ataxia, oculo-cutaneous telangiectagia and immune defects. These patients also present neuro-degeneration and radio-sensitivity. It is interesting that, although one third of the patients develop lymphoid or breast cancer there are others that present metabolic defects as insulin resistance (Jackson and Bartek, 2009).

Ataxia-telangiectagia like disorder (ATLD) in contrast to AT, does not present cancer predisposition, immune-deficiency or telangiectagia. ATLD presents, on the other hand cerebellar atrophy and radio-sensitivity. This disorder is associated in humans with mutations in the Mre11 gene. All point mutations associated with ATLD are located in a critical location of interaction with Nbs1 (Schiller et al., 2012). In mice,

the same mutations lead to increased cancer predisposition and elimination of the full protein is lethal (Bohgaki et al., 2010).

Table 2. Alleles of the MRE11 complex in mice. Adapted from Stracker and Petrini, 2011.

Allele*	Allele type	Phenotypes
hNbs1$^{657\Delta5}$	Transgene, N-terminal truncation	S/G2 checkpoint defects, damage sensitivity, chromosomal instability, reduced ATM activity, impaired T cell development, subfertility
hNbs1^{H45A}	Transgene, point mutation	S/G2 checkpoint defects, reduced ATM activity
hNbs1$^{S278/343A}$	Transgene, point mutations	None described
hNbs1^{tr735}	Transgene, C-terminal truncation	Apoptosis defect, reduced ATM activity
Mre11$^{\Delta}$ (*Mre11a*$^{tm2.1Dpf}$)	Conditional deletion	Embryonic lethality, reduced class switch recombination and DSB repair in B cells (CD19–Cre promoters)
Mre11^{ATLD1} (*Mre11a*^{tm1Jpt})	C-terminal truncation	S/G2 checkpoint defects, damage sensitivity, chromosomal instability, reduced ATM activation and activity, defective apoptosis, reduced fertility
Mre11^{H129N} (*Mre11a*^{tm1Dpf})	Conditional allele with point mutation	Embryonic lethality, reduced class switch recombination in B cells (CD19–Cre), DNA repair defects and damage sensitivity
Nbs1$^{\Delta6}$ (*Nbn*^{tm1Md}) and *Nbs1*$^{-}$ (*Nbn*^{tm1Zqw})	Targeted deletion	Early embryonic lethality
Nbs1$^{\Delta B}$ (*Nbn*^{tm1Jpt})	N-terminal truncation	S/G2 checkpoint defects, damage sensitivity, chromosomal instability, reduced ATM activity, subfertility
Nbs1$^{\Delta BC}$ (*Nbn*^{tm1Jpt})	N- and C-terminal truncations	S/G2 checkpoint defects, damage sensitivity, chromosomal instability, reduced ATM activity, subfertility, apoptosis defect
Nbs1$^{\Delta C}$ (*Nbn*$^{tm2.1Jpt}$)	C-terminal truncation	Apoptosis defect, S phase checkpoint defect, reduced ATM activity
Nbs1^{F6} (*Nbn*^{tm2Zqw}), *Nbs1*$^{\Delta6}$ (*Nbn*$^{tm1.1Md}$) and *Nbs1*$^{\Delta}$ (*Nbn*^{tm2Nus})	Conditional deletions	Reduced class switch recombination in B cells (CD19–Cre promoter), microcephaly, neuronal apoptosis, cerebellar defects and ataxia (nestin–Cre promoter), lymphopenia, T cell development defects (Lck–Cre promoter)
Nbs1^{m} (*Nbn*^{tm1Xu})	N-terminal truncation	S/G2 checkpoint defects, damage sensitivity, chromosomal instability, reduced ATM activity, subfertility, cancer
Rad50$^{\Delta}$ (*Rad50*^{tm1Jpt})	Targeted deletion	Early embryonic lethality
Rad50^{ind} and *Rad50*$^{-}$ (*Rad50*^{tm3Jpt})	Conditional deletion	Chromosomal instability and death in dividing cells (MX1–Cre, PCP2–Cre promoters)
Rad50^{S} (*Rad50*^{tm2Jpt})	Knock-in point mutation	Embryonic lethality, bone marrow failure, cancer predisposition, activated DDR, sensitivity to topoisomerase poisons

4.3) Mouse model

All full KO mice of individual proteins of MRE11 complex are lethal. This evidence demonstrates how crucial is the function of this complex in mammalian life (Bohgaki

et al., 2010). There are many mouse models created to mimic the defects in the abovementioned diseases. In Figure 10, the sequence affected in distinct models is presented, and Table 2 summarises the phenotypes of different models (Stracker and Petrini, 2011).

Experimental work presented in this Thesis related to Nbs1 was performed with $Nbs1^{\Delta B/\Delta B}$ mouse model (Williams et al., 2002). This mouse model presents a N-terminal truncation form of Nbs1. The phenotype of cells originated from $Nbs1^{\Delta B/\Delta B}$ presenting chromosomal instability and defects in cell cycle which mimic part of the NBS patients' cell defects. The mouse model does not, on the other hand, mimic neurological defects found in patients. Untill now, immunological defects associated to patients have neither been observed nor further assessed by experimental immune challenge.

Hypothesis and Objectives

Hypothesis

The role of DNA damage repair proteins is critical for pro-inflammatory activation of macrophages.

Objectives

1) To study the role of Trex1 in macrophages in autoimmune disease and inflammation.

2) To understand the function of Nbs1 in the normal macrophages inflammatory response.

Publications

Characterization of Trex1 Induction by IFN-γ in Murine Macrophages

Summary of "Characterization of Trex1 Induction by IFN-γ in Murine Macrophages" in Spanish

Caracterización de la inducción de Trex1 por IFN-γ en macrófagos de ratón

TREX1 es la 3'→5' exonucleasa más abundante en los mamíferos. Esta exonucleasa tiene actividad específica para ADNss. La deficiencia en TREX1 se ha relacionado con el desarrollo de enfermedades autoinmunes en ratones y en seres humanos, donde causa el síndrome de Aicardi-Goutieres. Además, se han asociado polimorfismos de TREX1 con el Lupus Eritematoso Sistémico. En base a los conocimientos que se tienen de estas enfermedades se supone que TREX1 actúa destruyendo el ADN endógeno. En este estudio, mostramos que TREX1 está regulado por el IFN-γ durante la activación de los macrófagos primarios. IFN-γ induce el aumento de la expresión de TREX1 con una cinética correspondiente a la de un gen de expresión temprana, esta inducción se produce a nivel de la transcripción. La vida media del ARNm es relativamente corta (70 min). El gen que codifica para TREX1 tiene un solo exón y un intrón de 260pb en la región del promotor del ARNm no traducido. Se detectaron tres inicios de la transcripción, pero el más importante es el que se localiza a -580pb. En experimentos de transfección transitoria utilizando el promotor TREX1, hemos encontrado dos secuencias dependientes de la activación por IFN-γ, así como una secuencia AP-1 que también depende de la inducción por el IFN-γ. Mediante el uso de la técnica de EMSA y con ensayos de inmunoprecipitación de cromatina, se ha determinado que STAT1 se une a las dos secuencias dependientes de IFN-γ. La necesidad de la participación de STAT1 para la inducción de TREX1 fue confirmada mediante la utilización de macrófagos provenientes de ratones knockout para Stat1. También hemos determinado que la proteína c-Jun se une a la secuencia AP-1, sin embargo son dispensables c-Fos, jun-B o CREB. Por lo tanto, nuestros resultados indican que el IFN-γ induce la expresión de la exonucleasa TREX1 través de STAT1 y c-Jun.

Characterization of *Trex1* Induction by IFN-γ in Murine Macrophages

Maria Serra, Sonia-Vanina Forcales, Selma Pereira-Lopes, Jorge Lloberas, and Antonio Celada

3′ Repair exonuclease (Trex1) is the most abundant mammalian 3′→5′ DNA exonuclease with specificity for ssDNA. Trex1 deficiency has been linked to the development of autoimmune disease in mice and humans, causing Aicardi-Goutières syndrome in the latter. In addition, polymorphisms in *Trex1* are associated with systemic lupus erythematosus. On the basis of all these observations, it has been hypothesized that Trex1 acts by digesting an endogenous DNA substrate. In this study, we report that *Trex1* is regulated by IFN-γ during the activation of primary macrophages. IFN-γ upregulates *Trex1* with the time course of an early gene, and this induction occurs at the transcription level. The half-life of mRNA is relatively short (half-life of 70 min). The coding sequence of *Trex1* has only one exon and an intron of 260 bp in the promoter in the nontranslated mRNA. Three transcription start sites were detected, the one at −580 bp being the most important. In transient transfection experiments using the *Trex1* promoter, we have found that two IFN-γ activation site boxes, as well as an adaptor protein complex 1 box, were required for the IFN-γ–dependent induction. By using EMSA assays and chromatin immune precipitation assays, we determined that STAT1 binds to the IFN-γ activation site boxes. The requirement of STAT1 for *Trex1* induction was confirmed using macrophages from *Stat1* knockout mice. We also establish that c-Jun protein, but not c-Fos, jun-B, or CREB, bound to the adaptor protein complex 1 box. Therefore, our results indicate that IFN-γ induces the expression of the Trex1 exonuclease through STAT1 and c-Jun. *The Journal of Immunology*, 2011, 186: 2299–2308.

The most abundant mammalian 3′→5′ DNA exonuclease that shows specificity for ssDNA is 3′ repair exonuclease (Trex1) (1). Trex1 mutations are associated with autoimmune diseases characterized by the chronic production of IFN in the absence of viral infections (2). *Trex1* knockout mice experience development of myocarditis and circulatory failure of inflammatory origin (3). In humans, mutations in *Trex1* cause Aicardi-Goutières syndrome (AGS) and autosomal retinal vasculopathy with cerebral leukodystrophy (4). Trex1-deficient cells from knockout mice or AGS patients accumulate ssDNA in the perinucleus and show cell cycle defects as a result of chronic checkpoint activation (5). Recently, it has been demonstrated that Trex1 participates in the digestion of DNA from retrotranscribed endogenous elements, and a prominent function for Trex1 in the IFN-stimulatory DNA response has been highlighted (6). Finally, cytosolic Trex1 suppresses IFN triggered by HIV, and in this way inhibits the innate immune response to HIV type 1 (7).

Macrophages play a key role in the immune response. These phagocytic cells are produced in the bone marrow and transported through blood as monocytes to distinct tissues. Most monocytes die through apoptosis; however, in the presence of certain cytokines or growth factors, they proliferate, differentiate into several cell types of macrophages (e.g., Kupffer cells, Langerhans cells, microglia), or become activated macrophages to develop their functions. At the inflammatory loci, the macrophage phagocytizes bacteria, removes cell debris, releases several mediators, presents Ags to T lymphocytes, and contributes to the resolution of inflammation (8).

IFN-γ, which is released by activated T lymphocytes, is the most potent activator of macrophages and induces the expression of >300 genes (9). IFN-γ exerts its effect by triggering the phosphorylation of STAT1, which then homodimerizes, thereby allowing it to interact with the IFN-γ activation site (GAS) (10, 11). Also, STAT1, in combination with p48 (IFN-stimulated gene factor 3γ), binds to the IFN-stimulated regulatory element (ISRE) sequence and transactivates genes that bear these sites in their promoter (12). Transcription factors of the IFN regulatory factor (IRF) family, such as IRF-1, IRF-2, and IFN consensus sequence-binding proteins, are induced by this pathway (13).

We have previously shown that the exonuclease activity of Trex1 is correlated to the binding preferences toward certain DNA sequences (14). To unravel how discrimination occurs, we determined the crystal structure of Trex1. When macrophages become activated, Trex1 is translocated to the nucleus but must first lose the C-terminal segment to do so. Through a proline-rich region in the surface, Trex1 interacts with proteins containing the WW2 domain. These observations suggest that Trex1 participates in more functional complexes than previously thought. Trex1 belongs to the exonuclease DEDDh family, whose members display low levels of sequence identity while possessing a common fold and active site organization. We demonstrate that lithium and sodium inhibit the exonucleolytic activity of Trex1 by inducing subtle rearrangements in active centers. Our analysis also revealed that a histidine residue (His124), highly conserved in the DEDDh family, is involved in the activity of Trex1 (15).

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Abbreviations used in this article: AGS, Aicardi-Goutières syndrome; CHX, cycloheximide; DBR, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; GAS, IFN-γ activation site; IRF, IFN regulatory factor; ISRE, IFN-stimulated regulatory element; SA-PMP, streptavidin-paramagnetic particle; Trex1, 3′ repair exonuclease; UTR, untranslated region.

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In this study, we show that, in macrophages, IFN-γ transcriptionally upregulates the expression of *Trex1* with the time course of an early gene. We determined the transcription start site of *Trex1*, and that two GAS boxes and an adaptor protein complex 1 box in the promoter are required for this induction.

Materials and Methods

Cell culture

Bone marrow-derived macrophages were isolated from 8-wk-old BALB/C mice (Charles River Laboratories, Wilmington, MA) as described previously (16). Bone marrow cells from the femora, tibia, and humerus were flushed and cultured in plastic tissue culture dishes (150 mm) in DMEM containing 20% FCS (PAA Laboratories, Pasching, Austria) and 30% of L cell conditioned media as a source of M-CSF. Medium was supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 7 d of culture, a homogeneous population of adherent macrophages was obtained (>99% Mac-1). *Stat1* knockout mice were kindly provided by Dr. Anna Planas (Consejo Superior de Investigaciones Cientificas-Institut d'Investigacions Biomèdiques August Pi i Sunyer, Barcelona, Spain) and Dr. Robert D. Schreiber (Washington University School of Medicine, St. Louis). For the experiments with these mice, we used the corresponding background mouse controls. Animal use was approved by the Animal Research Committee of the University of Barcelona (Procedure No. 2523). The RAW264.7 macrophage cell line (American Tissue Type Collection) was maintained in DMEM 10% heat-inactivated FCS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Human PBMCs were obtained from buffy coat preparations from healthy donors (Hospital Clinic, Barcelona, Spain). Mononuclear cells were isolated by density gradient centrifugation through Ficoll-Histopaque (Sigma-Aldrich Quimica S.A., Madrid, Spain), and monocytes were purified by adhesion to plastic (17). The bone marrow-derived dendritic cells were generated as described previously (18). For stimulation studies, saturating amounts of LPS (10 ng/ml) were used (19). LPS was obtained from Sigma-Aldrich (St. Louis, MO).

Reagents

rIFN-γ was obtained from R&D Systems (Minneapolis, MN). Actinomycin D, cycloheximide (CHX), LPS, and DBR (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside) were from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest purity grade available and were also purchased from Sigma-Aldrich. Deionized water that had been further purified with a Millipore Milli-Q system (Bedford, MA) was used.

Anti-Trex1–specific sera were obtained by immunizing rabbits with the mutant Trex1 H195A (14), which was performed by Vivotecnia (Madrid, Spain). Abs against STAT1, c-Fos, and phospho-c-Jun were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The monoclonal anti–diphospho-ERK-1/2 (clone MAPK-YT), anti–jun-B, and anti–β-actin Ab were from Sigma-Aldrich. The Abs against CREB were from Upstate Biotechnology (Lake Placid, NY), whereas those against tubulin were from Invitrogen (Camarillo, CA). Secondary HRP anti-mouse (MP Biomedicals, Irvine, CA) and anti-rabbit (Sigma-Aldrich) Abs were also used. All restriction enzymes were from Roche (Basel, Switzerland).

Quantitative RT-PCR analysis

Cells were washed twice with cold PBS, and total RNA was extracted with the EZ-RNA Kit (Biological Industries, Kibbutz Beit Haemek, Israel), following the manufacturer's instructions. For quantitative RT-PCR analysis, RNA was treated with DNAse (Roche) to remove contaminating DNA. For cDNA synthesis, 1 μg RNA and Moloney murine leukemia virus RT RNase H Minus, Point Mutant, oligo$(dT)_{15}$ primer and PCR nucleotide mix were used, following the manufacturer's instructions (Promega Corporation, Madison, WI). The primers used to amplify murine *Trex1* cDNA were 5′-CGTCAACGCTTCGATGACA-3′ and 5′-AGTCATAGCGGTCACCGTTGT-3′. Real-time monitoring of PCR amplification of cDNAs was performed using the SYBR Green PCR Core Reagents (Applied Biosystems) in the ABI Prism 7900 Detection System (Applied Biosystems). The relative quantification of gene expression was performed as described in the SYBR Green user's manual using β-actin (5′-ACTATTGGCAACGAGCGGTTC-3′ and 5′-AAGGAAGGCTGGAAAAGAGCC-3′). The threshold cycle was defined as the cycle number at which the fluorescence corresponding to the amplified PCR product is detected. The PCR arbitrary units of each gene were defined as the mRNA levels normalized to the β-actin expression level in each sample.

Western blot analysis

Total cytoplasmic extracts were made by lysing cells as described previously (20). SDS-PAGE was performed, and the gel was transferred to nitrocellulose membranes (Hybond-C; Amersham Biosciences, Buckinghamshire, U.K.). After blocking with milk, incubation with Trex1-specific serum and anti-rabbit secondary Ab was performed. Detection was done using ECL (Amersham), and the membrane was exposed to x-ray films (Agfa, Mortsel, Belgium). β-actin was used as a loading and transferring control.

Northern blot

Total RNA was extracted as described earlier. For mRNA extraction, polyATtract mRNA isolation system from Promega (Madison, WI) was used, following the manufacturer's instructions. In brief, total RNA was heated at 65°C for 10 min and incubated with a biotinylated-oligo(dT) probe. Streptavidin-paramagnetic particles (SA-PMPs) were added to the suspension, and the mixture was incubated for 10 min. SA-PMPs were recovered using a magnetic stand, supernatant was removed, and SA-PMP pellet was washed four times with 0.1× SSC. The mRNA was eluted by resuspending the final pellet in RNase-free water, capturing the SA-PMPs and recovering the supernatant.

Total RNA (20 μg) or mRNA (2 μg) was mixed with loading buffer (deionized formamide 0.5%, 1× buffer MOPS/EDTA, formaldehyde 6%, glycerol 0.13%, bromophenol blue 0.013%), denaturalized at 65°C, and separated by electrophoresis in an agarose/formaldehyde gel 1.5%. Samples were transferred by capillarity to a nylon membrane Hybond XL (Amersham) overnight in the presence of 10× SSC buffer (NaCl 1.5 M, sodium citrate 0.15 M; pH 7). RNA was fixed in the membrane by UV irradiation (150 mJ) in a Stratalinker (Bio-Rad).

As a probe, *Trex1* full-length cDNA (1300 bp) was cloned into pKS^+ vector from Stratagene (La Jolla, CA) and digested using MspA11 and EcoRI enzymes. This procedure produced a 1021-bp fragment comprising the ATG translation initiation codon to the poly A site. Fifty nanograms of the probe was labeled with 50 μCi α[^{32}P] dCTP from ICN Pharmaceuticals (Costa Mesa, CA) using 10 units of Klenow fragment (Pharmacia Biotech, Stockholm, Sweden) and the Oligolabeling Kit (Pharmacia Biotech). After the labeling, the probe was purified in a G50 Nick Column (Pharmacia Biotech), and the efficacy of the labeling was evaluated using the Packard TRiCARB 1500 (Packard Instrument, Meriden, CT).

Membranes were prehybridized for 4 h at 65°C in hybridization buffer (deionized formamide 20%, 4× SSC phosphate/EDTA, 5× Denhardt's solution, SDS 5%, dextran sulfate 10%, and 0.2 mg/ml denaturalized salmon sperm DNA). The labeled probe was then denaturalized at 95°C for 5 min and added at 2 × 10^6 cpm/ml to the hybridization buffer. Hybridization was performed for 18 h at 65°C, after which the membrane was sequentially washed during 30 min with 3× SSC 0.1% SDS, 1× SSC SDS 0.1%, and 0.1× SSC 0.1% SDS. The membrane was sealed in hybridization bags from Life Technologies (Invitrogen, Carlsbad, CA) and visualized by exposure to a photographic film (Kodak).

RACE-PCR

The genomic DNA of *Trex1* was obtained from a mouse library (129 SVJ) using the cDNA of *Trex1* as probe. The SMART RACE cDNA Amplification Kit from Clontech (Mountain View, CA) was used to recover the 5′-flanking regions of *Trex1* cDNAs. Two rounds of PCR were performed following the manufacturer's specifications. For cDNA synthesis, 1 μg total or poly A RNA and Superscript II (Life Technologies) enzyme were used. A first PCR was performed using the Universal Primer Mix as the forward primer, which consists of a Long Universal Primer: 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGT-3′ and a Short Universal Primer: 5′-CTAATACGACTCACTATAGGGC-3′. As reverse primers, *Trex1*-specific oligonucleotides located at −305 (5′-*Trex1* RACE nested) or −353 (rev st/d1) were used from the ATG translation initiation codon. The product of this PCR was used as template for a second PCR reaction using a Nested Universal Primer: 5′-AAGCAGTGGTAACAACGCAGAGT-3′ as the forward primer and *Trex1*-specific rev st/d1 (−353) as the reverse primer. The products of these PCRs were resolved by electrophoresis, purified using QiaExII (Qiagen, Düsseldorf, Germany), cloned in the pCR2.1 vector (Clontech), and sequenced.

Reporter plasmids

The *Trex1* promoter region was cloned from murine genomic DNA isolated from bone marrow-derived macrophages into plasmid pCR2.1 (Invitrogen) and subcloned directionally into pGL3 basic vector (Promega). The oligonucleotides used as forward primers were as follows: for the

350-bp construct, 5′-GGGTGGACCCAGGCAGTTTTACTTC-3′; for the 850-bp construct, 5′-GCACTTCCTGCTGAGAACAC-3′; for the 1.4-kb construct, 5′-GCCAGGGGTCAGCATGCTGATTC-3′; and for the 2.6-kb construct, 5′-GCAGGGCAGGACAGTCTCAACTG-3′. In all cases, 5′-ATG-CTGAGCCTGGGGAGGAGATGG-3′ was used as the reverse primer.

The pGl3-*Trex1*$_{-850}$ vector was used to introduce mutations of individual binding sites using the QuickChange Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). The GAS box from −690 to −679 was mutated using oligonucleotides 5′-CCT GCC CCT CTT CGC GAA TTA CCC TGA G-3′ and 5′-CTC AGG GTA ATT CGC GAA GAG GGG CAG G-3′. The adaptor protein complex 1 box from −675 to −669 was mutated with 5′-GGA ATT ACC CTG AGT TGT AGC TTT GGG CGG GGG-3′ and 5′-CCC CCG CCC AAA GCT ACA ACT CAG GGT AAT TCC-3′; the ISRE box from −337 to −325 was mutated using 5′-GGA CCC AGG CAG TTG ACC TTC CTC AGC CAC ACT G-3′ and 5′-CAG TGT GGC TGA GGA AGG TCA ACT GCC TGG GTC-3′; and the GAS box from −273 to −263 was mutated using 5′-GTG CTT GCT GCC TGC TTG GAG GTA AGT GTG GAG TAA GG-3′ and 5′-CCT TAC TCC ACA CTT ACC TCC AAG CAG GCA GCA AGC AC-3′. All plasmids were sequenced using Big Dye V.2 from Perkin Elmer (Wellesley, MA).

Transient transfection and dual reporter luciferase-renilla assays

For plasmid transfection, 5×10^4 RAW264.7 cells were seeded in 1 ml DMEM 10% FCS in 24-well plates. Cells were cotransfected with a renilla luciferase expression plasmid to verify uniformity of transfection. Transfection of 1 μg plasmid DNA (pGL3 constructs and pRL-TK-Renilla at a 100:1 ratio) per well was carried out using the JetPei-Man Kit from Genycell Biotech (Granada, Spain) and following the manufacturer's instructions. Twenty-four hours later, cells were stimulated with IFN-γ for 24 h or left untreated. Firefly luciferase and renilla activity was determined using Promega Dual Luciferase reporter assay system and on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) following the product instructions.

Nuclear extracts

Nuclear extracts were prepared from bone marrow-derived macrophages as described previously (21), with some modifications. For gel shift experiments, confluent cultures of macrophages were scraped and centrifuged (5 min at 1500 rpm, 4°C), and the pellet was rinsed twice with ice-cold PBS. The pellet was resuspended in 5 volumes of hypotonic buffer (10mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT) and centrifuged at 1500 rpm for 5 min at 4°C. The pellet was then resuspended in three volumes of hypotonic buffer and allowed to stand on ice for 10 min. The lysates were homogenized in a potter, and the homogenate was centrifuged at 5000 rpm for 20 min at 4°C to pellet crude nuclei. The nuclear pellet was resuspended in 1/2 volume of low-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% [v/v] glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT), followed by the addition of 1/2 volume of high-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 25% [v/v] glycerol, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). The crude nuclei were extracted at 4°C for 30 min with continuous stirring, followed by centrifugation at 14,000 rpm for 30 min. Supernatants were dialyzed with the PlusOne Mini Dialysis Kit (Amersham Biosciences, San Francisco, CA) in dialysis buffer (20 mM HEPES, pH 7.9, 20% v/v glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT) at 4°C. The extracts were cleared by centrifugation at 14,000 rpm for 20 min, and the supernatant was collected in aliquots and stored at −80°C until use. Protein concentrations were measured using the Bio-Rad Protein Assay Kit (Bio-Rad).

EMSA

EMSAs were performed as described previously (18). In brief, binding reactions were prepared with 10 μg of nuclear extracts and 20,000 cpm ^{32}P-labeled probe in the presence of 2 μg poly(deoxynosinic-deoxycytidylic) acid sodium, in a final volume of 15 μl containing 1× binding buffer (12 mM HEPES, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 0.12 mM EDTA, 0.3 mM PMSF, 0.3 mM DTT, 12% glycerol). Extracts and poly (deoxynosinic-deoxycytidylic) acid sodium were preincubated for 8 min. The radiolabeled probe was then added and incubated for 15 min at room temperature. Samples were loaded onto 8% acrylamide gel containing 5% glycerol and 0.25% Tris borate EDTA (TBE), and electrophorated at 4°C. Gels were dried and bands were visualized using a Phosphoimager (Molecular Dynamics, Sunnyvale, CA). For supershift experiments, after the binding reaction, 2 μg Abs was added and incubated for 30 min. For competition experiments, 100-fold excess of unlabeled primers was included in the binding reaction. The oligonucleotides used as probes in the assay were 5′-end labeled using T_4 polynucleotide kinase (USB Corporation, Cleveland, OH). All of them were synthesized by Genotek (Barcelona, Spain).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation analysis was performed as described previously (22). Approximately 2×10^7 of bone marrow-derived macrophages were grown on 15-cm^2 dishes and cross-linked to attached cells at room temperature for 20 min by addition of formaldehyde (to a final concentration of 1%). Cells were washed twice with PBS, then collected with scraper and centrifugation into 3 ml of 0.1 M Tris HCl, pH 9.4, 10 mM DTT. Cells were incubated at 30°C for 15 min and collected by centrifugation at 2000 × *g* for 5 min at 4°C, and the resulting pellet was resuspended sequentially in cold PBS, in buffer I (10 mM HEPES, pH 6.5, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and protease inhibitors: 1 mM PMSF, 1 mM iodomycine, 1 mM orthovanadate, 10 μg/ml aprotinin, 1 μg/ml leupeptin) and buffer II (10 mM HEPES, pH 6.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and protease inhibitors), and centrifuged at each step. Cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris HCl, pH 8.1, and protease inhibitors) and sonicated on ice using the Ikasonic U200S Control (Ika Labortecnik, Staufen, Germany) with 20 pulses of 10 s, 30% cycle, 30% amplitude. The size of the fragments obtained (between 200 and 1200 bp) was confirmed by electrophoresis. The soluble chromatin was cleared by centrifugation during 10 min at 16,000 × *g*, and the supernatant was diluted 10 times in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris HCl, pH 8.1, and protease inhibitors). For the control of input chromatin, 100 μl was saved.

Chromatin was precleared by incubation with 20 μg sonicated salmon sperm DNA (Amersham), 3 μg unspecific rabbit IgGs (Sigma-Aldrich), and 10 μl preimmune rabbit serum at 4°C overnight with 100 μl protein A Sepharose beads at 50%. After a short spin, the supernatant (precleared chromatin) was incubated for 8 h with 2 μg of each specific Ab. After that, 100 μl of 50% protein A Sepharose beads was added and the mixture was incubated at 4°C overnight. The beads were collected and washed sequentially for 10 min at 4°C with TSE I (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1), TSE II (500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris HCl, pH 8.1) and buffer III (0.25 LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris HCl, pH 8.1). The beads were washed with TE buffer and eluted three times with 100 μl of 0.1 M $NaHCO_3$, 1% SDS. Cross-links were reversed by incubating samples and input chromatin at 65°C overnight, and DNA was purified with the GFX Purification Kit (Amersham), eluted with 30 μl H_2O, and assayed by quantitative PCR using the following primers: 5′-CCTCTTCGGGAATTACCCTGAG-3′ and 5′-CCTT-TTCTTGTCTTTCCCCAGTCC-3′. In some control experiments, we used a fragment of 2035 bp of the *I-Aβ* that contains an adaptor protein complex 1 box at −1722 bp from the start of transcription (18). The following primers were used: CAGAGGACAGGAGGGTGG and CCCGCCCTAC-CGAGCTTG.

Statistical analysis

The Student paired *t* test was used to detect statistical differences.

Results

IFN-γ induces Trex1 expression in macrophages

After interaction of IFN-γ with bone marrow-derived mouse macrophages, a large number of genes are upregulated or downregulated. In this study, we used quantitative real-time RT-PCR to determine the levels of *Trex1* in bone marrow-derived macrophages. With the exception of transfections, this type of macrophage was used in all the experiments. As a function of time after stimulation with IFN-γ, an increase in *Trex1* was detected (Fig. 1*A*). *Trex1* expression increased within the first hour of stimulation, reaching a maximum at 6 h, when it reached <5-fold the basal level. By 24 h of incubation, expression had begun to decrease. The induction of *Trex1* mRNA was accompanied by increase in the protein level, as confirmed by Western blotting (Fig. 1*A*). Trex1 protein was detected in resting macrophages, but its levels started to increase at about 6 h of stimulation with IFN-γ,

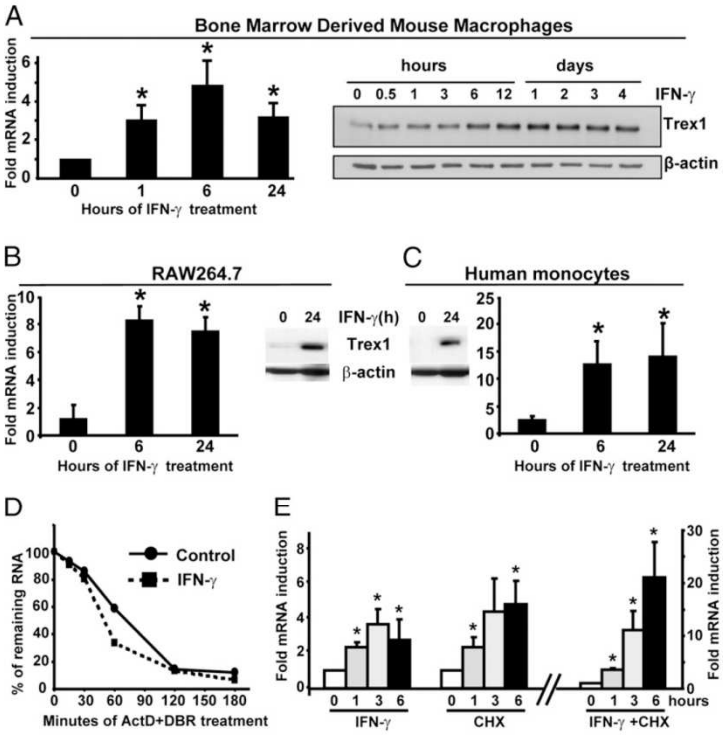

FIGURE 1. Trex1 mRNA and protein are induced by IFN-γ in bone marrow-derived macrophages. *Trex1* expression was analyzed by quantitative real-time PCR or by Western blotting in bone marrow-derived macrophages (*A*), in the murine macrophagic cell line RAW264.7 (*B*), and in human monocytes (*C*). *D*, *Trex1* stability was analyzed in macrophages treated or not treated with IFN-γ. After 3 h of stimulation with IFN-γ, actinomycin D (ActD) and DBR were added to the cell culture to block further mRNA synthesis, and samples were collected at the time points indicated. *Trex1* expression was analyzed by real-time PCR. Data are shown as the percentage of *Trex1* remaining in comparison with cells not treated with ActD or DBR. *E*, *Trex1* induction by IFN-γ does not require new protein synthesis. Macrophages were treated with protein translation inhibitor CHX for 30 min before stimulation with IFN-γ, and *Trex1* expression was determined as in *A*. All assays are representative of at least four independent experiments showing similar results. *A–C* and *E*, Each point was performed in triplicate and the results are shown as mean ± SD. $*p < 0.01$ in relation to the controls when all the independent experiments had been compared.

reaching a plateau at about 12 h. This maximum level was maintained during the 96-h time course studied. The decrease in mRNA was due to the short half-life; however, no decrease in protein levels was observed. This finding is attributed to the long half-life of the protein, as detected through chase and pulse experiments with radiolabeled thymidine (data not shown).

Bone marrow macrophages are difficult to transfect in some experiments. We therefore used the macrophage cell line RAW264.7. In these cells, IFN-γ induces Trex1 at mRNA (Fig. 1*B*) and protein level (Fig. 1*B*). Also, we tested the expression of Trex1 in human monocytes. Similar to murine cells, Trex1 was induced by IFN-γ (Fig. 1*C*).

To examine whether the increase in *Trex1* expression induced by IFN-γ treatment occurred at the transcriptional level or was due to mRNA stabilization, we determined the half-life of *Trex1* transcripts in cells treated or not treated with IFN-γ (Fig. 1*D*). Macrophages were treated with IFN-γ for 3 h, thereby inducing *Trex1*. Actinomycin D and DBR were then added at a concentration sufficient to block all further mRNA synthesis (5 and 20 μg/ml, respectively), as determined by [^{3}H]UTP incorporation (23). We then isolated mRNA from aliquots of cells at distinct intervals. Measurement of *Trex1* expression by quantitative RT-PCR allowed us to estimate that the half-life of this mRNA in resting cells is quite short, about 70 min (Fig. 1*D*). IFN-γ treatment did not modify the stability of *Trex1*, thus indicating that the induction of this gene in response to IFN-γ was at the transcription level.

Trex1 expression began to increase within the first hour of IFN-γ treatment. This observation suggests that *Trex1* is an early gene, and thus its induction may not require new protein synthesis. To confirm this hypothesis, we treated cells with IFN-γ in the presence of 10 μg/ml CHX. This treatment produced a >90% reduction in the incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material (23). Macrophages were treated with CHX 30 min before and during stimulation with IFN-γ; cells were then incubated in normal media and gene expression was analyzed. This short treatment with CHX was carried out because this substance is highly toxic for macrophages. CHX alone increased *Trex1* expression, and the combined stimulation of IFN-γ and CHX had a synergistic effect on the induction of the gene (Fig. 1*E*). These observations imply that the two stimuli induce *Trex1* by distinct mechanisms. Moreover, the finding that CHX treatment did not block IFN-γ–induced *Trex1* synthesis provides evidence that this induction is not dependent on de novo protein synthesis.

To analyze whether the inhibition of protein synthesis affected the half-life of *Trex1*, we quantified the half-life of this gene in resting macrophages and in IFN-γ–treated macrophages pretreated with CHX. Treatment with CHX dramatically increased the *Trex1* half-life in both resting and IFN-γ–activated macrophages (Fig. 2). This observation demonstrates the presence of a protein factor with short half-life that requires continuous synthesis and is responsible for *Trex1* destabilization. This observation would explain the synergistic effect of the combined stimulation with IFN-γ and CHX on *Trex1* induction.

Determination of a splicing process in Trex1

By comparing the genomic and *Trex1* cDNA sequences, we determined that the *Trex1* coding region is localized in a single exon. Regarding the 5′-untranslated region (UTR), *Trex1* cDNA lacked a 260-bp fragment, spanning from positions −267 to −7, which coincide with a splicing donor and a splicing acceptor site, respectively. Analysis of the genomic sequence revealed another possible splicing donor site at −243 and two possible splicing acceptor sites at −207 and +12 (Fig. 3*A*). To determine whether other cDNAs were produced by alternative splicing, we performed RT-PCR using a forward primer beginning at −370 (for St/d1) and a reverse primer beginning at +13 (rev *Trex1*-5′), and resolved by electrophoresis. Two bands were detected with calculated molecular masses of 400 and 120 bp. Both were cloned and sequenced.

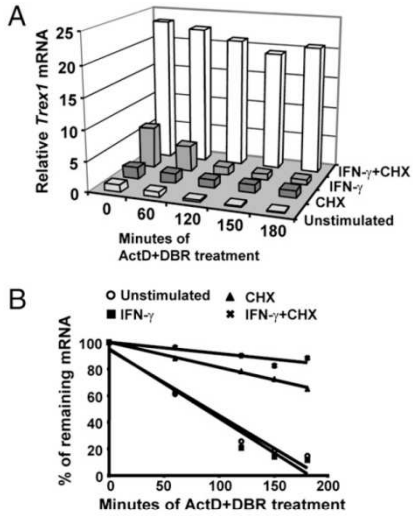

FIGURE 2. *Trex1* mRNA stability is dramatically increased by CHX treatment. *Trex1* mRNA stability was determined in resting macrophages, macrophages treated with IFN-γ, macrophages treated with CHX, or both IFN-γ and CHX. *Trex1* mRNA stability was determined as in Fig. 1*C*. *A*, Decay of *Trex1* mRNA expression is shown. *B*, Data are represented as the percentage of remaining mRNA at each time point of actinomycin D (ActD) + DBR in relation to untreated cells. This assay is representative of at least four independent experiments showing similar results.

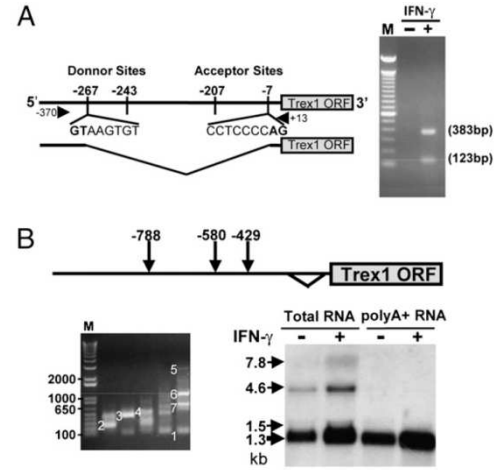

FIGURE 3. Processing of an intron in *Trex1* at the 5′-UTR. *A*, Scheme represents two putative splicing donor sites and two putative splicing acceptor sites in the *Trex1* promoter. Arrows at −370 and +13 indicate the oligonucleotides used. RT-PCR was performed as described in *Materials and Methods* to detect *Trex1* alternative splicings. The two bands obtained were cloned and sequenced. The higher band corresponds to unprocessed RNA, whereas the lower band (123 bp) corresponds to a splicing reaction involving the −267 donor site and the −7 splicing acceptor sites. M is the size marker. *B*, *Trex1* transcription initiation sites were determined by RACE-PCR (*left panel*). By cloning and sequencing the PCR products, we determined the bands 1, 2, and 3 as corresponding to three transcription start sites located at −429, −580, and −788 from the translation initiation sites. *Right panel* shows the analysis of Trex1 mRNA transcripts expressed in macrophages. Total RNA or poly A+ RNA was isolated from macrophages treated or not treated with IFN-γ and was analyzed by Northern blotting using *Trex1* cDNA as a probe. The major transcript has a calculated length of 1.3 kb, which corresponds to the initiation from the transcription start site located at −580. This assay is representative of at least four independent experiments showing similar results.

The 383-bp fragment corresponded to unprocessed RNA, whereas the 123-bp fragment corresponded to a molecule obtained by splicing using the donor site located at −267 bp and the acceptor site located at −7 bp. In conclusion, during *Trex1* maturation, a splicing process in the noncoding region eliminates an intron of 260 bp.

Determination of Trex1 transcription start sites

To determine the transcription start sites of *Trex1* in the mouse promoter, we performed a RACE-PCR procedure. A two-round PCR was designed using *Trex1* cDNA. The first reaction used the 5′ *Trex1* RACE nested (−305) or the rev st/d1 (−353) as the reverse primer. In both cases, the Universal Primer Mix primer (a mix of the Long Universal Primer and the Short Universal Primer) was used as the forward primer. Using the product of these PCRs as the template, we performed a second PCR with the Nested Universal Primer and the rev st/d1 primer. The products of these PCRs were resolved by electrophoresis and seven bands were obtained, which were cloned and sequenced. Three transcription start sites were detected, corresponding to positions −429 (producing a 1154-bp cDNA), −580 (producing a 1305-bp cDNA), and −788 (producing a 1513-bp cDNA) (Fig. 3*B*).

To determine the relative abundance of these transcript variants, we analyzed *Trex1* expression by Northern blotting. We purified total RNA and poly A^+ RNA from resting and IFN-γ–stimulated macrophages, and used *Trex1* cDNA as a probe. Total RNA transcripts of a calculated m.w. of 1.1, 1.3, 1.5, 4.6, and 7.8 kb were detected, with the 1.3-kb transcript clearly being the most abundant. Moreover, in poly A^+ RNA samples, only the 1.3-kb transcript was detected. Similar results were obtained when RNA from spleen or thymus was used. These results are supported by the studies of primer extension (data not shown). In conclusion, the main transcription start site in *Trex1* in macrophages is located at −580, whereas those located at −429 and −788 are minor (Fig. 3*B*).

Analysis of the Trex1 promoter sequence

To determine the putative elements responsible for the transcriptional regulation of *Trex1*, a fragment of ~3 kb upstream of the first ATG was sequenced and analyzed to detect possible regulatory boxes using the TFSEARCH and Transfac databases. The analysis of the 5′-proximal region reflects the absence of canonic TATA and CCAAT boxes. However, this region contains GC-rich sequences (Sp1 motives) typical of TATA-less promoters (Fig. 4). Sp1 boxes are implicated in the initiation of transcription in the absence of TATA boxes. The accumulation of various transcription start sites in a small region is also common in GC-rich sequences that promoted initiation of transcription. An initiation consensus element (Inr) was also detected between positions −180 and −185 bp (CCCTCA). Consensus binding sites for several transcription factors, such as GAS elements, NF-κB, upstream transcription factor, Lyf.1, PU.1, GATA, adaptor protein complex 1, and ISRE boxes, were localized between positions +1 and −1400 bp but not between −1400 and −3000 bp.

Functional analysis of the mouse Trex1 promoter in IFN-γ–mediated Trex1 induction

Next, analysis of the functional activity of the *Trex1* promoter sequence using the TFSEARCH database showed that the promoter region contains various putative sites for the binding of transcription factors involved in IFN-γ signal transduction, such as three GAS boxes (one from −1260 to −1252, one from −690 to

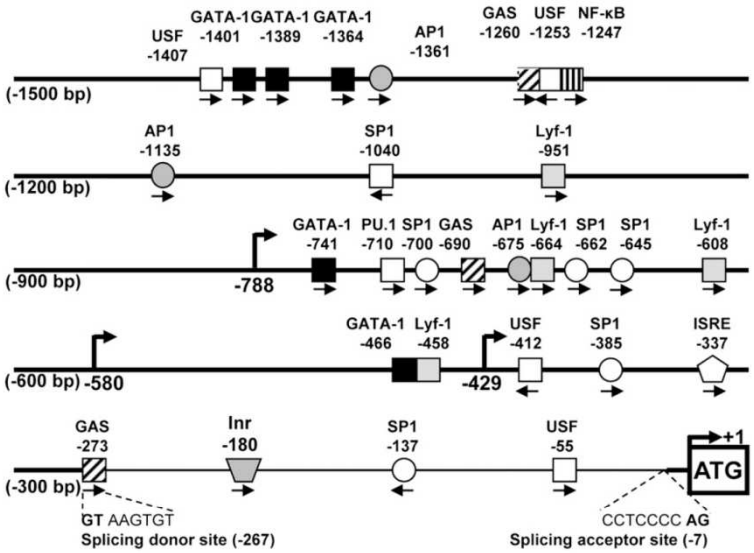

FIGURE 4. Putative consensus binding sites for transcription factors in the *Trex1* promoter. The sequence spanning 1500 bp upstream of *Trex1* translation start site was analyzed using TFSearch program, and putative boxes sharing >85% similarity with the consensus sequence for the binding of each transcription factor are shown. The transcription start sites detected by RACE-PCR and the splicing donor and acceptor sites are indicated by arrows.

−679, and one from −273 to −263) and an ISRE box (from −337 to −325). In addition, we detected one NF-κB box (−1247 to −1237) and three adaptor protein complex 1 boxes (−1361 to −1353; −1135 to −1126; −675 to −669; Fig. 4).

To delimitate the region responsible for IFN-γ induction, we linked vectors varying in the lengths of the 5′ sequence of the *Trex1* promoter to the luciferase reporter gene. These vectors were transfected into RAW264.7 macrophages and luciferase activity was measured. Each construct was cotransfected with the renilla expression vector. All luciferase activity values were normalized to the level of renilla expression to correct for any differences in transfection efficiency. In unstimulated macrophages, a construct comprising 350 bp of the promoter showed negligible activity (Fig. 5*A*). The longest fragment that we tested comprised 2.6 kb and showed no IFN-γ inducibility. However, the 1.4-kb construct and the 850-bp fragment were clearly induced by IFN-γ. Little induction was found in the 600-bp fragment. All these data suggest that the sequence between −350 and −600 is required for basal activity, whereas the region between −600 and −850 is needed for induction by IFN-γ.

Two GAS boxes and an adaptor protein complex 1 box are required for Trex1 promoter inducibility

To determine more precisely the areas in the *Trex1* promoter responsible for its induction by IFN-γ, we mutated some of the putative IFN-γ responsive elements by site-directed mutagenesis of the 850-bp construct and assayed activity as described earlier. Also, the adaptor protein complex 1 box located at −675 bp was mutated.

Mutation of the ISRE box at −337 had almost no effect, whereas mutation of the GAS boxes located at −273 and −690 caused a decrease in the promoter induction from 3.7-fold to 1.5- and 1.7-fold, respectively (Fig. 5*B*). These results demonstrate that both GAS boxes are required for maximal induction by IFN-γ, and

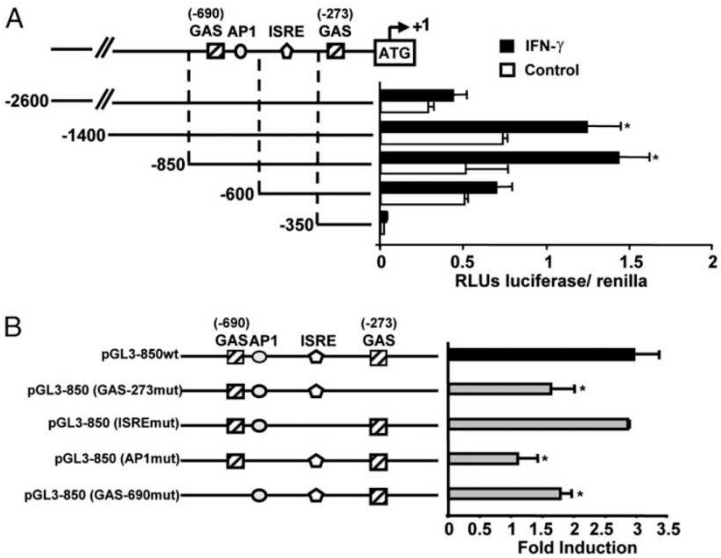

FIGURE 5. Characterization of the *Trex1* functional promoter. *A*, RAW264.7 macrophages were transiently transfected with pGL3 plasmids containing distinct deletions of the *Trex1* promoter region. At 24 h posttransfection, cells were stimulated with IFN-γ for a further 24 h and luciferase activity was tested. *B*, Plasmids containing the 850-bp promoter region of *Trex1* were used for site-directed mutagenesis, transfected in RAW264.7 cells that were stimulated with IFN-γ. Fold induction by IFN-γ is represented. *A* and *B*, A plasmid coding for renilla enzyme under the control of the *Bos* promoter was cotransfected, and renilla activity was used to control transfection. All assays are representative of at least four independent experiments showing similar results. Each point was performed in triplicate, and the results are shown as mean ± SD. *$p < 0.01$ in relation to the controls when all the independent experiments had been compared.

are consistent with the low induction of the 600-bp construct, which holds only one GAS box. Considering these experiments, the ISRE box plays a minimum role in the induction by IFN-γ. This finding can be expected because this motif is usually more implicated in the response to type I IFNs. Surprisingly, mutation of the adaptor protein complex 1 box abolished the IFN-γ induction of the promoter. This observation suggests a critical and unsuspected role of adaptor protein complex 1 factors in the regulation of the *Trex1* promoter (Fig. 5*B*).

STAT1 binds to the GAS boxes in response to IFN-γ

To determine the proteins that bind to the areas of interest in the promoter from BALB/C mice, we performed gel electrophoresis DNA binding assays. Nuclear extracts were prepared from cells treated with IFN-γ. When the extracts of these cell were incubated with a probe covering the GAS1 box located at −690 bp, a band was induced that was inhibited when anti-STAT1 Abs were added (Fig. 6*A*). This band is specific because it competed with an excess of cold probe and with a consensus GAS box, but not with an adaptor protein complex 1 box or with the mutated GAS box. All these observations demonstrate that STAT1 specifically binds the GAS box at position −690 bp in the *Trex1* promoter of macrophages in response to IFN-γ.

Similarly, a probe covering the GAS box located at −273 bp was retarded by extracts from IFN-γ–stimulated but not from resting macrophages (Fig. 6*A*, lower panel). The same extracts did not have the capacity to bind to a probe with a mutated GAS box. Moreover, binding to the wild-type GAS box was abolished with anti-STAT1 Abs, coinciding with the observation made for the GAS box at −690 bp. These observations demonstrate that STAT1 binds in vitro to two GAS boxes located in the *Trex1* promoter in response to stimulation with IFN-γ.

To confirm that the binding of STAT1 to the *Trex1* promoter also occurred in vivo, we performed chromatin immunoprecipitation assays, amplifying the area −522 to −693 bp. Whereas low levels of amplification were detected in the samples immunoprecipitated using total Igs, enhanced amplification of the *Trex1* promoter was observed in the sample from IFN-γ–stimulated macrophages immunoprecipitated using STAT1-specific Ab (Fig. 6*B*). The specificity of the reaction was checked by using unrelated Abs or a fragment of the promoter that does not contain the transcription start site (data not shown). This result confirmed that the binding of STAT1 to the *Trex1* promoter occurs in vivo in response to IFN-γ stimulation.

To further confirm the involvement of STAT1 in the regulation of *Trex1* by IFN-γ, macrophages from STAT1-deficient mice were stimulated with IFN-γ for a range of times. RNA was extracted and *Trex1* expression was detected by Northern blotting and quantitative real-time PCR. In comparison with what was observed in control cells, the induction of *Trex1* in macrophages derived from STAT1-deficient mice was impaired, once again confirming that STAT1 is required for *Trex1* induction in response to IFN-γ (Fig. 6*C*).

Phospho–c-Jun binds to the adaptor protein complex 1 box in response to IFN-γ

To determine the factors that bind to the adaptor protein complex 1 box in the *Trex1* promoter, we performed EMSAs. An induced band was detected; however, this band did not appear when the mutated adaptor protein complex 1 box was used (Fig. 7*A*, left panel). There was no inhibition or supershift when the binding was made in the presence of Abs against adaptor protein complex 1 factors CREB or jun-B, or with unspecific Igs, but the binding was eliminated with a phospho–c-Jun–specific Ab (Fig. 7*A*, right panel). This result confirms that a complex containing phospho–c-Jun binds the adaptor protein complex 1 box in the *Trex1* promoter in vitro in response to IFN-γ stimulation. By Western blot, we observed that IFN-γ induces the phosphorylation of c-Jun, as well

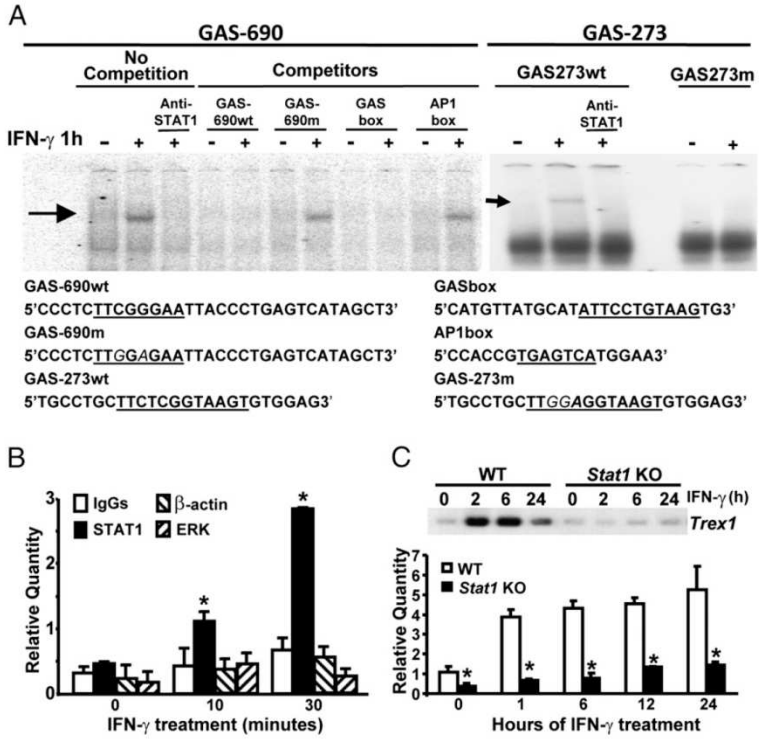

FIGURE 6. STAT1 binds to the *Trex1* promoter in response to IFN-γ treatment. *A*, In vitro binding of STAT1 to the *Trex1* promoter by EMSA. Nuclear extracts from macrophages stimulated with IFN-γ were used to detect the binding of two putative GAS boxes in the *Trex1* promoter. The cold competitors were used in a 100-fold excess. In some cases, anti-STAT1 Abs were added to the mixture of nuclear extracts and oligonucleotides. *B*, In vivo binding of STAT1 to the *Trex1* promoter was analyzed by chromatin immunoprecipitation. *C*, Induction of *Trex1* mRNA in STAT1-deficient macrophages is impaired. Macrophages from *Stat1* knockout mice and the corresponding wild-type counterparts were isolated and stimulated with IFN-γ for the times indicated. At the top, *Trex1* expression was analyzed by Northern blotting using Trex1 cDNA as a probe (see *Materials and Methods*). At the bottom, RNA was used for *Trex1* analysis by quantitative real-time PCR. All assays are representative of at least four independent experiments showing similar results. *B* and *C*, Each point was performed in triplicate and the results are shown as mean ± SD. $*p < 0.01$ in relation to the controls when all the independent experiments had been compared.

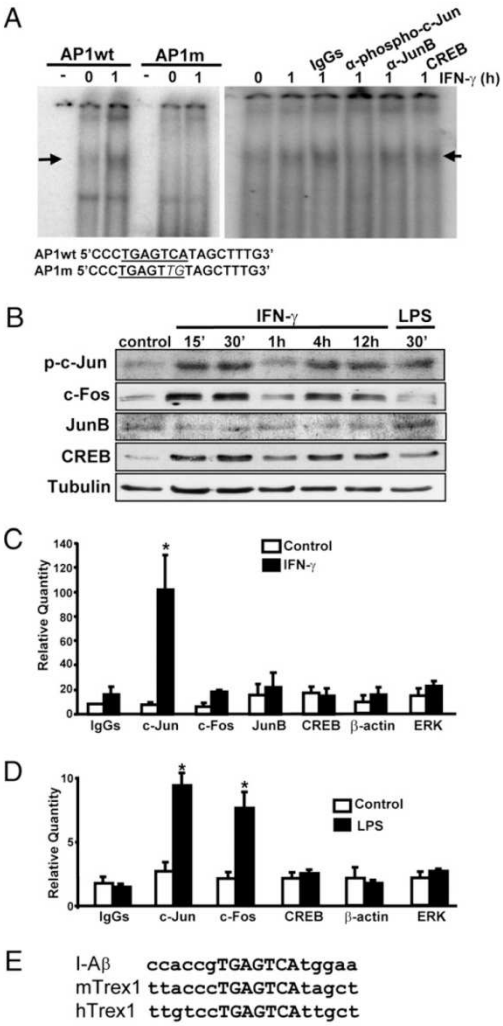

FIGURE 7. c-Jun binds to the *Trex1* promoter adaptor protein complex 1 box. *A*, In vitro binding of phospho–c-Jun to the *Trex1* promoter. Nuclear extracts from macrophages stimulated with IFN-γ for 1 h were tested for the binding to a nucleotide carrying the adaptor protein complex 1 box of the *Trex1* promoter. A specific Ab against phospho–c-Jun inhibited the binding (*right panel*). *B*, c-Jun is phosphorylated on IFN-γ treatment. Macrophages were stimulated with IFN-γ for the indicated times or with LPS (10 ng/ml) for 24 h. Total cell extracts were prepared and tested for the indicated components by Western blotting. Membranes were also tested for tubulin as a loading control. *C*, The binding of phospho–c-Jun to the *Trex1* promoter was determined by chromatin immunoprecipitation assays. *D*, The binding of phospho–c-Jun and c-Fos to the *I-Aβ* promoter was determined by chromatin immunoprecipitation assays. All assays are representative of at least four independent experiments showing similar results. *C* and *D*, Each point was performed in triplicate and the results are shown as mean ± SD. $^*p < 0.01$ in relation to the controls when all the independent experiments had been compared. *E*, Sequence alignments of the adaptor protein complex 1 boxes of *I-Aβ* and human and murine *Trex1*.

as the induction of c-Fos and CREB (Fig. 7*B*). No induction of jun-B was detected by the incubation of macrophages with IFN-γ (Fig. 7*B*). These findings confirm our previous observations (24). To check that the binding of c-Jun to the *Trex1* promoter also occurred in vivo, we performed a chromatin immunoprecipitation assay. Amplification of the *Trex1* promoter from BALB/C mice (area −522 to −693) was increased in samples from stimulated macrophages immunoprecipitated with c-Jun Ab, but not from unstimulated samples or samples immunoprecipitated with an irrelevant Ab (Fig. 7*C*). These observations thus confirm that the binding of c-Jun to the *Trex1* promoter in response to IFN-γ occurs in vivo. On the basis of these experiments, we conclude that neither c-Fos, jun-B, nor CREB binds to the *Trex1* promoter in vivo. Thus, c-Jun is probably bound to DNA as homodimer, as has been shown in other genes (25, 26). However, more experiments are required to confirm this hypothesis.

As a control for the c-Fos Ab, we performed experiments of chromatin immunoprecipitation using Abs against c-Jun and c-Fos, and the promoter of I-Aβ gene (18). The fragments of DNA were amplified and the amounts determined by quantitative PCR. The adaptor protein complex 1 box was precipitated in dendritic cells by Abs against both c-Jun and c-Fos (Fig. 7*D*). This observation suggests that the lack of binding of c-Fos to the Trex1 promoter is not due to a technical problem. Finally, the sequence alignment of the adaptor protein complex 1 boxes in the murine and human genes, as well as in the I-Aβ gene used as a control, showed perfect homology (Fig. 7*E*).

Discussion

In this study, we have shown that *Trex1* exonuclease expression in macrophages is regulated at the transcriptional level by IFN-γ. *Trex1* gene is located in position F2 in chromosome 9 of the mouse karyotype, and its protein coding region is found at a single exon, which is quite uncommon in eukaryote genes. The analysis of *Trex1* cDNA reveals the presence of a single intron upstream of the open reading frame, in the 5′-UTR. By the RACE-PCR technique, we have determined that at least three transcription start sites are active in the *Trex1* promoter region, and using Northern blotting, we have observed that the most abundantly expressed transcript in bone marrow macrophages is located −580 bp from the translation start site, and that it generates a transcript of 1.3 kb. *Trex1* promoter spans 850 bp from the main transcription start site and lacks the basic elements for transcription initiation, such as TATA and CCAAT boxes. However, an *Inr* sequence and several Sp1 (GC-rich) and upstream transcription factor motifs are found, which can functionally replace the TATA box sequence (27–29). Although this structure is typical of housekeeping genes, it has also been described in genes modulated by IFN-γ (30–33).

By blocking de novo protein synthesis, we have demonstrated that *Trex1* induction by IFN-γ does not require the synthesis of a protein factor. Instead, *Trex1* mRNA induction occurs with the timing of an early gene in the first hour of IFN-γ treatment. Moreover, CHX treatment alone was able to induce *Trex1* expression, thereby suggesting that a protein factor negatively regulates *Trex1* levels. Furthermore, CHX and IFN-γ treatment synergistically induced *Trex1*, thus demonstrating that the two treatments act through different mechanisms. By blocking transcription with actinomycin D, we determined that *Trex1* is unstable, showing a half-life of about 90 min in resting mouse macrophages. Treatment with IFN-γ did not enhance the half-life of this gene but rather caused a slight decrease. This observation implies that the increase in *Trex1* expression caused by IFN-γ is not due to an enhancement of mRNA half-life but to an increase in the transcription rate.

The most widely studied regulatory elements controlling mRNA stability are adenylate-uridylate-rich elements found mainly in the 3′-UTR of many genes coding cytokines and stress-response proteins (34). However, *Trex1* has a very short 3′-UTR spanning

only 45 bp, and these elements were not found in its sequence or in the 5′-UTR.

Unstable mRNAs are commonly found in the immune system and allow immediate activation of immune cells toward pathogens, transient mode of action, and swift recovery (34). In the case of *Trex1*, a tightly regulated recovery to basal mRNA levels after induction is crucial. Because of the catalytic activity of Trex1 nuclease, improper overexpression or activity of this protein may cause cell toxicity. In fact, Trex1 participates in genomic DNA digestion during granzyme-A–mediated cell death (35). Also, *Trex1* mutants with C-terminal truncations have been associated with the development of retinal vasculopathy with cerebral leukodystrophy (36). These mutants retain the catalytic activity of the nuclease but lose the domain that maintains the protein attached to the endoplasmic reticulum. Consequently, the protein diffuses throughout the cell. This improper localization has detrimental effects on endothelial cells and causes the characteristic necrosis of the small blood vessels observed in retinal vasculopathy with cerebral leukodystrophy patients (36).

The most well-known signaling pathway responsible for transcription regulation by IFN-γ is the one that involves JAKs and STAT1 transcription factor (JAK1–STAT1 pathway), which recognizes conserved GAS elements in the promoters of IFN-γ–regulated genes (10, 37). The *Trex1* promoter holds two GAS elements that bind STAT1 on IFN-γ treatment and that are required to induce *Trex1* promoter activity. Apart from these two GAS boxes, in this study, we show that a functional adaptor protein complex 1 box is also necessary for *Trex1* promoter induction. This box corresponds to the consensus octameric element TGACGTCA, and we have demonstrated that this element binds phospho–c-Jun on IFN-γ treatment. The members of the adaptor protein complex 1 family of NFs can form homodimeric or heterodimeric protein complexes, which transduce distinct signals and exert discrete transcriptional responses on various promoter targets (38). In fact, the heterogeneity in dimer composition is the main determinant of the functional diversification of adaptor protein complex 1 complexes. Whether phospho–c-Jun binds to the *Trex1* promoter as homodimer or heterodimer remains to be studied. In a large-scale identification of promoters bound by adaptor protein complex 1 factors in response to genotoxic stress, a heterodimer comprising c-Jun and the transcription factor ATF-2 was found to bind *Trex1* promoter in vivo (39). Given that the formation of c-Jun/ATF-2 heterodimers is widely induced by cytokines, as well as by oncogenes, viral infection, and several kinds of cellular stress factors (40), it is tempting to hypothesize that the heterodimer c-Jun/ATF2 also regulates the *Trex1* promoter in response to IFN-γ.

It has recently been shown that in fibroblasts, *Trex1* is induced by genotoxic stress and is regulated by the same adaptor protein complex 1 box that we describe in this study (41). However, the transcription factors that bind to this box in fibroblasts are c-Jun and c-Fos, and in macrophages only c-Jun was present, as shown by the chromatin immunoprecipitation assay. These differences may be related to either to the various stimuli used to induce *Trex1* expression or to the cellular models used. We have observed that in the promoter of I-Aβ, LPS induces the binding of c-Jun and c-Fos to an adaptor protein complex 1 box in dendritic cells, whereas in B lymphocytes, the complexes that bound the same sequence are formed by c-Jun but not by c-Fos (18).

A comparison of the mouse and human *Trex1* promoter shows that the adaptor protein complex 1 binding site is identical (41). Similar comparison was made for the two GAS boxes that are critical for the induction of *Trex1* in macrophages, and we found that they are also present in the human promoter. The observation that these elements are conserved between the human and mouse genomes reinforces their role as critical regulatory areas.

IFN-γ is the most potent activator of macrophages; therefore, the data shown in this study reflect the requirement for *Trex1* upregulation during activation of these cells. IFNs induce about 300 genes with diverse functions, the fight against viral infection being one of the major functions (9). To our knowledge, this is the first report of the induction of a DNA-specific exonuclease by proinflammatory stimulation. It has been reported that the ssRNA-specific IFN-stimulated gene 20 nuclease is induced by type I and II IFN treatments (42). According to several studies, this protein shows antiviral activity against RNA genomic viruses, such as vesicular stomatitis virus, influenza virus, and encephalomyocarditis virus. This activity is dependent on its exonuclease activity; therefore, IFN-stimulated gene 20 may act directly by degrading viral RNA (43). Similarly, Trex1 could be involved in the antiviral response against DNA genomic viruses. However, increased sensitivity to viral infection has not been reported in *Trex1* knockout mice or in AGS patients. In Trex1-deficiency models, autoimmunity caused by chronic production of IFN is observed in the absence of viral infection (3, 44). Moreover, mutations in *Trex1* are associated with the development of systemic lupus erythematosus (45). These observations lead to the hypothesis that Trex1 substrate is an endogenous nucleic acid species rather than a virus. In this context, the capacity of Trex1 to digest DNA from retrotranscribed elements and the accumulation of these elements in Trex1-deficient cells has recently been reported (6).

In this study, we show that Trex1 protein expression is regulated at the transcriptional level by a proinflammatory stimulus such as IFN-γ. Although the induction of *Trex1* by IFN may be explained by the need for Trex1 upregulation during viral infection or accumulation of endogenous nucleic acids, other proinflammatory stimuli such as LPS or TNF-α were found to induce *Trex1* expression in macrophages (M. Tartakis, M. Serra, J. Lloberas, and A. Celada, manuscript in preparation). These observations suggest an unknown role for Trex1 during macrophage activation that may not be related to antiviral activity. We have recently demonstrated that Trex1 interacts in vivo with the WW domain of the CA150 factor, a negative regulator of transcription elongation (14). In addition, Trex1-deficient cells have an impaired proliferative capacity and abnormal cell cycle checkpoint activation as a result of the chronic activation of the ATM kinase and destabilization of the cell cycle checkpoint kinase CHK2 (5). Moreover, the observation that human Trex1 is coded in the same open reading frame as ATRIP (ATR interacting protein), a key component of the cell cycle checkpoint machinery that is activated in response to replication stress and the presence of cellular ssDNA, further supports the notion that Trex1 is involved in these processes. On the basis of all these observations, we propose that Trex1 exerts an as yet unidentified function in other cellular pathways involving nucleic acid processing, such as DNA replication, DNA damage repair, and DNA transcription, in the context of inflammation and macrophage biology.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Perrino, F. W., H. Miller, and K. A. Ealey. 1994. Identification of a 3′→5′-exonuclease that removes cytosine arabinoside monophosphate from 3′ termini of DNA. *J. Biol. Chem.* 269: 16357–16363.
2. Rigby, R. E., A. Leitch, and A. P. Jackson. 2008. Nucleic acid-mediated inflammatory diseases. *Bioessays* 30: 833–842.
3. Morita, M., G. Stamp, P. Robins, A. Dulic, I. Rosewell, G. Hrivnak, G. Daly, T. Lindahl, and D. E. Barnes. 2004. Gene-targeted mice lacking the Trex1 (DNase III) 3′→5′ DNA exonuclease develop inflammatory myocarditis. *Mol. Cell. Biol.* 24: 6719–6727.
4. Crow, Y. J., and J. Rehwinkel. 2009. Aicardi-Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. *Hum. Mol. Genet.* 18(R2): R130–R136.
5. Yang, Y. G., T. Lindahl, and D. E. Barnes. 2007. Trex1 exonuclease degrades ssDNA to prevent chronic checkpoint activation and autoimmune disease. *Cell* 131: 873–886.
6. Stetson, D. B., J. S. Ko, T. Heidmann, and R. Medzhitov. 2008. Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134: 587–598.
7. Yan, N., A. D. Regalado-Magdos, B. Stiggelbout, M. A. Lee-Kirsch, and J. Lieberman. 2010. The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. *Nat. Immunol.* 11: 1005–1013.
8. Celada, A., and C. Nathan. 1994. Macrophage activation revisited. *Immunol. Today* 15: 100–102.
9. Boehm, U., T. Klamp, M. Groot, and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* 15: 749–795.
10. Bach, E. A., M. Aguet, and R. D. Schreiber. 1997. The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu. Rev. Immunol.* 15: 563–591.
11. Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8: 958–969.
12. Darnell, J. E., Jr. 1998. Studies of IFN-induced transcriptional activation uncover the Jak-Stat pathway. *J. Interferon Cytokine Res.* 18: 549–554.
13. Chatterjee-Kishore, M., K. L. Wright, J. P. Ting, and G. R. Stark. 2000. How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J.* 19: 4111–4122.
14. Brucet, M., J. Querol-Audí, M. Serra, X. Ramirez-Espain, K. Bertlik, L. Ruiz, J. Lloberas, M. J. Macias, I. Fita, and A. Celada. 2007. Structure of the dimeric exonuclease TREX1 in complex with DNA displays a proline-rich binding site for WW Domains. *J. Biol. Chem.* 282: 14547–14557.
15. Brucet, M., J. Querol-Audí, K. Bertlik, J. Lloberas, I. Fita, and A. Celada. 2008. Structural and biochemical studies of TREX1 inhibition by metals. Identification of a new active histidine conserved in DEDDh exonucleases. *Protein Sci.* 17: 2059–2069.
16. Celada, A., P. W. Gray, E. Rinderknecht, and R. D. Schreiber. 1984. Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J. Exp. Med.* 160: 55–74.
17. Celada, A., R. Allen, I. Esparza, P. W. Gray, and R. D. Schreiber. 1985. Demonstration and partial characterization of the interferon-gamma receptor on human mononuclear phagocytes. *J. Clin. Invest.* 76: 2196–2205.
18. Casals, C., M. Barrachina, M. Serra, J. Lloberas, and A. Celada. 2007. Lipopolysaccharide up-regulates MHC class II expression on dendritic cells through an AP-1 enhancer without affecting the levels of CIITA. *J. Immunol.* 178: 6307–6315.
19. Fenton, M. J., and D. T. Golenbock. 1998. LPS-binding proteins and receptors. *J. Leukoc. Biol.* 64: 25–32.
20. Xaus, J., M. Mirabet, J. Lloberas, C. Soler, C. Lluis, R. Franco, and A. Celada. 1999. IFN-gamma up-regulates the A2B adenosine receptor expression in macrophages: a mechanism of macrophage deactivation. *J. Immunol.* 162: 3607–3614.
21. Celada, A., M. Shiga, M. Imagawa, J. Kop, and R. A. Maki. 1988. Identification of a nuclear factor that binds to a conserved sequence of the I-A beta gene. *J. Immunol.* 140: 3995–4002.
22. Sebastián, C., M. Serra, A. Yeramian, N. Serrat, J. Lloberas, and A. Celada. 2008. Deacetylase activity is required for STAT5-dependent GM-CSF functional activity in macrophages and differentiation to dendritic cells. *J. Immunol.* 180: 5898–5906.
23. Celada, A., and R. Maki. 1989. Evidence for multiple major histocompatibility class II X-box binding proteins. *Mol. Cell. Biol.* 9: 5219–5222.
24. Valledor, A. F., E. Sánchez-Tilló, L. Arpa, J. M. Park, C. Caelles, J. Lloberas, and A. Celada. 2008. Selective roles of MAPKs during the macrophage response to IFN-gamma. *J. Immunol.* 180: 4523–4529.
25. Eferl, R., and E. F. Wagner. 2003. AP-1: a double-edged sword in tumorigenesis. *Nat. Rev. Cancer* 3: 859–868.
26. van Dam, H., and M. Castellazzi. 2001. Distinct roles of Jun : Fos and Jun : ATF dimers in oncogenesis. *Oncogene* 20: 2453–2464.
27. Tamura, T., and K. Mikoshiba. 1991. Role of a GC-rich motif in transcription regulation of the adenovirus type 2 IVa2 promoter which lacks typical TATA-box element. *FEBS Lett.* 282: 87–90.
28. Boisclair, Y. R., A. L. Brown, S. Casola, and M. M. Rechler. 1993. Three clustered Sp1 sites are required for efficient transcription of the TATA-less promoter of the gene for insulin-like growth factor-binding protein-2 from the rat. *J. Biol. Chem.* 268: 24892–24901.
29. Lu, J., W. Lee, C. Jiang, and E. B. Keller. 1994. Start site selection by Sp1 in the TATA-less human Ha-ras promoter. *J. Biol. Chem.* 269: 5391–5402.
30. Zahedi, K., A. E. Prada, and A. E. Davis, III. 1994. Transcriptional regulation of the C1 inhibitor gene by gamma-interferon. *J. Biol. Chem.* 269: 9669–9674.
31. Grötzinger, T., K. Jensen, and H. Will. 1996. The interferon (IFN)-stimulated gene Sp100 promoter contains an IFN-gamma activation site and an imperfect IFN-stimulated response element which mediate type I IFN inducibility. *J. Biol. Chem.* 271: 25253–25260.
32. Kuhen, K. L., J. W. Vessey, and C. E. Samuel. 1998. Mechanism of interferon action: identification of essential positions within the novel 15-base-pair KCS element required for transcriptional activation of the RNA-dependent protein kinase pkr gene. *J. Virol.* 72: 9934–9939.
33. George, C. X., and C. E. Samuel. 1999. Characterization of the 5′-flanking region of the human RNA-specific adenosine deaminase ADAR1 gene and identification of an interferon-inducible ADAR1 promoter. *Gene* 229: 203–213.
34. Khabar, K. S. 2007. Rapid transit in the immune cells: the role of mRNA turnover regulation. *J. Leukoc. Biol.* 81: 1335–1344.
35. Chowdhury, D., P. J. Beresford, P. Zhu, D. Zhang, J. S. Sung, B. Demple, F. W. Perrino, and J. Lieberman. 2006. The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. *Mol. Cell* 23: 133–142.
36. Richards, A., A. M. van den Maagdenberg, J. C. Jen, D. Kavanagh, P. Bertram, D. Spitzer, M. K. Liszewski, M. L. Barilla-Labarca, G. M. Terwindt, Y. Kasai, et al. 2007. C-terminal truncations in human 3′-5′ DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. *Nat. Genet.* 39: 1068–1070.
37. Platanias, L. C. 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* 5: 375–386.
38. Shaulian, E., and M. Karin. 2002. AP-1 as a regulator of cell life and death. *Nat. Cell Biol.* 4: E131–E136.
39. Hayakawa, J., S. Mittal, Y. Wang, K. S. Korkmaz, E. Adamson, C. English, M. Ohmichi, M. Omichi, M. McClelland, and D. Mercola. 2004. Identification of promoters bound by c-Jun/ATF2 during rapid large-scale gene activation following genotoxic stress. *Mol. Cell* 16: 521–535.
40. Shaulian, E., and M. Karin. 2001. AP-1 in cell proliferation and survival. *Oncogene* 20: 2390–2400.
41. Christmann, M., M. T. Tomicic, D. Aasland, N. Berdelle, and B. Kaina. 2010. Three prime exonuclease I (TREX1) is Fos/AP-1 regulated by genotoxic stress and protects against ultraviolet light and benzo(a)pyrene-induced DNA damage. *Nucleic Acids Res.* 38: 6418–6432.
42. Gongora, C., G. Degols, L. Espert, T. D. Hua, and N. Mechti. 2000. A unique ISRE, in the TATA-less human Isg20 promoter, confers IRF-1-mediated responsiveness to both interferon type I and type II. *Nucleic Acids Res.* 28: 2333–2341.
43. Degols, G., P. Eldin, and N. Mechti. 2007. ISG20, an actor of the innate immune response. *Biochimie* 89: 831–835.
44. Crow, Y. J., B. E. Hayward, R. Parmar, P. Robins, A. Leitch, M. Ali, D. N. Black, H. van Bokhoven, H. G. Brunner, B. C. Hamel, et al. 2006. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutières syndrome at the AGS1 locus. *Nat. Genet.* 38: 917–920.
45. Lee-Kirsch, M. A., M. Gong, D. Chowdhury, L. Senenko, K. Engel, Y. A. Lee, U. de Silva, S. L. Bailey, T. Witte, T. J. Vyse, et al. 2007. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat. Genet.* 39: 1065–1067.

The Exonuclease Trex1 Restrains Macrophage Proinflammatory Activation

Summary of "The Exonuclease Trex1 Restrains Macrophage Proinflammatory Activation" in Spanish

La exonucleasa Trex1 restringe la activación proinflamatoria del macrófago

TREX1 es la exonucleasa más abundante en células de mamífero. Las mutaciones en el gen TREX1 están relacionadas con el desarrollo del síndrome de Aicardi-Goutieres, una enfermedad inflamatoria del cerebro, así como también con el Lupus Eritematoso Sistémico. En los casos clínicos y en el modelo murino deficiente para Trex1, la producción crónica de interferones de tipo I juega un papel central en el desarrollo de la patología. En este estudio hemos demostrado que ratones Trex1$^{-/-}$ presentan características inflamatorias en diferentes órganos, incluyendo el cerebro. Trex1 se induce en los macrófagos como respuesta a los estímulos proinflamatorios, incluyendo los ligandos de TLR7 y TLR9. Nuestros resultados muestran que, en ausencia de Trex1, los macrófagos muestran una respuesta proinflamatoria más exacerbada. Más concretamente, después de la estimulación proinflamatoria, los macrófagos Trex1$^{-/-}$ muestran un aumento de la producción de TNF-α e IFN-β, niveles más elevados de CD86 y un aumento de la presentación de antígenos así como una disminución de la fagocitosis de cuerpos apoptóticos de linfocitos T. Estos resultados evidencian una función desconocida hasta ahora de Trex1 como regulador negativo de la activación inflamatoria de los macrófagos y demuestran que estas células juegan un papel esencial en las enfermedades asociadas con mutaciones en el gen Trex1. Esta función de Trex1 Esto contribuye a la comprensión del papel de los procesos inflamatorios en estas enfermedades.

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The Exonuclease Trex1 Restrains Macrophage Proinflammatory Activation

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The three-prime repair exonuclease 1 (TREX1) is the most abundant exonuclease in mammalian cells. Mutations in *Trex1* gene are being linked to the development of Aicardi–Goutières syndrome, an inflammatory disease of the brain, and systemic lupus erythematosus. In clinical cases and in a *Trex1*-deficient murine model, chronic production of type I IFN plays a pathogenic role. In this study, we demonstrate that *Trex1*$^{-/-}$ mice present inflammatory signatures in many different organs, including the brain. Trex1 is highly induced in macrophages in response to proinflammatory stimuli, including TLR7 and TLR9 ligands. Our findings show that, in the absence of Trex1, macrophages displayed an exacerbate proinflammatory response. More specifically, following proinflammatory stimulation, *Trex1*$^{-/-}$ macrophages exhibited an increased TNF-α and IFN-α production, higher levels of CD86, and increased Ag presentation to CD4$^+$ T cells, as well as an impaired apoptotic T cell clearance. These results evidence an unrevealed function of the Trex1 as a negative regulator of macrophage inflammatory activation and demonstrate that macrophages play a major role in diseases associated with Trex1 mutations, which contributes to the understanding of inflammatory signature in these diseases. *The Journal of Immunology*, 2013, 191: 000–000.

Three-prime repair exonuclease 1 (TREX1) is a homodimeric exonuclease (1–3) that degrades DNA in a 3′→5′ manner (4). TREX1 activity has a propensity toward certain DNA sequences and requires magnesium and manganese in the active site for proper functioning (5). In humans, mutations in TREX1 are associated with the Aicardi–Goutières syndrome (AGS), a neurologic disease characterized by chronic production of IFN in the CNS (6). Such mutations are also associated with familial chilblain lupus (7) and with systemic lupus erythematosus (SLE), both of which have partial symptom overlap with AGS phenotype (8). About 0.5% of SLE patients have mutations in TREX1, which indicates that mutations in this gene are the most commonly known monogenic cause for SLE (9).

The mechanism by which TREX1 deficiency leads to a disease is still not entirely clear. The mouse model, *Trex1*$^{-/-}$, has a dramatically reduced life expectancy due to the development of inflammatory myocarditis (10). An increase of intracellular DNA triggers IFN-αβ production that causes a so-called antiviral state (11), which, in turn, leads to autoimmunity. There are currently two predominant explanations for DNA origin. First, it has been shown that TREX1 deficiency impairs G_1/S cell cycle transitions. This, in turn, leads to the accumulation of ssDNA produced in S phase (12). Second, alternative evidence shows that ssDNA originates from the replication of endogenous retroelements being accumulated in Trex1-deficient mice (13). In fact, it has been found that Trex1 also neutralizes part of the function of endogenous retroelements in synovial fibroblasts, which in consequence leads to rheumatoid arthritis when Trex1 is not present (14).

The IFN-αβ production, which contributes to the above-mentioned, has been shown to be dependent on stimulator of IFN genes (STING) pathway (15). In addition, according to further studies, TBK1, IFN regulatory factor (IRF)3, and IRF7 are also required for the expression of the antiviral genes when TREX1 is absent or nonfunctional (16).

Pattern-recognition receptors when triggered lead to an innate immune activation by inducing cytokine and IFN expression (17). More precisely, in the case of nucleotides being the trigger, TLR7 recognizes ssRNA, whereas TLR9 recognizes hypomethylated CpG DNA. Both TLR7 and TLR9 trigger IRF7 and NF-κB and induce the expression of type I IFN and cytokines, respectively. In SLE and other autoimmune diseases, a plethora of immune cells are dysfunctional, including T and B cells, which together result in high levels of circulating autoreactive Abs (18). Macrophages from SLE patients are more active, with increased cytokine production and an increase in the ability to present self-Ags to T cells, together with a decreased ability to perform apoptotic clearance (19).

Our work shows that, in mice, Trex1 has a different tissue distribution with higher expression in macrophages after proinflammatory activation when comparing with other cell types as B and T cells. In the current investigation, we examined the role of Trex1 in activated macrophages. In brief, our findings show that Trex1 is required for the correct function of macrophages. Defi-

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Abbreviations used in this article: AGS, Aicardi–Goutières syndrome; BMDDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; IRF, IFN regulatory factor; qPCR, quantitative PCR; SLE, systemic lupus erythematosus; STING, stimulator of IFN gene; TREX1, three-prime repair exonuclease 1; WT, wild type.

ciency results in a more proinflammatory phenotype characterized by increased cytokine production and increased capacity to activate T cells. Furthermore, the loss of Trex1 weakens the macrophage ability to perform an apoptotic clearance. This lack of autoregulation may explain the generalized inflammation associated with deficiency.

Materials and Methods

Mice

C57BL6J mice were purchased at Charles River Laboratories (Wilmington, MA). *Trex1*$^{-/-}$ mice (10) were provided by D. Bonthron (University of Leeds). OTII mice expressing transgenic TCRs specific for I-A^{b} plus OVA$_{323–339}$ were provided by J. L. Rodriguez (Centro de Investigaciones Biologicas, Consejo Superior de Investigaciones Cientificas, Madrid, Spain). The generation of *Tlr9*-deficient mice has been described earlier (20). Mice were at least eight generations C57BL6J; therefore, C57BL6J were used as wild type (WT). All mice were maintained and used in a specific pathogen-free facility at the Parc Cientific de Barcelona or the Biological Resource Centre in Singapore. The care and use of laboratory animals conformed to the National Institutes of Health guidelines, and all experimental procedures conformed to an Institutional Animal Care and Use Committee–approved animal protocol (Animal Research Committee of the Government of Catelonia, number 2523; A*STAR BRC IACUC 100539).

Reagents

Murine rIFN-γ, rIFN-α, rTNF-α, rIL-4, and rIL-10 were purchased from R&D Systems (Minneapolis, MN). CpGB and R848 were obtained from InvivoGen (San Diego, CA). OVA was purchased from Hyglos. All other chemicals used were of the highest available purity grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Histochemistry

Animals were euthanized, and tissues were fixed in 4% paraformaldehyde and paraffin embedded. H&E sections were measured, and the grade of mononuclear inflammatory infiltration was semiquantified by using an ordinal scale as follows: −, minimal or no evidence of mononuclear infiltration; +, mild mononuclear infiltration; ++, moderate to severe mononuclear infiltration; and +++, severe mononuclear infiltration.

Cell culture and purification

Mouse fibroblasts (L929 cell line from American Type Culture Collection) were maintained in DMEM 10% heat-inactivated FCS supplemented with 2-ME and flutamax from Invitrogen (Carlsbad, CA), supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Bone marrow–derived macrophages (BMDM) were generated from 6- to 8-wk-old mice. Bone marrow cells from femora and tibia were flushed and cultured in plastic tissue culture dishes (150 mm) in DMEM containing 20% FCS (PAA Laboratories, Pasching, Austria) and 30% of L-cell conditioned media as a source of M-CSF (21). Media was supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 7 d of culture, a homogeneous population of adherent macrophages was obtained (>99% CD11b and F4/80). To obtain bone marrow–derived dendritic cells (BMDDC), bone marrow cells were incubated in DMEM containing 10% FCS with GM-CSF (20 ng/ml). On days 3, 6, and 8, fresh media was added to cells. On day 10, cells were recovered (22).

Peritoneal macrophages were obtained by lavage following euthanasia. Cells were cultured in 10% FCS DMEM for 3 h at 37°C to enable adherence of macrophages. All nonattached cells were washed using warm DMEM. Purity of the macrophage population was determined by flow cytometry (>90% CD11b and F4/80).

T and B cells were sorted from fresh spleen, using CD3-allophycocyanin Cy7 and B220-AF700, respectively, from eBioscience. Sorting was performed using a BD FACSAria SORP.

RNA extraction and real-time RT-PCR

Total RNA was extracted with Tri Reagent, purified, and DNase treated with PureLink RNA Mini Kit, as described by the manufacturer (Ambion, Life Technologies). For cDNA synthesis, 400 ng total RNA and Moloney murine leukemia virus reverse-transcriptase RNase H Minus, Point Mutant, oligo $(dT)_{15}$ primer, and PCR nucleotide mix were used, as described by the manufacturer (Promega). Quantitative PCR (qPCR) was performed in triplicate using the SYBR Green Master Mix (Applied Biosystems) in a final volume of 10 μl using a 7900 HT Fast Real Time PCR System (Applied Biosystems). Data were normalized to the housekeeping gene, *hprt1* and/or *114*. Data are expressed as relative mRNA levels compared with the untreated control. Sequences of primers used are detailed in Supplemental Table I.

Western blot protein analysis

Cells were lysed, as described (23), in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM HEPES [pH 7.5], 150 mM NaCl, protease inhibitors, and 1 mM sodium ortovanadate). After 20 min of rotation at 4°C, cell extracts were centrifuged at 12,000 × *g* and supernatants were kept at −80°C. Protein concentration was measured by the Bio-Rad protein analysis kit. A total of 20 μg protein extracts was heated at 95°C in Laemmli SDS loading buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Amersham). Membranes were blocked with PBS with 5% of dry milk for 1 h and then incubated with Abs against Trex1 and β-actin. Secondary Abs were peroxidase-labeled anti-mouse. ECL (Amersham) was used for detection.

TNF-α and IL-6 were detected in supernatant using Mouse ELISA Ready-SET-Go (eBioscience).

Flow cytometry

On day 7, BMDM were collected and 5 × 10^{5} cells were used to assess the phenotype with flow cytometry using anti–CD115-PE, anti–F4/80-PECy5, anti–CD45-PECy7, anti–CD11b-allophycocyanin, and anti–GR1-allophycocyanin Cy7 Abs from eBioscience plus DAPI to gate out dead cells. To determine changes in expression of surface markers after activation, cells were plated in 12-well plates, left to adhere, and treated with different stimuli for 21 h. Anti–CD86-PECy7 and anti–CD40-allophycocyanin were used to detect activation levels after stimulation. Samples were acquired in BD LSRFortessa, BD FACSCanto II, or Gallios flow cytometer from Beckman Coulter depending on experimental design and availability.

Phagocytosis

Jurkat cells were washed three times in plain RPMI 1640 media and stained with CFSE (Molecular Probes, Life Technologies) at 1.25 μM for 10 min. For inducing apoptosis, labeled cells were incubated with 30 μM etoposide for 18 h. Apoptosis level was assessed with the Annexin V Apoptosis Detection Kit APC (eBioscience) (Supplemental Fig. 1). Apoptotic cells were incubated with stimulated macrophages at a ratio 4:1 (apoptotic cells: macrophage) after 1 h, media was discarded, and macrophages were collected by scraping on ice. Macrophages were stained on ice with specific Abs anti–CD45-PECY7, anti–CD11b-allophycocyanin Cy7, or anti–Ly108-PE plus DAPI to distinguish live and dead cells. Macrophages and T cells were acquired and analyzed using FlowJo. The percentage of CD11b- and CFSE-positive macrophages was used as a measure of phagocytosis.

T cell proliferation

Macrophages were plated in 96-well plate at a concentration of 5 × 10^{4} cells/well. Macrophages were left to attach for 4 h and stimulated for 18 h with LPS, CpGB, or R848. Media was removed, and cells were washed once and then pulsed with 100 μg/ml OVA for 3 h; then 5 × 10^{5} of CFSE-stained splenocytes from OTII mice were added to each well. After 5-d incubation period, the proliferation of T cells was assessed by flow cytometry. Cells were stained with anti–CD4-PerCpCy5.5, anti–Vα2-allophycocyanin, and anti–CD45-PECy7, as well as with DAPI for discrimination between live and dead cells.

Statistical analysis

Data were analyzed by using a two-tailed Student *t* test for comparing two groups or ANOVA for multiple groups. Bonferroni post hoc correction was used to compare pairs. Statistical analysis was performed with GraphPad Software Prism 4.

Results

Trex1$^{-/-}$ *mice have systemic inflammation*

Trex1$^{-/-}$ mice were originally reported to have a mortality rate of 50% at 20 wk of age, due to myocarditis (10). More recently, the $t_{1/2}$ has been reported to be just 10 wk, with multiple organs exhibiting extensive inflammation (15). This may suggest that

housing conditions may alter the pathological impact of *Trex1* deficiency.

Given the difference in pathology between the studies, we undertook an anatomopathological study of *Trex1*$^{-/-}$ mice to better understand the phenotypes present when these knockout mice were kept in our specific pathogen-free animal facility. To that end, *Trex1*$^{-/-}$ and WT littermates (controls) were euthanized at 6–8 wk old, and the extent of mononuclear infiltration was measured in different tissues using H&E staining (Fig. 1A, Table I). Examination of the heart demonstrated severe diffuse lymphocytic infiltration (Fig. 1A). This was more prominent in the myocardium of the left ventricle and atrium and less so in the right ventricle (data not shown). We also observed a moderate to severe mononuclear inflammatory infiltration in multiple organs, including the lung, the liver, the smooth muscle of the uterus, and the salivary gland with periductal infiltration. Inflammatory infiltration was minimal to mild in other tissues and organs, including skeletal muscle, lamina propia of glandular stomach, and lamina propia of urinary bladder, kidney, pancreas, and brain of *Trex1*$^{-/-}$ mice.

Given the fact that humans with AGS present severe encephalopathy, we decided to examine the brain further, hypothesizing that the effects of Trex1 deletion may be evident prior to pathological presentation. We determined that brain tissue from *Trex1*$^{-/-}$ mice had increased expression of *Il1β*, *Tnfα*, *Nos2*, and *cxcl10* compared with controls (Fig. 1B).

Trex1 is highly expressed in macrophages after proinflammatory activation

Due to the varying levels of cellular infiltration and inflammation in different organs, we decided to define in detail the anatomical distribution of *Trex1*. RNA expression of different C57BL6J mouse tissues was determined by qPCR. Our results show that *Trex1* is expressed in all tissues tested to some degree, and that the spleen, thymus, and uterus express the higher levels when compared with the remaining tissues examined (Fig. 2A).

Given the differing levels of Trex1 within tissues, we decided to examine the expression in purified cell populations using qPCR.

Table I. Histological mononuclear infiltration observed in the different tissues and organs of Trex1$^{-/-}$ animals

Tissue/Organ	Animal 1	Animal 2	Animal 3	Mean
Psoas muscle	+	+	+	+
Masseter muscle	+	+	+	+
Brachial muscle	+	−	−	− to +
Salivary gland	++	−	+	+ to ++
Lung	++	++	+	++
Brown adipose tissue	+++	++	++	++ to +++
Heart	+++	+++	++	+++
Pancreas	++	−	+	+
Gut	−	−	−	−
Glandular stomach	+	+	+	+
Nonglandular stomach	−	−	−	−
Liver	++	+	+	+ to ++
Gall bladder	−	−	−	−
Kidney	+	−	+	+
Urinary bladder	+	−	+	+
Uterus	++	ND	ND	++
Coagulative gland	ND	+	+	+
Brain	−	−	−	−

Four intensity levels have been defined, as follows: −, absence of mononuclear infiltration; +, mild mononuclear infiltration; ++, moderate to severe mononuclear infiltration; and +++, severe mononuclear infiltration.

Furthermore, because *Trex1* deletion is associated with systemic inflammation, we determined expression following stimulation. *Trex1* was absent in a murine fibroblast cell line, L929, but was detected in splenic B and T cells, peritoneal macrophages, BMDDCs, and BMDMs (Fig. 2B). As a contrast, *Trex1* levels were significantly higher in all immune cells. This suggests the importance of Trex1 expression in the immune cells analyzed.

We have shown previously that *Trex1* is upregulated in macrophages following stimulation with IFN-γ (24). We therefore sought to determine whether other inflammatory stimuli could modulate Trex1 expression across multiple cell types. Interestingly, expression in T cells and fibroblasts was unaffected by all stimuli used (Fig. 2B). However, incubation with type I IFN (IFN-α) resulted in a significant upregulation of Trex1 mRNA in purified B cells, peritoneal macrophages, BMDMs, and BMDDCs

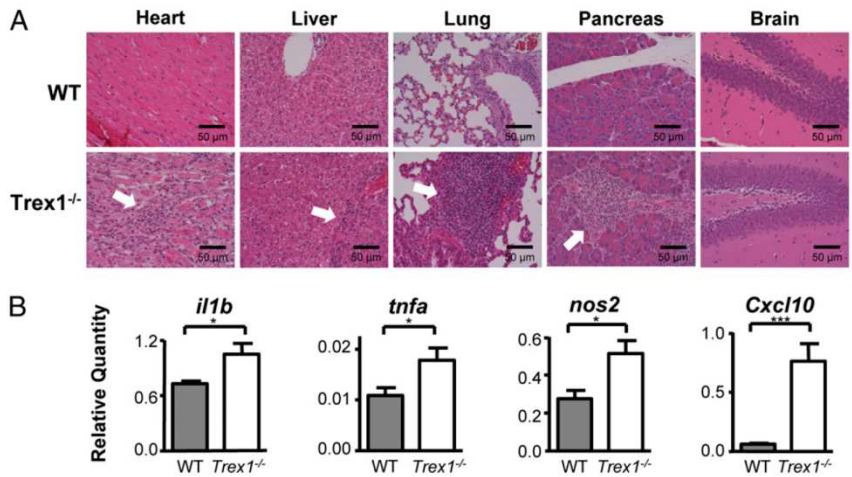

FIGURE 1. *Trex1*$^{-/-}$ mice develop multiple organ inflammation. (**A**) Representative H&E tissue sections are shown for both WT and *Trex1*$^{-/-}$ mice. Mononuclear infiltration (indicated with the white arrows) can be observed in heart, liver, lung, and pancreas sections from *Trex*$^{-/-}$ mice compared with WT, but not brain. Scale bars, 50 μm. (**B**) RNA was extracted from brains of *Trex1*$^{-/-}$ mice and controls. The level of RNA expression of the indicated genes was determined using qPCR. *Trex1*$^{-/-}$ mice brains present higher expression of proinflammatory genes in comparison with controls. All assays are representative of at least three independent experiments showing similar results. The results shown are mean ± SD. $*p < 0.05$, $***p < 0.001$ in relation to the controls when all the independent experiments have been compared.

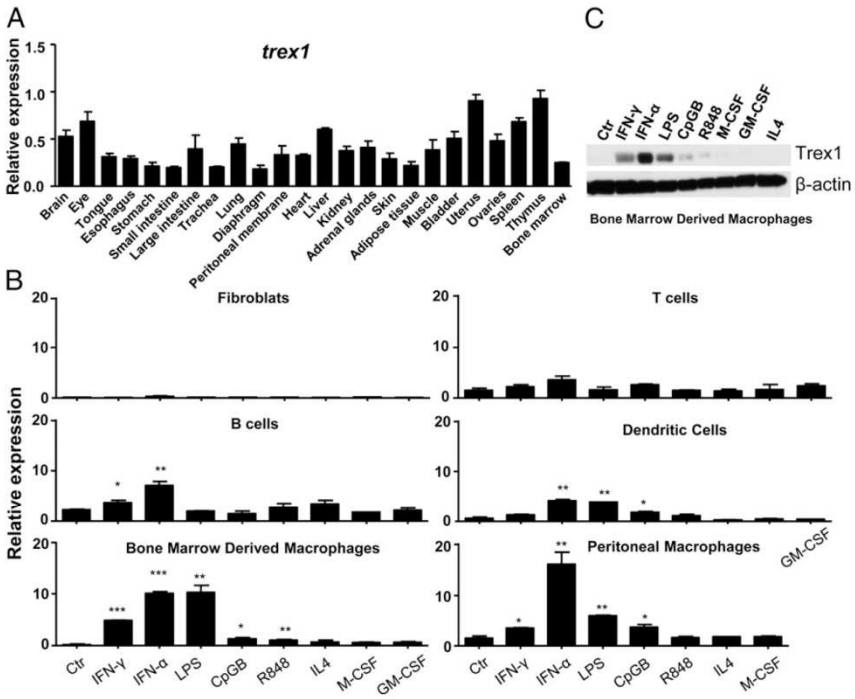

FIGURE 2. Trex1 is widely expressed in immune cells and upregulated after inflammatory stimulation in macrophages. (**A**) *Trex1* shows tissue-specific distribution. Tissues from three different C57BL6J mice were used to obtain RNA, and Trex1 expression was determined by qPCR. (**B**) *Trex1* mRNA expression in different cells under distinct conditions. Total RNA was isolated from different cells stimulated with the stimulus indicated during 6 h. (**C**) Trex1 protein in BMDM. Total protein extracts were prepared from BMDM stimulated for 24 h with the stimulus indicated and tested by Western blotting for Trex1 and β-actin expression. All assays are representative of at least three independent experiments showing similar results. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in relation to the controls when all the independent experiments have been compared.

(Fig. 2B). Stimulation with type II IFN (IFN-γ) resulted in an increase in Trex1 expression in macrophages and B cells, but not in DCs. Dysregulation of several TLRs has been associated with the development of autoimmunity, particularly SLE. Stimulation of macrophages or DCs, but not B cells, with ligands to TLR4 (LPS), TLR7 (R848), or TLR9 (CpG-B) resulted in increased levels of Trex1, demonstrating that multiple proinflammatory stimuli can regulate Trex1 expression. However, incubation with anti-inflammatory stimuli such as IL-4, or growth factors, M-CSF, or GM-CSF had no effect on all cell types tested. Because mRNA expression may not reflect actual protein changes, we analyzed expression in BMDM using Western blot (Fig. 2C). To confirm that we did not have endotoxin contamination, we used polymyxin B while stimulating BMDM with IFN-γ, IFN-α, and LPS (as positive control) (Supplemental Fig. 2). Our data confirmed an upregulation in Trex1 protein following IFN-γ, IFN-α, and TLR stimulation.

Stimulation of TLR9 induces Trex1 in macrophages

TLR9 is one of the four intracellular pathogen-associated molecular pattern receptors that recognize pathogenic nucleic acids, specifically CpG islands present in bacteria and virus, but not in mammalian DNA. This TLR activates the immune system signaling through MyD88 activating NF-κB and IRF transcription factors (3). TLR9 deficiency has also been shown to drive SLE pathogenesis in murine models (25, 26). Furthermore, the data described above demonstrate that TLR9 signaling modulates Trex1 expression, suggesting the possibility that they exist in a regulatory feedback loop. Therefore, we went on to verify whether there was dysregulated Trex1 expression in the TLR9-deficient mice. As expected, upregulation of Trex1 by CpGB was abolished in TLR9$^{-/-}$ macrophages (Fig. 3). However, the increase in Trex1 expression following IFN-γ and LPS was unaltered in TLR9-deficient macrophages (Fig. 3A). Interestingly, we did detect an inhibition of the increase in IFN-β and TNF-α following LPS and CpG stimulation, respectively (Fig. 3B, 3C).

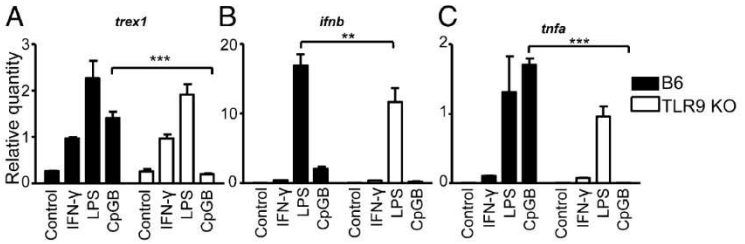

FIGURE 3. Trex1 is upregulated by TLR9 direct stimulation. Macrophages from *TLR9*$^{-/-}$ and control mice were treated for 6 h with different proinflammatory stimuli, and *trex1* (**A**), *ifnβ* (**B**), and *tnfα* (**C**) expression was determined. All assays are representative of at least three independent experiments showing similar results. Each point was performed in triplicate, and the results are shown as mean ± SD. $**p < 0.01$, $***p < 0.001$ in relation to the controls when all the independent experiments have been compared.

Trex1$^{-/-}$ macrophages exhibit an exacerbated proinflammatory response

In view of the significantly higher induction of Trex1 in macrophages compared with other immune cell types, we decided to examine whether the lack of Trex1 in this population could affect phenotype and function. We analyzed the surface expression of maturation markers in *Trex1$^{-/-}$* and control BMDMs, which are a homogeneous population of primary quiescent cells. There were no significant differences in the surface expression of CD11b, F4/80, CD115 (also known as M-CSFR), and GR-1 (also Ly6G) if Trex1 was present or not (Fig. 4A).

We then examined the functional cytokine response of Trex1-deficient BMDMs to TLR4, TLR7, and TLR9 stimulation. LPS stimulation revealed an increase in IL-1β mRNA with a concomitant decrease in IFN-β (Fig. 4B). There were no measurable differences in the release of TNF-α or IL-6 following LPS stimulation (Fig. 4C). Further examination revealed that the increased production of TNF-α following TLR9 stimulation was augmented in BMDMs lacking Trex1 (Fig. 4C). Incubation with the TLR7 ligand (R848) resulted in increased IFN-β expression in *Trex1$^{-/-}$* BMDMs compared with controls (Fig. 4B). The other cytokines tested did not show any significant differences. Analysis of cell surface costimulation molecules revealed a higher upregulation of CD86 following LPS stimulation in *Trex1$^{-/-}$* BMDMs compared with controls. There were no detectable differences following TLR7 or TLR9 incubation (Fig. 4D). The presence of Trex1 did not modify the levels of CD40 following any TLR stimulation (Fig. 4D). These results suggest that Trex1 has an important role in macrophage response to proinflammatory activation.

Trex1 represses Ag presentation by activated macrophages

As absence of Trex1 in macrophages induced higher expression of CD86 (a costimulator protein in APCs), we decided to determine the ability of Trex1-deficient macrophages to induce T cell proliferation following Ag processing and presentation. To that end, we pulsed previously stimulated macrophages with OVA for 3 h. Thereafter, we added splenocytes from OTII mice (mice with a transgenic TCR that specifically recognizes the OVA 323–339 peptide) that were previously stained with CFSE. T cell proliferation was assessed by flow cytometry on day 5. We determined that, whereas there were no differences in naive macrophage Ag presentation between control and Trex1-deficient mice, macrophages from *Trex1$^{-/-}$* mice induce significantly more T cell proliferation after stimulation with LPS and R848 (Fig. 5). These findings demonstrate the importance of Trex1 in macrophage Ag presentation that is exacerbated after specific TLR stimulation.

Trex1 is necessary for efficient phagocytosis by activated macrophages

A defect in macrophage clearance of apoptotic cells has been described in SLE patients and murine models (27). Therefore, we decided to analyze whether and to what extent the lack of Trex1 would affect this function. Macrophages from *Trex1$^{-/-}$* and control mice were stimulated with different proinflammatory stimuli and thereafter further incubated with apoptotic CFSE-labeled T cells for 1 h, as described in *Materials and Methods*. Our results show that macrophages lacking Trex1 have an impaired phagocytic ability following overnight incubation with TLR4, TLR7, and TLR9 ligands (Fig. 6). These treatments did not affect significantly the expression of macrophage markers CD11b and Ly108. To determine whether the change in apoptotic clearance could impact the levels of released nucleic acids, we measured the DNA present in the supernatant following incubation with apoptotic cells (Supplemental Fig. 3). No significant difference was found. These data, together with our results described above, suggest that Trex1 controls the response of macrophages to TLR stimulation and has an important role in the resolution of inflammation.

Discussion

Deficiency of Trex1 in human patients and murine models is characterized by systemic inflammation, which includes autoim-

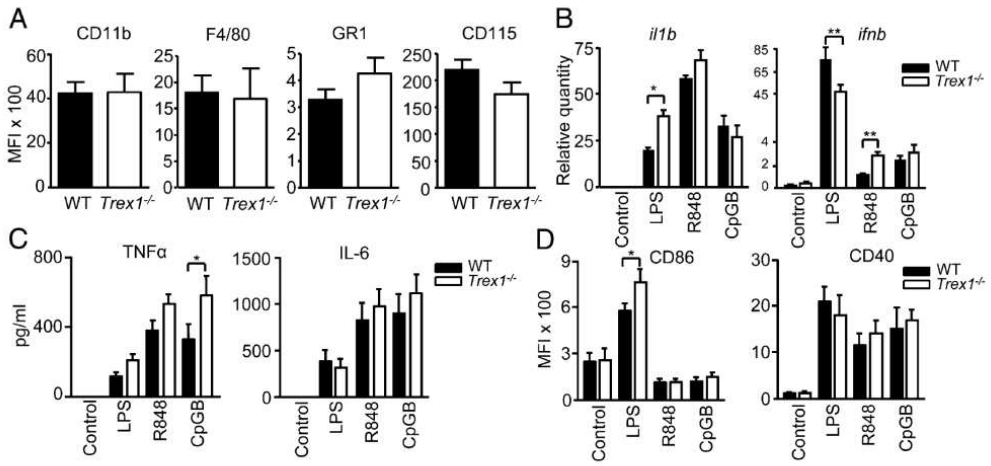

FIGURE 4. Macrophages from *Trex1$^{-/-}$* mice produce higher levels of inflammatory cytokines in response to TLR ligands. (**A**) Macrophages obtained from *Trex1$^{-/-}$* and control mice present similar phenotype. CD11b, F4/80, CD115, and GR1 expression were measured by flow cytometry. (**B**) Macrophages from *Trex1$^{-/-}$* mice upregulate *il1β* to a greater extent, but regulate *ifnβ* to a lesser extent following TLR4 simulation. Incubation with R848 (TLR7 ligation) resulted in an increase of *ifnβ* by *Trex1$^{-/-}$* macrophages. Levels of expression were determined by qPCR at 9 and 3 h for *il1β* and *ifnβ*, respectively. (**C**) Macrophages from *Trex1$^{-/-}$* produce more TNF-α after 24-h stimulation. Supernatants were collected, and the levels of TNF-α and IL-6 were measured. (**D**) Increased expression of CD86 in macrophages of *Trex1$^{-/-}$* mice after LPS treatment. The expression of CD86 and CD40 was determined with flow cytometry after 24-h stimulation. All assays are representative of at least three independent experiments showing similar results. Results are shown as mean ± SD. $*p < 0.05$, $**p < 0.01$ in relation to the controls when all the independent experiments have been compared.

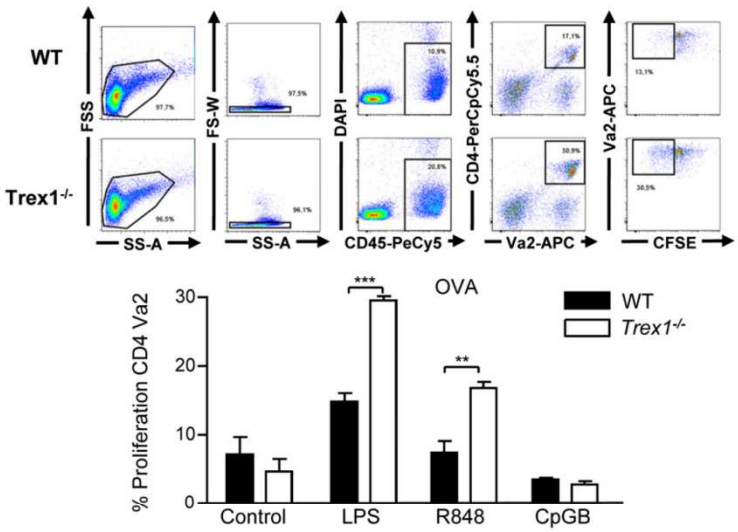

FIGURE 5. Activated macrophages from *Trex1*$^{-/-}$ mice induce increased T cell proliferation. Macrophages were stimulated for 24 h, incubated with OVA for 3 h, and then cocultured with splenocytes from OT-II mice for 5 d. Floating cells were collected, stained, and analyzed by FACS. In the *top panels*, an example is shown of the gating strategy. First and second panel gates for single cells are followed by a third panel gate where only CD45 live cells are selected. From these, the positive CD4 cells with TCR Vα2 chain were gated, and the percentage of T cell proliferating cells was determined by the loss of CFSE fluorescence, as this staining is diluted after cell proliferation. In the *bottom panels*, the mean ± SD of three independent experiments is shown. $^{**}p < 0.01$, $^{***}p < 0.001$ in relation to the controls.

munity (11). In the studies presented in this work, we demonstrate that Trex1 plays an important regulatory role in activated macrophages. Deficiency results in an increased activated proinflammatory phenotype associated with an enhanced Ag presentation. Furthermore, *Trex1*$^{-/-}$ mature macrophages exhibit a defect in the phagocytosis of apoptotic cells, which is an important mechanism for the resolution of inflammation.

Our findings also confirm and extend previous observations that *Trex1*$^{-/-}$ mice present severe inflammation at multiple sites (15), including the brain, despite measureable monocyte infiltration in this organ. In fact, recently, it has been demonstrated that in vitro IFN-α treatment promotes astrocyte activation, supporting the idea of inflammation as a key feature in the pathogenesis of AGS (28). We also showed that Trex1 has a tissue-specific distribution with the highest levels in spleen, thymus, and uterus. This may explain why the level of tissue inflammation in the mice varies across various tissues and why cardiac disease is the most frequently described trait of Trex1-deficient mice.

Examination of cellular expression revealed high levels of Trex1 in immune cells, which were highly induced by proinflammatory stimuli and not by anti-inflammatory cytokines or growth factors in macrophages. This is concurrent with the observation made in human monocyte-derived macrophages in which Trex1 was also upregulated after proinflammatory stimuli (29). Additionally, it has also been reported that Trex1 is upregulated by genotoxic drugs (30, 31), indicating that Trex1 is needed for both pathogen DNA degradation and also small strands of nuclear DNA originated from genotoxicity. This suggests that, under the normal physio-

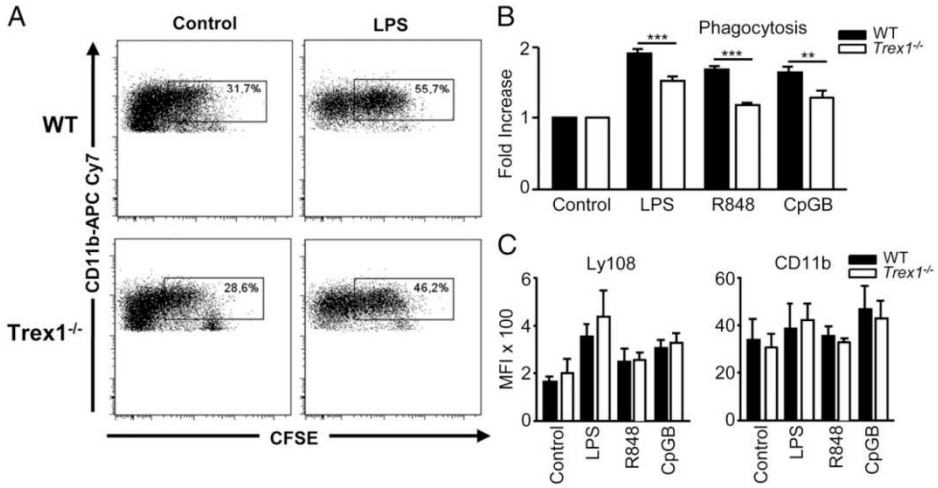

FIGURE 6. Apoptotic clearance is impaired in macrophages from *Trex1*$^{-/-}$ mice. (**A**) Apoptotic cells were labeled with CFSE (cytoplasmatic dye) and incubated with stimulated or control BMDM at a ratio of 4:1 for 1 h. Supernatant was removed and macrophages were collected, stained, and analyzed by flow cytometry. Macrophages that phagocyted apoptotic cells were detected as CFSE positive, when compared with the control (macrophages incubated with no CFSE-labeled cells). Phagocytosis values are presented as fold change of percentage of positive CFSE live macrophages. (**B**) The cumulative graphs of phagocytosis show a reduction in *Trex1*$^{-/-}$ macrophages following TLR stimulation. (**C**) The expression levels of macrophage surface markers are shown; no significant differences were seen here. All assays are representative of at least three independent experiments showing similar results. Results are shown as mean ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$ in relation to the controls when all the independent experiments have been compared.

logical environment, Trex1 is upregulated following immune or genotoxicity challenge to degrade an excess of cytoplasmatic DNA and also to regulate the host immune response. This hypothesis is based upon findings demonstrating that deficiency of Trex1 increases the production of proinflammatory cytokines such as TNF-α, IFN type 1, and IL-1β following TLR ligand exposure. These *Trex1*$^{-/-}$ TLR-activated macrophages have higher levels of surface CD86 and an increased capability in Ag presentation, which leads to activation of adaptive immune system with priming autoreactive T cells. The accumulation of DNA further triggers intracellular DNA receptors as STING (32) and in consequence overactivates macrophages. Furthermore, *Trex1*$^{-/-}$ macrophages present a reduced ability to phagocyte apoptotic material. All these facts may explain the exacerbated inflammatory phenotype in Trex1-associated diseases.

Excessive macrophage activation has numerous damaging effects, exemplified in septic shock, which can lead to multiple organ dysfunction syndrome and death through a surplus of TNF-α production among other cytokines (33). In other situations, persistence of proinflammatory activity results in the development of chronic inflammation and autoimmunity. This defect is arguably related to the excessive production of type I of IFN, signature of the AGS (6) that is also associated with autoimmune diseases.

A strong activation of macrophages could result in self-damage. LPS activation of macrophages can induce apoptosis through the autocrine production of TNF-α (34). To prevent undesirable effects, macrophages have several mechanisms to protect against excessive activation that prevents their self-destruction (35). For example, CDK inhibitor p21^{Waf1} is induced by IFN-γ and protects from apoptosis (23). In the absence of this CDK inhibitor, mice develop a lupus-like phenotype characterized by anti-DNA Abs and glomerulonephritis (36, 37). p21^{Waf1} is a negative regulator that curbs excessive macrophage activation by providing a negative feedback system of TNF-α and IL-1β production (38, 39). We propose a similar function for Trex1 as macrophage controller of activation.

Impaired macrophage function, specifically with regard to the clearance of dead cells, has been widely described in both patients and murine models of SLE (40). This is an important mechanism for the resolution of inflammation. Also, this process generates circulating DNA, which is often found increased in patients with SLE (41, 42). In our system, we did not observe any differences in the release of DNA by macrophages, despite the remarkable reduction in phagocytosis by *Trex1*$^{-/-}$ macrophages, indicating that these are two independent mechanisms. Trex1 is a highly processive exonuclease potentially affecting the digestion of apoptotic DNA engulfed by macrophages. Therefore, it is possible that the uptake of external apoptotic bodies may also accumulate outside the cell, affecting the net results. Furthermore, a recent publication has described dysregulation of lysosomes associated with Trex1 deficiency that may affect apoptotic cell processing (16). This would top with further activation of the macrophage, as it is known that macrophages switch to an anti-inflammatory profile after phagocytosis of apoptotic cells (43), and the lower phagocytic capacity could contribute to a delayed switch.

A percentage of SLE patients exhibits a loss in Trex1 function (8). In addition, we have shown that both TLR7 and TLR9 (19), which have been associated with the development of SLE, upregulate Trex1, and that, in its absence, responses to these TLR ligands result in an augmented inflammatory response.

Our data demonstrate that Trex1 plays a key role in the inflammatory processes by macrophages. These cells are scavengers for apoptotic cells and, in the absence of Trex1, unprocessed DNA could induce in a STING-dependent or independent fashion the production of cytokines and the exacerbation of the immune system explaining the inflammatory process. Our data suggest that this pathway may play a role in the development of autoimmune diseases.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Mazur, D. J., and F. W. Perrino. 2001. Structure and expression of the TREX1 and TREX2 3′—>5′ exonuclease genes. *J. Biol. Chem.* 276: 14718–14727.
2. Brucet, M., J. Querol-Audí, M. Serra, X. Ramírez-Espain, K. Bertlik, L. Ruiz, J. Lloberas, M. J. Macias, I. Fita, and A. Celada. 2007. Structure of the dimeric exonuclease TREX1 in complex with DNA displays a proline-rich binding site for WW domains. *J. Biol. Chem.* 282: 14547–14557.
3. Kawai, T., and S. Akira. 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 11: 373–384.
4. Mazur, D. J., and F. W. Perrino. 1999. Identification and expression of the TREX1 and TREX2 cDNA sequences encoding mammalian 3′—>5′ exonucleases. *J. Biol. Chem.* 274: 19655–19660.
5. Brucet, M., J. Querol-Audí, K. Bertlik, J. Lloberas, I. Fita, and A. Celada. 2008. Structural and biochemical studies of TREX1 inhibition by metals: identification of a new active histidine conserved in DEDDh exonucleases. *Protein Sci.* 17: 2059–2069.
6. Crow, Y. J., B. E. Hayward, R. Parmar, P. Robins, A. Leitch, M. Ali, D. N. Black, H. van Bokhoven, H. G. Brunner, B. C. Hamel, et al. 2006. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 cause Aicardi-Goutières syndrome at the AGS1 locus. *Nat. Genet.* 38: 917–920.
7. Rice, G., W. G. Newman, J. Dean, T. Patrick, R. Parmar, K. Flintoff, P. Robins, S. Harvey, T. Hollis, A. O'Hara, et al. 2007. Heterozygous mutations in TREX1 cause familial chilblain lupus and dominant Aicardi-Goutieres syndrome. *Am. J. Hum. Genet.* 80: 811–815.
8. Lee-Kirsch, M. A., M. Gong, D. Chowdhury, L. Senenko, K. Engel, Y. A. Lee, U. de Silva, S. L. Bailey, T. Witte, T. J. Vyse, et al. 2007. Mutations in the gene encoding the 3′-5′ DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat. Genet.* 39: 1065–1067.
9. Stewart, A. E., S. Dowd, S. M. Keyse, and N. Q. McDonald. 1999. Crystal structure of the MAPK phosphatase Pyst1 catalytic domain and implications for regulated activation. *Nat. Struct. Biol.* 6: 174–181.
10. Morita, M., G. Stamp, P. Robins, A. Dulic, I. Rosewell, G. Hrivnak, G. Daly, T. Lindahl, and D. E. Barnes. 2004. Gene-targeted mice lacking the Trex1 (DNase III) 3′—>5′ DNA exonuclease develop inflammatory myocarditis. *Mol. Cell. Biol.* 24: 6719–6727.
11. Tanoue, T., T. Yamamoto, and E. Nishida. 2002. Modular structure of a docking surface on MAPK phosphatases. *J. Biol. Chem.* 277: 22942–22949.
12. Yang, Y. G., T. Lindahl, and D. E. Barnes. 2007. Trex1 exonuclease degrades ssDNA to prevent chronic checkpoint activation and autoimmune disease. *Cell* 131: 873–886.
13. Stetson, D. B., J. S. Ko, T. Heidmann, and R. Medzhitov. 2008. Trex1 prevents cell-intrinsic initiation of autoimmunity. *Cell* 134: 587–598.
14. Neidhart, M., E. Karouzakis, G. G. Schumann, R. E. Gay, and S. Gay. 2010. Trex-1 deficiency in rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum.* 62: 2673–2679.
15. Owens, D. M., and S. M. Keyse. 2007. Differential regulation of MAP kinase signalling by dual-specificity protein phosphatases. *Oncogene* 26: 3203–3213.
16. Wang, Z., C. Zang, K. Cui, D. E. Schones, A. Barski, W. Peng, and K. Zhao. 2009. Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell* 138: 1019–1031.

17. Cao, W., C. Bao, E. Padalko, and C. J. Lowenstein. 2008. Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. *J. Exp. Med.* 205: 1491–1503.
18. Celhar, T., R. Magalhães, and A. M. Fairhurst. 2012. TLR7 and TLR9 in SLE: when sensing self goes wrong. *Immunol. Res.* 53: 58–77.
19. Byrne, J. C., J. Ní Gabhann, E. Lazzari, R. Mahony, S. Smith, K. Stacey, C. Wynne, and C. A. Jefferies. 2012. Genetics of SLE: functional relevance for monocytes/macrophages in disease. *Clin. Dev. Immunol.* 2012: 582352.
20. Burkatovskaya, M., G. P. Tegos, E. Swietlik, T. N. Demidova, A. P Castano, and M. R. Hamblin. 2006. Use of chitosan bandage to prevent fatal infections developing from highly contaminated wounds in mice. *Biomaterials* 27: 4157–4164.
21. Celada, A., P. W. Gray, E. Rinderknecht, and R. D. Schreiber. 1984. Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J. Exp. Med.* 160: 55–74.
22. Lutz, M. B., N. Kukutsch, A. L. Ogilvie, S. Rössner, F. Koch, N. Romani, and G. Schuler. 1999. An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J. Immunol. Methods* 223: 77–92.
23. Xaus, J., M. Cardó, A. F. Valledor, C. Soler, J. Lloberas, and A. Celada. 1999. Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. *Immunity* 11: 103–113.
24. Lu, T. C., Z. Wang, X. Feng, P. Chuang, W. Fang, Y. Chen, S. Neves, A. Maayan, H. Xiong, Y. Liu, et al. 2008. Retinoic acid utilizes CREB and USF1 in a transcriptional feed-forward loop in order to stimulate MKP1 expression in human immunodeficiency virus-infected podocytes. *Mol. Cell. Biol.* 28: 5785–5794.
25. Santiago-Raber, M. L., I. Dunand-Sauthier, T. Wu, Q. Z. Li, S. Uematsu, S. Akira, W. Reith, C. Mohan, B. L. Kotzin, and S. Izui. 2010. Critical role of TLR7 in the acceleration of systemic lupus erythematosus in TLR9-deficient mice. *J. Autoimmun.* 34: 339–348.
26. Hasselgren, P. O. 2007. Ubiquitination, phosphorylation, and acetylation: triple threat in muscle wasting. *J. Cell. Physiol.* 213: 679–689.
27. Xu, G., Y. Zhang, L. Zhang, A. I. Roberts, and Y. Shi. 2009. C/EBPbeta mediates synergistic upregulation of gene expression by interferon-gamma and tumor necrosis factor-alpha in bone marrow-derived mesenchymal stem cells. *Stem Cells* 27: 942–948.
28. Cuadrado, E., M. H. Jansen, J. Anink, L. De Filippis, A. L. Vescovi, C. Watts, E. Aronica, E. M. Hol, and T. W. Kuijpers. 2013. Chronic exposure of astrocytes to interferon-α reveals molecular changes related to Aicardi-Goutieres syndrome. *Brain* 136: 245–258.
29. Cobos Jiménez, V., T. Booiman, S. W. de Taeye, K. A. van Dort, M. A. Rits, J. Hamann, and N. A. Kootstra. 2012. Differential expression of HIV-1 interfering factors in monocyte-derived macrophages stimulated with polarizing cytokines or interferons. *Sci. Rep.* 2: 763.
30. Christmann, M., M. T. Tomicic, D. Aasland, N. Berdelle, and B. Kaina. 2010. Three prime exonuclease I (TREX1) is Fos/AP-1 regulated by genotoxic stress and protects against ultraviolet light and benzo(a)pyrene-induced DNA damage. *Nucleic Acids Res.* 38: 6418–6432.
31. Tomicic, M. T., D. Aasland, T. Nikolova, B. Kaina, and M. Christmann. 2013. Human three prime exonuclease TREX1 is induced by genotoxic stress and involved in protection of glioma and melanoma cells to anticancer drugs. *Biochim. Biophys. Acta* 1833: 1832–1843.
32. Gall, A., P. Treuting, K. B. Elkon, Y. M. Loo, M. Gale, Jr., G. N. Barber, and D. B. Stetson. 2012. Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. *Immunity* 36: 120–131.
33. Casals-Casas, C., E. Alvarez, M. Serra, C. de la Torre, C. Farrera, E. Sánchez-Tilló, C. Caelles, J. Lloberas, and A. Celada. 2009. CREB and AP-1 activation regulates MKP-1 induction by LPS or M-CSF and their kinetics correlate with macrophage activation versus proliferation. *Eur. J. Immunol.* 39: 1902–1913.
34. Xaus, J., M. Comalada, A. F. Valledor, J. Lloberas, F. López-Soriano, J. M. Argilés, C. Bogdan, and A. Celada. 2000. LPS induces apoptosis in macrophages mostly through the autocrine production of TNF-alpha. *Blood* 95: 3823–3831.
35. Valledor, A. F., M. Comalada, L. F. Santamaría-Babi, J. Lloberas, and A. Celada. 2010. Macrophage proinflammatory activation and deactivation: a question of balance. *Adv. Immunol.* 108: 1–20.
36. Balomenos, D., J. Martín-Caballero, M. I. García, I. Prieto, J. M. Flores, M. Serrano, and C. Martínez-A. 2000. The cell cycle inhibitor p21 controls T-cell proliferation and sex-linked lupus development. *Nat. Med.* 6: 171–176.
37. Salvador, J. M., M. C. Hollander, A. T. Nguyen, J. B. Kopp, L. Barisoni, J. K. Moore, J. D. Ashwell, and A. J. Fornace, Jr. 2002. Mice lacking the p53-effector gene Gadd45a develop a lupus-like syndrome. *Immunity* 16: 499–508.
38. Scatizzi, J. C., M. Mavers, J. Hutcheson, B. Young, B. Shi, R. M. Pope, E. M. Ruderman, D. S. Samways, J. A. Corbett, T. M. Egan, and H. Perlman. 2009. The cyclin dependent kinase domain of p21 is a suppressor of IL-1β-mediated inflammation in activated macrophages. *Eur. J. Immunol.* 39: 820-825.
39. Trakala, M., C. F. Arias, M. I. García, M. C. Moreno-Ortiz, K. Tsilingiri, P. J. Fernández, M. Mellado, M. T. Díaz-Meco, J. Moscat, M. Serrano, et al. 2009. Regulation of macrophage activation and septic shock susceptibility via p21(WAF1/CIP1). *Eur. J. Immunol.* 39: 810-819.
40. Szwench, E., M. P. Czkowska, K. Marczewski, A. Klisiewicz, I. Micha Owska, I. Ciuba, M. Januszewicz, A. Prejbisz, P. Hoffman, and A. Januszewicz. 2011. Phaeochromocytoma in a 86-year-old patient presenting with reversible myocardial dysfunction. *Blood Press* 20: 383-386.
41. De Vlas, S. J., D. Engels, A. L. Rabello, B. F. Oostburg, L. Van Lieshout, A. M. Polderman, G. J. Van Oortmarssen, J. D. Habbema, and B. Gryseels. 1997. Validation of a chart to estimate true *Schistosoma mansoni* prevalences from simple egg counts. *Parasitology* 114: 113–121.
42. Gaipl, U. S., L. E. Munoz, G. Grossmayer, K. Lauber, S. Franz, K. Sarter, R. E. Voll, T. Winkler, A. Kuhn, J. Kalden, et al. 2007. Clearance deficiency and systemic lupus erythematosus (SLE). *J. Autoimmun.* 28: 114–121.
43. Cvetanovic, M., J. E. Mitchell, V. Patel, B. S. Avner, Y. Su, P. T. van der Saag, P. L. Witte, S. Fiore, J. S. Levine, and D. S. Ucker. 2006. Specific recognition of apoptotic cells reveals a ubiquitous and unconventional innate immunity. *J. Biol. Chem.* 281: 20055–20067.

Supplemental material

The exonuclease Trex1 restrains the macrophage pro-inflammatory activation

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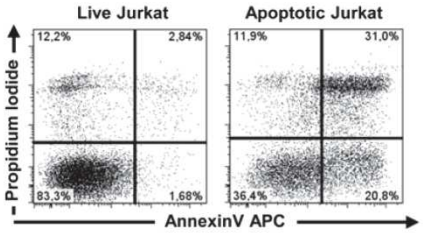
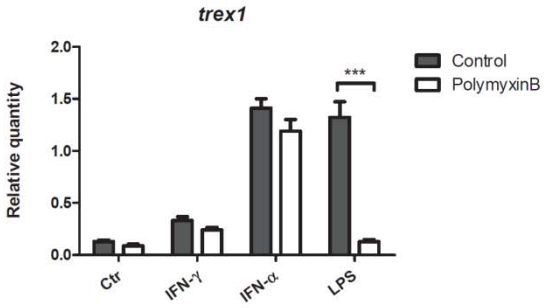
Supplemental Table 1. Sequence of primers used in qPCR.

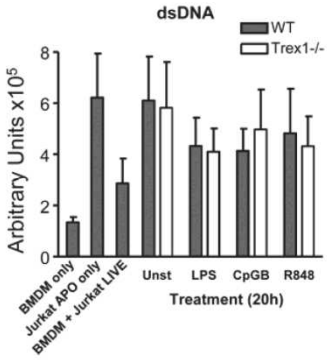
Gene	NCBI reference	Forward primer (5' to 3')	Reverse primer (5' to 3')
hprt1	NM_0133556.2	ATCATTATGCCGAGGATTTGG	GCAAAGAACTTATAGCCCCC
l14	NM_026732.2	TCCCAGGCTGTTAACGCGGT	GCGCTGGCTGAATGCTCTG
trex1	NM_001012236.1	TTCCTCAGCCACACTGCT	AGAGCTTGTCCACCACACG
ifnb1	NM_010510.1	AAGAGTTACACTGCCTTTGCCATC	CACTGTCTGCTGGTGGAGTTCATC
il1b	NM_008361.3	TGGGCCTCAAAGGAAAGAAT	CAGGCTTGTGCTCTGCTTGT
tnfa	NM_013693	CCAGACCCTCACACTCAGATC	CACTTGGTGGTTTGCTACGAC
nos2	NM_010927	GCCACCAACAATGGCAACA	CGTACCGGATGAGCTGTGAATT
cxcl10/ip10	NM_021274.2	CAACTGCATCCATATCGATGA	TTAGATTCCGGATTCAGACATCTC



Supplemental Figure 1. **Induction of apoptosis of Jurkat cells by incubation with Etoposide.** Annexin V/IP staining was done to test and quantify the level of apoptosis of the cells used in the phagocytosis assay. All assays are representative of at least three independent experiments showing similar results.



Supplemental Figure 2. Polymyxin B did not modified *trex1* expression in macrophages treated with interferons. BMDM were treated with IFN-γ, IFN-α or LPS in the presence or absence of polymyxin B (50μg/ml) and *trex1* expression was determined. As control, we treated macrophages with LPS alone or in the presence of polymyxin B. As expected, the drug inhibited the *trex1* induction mediated by LPS. All assays are representative of at least three independent experiments showing similar results. Results are shown as mean ± SD. ***$p<0.001$ in relation to the controls when all the independent experiments have been compared.



Supplemental Figure 3. Trex1 did not modify the amounts of dsDNA present in the supernatants after phagocytosis. The supernatant of macrophages after the phagoctytosis assay was collected and centrifuged for 10 min at 1500 rpm, the pellet was discarded and the levels of dsDNA were measured with Quant-iT™ PicoGreen® Molecular Probes, Invitrogen. All assays are representative of at least three independent experiments showing similar results.

Nbs1 is Essential for Macrophages Differentiation and Inflammatory Response

Summary of "Nbs1 is Essential for Macrophages Differentiation and for Inflammatory Response" in Spanish

Nbs1 es esencial para la diferenciación de los macrófagos y modula la respuesta inflamatoria

La proteína del síndrome de rotura de Nijmegen 1 (Nbs1) repara rupturas de doble cadena del ADN. Las mutaciones en el gen que codifica para Nbs1 se asocian con el síndrome de rotura de Nijmegen (NBS), una enfermedad hereditaria que se caracteriza por microcefalia, aumento de la incidencia de cáncer e inmunodeficiencia. Para entender mejor los procesos de inmunodeficiencia en el NBS hemos estudiado la actividad funcional de Nbs1 en los macrófagos. La expresión de esta proteína en los macrófagos se incrementa tras activarlos con estímulos proliferativos y pro-inflamatorios como IFN-γ y LPS. Los macrófagos obtenidos de ratones $Nbs1^{\Delta B/\Delta B}$ (carecen de una parte del gen de Nbs1), presentan una diferenciación tardía asociada con un defecto en la proliferación, que se puede observar tanto en condición de hiperoxia como de hipoxia. La disminución de la proliferación inducida por el factor M-CSF en los macrófagos $Nbs1^{\Delta B/\Delta B}$ no está relacionada con una mayor tasa de inducción de la apoptosis. Los macrófagos $Nbs1^{\Delta B/\Delta B}$ presentan un aumento del daño en el ADN caracterizada por el aumento de la expresión de γH2AX y de $p21^{waf-1}$, además presentan un aumento del marcador pro-inflamatorio CD80. Estos resultados obtenidos in vitro se correlacionan con la disminución del número de monocitos y macrófagos en la sangre y en la cavidad peritoneal de los ratones $Nbs1^{\Delta B/\Delta B}$ de 28 semanas de edad. Por último, en el modelo de inflamación inducida por DFNB en la oreja de ratones $Nbs1^{\Delta B/\Delta B}$, se observa una reacción mayor del aumento del grosor de la oreja y un perfil de expresión de genes preferentemente pro-inflamatorios. En conjunto, estos resultados muestran que Nbs1 es necesario para la diferenciación de los macrófagos y que la ausencia de la proteína funcional induce un estado pro-inflamatorio, que además puede ayudar a explicar el fenotipo de inmunodeficiencia presente en el NBS

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Nbs1 is Essential for Macrophages Differentiation and for Inflammatory Response

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Running title: Nbs1 and macrophage proliferation, and immune response

Keywords: Nbs1, Mre11 complex, macrophage, inflammation and cell cycle

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Abstract

Nijmegen breakage syndrome 1 (Nbs1) is a double-strand break (DSB) DNA damage repair protein. Mutations in the gene encoding Nbs1 are associated to Nijmegen breakage syndrome (NBS) in humans, which is a rare autosomal recessive inherited disorder characterised by microcephaly, increased cancer incidence and immunodeficiency. To better understand the immunodeficiency in NBS we studied the functional activity of Nbs1 in macrophages. We determined that proliferative and pro-inflammatory (IFN-γ and LPS) stimuli lead to Nbs1 up-regulation in these immune cells. Furthermore, macrophages derived from mice lacking a full Nbs1 protein ($Nbs1^{\Delta B/\Delta B}$ mice) presented a delayed differentiation associated with a defect in proliferation, both in hyperoxia and hypoxia conditions. M-CSF dependent decrease in proliferation in $Nbs1^{\Delta B/\Delta B}$ macrophages proved to be unrelated to apoptosis. These results were consistent with a decrease in the number of monocytes and macrophages in blood and peritoneum cavity of 28-week-old $Nbs1^{\Delta B/\Delta B}$ mice. Furthermore, $Nbs1^{\Delta B/\Delta B}$ macrophages presented an increase in DNA damage (γH2AX and $p21^{waf-1}$), as well as an increase of the pro-inflammatory CD80 marker. We demonstrate that $Nbs1^{\Delta B/\Delta B}$ mice upon DNFB induced inflammation presented an increased ear thickness and a skewed pro-inflammatory gene expression pattern. The above findings show that Nbs1 is necessary for macrophages differentiation and that the absence of the full Nbs1 protein induces a pro-inflammatory stage. This observation can be consequential in explaining the NBS immunodeficiency phenotype.

Introduction

Endogenous or environmental agents, such as DNA replication, reactive oxygen species (ROS) and ultraviolet (UV) light can cause DNA damage (Jackson and Bartek 2009). Accumulation of DNA lesions leads to genomic instability, which in turn causes human disease and, among others, infertility. DNA damage response (DDR) mechanisms prevent the accumulation of these lesions and are specialised in their repair (Papamichos-Chronakis and Peterson 2013). DNA damage can take many forms, however, double-strand breaks (DSB) are one of the most harmful. This DNA damage can be repaired both by non-homologous DNA end-joining (NHEJ) and homologous recombination (HR) (Bohgaki, Bohgaki and Hakem 2010). HR occurs when chromatid sister strand is available and originates a more reliable correction than that of NHEJ. In this regard, MRE11 complex that is composed of meiotic recombination 11 homolog A (Mre11), Nijmegen breakage syndrome 1 (Nbs1) and Rad50, has a crucial role in detecting and effecting the repair of the DSB and DNA damage signaling (Stracker et al. 2013). Once MRE11 complex recognises DSB, Ataxia telangiectasia mutated (ATM) is activated by phosphorylation. As a result, the DNA damage the repair and signaling is initiated by recruiting other proteins, such as checkpoint kinase 2 (CHK2) (Stracker and Petrini 2008).

MRE11 complex and ATM are crucial molecules for the proper functioning of humans. That is because mutations in these genes originate different but, in terms of phenotype, very similar syndromes that affect nervous and immune systems, as well as increase cancer incidence (Bohgaki, Bohgaki and Hakem 2010). Mutations in ATM are associated with ataxia telangiectasia (AT) syndrome characterised by cerebellar ataxia, telangiectasia, immunodeficiency with low numbers of B- and T-cells and predisposition to malignancy (Driessen et al. 2013; Chun and Gatti 2004). When mutations arise in gene coding Mre11, humans are affected by ataxia telangiectasia-like disorder syndrome (ATLD), which is similar to AT, though less severe (Stewart et al. 1999). Nbs1 mutations originate Nijmegen breakage syndrome (NBS) which is characterised by microcephaly, growth and mental retardation and immunodeficiency (Weemaes et al. 1981), as well as predisposition to cancer (Varon et al. 2000). A similar disorder, NBS-like syndrome, arises from Rad50 mutations (Waltes et al. 2009). The abovementioned syndromes are relatively similar and highlight the importance of a proper DSB repair and signaling in the development and functioning

of the nervous and immune systems. Nbs1 is the link between the proper function of MRE11 complex and ATM. Nbs1 is composed of a Forkhead-associated (FHA) and two BRCA1 C-Terminal (BRCT) domains in the N terminal and Mre11 binding domain and PI3K-related protein kinase (PIKK) domain. The later interacts with ATM (Stracker and Petrini 2011).

Immunological defects in NBS are to date poorly understood. Until recently, T- and B-cells were the major focus and considered to be a plausible explanation of the immunodeficiency in NBS patients, since these cells endogenously produce DSB in VDJ recombination (van der Burg et al. 2010; Piątosa et al. 2012). Little is known about the relevance and role of macrophages in NBS immunodeficiency.

Macrophages play a key role in both the innate and adaptive immune systems. They are highly plastic cells and produce high amounts of ROS upon pro-inflammatory activation (Khansari, Shakiba and Mahmoudi 2009). Though macrophages originate, among others, in the bone marrow, recent research has shown that they can undergo local proliferation to maintain homeostasis and to fight pathogenic intruders (Jenkins et al. 2011). This indicates that a proper control of DNA damage repair and proliferation is important in macrophages, as well as in other highly proliferative immune cells, such as T- and B-cells.

Our hypothesis was that impaired Nbs1 may diminish macrophages' ability to tolerate DNA damage and hence impair their proper cell cycle progression and function, and as a result, that would cause an imbalanced immune response and contribute to NBS immunodeficiency. To test this hypothesis, we analysed the role of MRE11 complex in macrophages behavior under inflammatory conditions. To that end, we used an $Nbs1^{\Delta B/\Delta B}$ (Williams et al. 2002) mouse model that expressed a truncated form of Nbs1 lacking N-terminal, which is necessary for cell cycle checkpoint in DNA damage response (Difilippantonio et al. 2007).

Our results demonstrate that Nbs1 is required for correct macrophages differentiation, their proliferation and inflammatory response. This conclusion is relevant for explaining the immunodeficiency in NBS patients.

Material and Methods

Mice

Balb/c mice were purchased from Charles Rivers. Nbs1$^{\Delta B/\Delta B}$ mutant mice (Williams et al. 2002) and other mice were maintained and used in a Specific Pathogen Free facility at *Parc Cientific de Barcelona*. Laboratory animals were maintained and used and experimental procedures were performed in accordance with the IACUC approved animal protocol (Animal Research Committee of the Government of Catalonia, number 2523).

Reagents

Murine recombinant IFN-γ and IL-4 of R&D Systems (Minneapolis, MN) were used. All chemicals used were of the highest available purity grade and were purchased from Sigma-Aldrich (St Louis, MO), unless explicitly stated otherwise.

Bone marrow-derived macrophages culture

Bone marrow-derived macrophages (BMDM) were generated from 6-12 week-old mice. Femora and tibia bone marrow cells were flushed and cultured in plastic tissue culture dishes (150 mm) in DMEM containing 20% FCS (Gibco, Invitrogen) and 30% of L-cell conditioned media as M-CSF source. Media was supplemented with Penicillin 100U/ml and Streptomycin 100µg/ml. Bone marrow was incubated at 37ºC in a humidified 5% CO_2 atmosphere (Celada et al. 1996). Homogeneous population of adherent macrophages was obtained after seven days of culture (>99% CD11b and F4/80).

Western blot protein analysis

To obtain protein cells were incubated in lysis buffer (1% Triton X-100, 10% glycerol, 50 mM Hepes pH 7.5, 150 mM NaCl, protease inhibitors and 1mM sodium ortovanadate) rotating for 20 minutes at 4ºC (Xaus et al. 1999). Cell extracts were centrifuged at 12,000xg and supernatants were kept at -80ºC. Protein concentration was measured by BioRad protein analysis kit. For the western blot, 20µg of protein extracts were heated to 95ºC in Laemmli SDS loading buffer, resolved by SDS-PAGE and transferred to PVDF membranes (Amersham). Membranes were blocked with PBS with 5% of dry milk for 1 hour and subsequently incubated with specific

antibodies; β-actin was used as loading control. Secondary antibodies peroxidase labelled anti-mouse and anti-rabbit were used. ECL (Amersham) was used for detection.

RNA extraction and Real-Time RT-PCR

Total RNA was extracted, purified and DNase treated with PureLink^{TM} RNA Mini Kit as described by the manufacturer (Ambion, Life Technologies). For cDNA synthesis, 400ng of total RNA and M-MLV Reverse transcriptase RNase H Minus, Point Mutant, oligo $(dT)_{15}$ primer and PCR Nucleotide mix were used, as described by the manufacturer (Promega). qPCR was performed in triplicate using the SYBR Green Master Mix (Applied Biosystems) in a final volume of 10µl using a 7900 HT Fast Real Time PCR System (Applied Biosystems) as described previously. Data were normalised to the housekeeping gene, *hprt1* and/or *l14.* Data are expressed as relative mRNA levels compared to the untreated control.

Cell cycle and DNA content

Macrophages were collected at day 6. 10^6cells were plated in 60 mm petri dish with 3 ml of 10% FCS DMEM and incubated for 16 hours before the addition of stimulants. Cells were collected after 24 hours stimulation fixed with 95% EtOH, incubated with propidium iodide (PI) and Rnase A and later analysed by flow citometry. Cell cycle distributions were analysed using FlowJo 7.6 software.

Proliferation assay

Cell proliferation was measured as previously described (Pascual-García et al. 2011). 10^5/well quiescent macrophages were stimulated with M-CSF and incubated for 24 hours either in normal O_2 conditions or in hypoxia 1%O_2. 6 hours before the end of the treatment $^{[3H]}$thymidine (1 µCi/ml; ICN Pharmaceuticals) was added. After the treatment, cells were fixed with 70% methanol, washed three times with 10% TCA, and lysed in 1% SDS 0,3 M NaOH solution. Radioactivity was counted by liquid scintillation using a 1400 Tri-Carb Packard counter.

Inflammation *in vivo* assay

0,5% 2,4-dinitorfluorobenezene (DNFB) dissolved in acetone was applied on the right ear of each mouse and acetone on the left ear as control(Bonneville et al. 2007).

Animals were sacrificed on day 4, 7 and 10 after the application. Ears, peritoneal cavity cells and blood were obtained from the animals. Ears were used for both histology and RNA, blood and peritoneal cavity cells were collected and stained with different antibodies to determine the percentage to T-cells, B-cells and macrophages by flow cytometry.

Histology

Ears were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Ear sections were stained with hematoxylin and eosin. Images were collected with Nikon E800 microscope and maximum ear thickness measurements were calculated with Fiji software.

Flow cytometry

On day 7, BMDM were collected and 5 x 10^5 cells were used to assess the phenotype with flow cytometry using anti-CD115-PE, anti-F4/80-PECy5, anti-CD45-PECy7, anti-CD11b-APC and anti-GR1-APCCy7 antibodies from eBioscience plus DAPI to gate out dead cells. To determine changes in expression of surface markers after activation cells were plated in 12 well plates, left to adhere and treated with different stimuli for 24 hours. Anti-CD86-PECy7 and anti-CD40-APC were used to detect activation levels after stimulation. Samples were acquired in Gallios™ Flow Cytometer from Beckman Coulter depending on experimental design and availability.

Statistical analysis

Data was analysed by using a two-tailed Student t-test comparing two groups or ANOVA for multiple groups. Bonferroni post-hoc correction was used to compare pairs. Statistical analysis was performed with GraphPad Software Prism 5.

Results

Pro-inflammatory and M-CSF stimulation induces Nbs1 expression in macrophages.

Nbs1 has been reported to be expressed in the red pulp of the mouse spleen (Wilda et al. 2000) that is characterised by macrophages' presence. Initially, we determined if the expression of Nbs1 in macrophages varied depending on the different stimulation of macrophages. We obtained bone marrow from WT mice and differentiated it *in vitro* to bone marrow-derived macrophages (BMDM). These cells are a population of primary homogeneous and quiescent macrophages that can be induced to proliferate by growth factors or that can be classically and alternatively activated by cytokines. BMDM were incubated with different pro-inflammatory (IFN-γ and LPS), anti-inflammatory (IL-4) and proliferative stimuli (M-CSF and GM-CSF) and the levels of Nbs1 were determined. Although there were no significant changes in mRNA levels (Figure 1A), protein levels were higher after IFN-γ, LPS and M-CSF treatment than after other treatments (Figure 1B). To discard the possibility of different mRNA transcripts, we conducted a qualitative PCR to determine the levels of expression of four sections of 2491bp mRNA (NM_013752.3). We obtained the same level of expression for all sections and different stimuli (data not shown). Since no change in the total Nbs1 expression has been reported earlier, we undertook to determine whether other DNA damage protein expression in macrophages was changed after pro-inflammatory and M-CSF stimulation. Examined proteins (ATM, Ku80 and CHK2) did not have expression differences (Figure 1C).

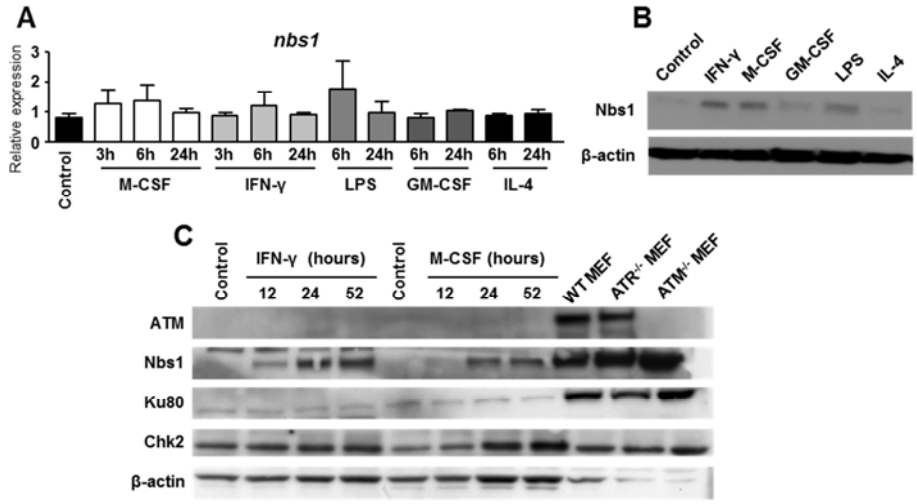

Figure 1. Nbs1 is induced in macrophages after pro-inflammatory and M-CSF stimulation. Seven-day BMDM were starved of growth factors for 16-18 hours and then incubated with different stimuli. IFN-γ (10ng/ml) and LPS (10ng/ml) were used as pro-inflammatory stimuli, IL-4 (10ng/ml) as anti-inflammatory stimuli and M-CSF and GM-CSF (10ng/ml) as growth factors. (A) RNA expression was determined at 3 hours, 6 hours and 24 hours. (B) Protein expression was determined after 24 hours of stimulation. (C) Expression of other DNA damage response proteins was determined in macrophages. WT, $ATM^{-/-}$ and $ATR^{-/-}$ mouse embryonic fibroblasts were used as control.

Macrophages from $Nbs1^{\Delta B/\Delta B}$ mice showed delayed differentiation.

Macrophages are differentiated in bone marrow due to the effect of M-CSF. We undertook to examine differentiation markers of BMDM originated from $Nbs1^{\Delta B/\Delta B}$ mice. Bone marrow was extracted from 6 to 10 week-old WT and $Nbs1^{\Delta B/\Delta B}$ mice. Bone marrow cells were counted prior to incubation in M-CSF rich media for differentiation. After seven days, differentiated macrophages were collected and counted. Although the number of bone marrow cells obtained from WT and $Nbs1^{\Delta B/\Delta B}$ mice did not differ, the amount of macrophages obtained after seven days of differentiation was reduced to approximately half (data not shown) in the $Nbs1^{\Delta B/\Delta B}$ population compared to WT mice.

Following counting, half a million seven-day BMDM were stained with specific antibodies, such as CD11b, F4/80 and Ly6C markers of macrophages differentiation.

Macrophages from Nbs1$^{\Delta B/\Delta B}$ express lower levels of CD11b and F4/80, and higher levels of Ly6C (Figure 2). This indicates that Nbs1$^{\Delta B/\Delta B}$ macrophages have a delayed maturation.

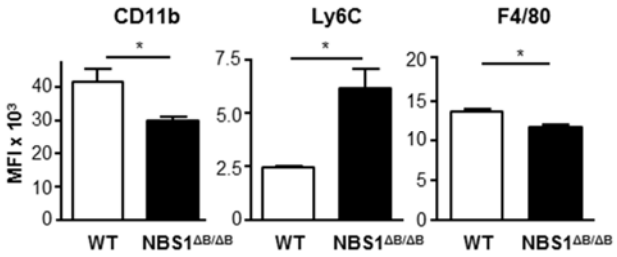

Figure 2. Nbs1$^{\Delta B/\Delta B}$ macrophages have delayed differentiation. Macrophages were differentiated from bone marrow of WT and Nbs1$^{\Delta B/\Delta B}$ mice. The surface expression of macrophages differentiation markers was determined. The Figure shows mean fluorescence intensity from the live single cells population.

Nbs1$^{\Delta B/\Delta B}$ macrophages have decreased proliferation capacity.

Macrophages' stimulation with M-CSF induces Nbs1 expression. Since this growth factor is crucial for macrophages differentiation and proliferation (Celada et al. 1996), we examined if the loss of Nbs1 function affected macrophages proliferation. To that end, BMDM were obtained from wild type (WT) and Nbs1$^{\Delta B/\Delta B}$ mice. These transgenic mice express a truncated form of Nbs1 protein that lacks part of N-terminal and the capacity to properly function (Williams et al. 2002). Once differentiated after seven days in the response to M-CSF, BMDM were plated and deprived of M-CSF for 16-18 hours. The cells in question were subsequently stimulated for 1 hour with the indicated stimuli (Figure 3), washed and further incubated with M-CSF for 24 hours. Proliferation was detected by incorporation of radioactive thymidine. A decreased proliferative capacity was observed in Nbs1$^{\Delta B/\Delta B}$ macrophages (Figure 3A). To discard the possibility that the differences observed were caused by environmental O_2, a hypoxia chamber (1% O_2) was used. The results obtained under hypoxic conditions were similar (Figure 3A) to those of the standard O_2 concentration in the environment.

Since macrophages proliferation was affected, we undertook to evaluate the cell cycle of proliferating macrophages. Upon cell cycle synchronisation by incubating macrophages without M-CSF for 18 hours, cells were cultured in M-CSF rich environment for 24 hours. Cells in question were subsequently fixed, permeabilised and the DNA was stained with propidium iodide. This cell cycle analysis demonstrated that the amount of macrophages in S phase from Nbs1$^{\Delta B/\Delta B}$ mice was reduced when compared to WT (Figure 3B). This observation proved correct in both hypoxia and hyperoxia conditions. These results corroborate the deficiency in proliferation and demonstrate that Nbs1 is crucial for macrophages proliferation and cell cycle progression *in vitro*.

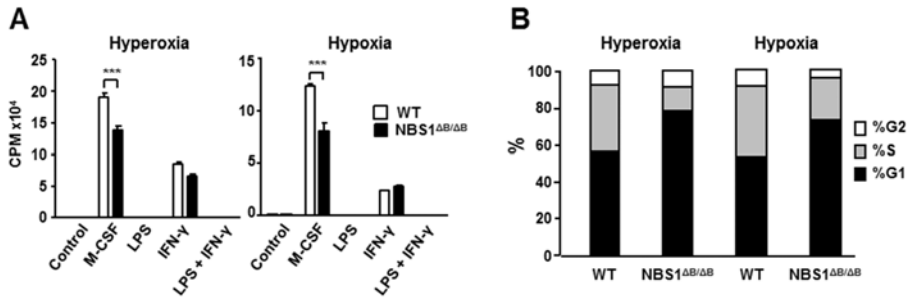

Figure 3. Nbs1$^{\Delta B/\Delta B}$ macrophages have reduced proliferation. Macrophages were starved of growth factor for 16-18 hours. Macrophages were further incubated for 1 hour with the indicated stimuli, washed and incubated for 24 hours with M-CSF. In (A), incubations were in hyperoxia conditions (20% O_2) and incubations were made in a hypoxia chamber (1% O_2). (B) Cell cycle analysis of proliferating macrophages was performed in hypoxia and hyperoxia conditions and assessed by flow cytometry.

Lack of functional Nbs1 protein does not affect induction of apoptosis in macrophages.

It has been reported that lack of a functional Nbs1 protein induces apoptosis in different cell types (Wan and Crowe 2012).Therefore, we undertook to test if apoptosis was also increased in macrophages that express truncated form of Nbs1. To that end, we incubated macrophages with different pro-inflammatory and proliferative stimuli for 24 hours. After incubation BMDM were collected and the content of sub-G1/G0 DNA,

which is a characteristic of apoptotic cells, was assessed. The measured levels of apoptosis were very low and no significant difference was found between WT and Nbs1$^{\Delta B/\Delta B}$ BMDM (Figure 4A). We also determined the mRNA expression of Bcl2, a pro-apoptotic gene. No difference was observed between distinct BMDM genotype (Figure 4B). These results demonstrate that lack of Nbs1 does not induce apoptosis in macrophages and that the decrease of proliferation is not related to an increase in apoptosis.

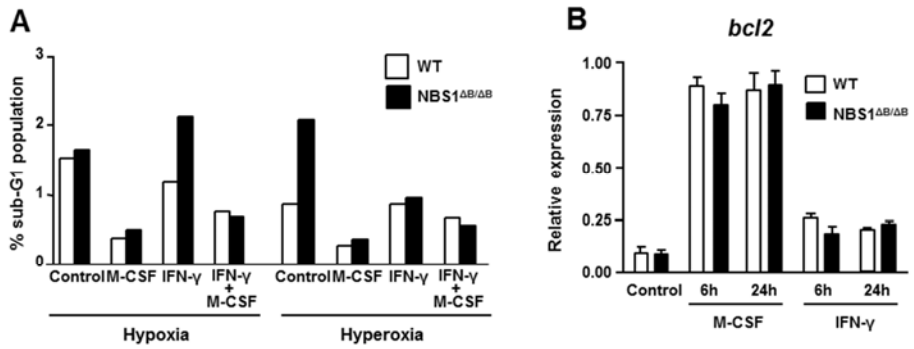

Figure 4. Nbs1 does not affect apoptosis induction by M-CSF and IFN-γ in macrophages. (A) Using flow cytometry he level of apoptosis from WT and Nbs1$^{\Delta B/\Delta B}$ macrophages stimulated 24 hours with M-CSF, IFN-γ was determined by flow cytometry. (B) The mRNA expression level of Bcl2 was determined after stimulation of both WT and Nbs1$^{\Delta B/\Delta B}$ macrophages by qPCR.

Nbs1$^{\Delta B/\Delta B}$ mice have less monocytes and macrophages in older mice.

To determine the consequences of decreased proliferation of Nbs1$^{\Delta B/\Delta B}$ macrophages *in vitro*, we assessed the number of circulating monocytes (precursors of macrophages) and local macrophages *in vivo*. To that end, we recovered blood, spleen and cells from peritoneal cavity of WT and Nbs1$^{\Delta B/\Delta B}$ mice that were 8 week and 28 weeks old. Half-a-million cells of each sample were stained with specific antibodies against T-cells (CD3), B-cells (B220), monocytes and macrophages (CD11b). No differences were observed in the percentage of these cells when compared to WT and Nbs1$^{\Delta B/\Delta B}$ younger mice. On the contrary, older Nbs1$^{\Delta B/\Delta B}$ mice presented a decrease in the percentage of monocytes and macrophages population in

blood and in peritoneal cavity (Figure 5). These findings suggest that *in vitro* data correlates with *in vivo* monocyte and macrophages generation. The differences identified in older mice suggest that DNA damage accumulates over time in monocytes and macrophages.

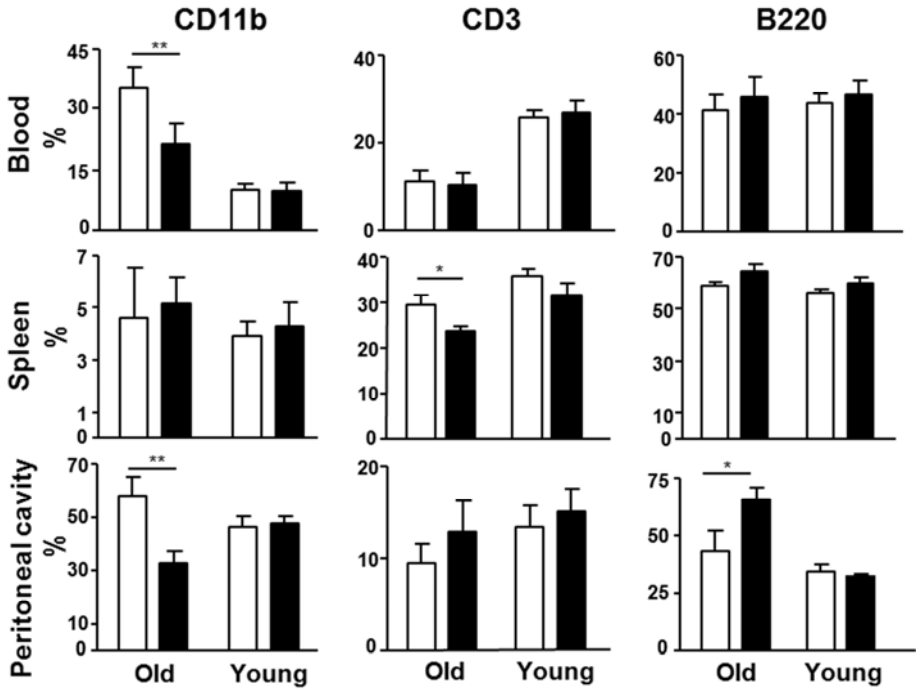

Figure 5. Decreased number of monocytes and macrophages in blood and peritoneum Nbs1$^{\Delta B/\Delta B}$ mice. Percentage of T-cells (CD3 positive), B-cells (B220 positive) and macrophages or monocytes (CD11b positive) in blood, spleen and peritoneum cavity was determined by flow cytometry. Total stained cells were gated for singlets, live and CD45^{+} cells. Shown percentages are relative to the total CD45^{+} live cell population. Mice were 28 weeks (Old) or 8 weeks (Young).

Nbs1$^{\Delta B/\Delta B}$ macrophages have higher levels of DNA damage.

Cell cycle arrest and lack of proliferation can be generated by an increase of DNA damage levels, as well as by the presence of cellular senescence. Since Nbs1$^{\Delta B/\Delta B}$ macrophages presented arrested cell cycle, we undertook to check whether markers

of DNA damage and senescence could be affected. To that end, macrophages were treated with IFN-γ and M-CSF, a pro-inflammatory and proliferative stimuli. After 24 hours of treatment macrophages were collected and γH2AX levels were determined by flow cytometry. Nbs1$^{\Delta B/\Delta B}$ macrophages expressed higher levels of γH2AX upon proliferative stimuli (Figure 6A).

Thereafter, we assessed p21^{waf-1} (hereinafter: p21) levels, another marker of DNA damage, as well as senescence marker. The level of p21 expression was determined by qPCR at different time intervals after IFN-γ and M-CSF stimuli. Both stimuli increased *p21* expression at different time intervals. Upon IFN-γ stimulation, *p21* expression reached its peak at 3 hours. On the other hand, M-CSF stimulation lead to *p21* maximum values of expression after 24 hours. In both cases, Nbs1$^{\Delta B/\Delta B}$ macrophages presented higher levels of *p21* (Figure 6B). These observations suggest that Nbs1$^{\Delta B/\Delta B}$ macrophages impair cell cycle progression and proliferation due to higher levels of DNA damage and senescence.

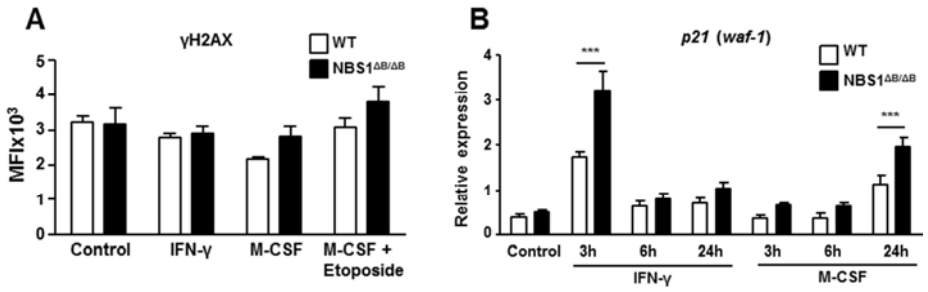

Figure 6. Macrophages from Nbs1$^{\Delta B/\Delta B}$ mice present higher levels of DNA damage. (A) The expression of γH2AX was determined by flow cytometry. Etoposide was used as control to induce DNA damage. (B) The expression of p21 was determined by qPCR.

Nbs1$^{\Delta B/\Delta B}$ macrophages express higher CD80 levels.

As Nbs1 is over-expressed after pro-inflammatory stimulation in macrophages we undertook to determine, by flow cytometry, whether the expression of antigen presentation surface molecules was modified. Although no differences were present in the expression levels of MHC II and CD86, CD80 expression was increased in

Nbs1$^{\Delta B/\Delta B}$ macrophages upon stimulation with IFN-γ (Figure 7). These findings further demonstrate Nbs1 role in macrophages pro-inflammatory response.

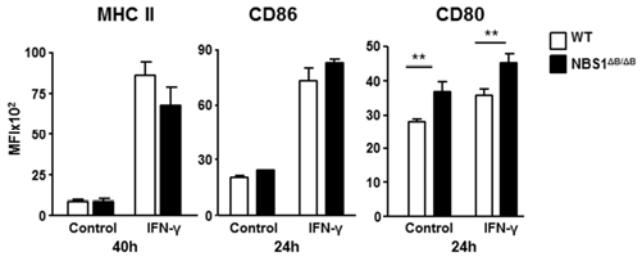

Figure 7. Nbs1$^{\Delta B/\Delta B}$ macrophages express higher levels of pro-inflammatory marker. The expression of different antigen presentation markers was determined by flow cytometry.

Nbs1$^{\Delta B/\Delta B}$ mice present an imbalanced inflammatory response *in vivo*.

The number and the functional activity of immune cells are important for a balanced immune response. To test *in vivo* immune response of Nbs1$^{\Delta B/\Delta B}$ mice, we have used DNFB ear irritation model (Bonneville et al. 2007). In this model, macrophages incorporated from circulating blood play a critical role in the increase of inflammation reaction to DNFB, as well as in the resolution of inflammation (unpublished observation). After treatment of the right ear with DNFB and of the left ear with acetone (control) mice were sacrificed at days 7 or 10 after the initial treatment. Mice ears were equally punched, weighted and collected for histology and RNA. Ears' thickness was measured after eosin-hematoxylin staining and levels of pro-inflammatory and anti-inflammatory genes were measured by qPCR. Our results showed a tendency for increased ear weight and a significant increase of thickness in Nbs1$^{\Delta B/\Delta B}$ DNFB ears when compared to WT (Figure 8A). qPCR results showed a substantial decrease in the levels of mannose receptor (anti-inflammatory marker) in Nbs1$^{\Delta B/\Delta B}$ (Figure 8B). These results demonstrate that Nbs1$^{\Delta B/\Delta B}$ mice have an impaired immune response with exacerbated pro-inflammatory response.

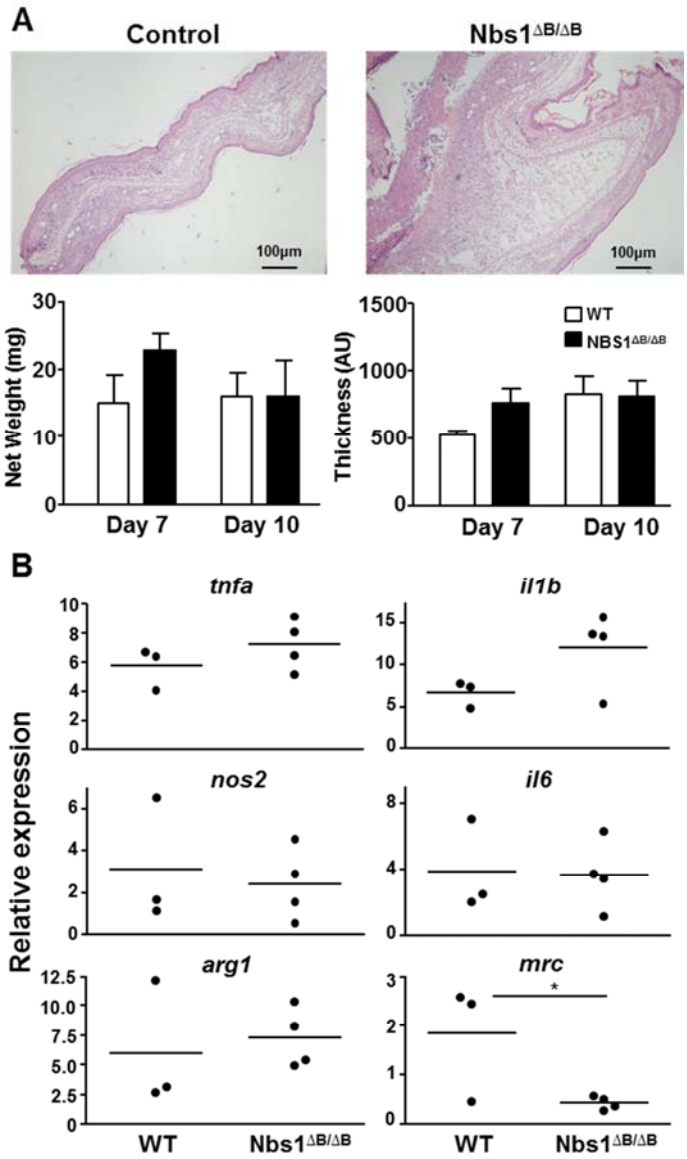

Figure 8. Nbs1$^{\Delta B/\Delta B}$ mice produce an exacerbated inflammation. A) DNFB treated ears are shown. The net ear weight is presented and was calculated by subtracting the weight of the DNFB treated right ear from the Acetone (control) treated left ear. The ear thickness measurements were taken from the histological images using Fiji, only DNFB treated ear thickness are shown. B) Panel of pro-inflammatory and anti-inflammatory markers expression by qPCR.

Discussion

Because NBS patients present immunodeficiency, in an attempt to gain knowledge of the immunological problems we characterized macrophages that express a truncated Nbs1 protein, which lacks N-terminal FHA and BRCT domain (Williams et al. 2002). Our results indicate that macrophages from these ($Nbs1^{\Delta B/\Delta B}$) mice have a defect in differentiation that is associated with reduced proliferation ability. The miss of functional NBS induce, as expected, in macrophages a DNA damage signature that is closely related to aging. Therefore, it is not surprising that defects observed *in vitro* are translated *in vivo* in a reduced amount of circulating monocytes and macrophages in older mice. We also demonstrate that $Nbs1^{\Delta B/\Delta B}$ mice have a disproportionate macrophages activation that tends to have a biased pro-inflammatory phenotype.

In most of the publications about Nbs1, the protein levels were unchanged. However, under our experimental conditions upon stimulation with pro-inflammatory stimuli and M-CSF, we observed that at protein level, Nbs1 is up-regulated in macrophages. Interestingly, no variations in the level of Nbs1 mRNA were observed. This suggests that the up-regulation of the protein occurs at post-transcriptional level. In different cell lines, a variation of the level of Nbs1 mRNA has been described related to an increase in c-myc (Chiang et al. 2003). This is not the case in macrophages because M-CSF that increases the levels of c-myc do not modified the Nbs1 mRNA. Further studies would be required to elucidate the mechanisms in macrophages that affect Nbs1 at the post-transcription level, i.e. block of protein degradation, inhibition of proteosomal activity, etc.

Under pro-inflammatory activation or proliferation in macrophages, Nbs1 was the only DNA damage protein that was expressed differently. No modifications were observed with ATR (data not shown), ATM, CHK2 and Ku80 (also a detector of DSB). This was an unexpected observation because many of the DNA damage proteins, such as Nbs1 are activated by phosphorylation (X Wu et al. 2000), and thus there is no need to strictly control their expression in the cell. These observations suggest that Nbs1 has an important and distinct function of the other DNA damage proteins during stress responses of the macrophages.

It has been shown that Nbs1 is crucial for the differentiation of lenses in eyes and that, in the absence of Nbs1, cataracts would appear at an early age (Yang et al. 2006). Furthermore, it has been demonstrated that Nbs1 regulates the differentiation of neural cells (Lee et al. 2007). These studies also showed defects in cell proliferation if Nbs1 is absent. Our results demonstrate that, differentiation of $Nbs1^{\Delta B/\Delta B}$ macrophages is defective together with impaired proliferation. Indeed, these evidences support, the observation that NBS patients-derived cells presented defects in intra-S-phase checkpoint control (Xiaohua Wu et al. 2004). In macrophages, cell cycle and differentiation are highly connected because PU1, an important transcription factor for differentiation, also has a role in macrophages proliferation. Furthermore, cell cycle can change the levels of expression of PU1 (Celada et al. 1996; Kueh et al. 2013). The above-mentioned observations justify the differentiation and proliferation defects found by us in $Nbs1^{\Delta B/\Delta B}$ macrophages.

Under our experimental conditions, we have not observed changes in apoptosis induction, that contradict previous findings where the lack of Nbs1 proved to lead to increased apoptosis in intestinal cells (Stracker et al., 2007). This could be explained by the fact that $Nbs1^{\Delta B/\Delta B}$ macrophages presented a higher expression of $p21^{waf\text{-}1}$ that protect these cells from apoptosis (Xaus et al. 1999).

Our results demonstrate that 28-week-old $Nbs1^{\Delta B/\Delta B}$ mice have a reduced percentage of monocytes and macrophages. These results contrast to a study showing that severe lymphopenia is present in a mouse model lacking full length Nbs1 protein only in T-cells, due to development malfunction (Saidi et al. 2010). This disparity in results can be attributed to the different mouse models. More specifically, $Nbs1^{\Delta B/\Delta B}$ expresses throughout all cells a truncated form of Nbs1 that only maintains a part of its functional activity (the full Nbs1 knock-out mice is lethal). It is possible that macrophages in a conditional Nbs1 knock-out mouse that targets only macrophages and monocytes would also present a more severe decrease of these leukocytes.

Until now, in NBS patients the immune cell populations that are known to be affected in blood are T and B lymphocytes, which are highly decreased. This observation was more pronounced in children under 2 years old than in older patients (Piątosa et al. 2012; van der Burg et al. 2010). It is hence plausible that mice could have a decrease in these populations at younger age than 8 weeks.

It is also important to note that monocytes are more prone to DNA damage than dendritic cells and macrophages with increased apoptosis upon reactive oxygen species (ROS) exposure due to impaired DSB repair (Bauer et al. 2011). This concurs with our findings, since Nbs1$^{\Delta B/\Delta B}$ mice are more prone to DSB, it is possible that over time DNA damage is accumulated in monocytes and that older mice present a reduced number of monocytes due to more extensive DNA damage and loss of proliferation capacity. In this sense, our work establishes a new perspective in the characteristic immunodeficiency of NBS, AT and related syndromes and demonstrates that monocytes and macrophages should not be neglected when assessing the role of immune cells in NBS and AT patients.

The fact that Nbs1 is up-regulated after pro-inflammatory stimulation as IFN-γ and LPS seems to be logical. Indeed, ROS production is a natural process that occurs in macrophages upon pro-inflammatory stimulation (Lonkar and Dedon 2011). Taking this into account, it will be possible that an increase of Nbs1 expression, in a pro-inflammatory environment, is a mechanism of repair, since ROS can damage both pathogens, as well as its own macrophages DNA. In this regard, a recent paper has demonstrated that DNA damage in germ cells triggered innate immunity and induced resistance to stress in somatic cells (Ermolaeva et al. 2013). Retroviruses require integration of their own genetic material in hosts genomes, and Nbs1 is used to help integration of this DNA (Xu 2006; Smith et al. 2008). In this context, it can also be argued that Nbs1 up-regulation occurs upon pro-inflammatory stimulation and that viruses have evolved to circumvent the existing DNA repair mechanisms of the host.

Macrophages can be skewed to a pro-inflammatory phenotype upon irradiation (Klug et al. 2013). A similar phenotype was presented in ATM kinase mutant flies that had escalated innate immune gene expression in glial cells (Petersen, Rimkus and Wassarman 2012). Taking these reports into account, we presume that an increase of CD80 expression, a pro-inflammatory gene, in Nbs1$^{\Delta B/\Delta B}$ macrophages occurs due to enhanced DNA damage. This could also explain our *in vivo* observations, whereby DNFB ear challenged Nbs1$^{\Delta B/\Delta B}$ mice present an exacerbated inflammation and bias cytokine expression towards a pro-inflammatory phenotype.

In conclusion, our results demonstrate that Nbs1 is a key molecule for macrophages differentiation, proliferation and inflammatory response. These observations can

further contribute to the understanding of the immunodeficiency in NBS and syndromes related to NBS.

References

Bohgaki, Toshiyuki, Miyuki Bohgaki, and Razqallah Hakem. 2010. “DNA Double-Strand Break Signaling and Human Disorders.” *Genome Integrity* 1 (1): 15. doi:10.1186/2041-9414-1-15.

Bonneville, Marlene, Cyril Chavagnac, Marc Vocanson, Aurore Rozieres, Josette Benetiere, Ingrid Pernet, Alain Denis, Jean-Francois Nicolas, and Ana Hennino. 2007. “Skin Contact Irritation Conditions the Development and Severity of Allergic Contact Dermatitis.” *The Journal of Investigative Dermatology* 127 (6) (June): 1430–1435. doi:10.1038/sj.jid.5700726.

Celada, A., F. E. Borràs, C. Soler, J. Lloberas, M. Klemsz, C. van Beveren, S. McKercher, and R. A. Maki. 1996. “The Transcription Factor PU.1 Is Involved in Macrophage Proliferation.” *The Journal of Experimental Medicine* 184 (1) (July 1): 61–69. doi:10.1084/jem.184.1.61.

Chun, Helen H, and Richard A Gatti. 2004. “Ataxia-Telangiectasia, an Evolving Phenotype.” *DNA Repair* 3 (8-9) (September): 1187–1196. doi:10.1016/j.dnarep.2004.04.010.

Difilippantonio, Simone, Arkady Celeste, Michael J Kruhlak, Youngsoo Lee, Michael J Difilippantonio, Lionel Feigenbaum, Stephen P Jackson, Peter J McKinnon, and André Nussenzweig. 2007. “Distinct Domains in Nbs1 Regulate Irradiation-Induced Checkpoints and Apoptosis.” *The Journal of Experimental Medicine* 204 (5) (May 14): 1003–1011. doi:10.1084/jem.20070319.

Driessen, Gertjan J, Hanna Ijspeert, Corry M R Weemaes, Ásgeir Haraldsson, Margreet Trip, Adilia Warris, Michiel van der Flier, et al. 2013. “Antibody Deficiency in Patients with Ataxia Telangiectasia Is Caused by Disturbed B- and T-Cell Homeostasis and Reduced Immune Repertoire Diversity.” *The Journal of Allergy and Clinical Immunology* 131 (5) (May): 1367–1375.e9. doi:10.1016/j.jaci.2013.01.053.

Jackson, Stephen P, and Jiri Bartek. 2009. “The DNA-Damage Response in Human Biology and Disease.” *Nature* 461 (7267) (October 22): 1071–1078. doi:10.1038/nature08467.

Jenkins, Stephen J, Dominik Ruckerl, Peter C Cook, Lucy H Jones, Fred D Finkelman, Nico van Rooijen, Andrew S MacDonald, and Judith E Allen. 2011. “Local Macrophage Proliferation, rather than Recruitment from the Blood, Is a Signature of TH2 Inflammation.” *Science (New York, N.Y.)* 332 (6035) (June 10): 1284–1288. doi:10.1126/science.1204351.

Khansari, Nemat, Yadollah Shakiba, and Mahdi Mahmoudi. 2009. “Chronic Inflammation and Oxidative Stress as a Major Cause of Age-Related Diseases and Cancer.” *Recent Patents on Inflammation & Allergy Drug Discovery* 3 (1) (January): 73–80.

Papamichos-Chronakis, Manolis, and Craig L. Peterson. 2013. “Chromatin and the Genome Integrity Network.” *Nature Reviews Genetics* 14 (1): 62–75. doi:10.1038/nrg3345.

Pascual-García, Mónica, José M Carbó, Theresa León, Jonathan Matalonga, Ruud Out, Theo Van Berkel, Maria-Rosa Sarrias, Francisco Lozano, Antonio Celada, and Annabel F Valledor. 2011. “Liver X Receptors Inhibit Macrophage Proliferation through Downregulation of Cyclins D1 and B1 and Cyclin-Dependent Kinases 2 and 4.” *Journal of Immunology (Baltimore, Md.: 1950)* 186 (8) (April 15): 4656–4667. doi:10.4049/jimmunol.1000585.

Piątosa, Barbara, Mirjam van der Burg, Katarzyna Siewiera, Małgorzata Pac, Jacques J M van Dongen, Anton W Langerak, Krystyna H Chrzanowska, and Ewa Bernatowska. 2012. “The Defect in Humoral Immunity in Patients with Nijmegen Breakage Syndrome Is Explained by Defects in Peripheral B Lymphocyte Maturation.” *Cytometry. Part A: The Journal of the*

International Society for Analytical Cytology 81 (10) (October): 835–842. doi:10.1002/cyto.a.22108.

Smith, Johanna A, Feng-Xiang Wang, Hui Zhang, Kou-Juey Wu, Kevin Jon Williams, and René Daniel. 2008. “Evidence That the Nijmegen Breakage Syndrome Protein, an Early Sensor of Double-Strand DNA Breaks (DSB), Is Involved in HIV-1 Post-Integration Repair by Recruiting the Ataxia Telangiectasia-Mutated Kinase in a Process Similar To, but Distinct From, Cellular DSB Repair.” *Virology Journal* 5: 11. doi:10.1186/1743-422X-5-11.

Stewart, G S, R S Maser, T Stankovic, D A Bressan, M I Kaplan, N G Jaspers, A Raams, P J Byrd, J H Petrini, and A M Taylor. 1999. “The DNA Double-Strand Break Repair Gene hMRE11 Is Mutated in Individuals with an Ataxia-Telangiectasia-like Disorder.” *Cell* 99 (6) (December 10): 577–587.

Stracker, T.H., Morales, M., Couto, S.S., Hussein, H., and Petrini, J.H.J. (2007). The carboxy terminus of NBS1 is required for induction of apoptosis by the MRE11 complex. Nature 447, 218–221.

Stracker, Travis H, and John H J Petrini. 2008. “Working Together and Apart: The Twisted Relationship of the Mre11 Complex and Chk2 in Apoptosis and Tumor Suppression.” *Cell Cycle (Georgetown, Tex.)* 7 (23) (December): 3618–3621.

Stracker, Travis H, and John H J Petrini. 2011. “The MRE11 Complex: Starting from the Ends.” *Nature Reviews. Molecular Cell Biology* 12 (2) (February): 90–103. doi:10.1038/nrm3047.

Stracker, Travis H, Ignasi Roig, Philip A Knobel, and Marko Marjanović. 2013. “The ATM Signaling Network in Development and Disease.” *Frontiers in Genetics* 4: 37. doi:10.3389/fgene.2013.00037.

Van der Burg, Mirjam, Malgorzata Pac, Magdalena A Berkowska, Bozenna Goryluk-Kozakiewicz, Anna Wakulinska, Bozenna Dembowska-Baginska, Hanna Gregorek, et al. 2010. “Loss of Juxtaposition of RAG-Induced Immunoglobulin DNA Ends Is Implicated in the Precursor B-Cell Differentiation Defect in NBS Patients.” *Blood* 115 (23) (June 10): 4770–4777. doi:10.1182/blood-2009-10-250514.

Varon, R, E Seemanova, K Chrzanowska, O Hnateyko, D Piekutowska-Abramczuk, M Krajewska-Walasek, J Sykut-Cegielska, K Sperling, and A Reis. 2000. “Clinical Ascertainment of Nijmegen Breakage Syndrome (NBS) and Prevalence of the Major Mutation, 657del5, in Three Slav Populations.” *European Journal of Human Genetics: EJHG* 8 (11) (November): 900–902. doi:10.1038/sj.ejhg.5200554.

Waltes, Regina, Reinhard Kalb, Magtouf Gatei, Amanda W Kijas, Markus Stumm, Alexandra Sobeck, Britta Wieland, et al. 2009. “Human RAD50 Deficiency in a Nijmegen Breakage Syndrome-like Disorder.” *American Journal of Human Genetics* 84 (5) (May): 605–616. doi:10.1016/j.ajhg.2009.04.010.

Weemaes, C M, T W Hustinx, J M Scheres, P J van Munster, J A Bakkeren, and R D Taalman. 1981. “A New Chromosomal Instability Disorder: The Nijmegen Breakage Syndrome.” *Acta Paediatrica Scandinavica* 70 (4) (July): 557–564.

Wilda, M, I Demuth, P Concannon, K Sperling, and H Hameister. 2000. “Expression Pattern of the Nijmegen Breakage Syndrome Gene, Nbs1, during Murine Development.” *Human Molecular Genetics* 9 (12) (July 22): 1739–1744.

Williams, Bret R., Olga K. Mirzoeva, William F. Morgan, Junyu Lin, Wesley Dunnick, and John H. J. Petrini. 2002. “A Murine Model of Nijmegen Breakage Syndrome.” *Current Biology* 12 (8) (April 16): 648–653. doi:10.1016/S0960-9822(02)00763-7.

Xaus, J, M Cardó, A F Valledor, C Soler, J Lloberas, and A Celada. 1999. “Interferon Gamma Induces the Expression of p21waf-1 and Arrests Macrophage Cell Cycle, Preventing Induction of Apoptosis.” *Immunity* 11 (1) (July): 103–113.

Disclosures

The authors declare that they have no financial conflicts of interest.

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Summary of Results and General Discussion

Summary of Results

The subject of the work presented, to obtain the degree of Ph.D., is about the role of two proteins (Trex1 and Nbs1) involved in the DNA metabolism. These proteins have acrucial function in the biology of macrophages during the immune response. The Thesis comprises three main parts. The first, characterises *Trex1* promoter and describes *Trex1* induction upon IFN-γ activation. The second, describes how and to what extent the lack of Trex1 in macrophages affects its function upon TLR stimulation and further explains the molecular mechanisms that could explain the development of autoimmunity in the absence of Trex1 function. Finally, the last, focuses primarily on Nbs1, assesses the importance of functional Nbs1 in macrophages and on the immune response *in vivo*.

When characterising Trex1 promoter, we revealed the presence of a single intron upstream of the open reading frame. We determined that the major transcription starting point is located −580 bp from the translation start site, generating 1.3 kb transcript. Furthermore, we demonstrated that Trex1 promoter stretches 850 base pairs from the main transcription start site and does not have TATA and CCAAT boxes. However, Inr sequence and Sp1 (GC-rich) factor motifs are found upstream of the transcription initiation point and are able to replace the function of the TATA box. Furthermore, we showed that IFN-γ induction of *Trex1* is independent of new protein synthesis. In addition, we established that *Trex1* IFN-γ induction behaves as an early gene and is unstable with a 90 minute half-life in both resting and stimulated macrophages.

Characterisation of macrophages lacking Trex1 (second part) demonstrates that when Trex1 is not present in these cells an exacerbated pro-inflammatory response is produced. Furthermore, we showed that this disequilibrium of macrophages during the immune response is marked by an increased production of pro-inflammatory cytokines, as well as by an exacerbated antigen presentation to T-cells and inability to

properly clear existing apoptotic cells. These *in vitro* findings were consistent with our *in vivo* observations of *Trex1*$^{-/-}$ mice generalised inflammation.

In the final part, we demonstrated for the first time, that upon stimulation with pro-inflammatory stimuli (IFN-γ and LPS) and M-CSF, Nbs1 is up-regulated in macrophages at protein level. This occurs at post-transcriptional level. Furthermore, macrophages that express a truncated form of Nbs1 (Williams et al. 2002) have an impaired differentiation that is accompanied with a reduced proliferation capacity but not an increase in apoptosis. These results *in vitro* are associated with *in vivo* findings. We find reduced numbers of monocytes and macrophages in older mice. In addition, we showed that Nbs1$^{\Delta B/\Delta B}$ macrophages present an increase in DNA damage markers and a higher expression of CD80 (pro-inflammatory macrophages marker). *In vivo,* Nbs1$^{\Delta B/\Delta B}$ mice have an unbalanced immune response that tends to biased pro-inflammatory phenotype with a substantial decrease of the expression of mannose receptor (anti-inflammatory marker) and an exacerbated ear thickness upon DNFB treatment.

General Discussion

Macrophages play a key role in the immune response. When these cells are activated by pro-inflammatory stimuli such as IFN-γ or LPS they develop a large battery of different instruments to destroy “non-self” particles. Indeed, part of the molecules produced by macrophages are able to induce DNA damage (Bauer et al., 2011; Koch et al., 2012). By doing that, macrophages are the most exposed, in the body to dangerous molecules that damage him-self (Lloberas and Celada, 2009; Valledor et al., 2010). Therefore, it seems logical that macrophages possess different mechanisms in order to be protected from destruction and survive in the inflammatory loci. $p21^{waf-1}$, for example, is induced by IFN-γ and protects macrophages from apoptosis (Xaus et al., 1999b).

Trex1 is a exonuclease that was first identified in mammal cells, and due to its role in DNA metabolism, was initially suggested to play a role in DNA damage repair and cancer (Höss et al., 1999; Mazur and Perrino, 1999). Subsequently, it was discovered, unexpectedly, that *Trex1*$^{-/-}$ mice which, were generated a few years later, did not present an increased cancer incidence. Instead, they presented an inflammatory myocarditis that lead to their premature death (Morita et al., 2004). Furthermore, *TREX1* mutations were later associated to a number of diseases that presented deregulation of the immune system, such as SLE and presented neurological defects in AGS (Chahwan and Chahwan, 2012).

Nbs1 is a 95kDa protein that was first identified because to it its interaction with Mre11 and Rad50 (MRE11 complex) (Carney et al., 1998). This complex is important for DSB detection and repair and has been conserved across the species. The genomic region encoding Nbs1 was described to be mutated in NBS patients (Saar et al., 1997). These patients are characterised with immunodeficiency, neural defects and increased cancer incidence (van der Burgt et al., 1996).

Given the above, in this Thesis, we demonstrate that both Trex1 and Nbs1, which together have a role in DNA metabolism, are crucial for the functioning of

macrophages. Our findings are in agreement with the immune defects present in humans with mutations in the genes encoding for Trex1 and Nbs1.

First, in the characterisation of Trex1 induction by IFN-γ we showed that *Trex1* up-regulation is dependent not only on STAT1, but also on the binding of c-Jun to AP-1 sequence in its promoter. Previous work by others supports these findings as the induction of Trex1 upon genotoxic stimuli was dependent also on c-Jun and c-Fos. It is possible that Trex1 up-regulation requires the coordination of different transcriptions factors (Christmann et al., 2010). Indeed, AP-1 transcription factors have been proven to be induced both after cytokine exposure and following genotoxic stress. Furthermore, our data, which demonstrates that Trex1 possesses a short-lived mRNA proves that Trex1 expression is tightly regulated. Notably, in the immune system it is frequent to encounter highly unstable mRNAs that are rapidly up-regulated but that are also quickly degraded. These features, induction and fast down-regulation of mRNA, are crucial for the phenotype change of macrophages and of other immune cells. For example, many cytokines, such as TNFα, are known to play a decisive role for pathogen removal, but can cause damage and chronic inflammation, if not down-regulated and thus they have their gene expression tightly controlled (Schott and Stoecklin, 2010). Furthermore, Trex1 mutations are associated with RVCL, where a deletion in the transmembrane domain produces Trex1 catalytic activity in an inappropriate cellular location inducing necrosis in small blood vessels (Richards et al., 2007). In brief, this suggests that an exacerbated activity of Trex1 can be harmful and toxic to the cells, and that tightly regulated expression mechanisms are necessary to maintain Trex1 expression and location within the cell under control.

Furthermore, in this Thesis it is also demonstrated that Trex1 is up-regulated by other pro-inflammatory stimuli, besides IFN-γ. The analysis of Trex1 promoter demonstrated that there are many different transcription factor binding sites present in this promoter. The presence, for example, of NF-kB binding site and ISRE allowed us to presume that the up-regulation of Trex1 expression in response to molecules such as IFN-α, LPS (TLR4 ligand), R848 (TLR7 and TLR8 ligand) and CpGB (TLR9 ligand) may be mediated through these transcription factors.

The findings from the third part of the results demonstrate that not only Trex1 but also Nbs1 is up-regulated in macrophages upon pro-inflammatory stimuli. In addition, our results show that Trex1 and Nbs1 are not induced by the anti-inflammatory cytokines,

such as IL-4. In this sense, the specific up-regulation of Trex1 and Nbs1 upon IFN-γ and LPS stimuli indicates that these proteins are crucial for the pro-inflammatory response of macrophages.

As referred to in the introduction section of this Thesis, macrophages are important for pathogen removal. Indeed, these innate immune cells rely on the acidification of phagolysosomes and on the production of high amounts of ROS and RNS. These highly reactive species are produced when macrophages are pro-inflammatorily activated and cause damage to pathogens. Together with pathogen damage, the surrounding environment and macrophages themselves are susceptible to damage (deRojas-Walker et al., 1995; Messmer et al., 1996). Our data corroborate the abovementioned statements, as pro-inflammatory stimuli in macrophages induce Nbs1 expression that, in turn, is crucial for the DNA damage repair and that Trex1 has been shown to be translocated to the nucleus upon DNA damage stimuli (Yang et al., 2007).

Furthermore, it is worthwhile to mention that, Trex1 is encoded in the same open reading frame as ATR interacting protein (ATRIP), i.e. a component for the repair of DNA breaks that is activated in response to replication stress and DNA damage (Figure 11). These data, taking into account the above-mentioned observations, suggest that Trex1 has a function in genotoxic stress management (Paper Cell Debora Barnis) repairing the endogenous DNA damaged.

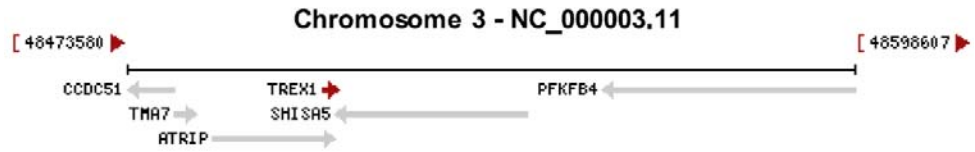

Figure 11. Position 3q21 of the human chromosomme 3. Trex1 and Atrip are closely encoded in the genome.

The detection of nucleic acids by the immune system is a cornerstone of innate immunity both for viral and bacterial pathogen removal. The presence of free DNA in the cell is believed to be sufficient to trigger the immune system through many

different intra-cellular receptors. In this regard, misguided accumulation and sensing of excessive free DNA can trigger autoimmunity (Crow and Rehwinkel, 2009; Gehrke et al., 2013).

In SLE, for example, the presence of self-DNA that is not degraded induces the production of type I IFN. There are multiple mechanisms to avoid the accumulation of DNA that can unsuitably trigger the activation of the immune system. DNase I, DNase II and Trex1 (also known as DNase III) are crucial for the degradation of undesirable DNA. Their function is shown to be compartment-specific: DNase I degrades extracellular DNA, while DNase II acts in the lysosome, and finally, Trex1 has been shown to have its main DNA degrading role in the cytoplasm. Both DNase I and DNase II are important in the degradation of apoptotic and necrotic DNA. Trex1, on the other hand, has been shown to degrade DNA from endogenous and exogenous retroviruses (Stetson et al., 2008; Yang et al., 2007). It is believed that it can degrade excessive DNA from genomic replication and DNA damage repair. The importance of these DNases in immune responses is demonstrated by the knock-out mice of these genes. DNase I knock-out mice have an SLE like phenotype, DNase II-deficient mice are lethal and Trex1$^{-/-}$ has a generalised inflammation that expands to all tissues (Atianand and Fitzgerald, 2013). In addition, *Trex1*$^{-/-}$ mice present a short half-life of up to 20 weeks.

Moreover, this Thesis demonstrates that Trex1 has specialised roles in macrophages biology. The absence of this exonuclease induced an exacerbated production of pro-inflammatory cytokines upon TLR stimulation. Macrophages from *Trex1*$^{-/-}$ mice were unable to properly clear apoptotic cells and showed an increased T-cell presentation (Figure 12). Interestingly, the bihaviour of *Trex1*$^{-/-}$ macrophages resembles SLE macrophages (Byrne et al., 2012). Furthermore, our results show that the stimulation of both TLR7 and TLR9, (mutations in these genes are also associated to SLE) increased expression of Trex1 in macrophages. Collectively, these observations provide evidences on the possible role of Trex1 function in both pro-inflammatory response and in SLE.

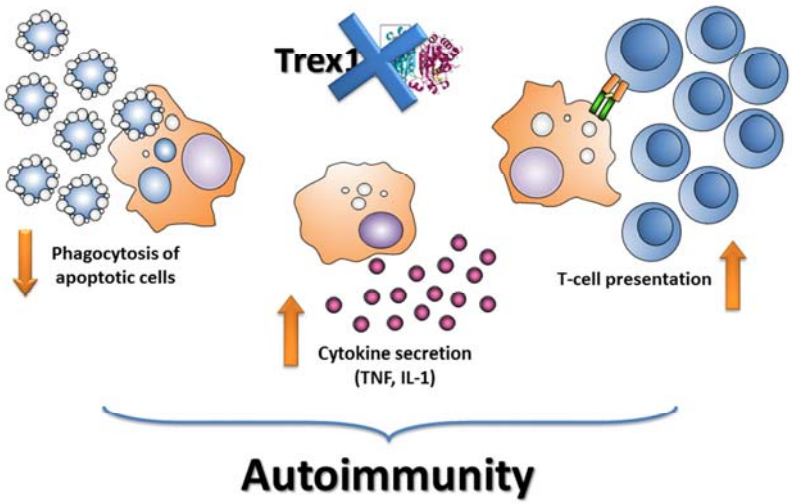

Figure 12. Loss of Trex1 function affects macrophages and induces autoimmunity. Macrophages that lack Trex1 have reduced apoptotic clearance ability, increase the pro-inflammatory cytokine expression and present more antigens to T-cells. All these modifications in macrophages enhance autoimmunity.

The analysis of Nbs1$^{\Delta B/\Delta B}$ macrophages demonstrated expected defects in differentiation and in proliferation. These observations correlated with the induction of expression of Nbs1 upon stimulation of macrophages by M-CSF. This growth factor is necessary for macrophages differentiation proliferation and survival, if not stimulated, macrophages undergo apoptosis (Xaus et al., 2001). Recently, it has been reported that the LPS treatment in mice can induce M-CSF expression which targets the bone marrow and helps hematopoietic stem cells chose their differentiation pathway towards the macrophages lineage, through modulation of PU.1 expression (Kueh et al., 2013).

Furthermore, Nbs1$^{\Delta B/\Delta B}$ macrophages showed an increased expression of CD80, which is a co-stimulatory molecule for T-cell antigen presentation and a marker of pro-inflammation. This observation was consistent with the skewed pro-inflammatory state of DNFB treated ears in Nbs1$^{\Delta B/\Delta B}$ mice (Figure 13). In relation to this, irradiation of macrophages has been demonstrated to alter macrophages' gene expression towards

a pro-inflammatory phenotype. This distortion could be plausibly attributed to the increase of DNA damage upon irradiation. Our findings of increased γH2AX expression (DNA damage marker) in Nbs1$^{\Delta B/\Delta B}$ macrophages demonstrate that for its pro-inflammatory response DNA damage regulation is very important.

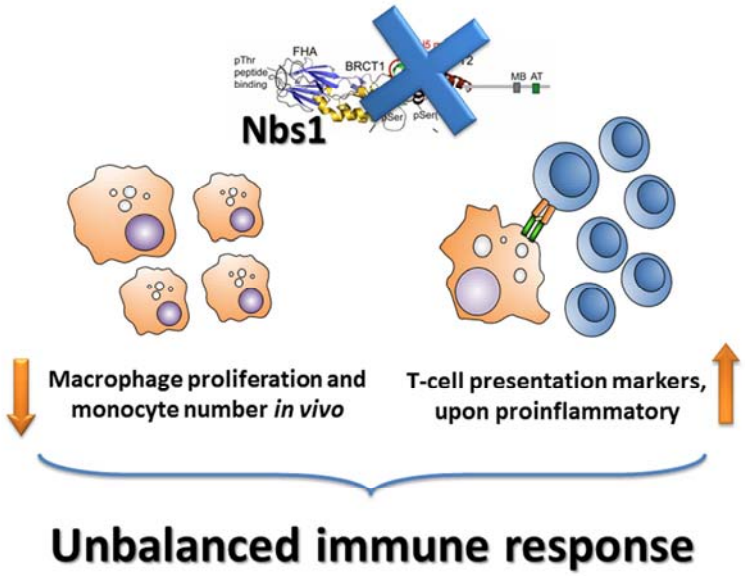

Figure 13. Nbs1 loss of function in macrophages deregulates them and originates an unbalanced immune system response.

Nbs1$^{\Delta B/\Delta B}$ mice presented a reduced monocyte population in blood of older mice. In this regard, we propose that the lack of Nbs1 impairs the repair of natural occurring DNA damage in the precursors of monocytes and thus lead to impaired proliferation and differentiation of these cells. Hematopoietic stem cells need to be preserved from damage to ensure continuous production of blood cells. Irregular amounts of ROS induce DNA damage in stem cells and trigger the response to repair (Ito et al., 2004; Yahata et al., 2011). If the damage is not repaired, it tends to be accumulated and impair cell renewal. Furthermore, it has been shown that monocytes have impaired DSB repair when compared to more differentiated cells such as macrophages and dendritic cells (Bauer et al., 2011).

DNA damage response is a complex system that comprises distinct molecules that sense and act upon the harmed DNA sequence. Irradiation (which generates DNA

damage) of macrophages has been proven to induce up-regulation of many antiviral interferon stimulated genes. In addition, the increase of these genes is dependent on ATM and that under these conditions, the infection of these macrophages by viruses is repressed (Mboko et al., 2012). DSB, can induce the expression of IFN in a NF-kB dependent manner and thus produce an immune response (Brzostek-Racine et al., 2011). Also, oxidation (from UV exposure) of cytoplasmic DNA through detection by cGAS and STING can increase the level of immune response (Gehrke et al., 2013). Even more, DNA damage sensor MRE11 complex recognizes cytosolic double-strand DNA and induces type I interferon by regulating STING trafficking (Kondo et al., 2013). It is also known that viruses, such as HIV, have evolved to highjack the cell and activate certain proteins of the DNA damage response to ease their integration in the hosts' genome and increase their replication (Koyama et al., 2013). These recent observations demonstrate how DNA damage and the immune system crosstalk and how proteins that were previously believed to have a unique role in DNA damage have also crucial functions in regulating the immune response.

Trex1 and Nbs1 have relevant DNA metabolism functions, and their mutations are associated to diseases with partial phenotype resemblance. How these mutations affect the function of different cells, including the macrophages is worth considering, as it can facilitate further generation of knowledge and eventually lead to the cures of specific diseases.

It is important to mention and discuss that both mutations in *NBS1* and *TREX1* in humans originate neurological defects besides immune failure as immunodeficiency and autoimmunity. It has been previously discussed that not only macrophages but also neurons have an increase of ROS production due to their high mitochondrial activity (Jackson and Bartek, 2009). Neurons are unable to proliferate and thus rest the majority of the time in the G0 phase of the cell cycle. These two characteristics of neurons increase their rates of DNA damage and decrease their ability to properly repair DNA damage. In the neurological context it was also proven that certain brain cells, such as astrocytes, are susceptible to type I IFN stimulation (Cuadrado et al., 2013) (that is produce upon accumulation of ssDNA in the cytoplasm). These evidences clarify the related role of Trex1 and Nbs1 in both DNA damage and cellular immune surveillance.

DNA replication and transcription produces ssDNA as a by-product. Indeed, at any given time one to two percent of the genomic DNA is present in this form (Bjursell et al., 1979). DNA resection during DSB repair leads to the creation of ssDNA in a Mre11-dependent manner. This ssDNA has been shown to activate ATM and subsequently activate DNA repair mechanism (Jazayeri et al., 2008). In addition, ssDNA can also be detected and activate a huge assortment of different free DNA detectors that lead to activate the immune system. In this sense, we believe that our findings support and extend the proposed model (Figure 14). The malfunction of both, Trex1 or Nbs1, can lead to the accumulation of ssDNA in the cell that triggers a signaling of alarm in the cell. On the other hand, the results presented in this thesis support evidences that the pro-inflammatory activation of macrophages induces DNA damage in these cells. This occurs either by an increased transcription, gene regulation or ROS production. In this context, Nbs1 and Trex1 are cellular modulators of the pro-inflammatory activation of macrophages. All these data suggest that the study of macrophages in autoimmune diseases and related NBS immune-deficiencies should not be neglected.

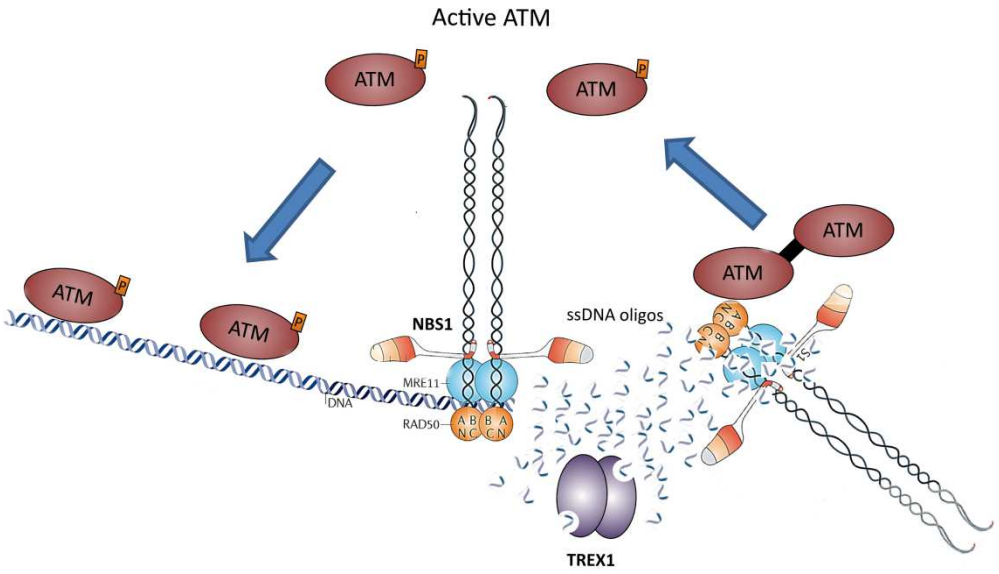

Figure 14. Proposed model of ssDNA generated from MRE11 complex when repairing DSB. This ssDNAis degraded by Trex1 restraining ATM activation (adapted from Jazayeri et al., 2008 and Stracker and Petrini, 2011).

Finally, we can conclude that Trex1 and Nbs1 are cornerstone proteins of macrophages pro-inflammatory function. Although the roles of these proteins in

macrophages are different, in essence, they both are similar because they are key elements for DNA metabolism. This further suggests that the tight regulation of the amounts of free DNA in macrophages is important in the pro-inflammatory but not in anti-inflammatory response. An excess of DNA damage can induce a dysfunction of macrophages, and both, Nbs1 and Trex1, are indispensable for this DNA repair.

Conclusions

1) Trex1 and Nbs1 are induced in macrophages upon pro-inflammatory stimuli.

2) Nbs1 is critical for macrophages differentiation and proliferation.

3) The absence of Trex1 or Nbs1 in macrophages leads to an exacerbated pro-inflammatory response similar to autoimmune disease.

Summary in Spanish

Trex1 y Nbs1 como Reguladores de la Respuesta Inflamatoria del Macrófago

Trex1 y Nbs1 como reguladores de la respuesta inflamatoria del macrófago

Introducción

Los monocitos y los macrófagos son células esenciales tanto en su papel efector como regulador del sistema inmunitario. Normalmente los monocitos circulantes en la sangre son los predecesores de los macrófagos tisulares, salvo en algunos casos donde los macrófagos se renuevan localmente como en el cerebro, la dermis y el bazo (Geissmann et al., 2010). Los macrófagos pertenecen al linaje mieloide del sistema inmunitario y se clasifican como efectores dentro de la respuesta inmunitaria innata; son responsables de muchas funciones tal como se ilustra en la Figura 1. Las funciones de los macrófagos están estrechamente relacionadas con su entorno, y su grado de diferenciación tisular depende en gran medida de las células residentes del tejido (Davies et al, 2013; Shi y Pamer, 2011). En cualquiera de estas funciones, tanto la imposibilidad de una correcta diferenciación como la inadecuada funcionalidad de estas células suponen un desequilibrio en la respuesta inmunitaria y en casos extremos el desarrollo de una patología. Entre estas funciones se debe destacar, por su importancia, el inicio y la resolución de la inflamación. Para llevar a cabo estas funciones, los macrófagos realizan un proceso de vigilancia inmunológica continuo para detectar en su entorno señales de daño o infección. Además de la eliminación de patógenos, los macrófagos también participan en la eliminación de partículas de polvo y de alérgenos en los pulmones (macrófagos alveolares), eliminan toxinas en el hígado (células de Kupffer), fagocitan eritrocitos senescentes del torrente sanguíneo en el bazo (macrófagos del bazo) y favorecen la tolerancia en el intestino (macrófagos residentes en el intestino) (Murray y Wynn, 2011).

TREX1 es la 3'-5' exonucleasa homodimerica más abundante presente en las células de mamíferos y presenta una actividad exonucleolítica específica para ssDNA (Mazur and Perrino, 1999). La deficiencia de TREX1 se ha confirmado que está relacionada con el desarrollo de enfermedades autoinmunes tales como el lupus eritematoso sistémico (LES), así como con el Síndrome de Aicardi Goutieres (AGS) (Crow and Rehwinkel, 2009). Por lo tanto los trabajos sobre TREX1 y en particular de sus mutaciones y deficiencias, son de primordial importancia debido a su influencia sobre estas patologías. De hecho, la presencia de mutaciones en TREX1 en humanos tiene la mayor correlación genética con el desarrollo de LES (Namjou et al., 2011). Tanto el LES como AGS, son dos enfermedades que se caracterizan por la producción crónica de interferón alfa (Crow and Rehwinkel, 2009; Fairhurst et al., 2008).

El Síndrome de roturas de Nijmegen (NBS) es una rara enfermedad autosómica recesiva causada por mutaciones en el gen NBS1(Antoccia et al., 2006). NBS1 es un miembro del complejo MRN, que está formado por las proteínas Mre11, Rad50 y Nbs1, y tiene un papel fundamental en la reparación del daño en el ADN y en la señalización (Stracker and Petrini, 2011). Los individuos que carecen de NBS1 se caracterizan por padecer microcefalia, retraso en el crecimiento, aumento de la tasa de tumores y también padecer inmunodeficiencia (Chrzanowska et al., 2012). Dado que los macrófagos juegan un papel crucial en la inmunidad tanto innata como adaptativa, sus perfiles de funcionalidad pueden ser de dos tipos: proinflamatorios (activación clásica), por ejemplo cuando se activan para combatir infecciones y agentes patógenos, y de fenotipo reparador (activación alternativa) cuando han de reparar el daño causado a los tejidos circundantes, ayudar a restablecer los capilares dañados (angiogénesis) y favorecer la cicatrización de los tejidos. Nuestra hipótesis apunta a que el papel de los macrófagos en la inmunodeficiencia de los pacientes que padecen el NBS se debe en parte a una disminución de la capacidad de estas células para eliminar o reparar el ADN defectuoso inducido por señales de estrés en el ADN cuando la proteína NBS1 no es funcional. En este trabajo estamos interesados en el papel de Nbs1 en los macrófagos durante la inflamación y en el Síndrome de roturas de Nijmegen, utilizando un modelo de ratón $Nbs1^{\Delta B/\Delta B}$ que carece del dominio

ForkHead-Associated (FHA) y de uno de los dos dominios BRCT (Breast Cancer Gene 1) del extremo N-terminal de esta proteína (Williams et al., 2002).

Hipótesis

El papel de las proteínas de reparación del ADN es fundamental para la activación pro-inflamatoria de los macrófagos.

Objetivos

1) Estudiar el papel de TREX1 en los macrófagos en la enfermedad autoinmune y la inflamación.

2) Comprender la función de Nbs1 en la respuesta normal de los macrófagos.

Resultados

Esta Tesis Doctoral trata del estudio del papel de las proteínas Trex1 y Nbs1 en los macrófagos, tanto en el mantenimiento de la integridad del ADN y en como condicionan la función de estas células que tienen un papel fundamental en la respuesta inmunitaria. La tesis está estructurada en tres partes principales. La primera parte trata de la caracterización del promotor que regula la expresión de Trex1 y describe su funcionalidad tras la inducción por IFN-γ. La segunda parte describe cómo y en qué medida la falta de Trex1 en macrófagos afecta a su función tras la estimulación de los receptores TLR y además trata de explicar los mecanismos moleculares que pueden estar involucrados en la inducción de la autoinmunidad al carecer de la función de Trex1. La última parte, se centra principalmente en Nbs1, y evalúa la importancia de la proteína Nbs1 funcional en la actividad de los macrófagos y en la respuesta inmunitaria en un modelo *in vivo*.

La caracterización de promotor Trex1, reveló la presencia de un solo intrón en dirección 5' respecto al marco de lectura abierto de Trex1. Se determinó que el principal punto de inicio de la transcripción se encuentra -580 pb del lugar de inicio de la traducción, generándose un transcrito de 1,3 kb. Además, hemos demostrado que el promotor de Trex1 se extiende hasta 850 pares de bases a 5' del principal sitio de inicio de la transcripción y no tiene cajas TATA y CCAAT. Sin embargo, se han hallado secuencias de Inr y Sp1 (ricas en GC) que se encuentran a 5' del punto de inicio de la transcripción y que son capaces de reemplazar la función de la caja TATA. Por otra parte, hemos demostrado que la inducción por IFN-γ de Trex1 es independiente de la síntesis de nuevas proteínas. Además, hemos establecido que la inducción de Trex1 por IFN-γ tiene un perfil de expresión de un gen temprano y su ARNm es inestable, con una vida media de 90 minutos tanto en macrófagos quiescentes como estimulados.

La caracterización de los macrófagos que carecen de Trex1 (segunda parte) demuestra que cuando Trex1 no está presente en estas células se produce una respuesta pro-inflamatoria exacerbada. Además, se demuestra que este desequilibrio en la respuesta inmunitaria de los macrófagos se caracteriza por un aumento de la producción de citocinas pro-inflamatorias, así como por una presentación de antígenos exacerbada a los linfocitos T y en la incapacidad de eliminación de cuerpos apoptóticos de una forma adecuada. Estos resultados *in vitro* están en consonancia con nuestras observaciones *in vivo* en los ratones *Trex1*$^{-/-}$ donde se aprecia una inflamación generalizada.

En la parte final de nuestros resultados, hemos demostrado por primera vez que la expresión de Nbs1 en los macrófagos estimulados con IFN-γ y LPS (pro-inflamatorios) y con M-CSF se realiza a nivel de la proteína, sin que se observen variaciones en la tasa de transcripción. Por otra parte, al caracterizar los macrófagos que expresan una proteína truncada de Nbs1 no funcional (Williams et al. 2002), encontramos que estos macrófagos tienen alterado el proceso de diferenciación acompañado por una reducida capacidad de proliferación sin que se incremente su tasa de apoptosis. Estos resultados obtenidos *in vitro* se correlacionan con los

resultados que hemos obtenido *in vivo*, donde hemos encontrado una reducción en el número de monocitos y macrófagos en ratones de mediana edad (28 meses).

Además, hemos demostrado que los macrófagos obtenidos de ratones $Nbs1^{\Delta B/\Delta B}$ presentan un aumento de los marcadores de daño en el ADN y una mayor expresión de CD80 (marcador de macrófagos pro-inflamatorio), *in vitro*. Además, hemos identificado que, *in vivo*, ratones $Nbs1^{\Delta B/\Delta B}$ tienen una respuesta inmunitaria desequilibrada que está sesgada hacia el fenotipo pro-inflamatorio con una disminución substancial de la expresión del receptor de manosa (marcador anti-inflamatorio). Para corroborar esta tendencia hemos aplicado el modelo de irritación en oreja con DFNB, que induce una inflamación aguda local, al ratón $Nbs1^{\Delta B/\Delta B}$ y el resultado ha sido una respuesta inflamatoria exacerbada en comparación con el control WT.

Discusión

Los pacientes con deficiencia de Trex1 y en los correspondientes modelos murinos se caracteriza por la inducción de una inflamación sistémica que viene asociada con los fenómenos de autoinmunidad (Crow and Rehwinkel, 2009). En los trabajos que presento demostramos que Trex1 juega un importante papel regulador en los macrófagos activados. El fenotipo más pro-inflamatorio de los macrófagos *Trex1*$^{-/-}$ activados y el asociado al incremento de la presentación de antígeno a linfocitos T frente a macrófagos WT, indica que los macrófagos necesitan Trex1 para regular su activación. Además, demostramos que los macrófagos *Trex1*$^{-/-}$ exhiben un defecto en la fagocitosis de las células apoptóticas, y por lo tanto se ve afectado un mecanismo esencial para la resolución de la inflamación.

Estos datos demuestran que TREX1 juega un papel clave en las funciones que llevan a cabo los macrófagos en los procesos inflamatorios. Estas células son "scavengers" para las células apoptóticas y, en ausencia de Trex1, el ADN sin procesar podría inducir, de una manera dependiente o independiente de STING, la producción de

citocinas y la exacerbación del sistema inmunitario que subyace en el proceso inflamatorio. Nuestros datos sugieren que esta vía puede desempeñar un papel relevante en el desarrollo de enfermedades autoinmunes.

Los pacientes con el NBS se caracterizan por un aumento de la incidencia de cáncer, inmunodeficiencia y microcefalia y esto está relacionado con mutaciones en el gen Nbs1. Aquí caracterizamos el fenotipo de los macrófagos que tienen una proteína Nbs1 truncada que carece de los dominios FHA y BRCT. Nuestros resultados indican que los macrófagos de los ratones $Nbs1^{\Delta B/\Delta B}$ tienen una capacidad reducida para proliferar tanto *in vitro* como *in vivo*. Como consecuencia de la reducida cantidad de monocitos y macrófagos circulantes en ratones $Nbs1^{\Delta B/\Delta B}$ se inducen respuestas inmunitarias desreguladas. Nuestros resultados dan una nueva visión al papel de NBS1 en los macrófagos y la importancia potencial de los macrófagos en la inmunodeficiencia asociada al NBS.

La replicación del ADN en la fase S del ciclo celular y la transcripción génica produce ADNss como un subproducto. De hecho, en todo momento entre el uno y el dos por ciento del ADN está presente en esta forma (Bjursell et al., 1979). Además, la resección del ADN durante la reparación de las roturas de doble cadena (DSB) conduce a la creación de ADNss dependiente de la acción del complejo MRE. Este ADNss se ha demostrado que es capaz de activar ATM y posteriormente activar el mecanismo de reparación del ADN (Jazayeri et al., 2008). Además, la presencia de ADNss también puede ser detectada por la enorme variedad de receptores de ADN citoplasmáticos (p.e. NLRP's) y activarlos induciendo señales de stress en la célula dañada que a su vez activará al sistema inmunitario. En este sentido creemos que estos hallazgos apoyan y amplían el anterior modelo propuesto (Jazayeri et al., 2008), donde el funcionamiento incorrecto tanto TREX1 como de Nbs1, puede conducir a la acumulación de ADNss en la célula y que est fenómeno provoque una señalización de alarma en la célula. Por otro lado los resultados presentados en esta tesis también apoyan evidencias de que la activación de perfil pro-inflamatorio de los macrófagos induce daño en el ADN en estas células, ya sea por el aumento de la transcripción, la regulación de genes o por la producción de ROS, y que Nbs1 y TREX1 son

moduladores celulares de la activación pro-inflamatoria de los macrófagos. Además, en esta tesis sugerimos que el estudio del papel de los macrófagos en la enfermedad autoinmune y en la immunodefiencia presente en el NBS han de ser tenidas en cuenta.

Tomando en consideración lo anteriormente expuesto podemos concluir que Trex1 y Nbs1 son proteínas fundamentales de la función pro-inflamatoria de los macrófagos. Aunque las funciones de estas proteínas en los macrófagos son diferentes, en esencia, ambas están relacionadas ya que son indispensables para el metabolismo del ADN. Esto sugiere, además, que la regulación estricta de las cantidades de ADN libre en los macrófagos es importante en la respuesta pro-inflamatoria pero no en la respuesta anti-inflamatoria, por lo tanto ambas proteínas son indispensables para la regulación de la respuesta pro-inflamatoria.

Conclusiones

1) Los estímulos pro-inflamatorios inducen Trex1 y Nbs1 en los macrófagos.
2) Nbs1 es necesario para la diferenciación y proliferación de los macrófagos.
3) La ausencia de Trex1 o Nbs1 en los macrófagos conduce a una respuesta pro-inflamatoria exacerbada similar a la que se presenta en las enfermedades autoinmunes.

Bibliography

Aderem, A., and Underhill, D.M. (1999). Mechanisms of phagocytosis in macrophages. Annu. Rev. Immunol. *17*, 593–623.

Allgayer, J., Kitsera, N., Lippen, C. von der, Epe, B., and Khobta, A. (2013). Modulation of base excision repair of 8-oxoguanine by the nucleotide sequence. Nucleic Acids Res. *41*, 8559–8571.

Antoccia, A., Kobayashi, J., Tauchi, H., Matsuura, S., and Komatsu, K. (2006). Nijmegen breakage syndrome and functions of the responsible protein, NBS1. Genome Dyn. *1*, 191–205.

Atianand, M.K., and Fitzgerald, K.A. (2013). Molecular basis of DNA recognition in the immune system. J. Immunol. Baltim. Md 1950 *190*, 1911–1918.

Auffray, C., Sieweke, M.H., and Geissmann, F. (2009). Blood monocytes: development, heterogeneity, and relationship with dendritic cells. Annu. Rev. Immunol. *27*, 669–692.

Bach, E.A., Aguet, M., and Schreiber, R.D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. Annu. Rev. Immunol. *15*, 563–591.

Bauer, M., Goldstein, M., Christmann, M., Becker, H., Heylmann, D., and Kaina, B. (2011). Human monocytes are severely impaired in base and DNA double-strand break repair that renders them vulnerable to oxidative stress. Proc. Natl. Acad. Sci. U. S. A. *108*, 21105–21110.

Biswas, S.K., and Mantovani, A. (2010). Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat. Immunol. *11*, 889–896.

Bjursell, G., Gussander, E., and Lindahl, T. (1979). Long regions of single-stranded DNA in human cells. Nature *280*, 420–423.

Bohgaki, T., Bohgaki, M., and Hakem, R. (2010). DNA double-strand break signaling and human disorders. Genome Integr. *1*, 15.

Borden, E.C., Sen, G.C., Uze, G., Silverman, R.H., Ransohoff, R.M., Foster, G.R., and Stark, G.R. (2007). Interferons at age 50: past, current and future impact on biomedicine. Nat. Rev. Drug Discov. *6*, 975–990.

Boulton, S.J., and Jackson, S.P. (1996). Saccharomyces cerevisiae Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. EMBO J. *15*, 5093–5103.

Brucet, M., Marqués, L., Sebastián, C., Lloberas, J., and Celada, A. (2004). Regulation of murine Tap1 and Lmp2 genes in macrophages by interferon gamma is mediated by STAT1 and IRF-1. Genes Immun. *5*, 26–35.

Brucet, M., Querol-Audí, J., Serra, M., Ramirez-Espain, X., Bertlik, K., Ruiz, L., Lloberas, J., Macias, M.J., Fita, I., and Celada, A. (2007). Structure of the dimeric exonuclease TREX1 in complex with DNA displays a proline-rich binding site for WW Domains. J. Biol. Chem. *282*, 14547–14557.

Brucet, M., Querol-Audí, J., Bertlik, K., Lloberas, J., Fita, I., and Celada, A. (2008). Structural and biochemical studies of TREX1 inhibition by metals. Identification of a new active histidine conserved in DEDDh exonucleases. Protein Sci. Publ. Protein Soc. *17*, 2059–2069.

Brzostek-Racine, S., Gordon, C., Van Scoy, S., and Reich, N.C. (2011). The DNA damage response induces IFN. J. Immunol. Baltim. Md 1950 *187*, 5336–5345.

Van der Burgt, I., Chrzanowska, K.H., Smeets, D., and Weemaes, C. (1996). Nijmegen breakage syndrome. J. Med. Genet. *33*, 153–156.

Byrne, J.C., Ní Gabhann, J., Lazzari, E., Mahony, R., Smith, S., Stacey, K., Wynne, C., and Jefferies, C.A. (2012). Genetics of SLE: functional relevance for monocytes/macrophages in disease. Clin. Dev. Immunol. *2012*, 582352.

Carney, J.P., Maser, R.S., Olivares, H., Davis, E.M., Le Beau, M., Yates, J.R., 3rd, Hays, L., Morgan, W.F., and Petrini, J.H. (1998). The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. Cell *93*, 477–486.

Celada, A., and Schreiber, R.D. (1987). Internalization and degradation of receptor-bound interferon-gamma by murine macrophages. Demonstration of receptor recycling. J. Immunol. Baltim. Md 1950 *139*, 147–153.

Celada, A., Gray, P.W., Rinderknecht, E., and Schreiber, R.D. (1984). Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. J. Exp. Med. *160*, 55–74.

Celada, A., Borràs, F.E., Soler, C., Lloberas, J., Klemsz, M., Beveren, C. van, McKercher, S., and Maki, R.A. (1996). The transcription factor PU.1 is involved in macrophage proliferation. J. Exp. Med. *184*, 61–69.

Chahwan, C., and Chahwan, R. (2012). Aicardi–Goutieres syndrome: from patients to genes and beyond. Clin. Genet. *81*, 413–420.

Chowdhury, D., Beresford, P.J., Zhu, P., Zhang, D., Sung, J.-S., Demple, B., Perrino, F.W., and Lieberman, J. (2006). The exonuclease TREX1 is in the SET complex and acts in concert with NM23-H1 to degrade DNA during granzyme A-mediated cell death. Mol. Cell *23*, 133–142.

Christmann, M., Tomicic, M.T., Aasland, D., Berdelle, N., and Kaina, B. (2010). Three prime exonuclease I (TREX1) is Fos/AP-1 regulated by genotoxic stress and protects against ultraviolet light and benzo(a)pyrene-induced DNA damage. Nucleic Acids Res. *38*, 6418–6432.

Chrzanowska, K.H., Gregorek, H., Dembowska-Bagińska, B., Kalina, M.A., and Digweed, M. (2012). Nijmegen breakage syndrome (NBS). Orphanet J. Rare Dis. *7*, 13.

Civril, F., Deimling, T., de Oliveira Mann, C.C., Ablasser, A., Moldt, M., Witte, G., Hornung, V., and Hopfner, K.-P. (2013). Structural mechanism of cytosolic DNA sensing by cGAS. Nature *498*, 332–337.

Comalada, M., Xaus, J., Sánchez, E., Valledor, A.F., and Celada, A. (2004). Macrophage colony-stimulating factor-, granulocyte-macrophage colony-stimulating factor-, or IL-3-dependent survival of macrophages, but not proliferation, requires the expression of p21(Waf1) through the phosphatidylinositol 3-kinase/Akt pathway. Eur. J. Immunol. *34*, 2257–2267.

Cooper, M.D., and Alder, M.N. (2006). The evolution of adaptive immune systems. Cell *124*, 815–822.

Coornaert, B., Carpentier, I., and Beyaert, R. (2009). A20: central gatekeeper in inflammation and immunity. J. Biol. Chem. *284*, 8217–8221.

Crow, Y.J., and Rehwinkel, J. (2009). Aicardi-Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. Hum. Mol. Genet. *18*, R130–136.

Cuadrado, E., Jansen, M.H., Anink, J., De Filippis, L., Vescovi, A.L., Watts, C., Aronica, E., Hol, E.M., and Kuijpers, T.W. (2013). Chronic exposure of astrocytes to interferon-α reveals molecular changes related to Aicardi-Goutieres syndrome. Brain J. Neurol. *136*, 245–258.

Cullell-Young, M., Barrachina, M., López-López, C., Goñalons, E., Lloberas, J., Soler, C., and Celada, A. (2001). From transcription to cell surface expression, the induction of MHC class II I-A alpha by interferon-gamma in macrophages is regulated at different levels. Immunogenetics *53*, 136–144.

Daniels, D.S., Woo, T.T., Luu, K.X., Noll, D.M., Clarke, N.D., Pegg, A.E., and Tainer, J.A. (2004). DNA binding and nucleotide flipping by the human DNA repair protein AGT. Nat. Struct. Mol. Biol. *11*, 714–720.

Davies, L.C., Jenkins, S.J., Allen, J.E., and Taylor, P.R. (2013). Tissue-resident macrophages. Nat. Immunol. *14*, 986–995.

Decottignies, A. (2013). Alternative end-joining mechanisms: a historical perspective. Front. Genet. *4*, 48.

Delacôte, F., and Lopez, B.S. (2008). Importance of the cell cycle phase for the choice of the appropriate DSB repair pathway, for genome stability maintenance: the trans-S double-strand break repair model. Cell Cycle Georget. Tex *7*, 33–38.

deRojas-Walker, T., Tamir, S., Ji, H., Wishnok, J.S., and Tannenbaum, S.R. (1995). Nitric oxide induces oxidative damage in addition to deamination in macrophage DNA. Chem. Res. Toxicol. *8*, 473–477.

Déry, U., Coulombe, Y., Rodrigue, A., Stasiak, A., Richard, S., and Masson, J.-Y. (2008). A Glycine-Arginine Domain in Control of the Human MRE11 DNA Repair Protein. Mol. Cell. Biol. *28*, 3058–3069.

Desmet, C.J., and Ishii, K.J. (2012). Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nat. Rev. Immunol. *12*, 479–491.

Fairhurst, A.-M., Mathian, A., Connolly, J.E., Wang, A., Gray, H.F., George, T.A., Boudreaux, C.D., Zhou, X.J., Li, Q.-Z., Koutouzov, S., et al. (2008). Systemic IFN-alpha drives kidney nephritis in B6.Sle123 mice. Eur. J. Immunol. *38*, 1948–1960.

Fragkos, M., Jurvansuu, J., and Beard, P. (2009). H2AX is required for cell cycle arrest via the p53/p21 pathway. Mol. Cell. Biol. *29*, 2828–2840.

Gall, A., Treuting, P., Elkon, K.B., Loo, Y.-M., Gale, M., Jr, Barber, G.N., and Stetson, D.B. (2012). Autoimmunity initiates in nonhematopoietic cells and progresses via lymphocytes in an interferon-dependent autoimmune disease. Immunity *36*, 120–131.

Gehrke, N., Mertens, C., Zillinger, T., Wenzel, J., Bald, T., Zahn, S., Tüting, T., Hartmann, G., and Barchet, W. (2013). Oxidative damage of DNA confers resistance to cytosolic nuclease TREX1 degradation and potentiates STING-dependent immune sensing. Immunity *39*, 482–495.

Geissmann, F., Manz, M.G., Jung, S., Sieweke, M.H., Merad, M., and Ley, K. (2010). Development of monocytes, macrophages and dendritic cells. Science *327*, 656–661.

Hasan, M., Koch, J., Rakheja, D., Pattnaik, A.K., Brugarolas, J., Dozmorov, I., Levine, B., Wakeland, E.K., Lee-Kirsch, M.A., and Yan, N. (2013). Trex1 regulates lysosomal biogenesis and interferon-independent activation of antiviral genes. Nat. Immunol. *14*, 61–71.

Hedrich, C.M., Fiebig, B., Hauck, F.H., Sallmann, S., Hahn, G., Pfeiffer, C., Heubner, G., Lee-Kirsch, M.A., and Gahr, M. (2008). Chilblain lupus erythematosus--a review of literature. Clin. Rheumatol. *27*, 949–954.

Hoebe, K., Janssen, E., and Beutler, B. (2004). The interface between innate and adaptive immunity. Nat. Immunol. *5*, 971–974.

Horan, K.A., Hansen, K., Jakobsen, M.R., Holm, C.K., Søby, S., Unterholzner, L., Thompson, M., West, J.A., Iversen, M.B., Rasmussen, S.B., et al. (2013). Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors. J. Immunol. Baltim. Md 1950 *190*, 2311–2319.

Höss, M., Robins, P., Naven, T.J., Pappin, D.J., Sgouros, J., and Lindahl, T. (1999). A human DNA editing enzyme homologous to the Escherichia coli DnaQ/MutD protein. EMBO J. *18*, 3868–3875.

Hume, D.A., and MacDonald, K.P.A. (2012). Therapeutic applications of macrophage colony-stimulating factor-1 (CSF-1) and antagonists of CSF-1 receptor (CSF-1R) signaling. Blood *119*, 1810–1820.

Ito, K., Hirao, A., Arai, F., Matsuoka, S., Takubo, K., Hamaguchi, I., Nomiyama, K., Hosokawa, K., Sakurada, K., Nakagata, N., et al. (2004). Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. Nature *431*, 997–1002.

Jackson, S.P., and Bartek, J. (2009). The DNA-damage response in human biology and disease. Nature *461*, 1071–1078.

Jazayeri, A., Balestrini, A., Garner, E., Haber, J.E., and Costanzo, V. (2008). Mre11-Rad50-Nbs1-dependent processing of DNA breaks generates oligonucleotides that stimulate ATM activity. EMBO J. *27*, 1953–1962.

Kass, E.M., and Jasin, M. (2010). Collaboration and competition between DNA double-strand break repair pathways. FEBS Lett. *584*, 3703–3708.

Kavanagh, D., Spitzer, D., Kothari, P.H., Shaikh, A., Liszewski, M.K., Richards, A., and Atkinson, J.P. (2008). New roles for the major human 3'-5' exonuclease TREX1 in human disease. Cell Cycle Georget. Tex *7*, 1718–1725.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat. Immunol. *11*, 373–384.

Kearney, S., Delgado, C., and Lenz, L.L. (2013). Differential effects of type I and II interferons on myeloid cells and resistance to intracellular bacterial infections. Immunol. Res. *55*, 187–200.

Kerur, N., Veettil, M.V., Sharma-Walia, N., Bottero, V., Sadagopan, S., Otageri, P., and Chandran, B. (2011). IFI16 Acts as a Nuclear Pathogen Sensor to Induce the Inflammasome in Response to Kaposi Sarcoma-Associated Herpesvirus Infection. Cell Host Microbe *9*, 363–375.

Khansari, N., Shakiba, Y., and Mahmoudi, M. (2009). Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. Recent Pat. Inflamm. Allergy Drug Discov. *3*, 73–80.

Klug, F., Prakash, H., Huber, P.E., Seibel, T., Bender, N., Halama, N., Pfirschke, C., Voss, R.H., Timke, C., Umansky, L., et al. Low-Dose Irradiation Programs Macrophage Differentiation to an iNOS+/M1 Phenotype that Orchestrates Effective T Cell Immunotherapy. Cancer Cell.

Kobayashi, J., Tauchi, H., Sakamoto, S., Nakamura, A., Morishima, K., Matsuura, S., Kobayashi, T., Tamai, K., Tanimoto, K., and Komatsu, K. (2002). NBS1 localizes to gamma-H2AX foci through interaction with the FHA/BRCT domain. Curr. Biol. CB *12*, 1846–1851.

Koch, M., Mollenkopf, H.-J., Klemm, U., and Meyer, T.F. (2012). Induction of microRNA-155 is TLR- and type IV secretion system-dependent in macrophages and inhibits DNA-damage induced apoptosis. Proc. Natl. Acad. Sci. U. S. A. *109*, E1153–1162.

Kondo, T., Kobayashi, J., Saitoh, T., Maruyama, K., Ishii, K.J., Barber, G.N., Komatsu, K., Akira, S., and Kawai, T. (2013). DNA damage sensor MRE11 recognizes cytosolic double-stranded DNA and induces type I interferon by regulating STING trafficking. Proc. Natl. Acad. Sci. U. S. A. *110*, 2969–2974.

Koyama, T., Sun, B., Tokunaga, K., Tatsumi, M., and Ishizaka, Y. (2013). DNA damage enhances integration of HIV-1 into macrophages by overcoming integrase inhibition. Retrovirology *10*, 21.

Kueh, H.Y., Champhekhar, A., Nutt, S.L., Elowitz, M.B., and Rothenberg, E.V. (2013). Positive feedback between PU.1 and the cell cycle controls myeloid differentiation. Science *341*, 670–673.

Kumagai, Y., Takeuchi, O., and Akira, S. (2008). TLR9 as a key receptor for the recognition of DNA. Adv. Drug Deliv. Rev. *60*, 795–804.

Lamarche, B.J., Orazio, N.I., and Weitzman, M.D. (2010). The MRN complex in double-strand break repair and telomere maintenance. FEBS Lett. *584*, 3682–3695.

Langerak, P., and Russell, P. (2011). Regulatory networks integrating cell cycle control with DNA damage checkpoints and double-strand break repair. Philos. Trans. R. Soc. B Biol. Sci. *366*, 3562–3571.

Lawrence, T., and Natoli, G. (2011). Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat. Rev. Immunol. *11*, 750–761.

Li, X.-D., Wu, J., Gao, D., Wang, H., Sun, L., and Chen, Z.J. (2013). Pivotal roles of cGAS-cGAMP signaling in antiviral defense and immune adjuvant effects. Science *341*, 1390–1394.

Lloberas, J., and Celada, A. (2009). p21(waf1/CIP1), a CDK inhibitor and a negative feedback system that controls macrophage activation. Eur. J. Immunol. *39*, 691–694.

Lonkar, P., and Dedon, P.C. (2011). Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates. Int. J. Cancer J. Int. Cancer *128*, 1999–2009.

Lu, Y.-C., Yeh, W.-C., and Ohashi, P.S. (2008). LPS/TLR4 signal transduction pathway. Cytokine *42*, 145–151.

Mason, P.A., and Cox, L.S. (2012). The role of DNA exonucleases in protecting genome stability and their impact on ageing. Age Dordr. Neth. *34*, 1317–1340.

Mazur, D.J., and Perrino, F.W. (1999). Identification and expression of the TREX1 and TREX2 cDNA sequences encoding mammalian 3'-->5' exonucleases. J. Biol. Chem. *274*, 19655–19660.

Mboko, W.P., Mounce, B.C., Wood, B.M., Kulinski, J.M., Corbett, J.A., and Tarakanova, V.L. (2012). Coordinate regulation of DNA damage and type I interferon responses imposes an antiviral state that attenuates mouse gammaherpesvirus type 68 replication in primary macrophages. J. Virol. *86*, 6899–6912.

Messmer, U.K., Reed, U.K., and Brüne, B. (1996). Bcl-2 protects macrophages from nitric oxide-induced apoptosis. J. Biol. Chem. *271*, 20192–20197.

Miller, S.I., Ernst, R.K., and Bader, M.W. (2005). LPS, TLR4 and infectious disease diversity. Nat. Rev. Microbiol. *3*, 36–46.

Morita, M., Stamp, G., Robins, P., Dulic, A., Rosewell, I., Hrivnak, G., Daly, G., Lindahl, T., and Barnes, D.E. (2004). Gene-targeted mice lacking the Trex1 (DNase III) 3'-->5' DNA exonuclease develop inflammatory myocarditis. Mol. Cell. Biol. *24*, 6719–6727.

Mossadegh-Keller, N., Sarrazin, S., Kandalla, P.K., Espinosa, L., Stanley, E.R., Nutt, S.L., Moore, J., and Sieweke, M.H. (2013). M-CSF instructs myeloid lineage fate in single haematopoietic stem cells. Nature *497*, 239–243.

Mosser, D.M. (2003). The many faces of macrophage activation. J. Leukoc. Biol. *73*, 209–212.

Mosser, D.M., and Edwards, J.P. (2008). Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. *8*, 958–969.

Müller, U., Steinhoff, U., Reis, L.F., Hemmi, S., Pavlovic, J., Zinkernagel, R.M., and Aguet, M. (1994). Functional role of type I and type II interferons in antiviral defense. Science *264*, 1918–1921.

Murray, P.J., and Wynn, T.A. (2011). Protective and pathogenic functions of macrophage subsets. Nat. Rev. Immunol. *11*, 723–737.

Namjou, B., Kothari, P.H., Kelly, J.A., Glenn, S.B., Ojwang, J.O., Adler, A., Alarcón-Riquelme, M.E., Gallant, C.J., Boackle, S.A., Criswell, L.A., et al. (2011). Evaluation of the TREX1 gene in a large multi-ancestral lupus cohort. Genes Immun. *12*, 270–279.

Oka, T., Hikoso, S., Yamaguchi, O., Taneike, M., Takeda, T., Tamai, T., Oyabu, J., Murakawa, T., Nakayama, H., Nishida, K., et al. (2012). Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. Nature *485*, 251–255.

Okabe, Y., Kawane, K., Akira, S., Taniguchi, T., and Nagata, S. (2005). Toll-like receptor-independent gene induction program activated by mammalian DNA escaped from apoptotic DNA degradation. J. Exp. Med. *202*, 1333–1339.

Paludan, S.R., and Bowie, A.G. (2013). Immune Sensing of DNA. Immunity *38*, 870–880.

Piątosa, B., van der Burg, M., Siewiera, K., Pac, M., van Dongen, J.J.M., Langerak, A.W., Chrzanowska, K.H., and Bernatowska, E. (2012). The defect in humoral immunity in patients with Nijmegen breakage syndrome is explained by defects in peripheral B lymphocyte maturation. Cytometry A *81A*, 835–842.

Postel-Vinay, S., Vanhecke, E., Olaussen, K.A., Lord, C.J., Ashworth, A., and Soria, J.-C. (2012). The potential of exploiting DNA-repair defects for optimizing lung cancer treatment. Nat. Rev. Clin. Oncol. *9*, 144–155.

Rice, G.I., Forte, G.M.A., Szynkiewicz, M., Chase, D.S., Aeby, A., Abdel-Hamid, M.S., Ackroyd, S., Allcock, R., Bailey, K.M., Balottin, U., et al. (2013). Assessment of interferon-related biomarkers in Aicardi-Goutières syndrome associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, and ADAR: a case-control study. Lancet Neurol. *12*, 1159–1169.

Richards, A., van den Maagdenberg, A.M.J.M., Jen, J.C., Kavanagh, D., Bertram, P., Spitzer, D., Liszewski, M.K., Barilla-Labarca, M.-L., Terwindt, G.M., Kasai, Y., et al. (2007). C-terminal truncations in human 3'-5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. Nat. Genet. *39*, 1068–1070.

Robertson, A.B., Klungland, A., Rognes, T., and Leiros, I. (2009). DNA repair in mammalian cells: Base excision repair: the long and short of it. Cell. Mol. Life Sci. CMLS *66*, 981–993.

Saar, K., Chrzanowska, K.H., Stumm, M., Jung, M., Nürnberg, G., Wienker, T.F., Seemanová, E., Wegner, R.D., Reis, A., and Sperling, K. (1997). The gene for the ataxia-telangiectasia variant, Nijmegen breakage syndrome, maps to a 1-cM interval on chromosome 8q21. Am. J. Hum. Genet. *60*, 605–610.

Sancar, A., Lindsey-Boltz, L.A., Ünsal-Kaçmaz, K., and Linn, S. (2004). Molecular Mechanisms of Mammalian Dna Repair and the Dna Damage Checkpoints. Annu. Rev. Biochem. *73*, 39–85.

Santamaría, D., Barrière, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., Cáceres, J.F., Dubus, P., Malumbres, M., and Barbacid, M. (2007). Cdk1 is sufficient to drive the mammalian cell cycle. Nature *448*, 811–815.

Schiller, C.B., Lammens, K., Guerini, I., Coordes, B., Feldmann, H., Schlauderer, F., Möckel, C., Schele, A., Strässer, K., Jackson, S.P., et al. (2012). Structure of Mre11–Nbs1 complex yields insights into ataxia-telangiectasia–like disease mutations and DNA damage signaling. Nat. Struct. Mol. Biol. *19*, 693–700.

Schipler, A., and Iliakis, G. (2013). DNA double-strand-break complexity levels and their possible contributions to the probability for error-prone processing and repair pathway choice. Nucleic Acids Res. *41*, 7589–7605.

Schoenborn, J.R., and Wilson, C.B. (2007). Regulation of Interferon-γ During Innate and Adaptive Immune Responses. In Advances in Immunology, Frederick W. Alt, ed. (Academic Press), pp. 41–101.

Schott, J., and Stoecklin, G. (2010). Networks controlling mRNA decay in the immune system. Wiley Interdiscip. Rev. RNA *1*, 432–456.

Schulz, C., Gomez Perdiguero, E., Chorro, L., Szabo-Rogers, H., Cagnard, N., Kierdorf, K., Prinz, M., Wu, B., Jacobsen, S.E.W., Pollard, J.W., et al. (2012). A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science *336*, 86–90.

Serhan, C.N., and Savill, J. (2005). Resolution of inflammation: the beginning programs the end. Nat. Immunol. *6*, 1191–1197.

Shi, C., and Pamer, E.G. (2011). Monocyte recruitment during infection and inflammation. Nat. Rev. Immunol. *11*, 762–774.

Simon, A.K., and Ballabio, A. (2013). T. rex attacks the lysosome. Nat. Immunol. *14*, 10–12.

Smith, C.D., and Cyr, M. (1988). The history of lupus erythematosus. From Hippocrates to Osler. Rheum. Dis. Clin. North Am. *14*, 1–14.

Spiegel, S., and Milstien, S. (2011). The outs and the ins of sphingosine-1-phosphate in immunity. Nat. Rev. Immunol. *11*, 403–415.

Stetson, D.B., Ko, J.S., Heidmann, T., and Medzhitov, R. (2008). Trex1 prevents cell-intrinsic initiation of autoimmunity. Cell *134*, 587–598.

Stracker, T.H., and Petrini, J.H.J. (2011). The MRE11 complex: starting from the ends. Nat. Rev. Mol. Cell Biol. *12*, 90–103.

Thomas, D.D., Ridnour, L.A., Isenberg, J.S., Flores-Santana, W., Switzer, C.H., Donzelli, S., Hussain, P., Vecoli, C., Paolocci, N., Ambs, S., et al. (2008). The chemical biology of nitric oxide: implications in cellular signaling. Free Radic. Biol. Med. *45*, 18–31.

Thompson, M.R., Kaminski, J.J., Kurt-Jones, E.A., and Fitzgerald, K.A. (2011). Pattern Recognition Receptors and the Innate Immune Response to Viral Infection. Viruses *3*, 920–940.

Underhill, D.M., and Goodridge, H.S. (2012). Information processing during phagocytosis. Nat. Rev. Immunol. *12*, 492–502.

Unterholzner, L. (2013). The interferon response to intracellular DNA: Why so many receptors? Immunobiology *218*, 1312–1321.

Valledor, A.F., Borràs, F.E., Cullell-Young, M., and Celada, A. (1998). Transcription factors that regulate monocyte/macrophage differentiation. J. Leukoc. Biol. *63*, 405–417.

Valledor, A.F., Comalada, M., Xaus, J., and Celada, A. (2000). The differential time-course of extracellular-regulated kinase activity correlates with the macrophage response toward proliferation or activation. J. Biol. Chem. *275*, 7403–7409.

Valledor, A.F., Arpa, L., Sánchez-Tilló, E., Comalada, M., Casals, C., Xaus, J., Caelles, C., Lloberas, J., and Celada, A. (2008). IFN-{gamma}-mediated inhibition of MAPK phosphatase expression results in prolonged MAPK activity in response to M-CSF and inhibition of proliferation. Blood *112*, 3274–3282.

Valledor, A.F., Comalada, M., Santamaría-Babi, L.F., Lloberas, J., and Celada, A. (2010). Macrophage proinflammatory activation and deactivation: a question of balance. Adv. Immunol. *108*, 1–20.

Vyas, J.M., Van der Veen, A.G., and Ploegh, H.L. (2008). The known unknowns of antigen processing and presentation. Nat. Rev. Immunol. *8*, 607–618.

Williams, B.R., Mirzoeva, O.K., Morgan, W.F., Lin, J., Dunnick, W., and Petrini, J.H.J. (2002). A Murine Model of Nijmegen Breakage Syndrome. Curr. Biol. *12*, 648–653.

Xaus, J., Valledor, A.F., Cardó, M., Marquès, L., Beleta, J., Palacios, J.M., and Celada, A. (1999a). Adenosine inhibits macrophage colony-stimulating factor-dependent proliferation of macrophages through the induction of p27kip-1 expression. J. Immunol. Baltim. Md 1950 *163*, 4140–4149.

Xaus, J., Cardó, M., Valledor, A.F., Soler, C., Lloberas, J., and Celada, A. (1999b). Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. Immunity *11*, 103–113.

Xaus, J., Comalada, M., Valledor, A.F., Cardó, M., Herrero, C., Soler, C., Lloberas, J., and Celada, A. (2001). Molecular mechanisms involved in macrophage survival, proliferation, activation or apoptosis. Immunobiology *204*, 543–550.

Yahata, T., Takanashi, T., Muguruma, Y., Ibrahim, A.A., Matsuzawa, H., Uno, T., Sheng, Y., Onizuka, M., Ito, M., Kato, S., et al. (2011). Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. Blood *118*, 2941–2950.

Yan, N., Regalado-Magdos, A.D., Stiggelbout, B., Lee-Kirsch, M.A., and Lieberman, J. (2010). The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. Nat. Immunol. *11*, 1005–1013.

Yan, Z., Zak, R., Luxton, G.W.G., Ritchie, T.C., Bantel-Schaal, U., and Engelhardt, J.F. (2002). Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. J. Virol. *76*, 2043–2053.

Yang, Y.-G., Lindahl, T., and Barnes, D.E. (2007). Trex1 exonuclease degrades ssDNA to prevent chronic checkpoint activation and autoimmune disease. Cell *131*, 873–886.

Yu, T., Yi, Y.-S., Yang, Y., Oh, J., Jeong, D., and Cho, J.Y. (2012). The pivotal role of TBK1 in inflammatory responses mediated by macrophages. Mediators Inflamm. *2012*, 979105.

Zhang, Z., Yuan, B., Bao, M., Lu, N., Kim, T., and Liu, Y.-J. (2011). The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nat. Immunol. *12*, 959–965.

Directors' Report

In connection with the doctoral thesis entitled "**Trex1 and Nbs1 as Regulators of the Macrophage Inflammatory Response**" by Selma Patrícia Pereira Lopes (hereinafter: Candidate) and directed by Dr. Antonio Celada Cotarelo and Dr. Jorge Lloberas Cavero, we report that the items that form the experimental part of the doctoral thesis:

"Characterization of TREX1 induction by IFN-γ in macrophages" was carried out in part by the Candidate although part of the results were used in the doctoral thesis of Maria Serra Sarasa "Papel de la exonucleasa TREX1 en el procesamiento del ADN monocatenario e implicaciones en la activación de los macrófagos", 2009. Part of the article was written by the Candidate.

Impact factor: 5,520

"The Exonuclease Trex1 Restrains the macrophage Pro-inflammatory Activation" was carried out in majority by the Candidate. The article was written by the Candidate.

Impact factor: 5,520

"Nbs1 is essential for macrophage differentiation and modulates inflammatory responses." was carried out predominantly by the Candidate. This manuscript was also written fully by the Candidate. The submission of this article is pending.

Dr. Antonio Celada Cotarelo Dr. Jorge Lloberas Cavero

Annexes

Collaborative work done during the PhD thesis

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Deacetylation of C/EBPβ is required for IL-4-induced *arginase-1* expression in murine macrophages

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The amount of arginine available at inflammatory loci is a limiting factor for the growth of several cells of the immune system. IL-4-induced activation of macrophages produced arginase-1, which converts arginine into ornithine, a precursor of polyamines and proline. Trichostatin A (TSA), a pan-inhibitor of histone deacetylases (HDACs), inhibited IL-4-induced *arginase-1* expression. TSA showed promoter-specific effects on the IL-4-responsive genes. While TSA inhibited the expression of *arginase-1*, *fizz1*, and *mrc1*, other genes, such as *ym,1* *mgl1*, and *mgl2*, were not affected. The inhibition of *arginase-1* occurred at the transcriptional level with the inhibition of polymerase II binding to the promoter. IL-4 induced STAT6 phosphorylation and binding to DNA. These activities were not affected by TSA treatment. However, TSA inhibited C/EBPβ DNA binding. This inhibitor induced acetylation on lysine residues 215–216, which are critical for DNA binding. Finally, using macrophages from STAT6 KO mice we showed that STAT6 is required for the DNA binding of C/EBPβ. These results demonstrate that the acetylation/deacetylation balance strongly influences the expression of *arginase-1*, a gene of alternative activation of macrophages. These findings also provide a molecular mechanism to explain the control of gene expression through deacetylase activity.

Keywords: Alternative activation · *Arginase-1* · Gene regulation · Histone deacetylases (HDACs) · Macrophages · STAT6 · Trichostatin A (TSA)

Introduction

In processes, such as wound healing, angiogenesis, and host defense against parasites, the phenotype of macrophages is related to alternative activation [1, 2], which includes arginase-1 expression. This enzyme converts arginine to ornithine, a precursor of polyamines and proline. This conversion is critical to induce the growth and proliferation of several cell types in damaged tissues, the production of the extracellular matrix, and tissue remodeling [3]. An excess of this type of activation contributes to fibrosis in some pathological conditions [4]. In fact, the catabolism of arginine by macrophages has emerged as a critical mechanism for the regulation of the immune response in several parasitic diseases [5–7]. Moreover, high arginase-1 expression has been associated with a variety of conditions, such as cancer, asthma, psoriasis, cardiovascular disease, and also pregnancy [8]. The consumption of arginine by macrophages in the inflammatory loci may impair the growth of other immune cells and thus result in potent immune suppressive activity.

Recently, considerable research effort has been devoted in macrophages to the regulation of genes in their natural setting, namely the chromatin substrate [9]. The recruitment of histone acetyl-transferases (HATs) and histone deacetylases (HDACs) to the transcriptional machinery is a key element in the dynamic regulation of genes. HAT activity promotes the acetylation of histone

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proteins (particularly H3 and H4), a process that leads to relaxed chromatin structure, thus binding core transcription machinery to DNA and resulting in the initiation of the transcription of several genes. In contrast, HDACs cause the deacetylation of histones, chromosomal condensation, and gene repression [10]. However, despite the common involvement of acetylation/deacetylation in general transcription, deacetylation is associated with activation of the transcription of particular genes, as demonstrated by the observation that histone hyperacetylation inhibits the expression of some genes [11]. Thus, a proper balance between HAT and HDAC activity is required to control chromatin accessibility to specific or general transcription factors [10]. Moreover, in addition to histones, these enzymes can also modify an extended group of proteins, including transcription factors, mitochondrial proteins, RNA-splicing factors, structural proteins, and chaperones. Acetylation affects the affinity of these molecules to bind to other proteins or DNA and can change their half-life, subcellular localization, and even their enzymatic activity. Thus acetylation provides a myriad of potential mechanisms to modulate gene expression. Interestingly, most nonhistone proteins targeted by acetylation are relevant in the regulation of immune functions, cell proliferation, and tumorigenesis [12, 13]. For these reasons, several specific HDAC inhibitors have emerged as anticancer drugs and more recently, due to their suppressive effect on the expression of proinflammatory mediators, as anti-inflammatory agents [14]. These compounds are effective in a variety of Th1-dependent inflammatory diseases, such as ulcerative colitis and rheumatoid arthritis, thereby suggesting that cellular HDAC activity exacerbates the inflammatory process [15]. The in vitro gene-specific regulation caused by HDAC inhibitors in macrophages has been described in the context of LPS response (classical activation). By means of genome-wide microarray analysis, it has been shown that trichostatin A (TSA), a new potential HDAC inhibitor for human diseases, modifies the macrophage transcriptome, inhibiting about 32% of all LPS-induced genes and counterregulating a significant percentage of LPS-repressed genes [16,17]. Recently, it was shown that histone deacetylase 3 is an epigenomic brake of alternative activation of macrophages as it blocks the IL-4-dependent induction of arginase-1 [18]. Also, in a macrophagic cell line, it has been reported that TSA leads to a concentration-dependent suppression of the arginase-1 expression induced by the cAMP analog Br-cAMP [19].

Given the important role of *arginase-1* in the regulation of immune response [8], here we studied the effect of TSA on the expression of this enzyme when induced by IL-4. Global acetylation induced by TSA had promoter-specific effects on IL-4-responsive genes. While *arginase-1*, *fizz 1*, and *mrc1* induction was inhibited, other genes such as *ym1* and *macrophage galactose N-acetyl-galactosamine-specific Lectin* CD301a, also called *mgl1,* showed increased expression. In the case of *mgl2,* no modifications were observed. To further extend our results, we studied the molecular mechanisms involved in TSA-mediated *arginase-1* inhibition. The induction of this gene by IL-4 requires a DNA response element composed by adjacent STAT6 and C/EBPβ transcription factor-binding sites. Moreover, acetylation on lysine residues 215–216 of C/EBPβ is an important regulator that modulates protein-DNA binding on the *arginase-1* promoter and, therefore, modulates IL-4-dependent *arginase-1* expression in macrophages.

Results

Not all the genes induced during the alternative activation of macrophages are downregulated by TSA

In our experiments, we used bone marrow-derived macrophages, a homogeneous population of primary and quiescent cells. Treatment of these cells with several cytokines causes several modifications that allow them to develop their functional activities [20]. To test whether deacetylase activity has any functional implication in IL-4-dependent arginase activity of macrophages, we used the pharmacological HDAC inhibitor TSA in our assays. The TSA concentration used (20 nM) did not induce cellular toxicity in our experimental conditions [21]. We studied whether acetylation is required for all the IL-4-dependent genes induced in macrophages. For this purpose, we explored the mRNA expression of several genes induced by IL-4 treatment in a dose- and time-dependent manner. As expected, *fizz1*, *mrc1, arginase-1, mgl1,* and *ym1* were strongly induced at 10 h of IL-4 treatment, a time-point at which expression was clearly detectable [22] (Fig. 1A and B). Although the treatment with TSA inhibited the expression of several of these genes, it was not a general phenomenon. The HDAG inhibitor significantly reduced *fizz1*, *mrc1,* and *arginase-1* expression (Fig. 1A), but not that of other genes such as *mgl1, mgl2* and *ym1* (Fig. 1B). The latter may be due to the positive role of histone acetylation in transcription. These results confirm that deacetylation is required for the transcriptional activation of some IL-4-responsive genes, as previously reported in the context of LPS response [16, 17], and suggest that other nonhistone-related targets of HDACs are involved in the transcriptional regulation of these genes. Although all the genes induced by IL-4 are dependent on Stat-6, not all have the same transcriptional or post-transcriptional mechanisms of induction.

Deacetylase activity is required for IL-4-dependent arginase activity

IL-4-induced arginase activity in macrophages (Fig. 2A) [23]. Treatment with TSA significantly reduced this activity. However, of note, the treatment with TSA alone produced an increase in arginase activity. The assay for arginase activity was done with cell lysates, to which we added the substrate [24]. Under these conditions, we were unable to differentiate the arginase activity caused by arginase-1 located in the cytosol or by arginase-2 in the mitochondria. Therefore, we studied the effects of TSA on the expression of each enzyme. Real-time PCR analysis of both mRNAs showed that TSA exerted different effects on the two arginase isoforms. Although *arginase-1* mRNA-induced expression by IL-4 or 8-Br-cAMP was significantly inhibited (Fig. 2B), expression of

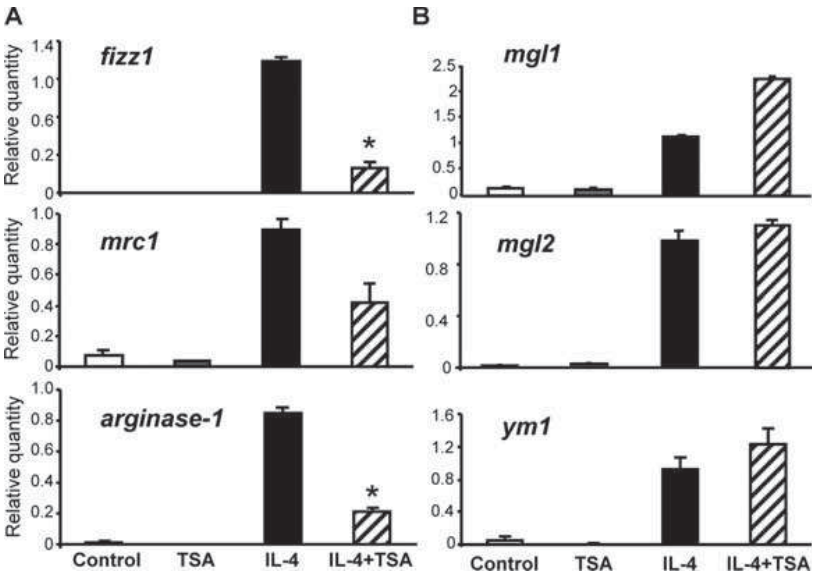

Figure 1. TSA regulates the transcription of some IL-4-induced genes in macrophages. Cells were treated with IL-4 (10 ng/mL) or IL-4 plus TSA (20 nM) for 10 h and gene expression was measured by real-time PCR assay. (A) Expression of *fizz1*, *mrc1*, and *arginase-1*. (B) Expression of *mgl1*, *mgl2*, and *ym1*. Each point was performed in triplicate and the results are shown as mean + SD. All assays are representative of at least four independent experiments. $^{*}p < 0.01$, in relation to the controls when all the independent experiments were compared., nonparametric Wilcoxon test.

arginase-2 was not (Fig. 2C). This observation could explain why TSA treatment increased arginase activity. Thus, taken together, these results showed that these two arginase isoforms are regulated by a specific acetylation/deacetylation mechanism in different manners in response to IL-4 and other stimuli.

TSA prevents RNA polymerase II recruitment to arginase-1 promoter

To determine whether the decrease in *arginase-1* mRNA was due to an inhibition of the mRNA production or to an increase in its degradation, we measured the rate of mRNA degradation. Macrophages were treated with IL-4 for 6 h and then with DRB [25] at a concentration sufficient to block all further RNA synthesis, as determined by $^{(3H)}$UTP incorporation [26]. RNA was isolated from aliquots of cells at different times after the addition of DRB and actinomycin D. This approach allowed us to estimate the half-life of *arginase-1* mRNA [27]. Under these conditions, the mRNA was stable (Fig. 3A). After 6 h of DRB and actinomycin D treatment, there were no modifications for the half-life of *arginase-1* mRNA. The treatment with TSA simultaneously with IL-4 did not modify the half-life of *arginase-1* (Fig. 3A). As a control, we determined the half-life of the c-myc mRNA. The mRNA of this protoncogene was very unstable with a half-life of less than 1 h [28]. When we extrapolated the amounts of *c-myc* RNA, we obtained a t1/2 of around 30 min (Fig. 3A). These results demonstrate that the reduced levels of mRNA were not due to a decrease in the half-life of RNA but to an inhibition at the transcriptional level.

Because HATs and HDACs play a crucial role in the formation of transcription pre-initiation complexes, we next tested the effect of TSA on the recruitment of RNA polymerase II upon IL-4 stimulation of macrophages. Using chromatin immunoprecipitation, we found that RNA polymerase II was recruited to the *arginase-1* promoter after 3 or 6 h of IL-4 treatment. However, this recruitment did not occur when the cells were treated with TSA (Fig. 3B). The specificity of the reaction was checked by using unrelated antibodies or a fragment of the coding region (data not shown). This observation indicates that TSA inhibits IL-4-mediated transcription by blocking the recruitment of the basal complex machinery. Taken together, these results suggest that deacetylase activity is required to recruit RNA polymerase II and activate the transcription of IL-4-dependent *arginase-1* induction in macrophages.

A detailed analysis of the time course of *arginase-1* expression showed that it increased rapidly after IL-4 treatment, reaching a maximum between 6 and 10 h and then progressively diminishing, but still detectable 24 h after IL-4 activation (Fig. 3C). When TSA was administrated before or at the same time as IL-4, the expression of *arginase-1* was blocked (Fig. 3C). It is known that *arginase-1* expression is dependent on new protein synthesis, although the required protein remains unknown [29]. To exclude that TSA inhibited *arginase-1* expression by blocking the induction of this unknown protein, TSA was added after 3 h of IL-4 treatment. Even when transcription was initiated, the addition of TSA reduced *arginase-1* induction (Fig. 3D). These data allow us to conclude that the TSA acts directly on *arginase-1* transcription machinery and not through the inhibition of a protein necessary for the transcription.

Deacetylase activity is not required by STAT6 binding to the arginase-1 promoter

The transcription of *arginase-1* induced by IL-4 in macrophages is regulated by a composed element, placed about 3 kb upstream of the start transcription site, which binds STAT6 and C/EBPβ [30]

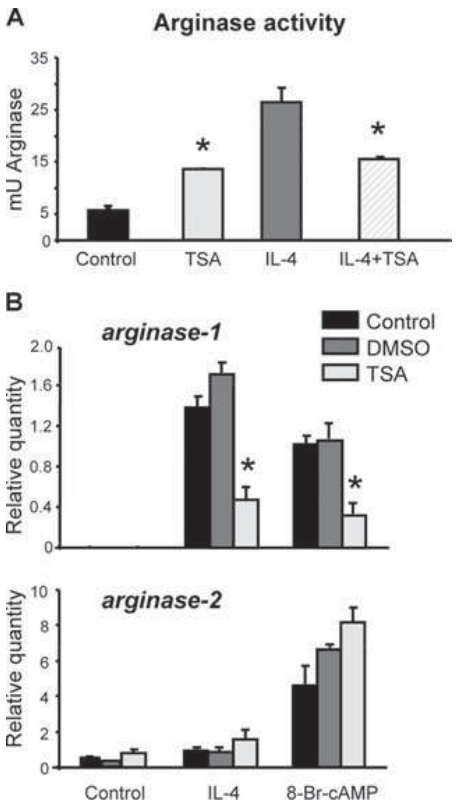

Figure 2. TSA impairs the arginase-1 activity induced by IL-4 in macrophages. (A) Macrophages were cultured for 24 h in the presence of IL-4 and/or TSA and arginase activity was determined. (B) *arginase*-1 and (C) *arginase*-2 expression was analyzed by real-time PCR. In this case, macrophages were left untreated or were treated with either DMSO, as a vehicle control, or TSA for 1 h. They were then stimulated or not with IL-4 or 8-Br-cAMP (100 μM) for 10 h. Each point was performed in triplicate and the results are shown as mean + SD. All assays are representative of at least four independent experiments. $^{*}p < 0.01$, in relation to the controls when all the independent experiments were compared; nonparametric Wilcoxon test.

(Fig. 4A). A similar element is present in other genes induced by IL-4 and whose expression is inhibited by TSA, such as *Fizz1* [31]. *Ym1,* which is not downregulated by TSA, has no C/EBPβ-binding sites adjacent to STAT6 boxes [32]. Therefore, we concentrated our efforts on determining whether TSA acts on the transcription factors that bound to the precise area in the promoter.

STAT6 plays a critical role in the *arginase-1* expression induced by IL-4 in macrophages since this gene is not expressed in macrophages from STAT6 KO mice [22]. For this reason, we explored the potential role of deacetylase activity on the STAT6 transduction pathway. In this regard, it is known that IL-4 stimulation promotes STAT6 phosphorylation, thereby allowing its dimerization and nuclear translocation [30, 33, 34]. TSA treatment did not modify the IL-4-induced phosphorylation of STAT6, as shown by western blot with antibodies against tyrosine-phosphorylated STAT6 (Fig. 4B). Moreover, the in vitro DNA-binding capacity of STAT6 was tested by EMSA assay (Fig. 4C). As a probe, we used a sequence of the *arginase-1* promoter, which includes the STAT6-binding element placed 2.86 Kb upstream of the transcription start site [30]. While the nuclear extracts from untreated macrophages did not bind to this probe, a shift was observed when we used those from IL-4-treated macrophages. This band was specific because treatment with 100-fold excess of cold oligonucleotide eliminated the binding but not the competition with an oligonucleotide that contains the mutated STAT6 box. In support of the results on the STAT6 phosphorylation, the in vitro DNA binding of STAT6 was not modified by TSA treatment (Fig. 4B).

To confirm the results, we performed DNA-binding assays in vivo using the chromatin immunoprecipitation technique. The treatment of macrophages with IL-4 induced an increase in the binding of STAT6 (Fig. 4D). However, this activity was not affected by TSA treatment. Therefore, these results indicate that TSA does not affect the IL-4-STAT6 pathway involved in *arginase-1* expression in macrophages.

Deacetylase activity is required for C/EBPβ binding to an enhancer element of arginase-1

So far, we can exclude that TSA affects STAT6. Therefore, we next tested the effect of TSA on the binding of C/EBPβ to the promoter of *arginase-1*. For this purpose, chromatin immunoprecipitation assays were performed. IL-4 treatment induced a strong binding of C/EBPβ to the *arginase-1* promoter while the addition of TSA abolished this binding (Fig. 5A). The attachment of C/EBPβ to DNA was abrogated in macrophages from STAT6 KO mice (Fig. 5B), suggesting that the cooperation between STAT6 and C/EBPβ binding to the *arginase-1* promoter is essential for C/EBPβ binding to this element.

C/EBPβ is regulated at multiple levels in several cell types [35–37]. Therefore, we first explored the effects of TSA in the mRNA and protein expression of C/EBPβ in IL-4-activated macrophages. IL-4 treatment did not modify *C/EBP*β (Fig. 5C) or protein levels (Fig. 5D). This result is consistent with the previous findings that discarded the involvement of early genes on the TSA inhibitory effect. To date, three well-known C/EBPβ isoforms have been described. These share the 145 C-terminal amino acids that contain the basic DNA-binding domain and the leucine zipper dimerization helix. Of these, LAP (liver activator protein) is a transcriptional activator whereas the shortest form LIP (liver inhibitor protein) acts as a transcriptional repressor [38]. It has been proposed that the LAP/LIP ratio determines the final outcome of C/EBPβ activity [39]. However, we did not observe changes in the percentage of expression of any of the isoforms between non-stimulated (starvation conditions) or IL-4-activated cells. Nor were changes detected after addition of TSA (Fig. 5 D).

Because C/EBPβ-induced expression is required for *arginase-1* expression in macrophages in response to activation with cAMP [40], we evaluated the role of TSA on the expression of this transcription factor upon 8-Br-cAMP treatment. After 3 or 6 h of this treatment, *C/EBP*β as well as protein levels were increased,

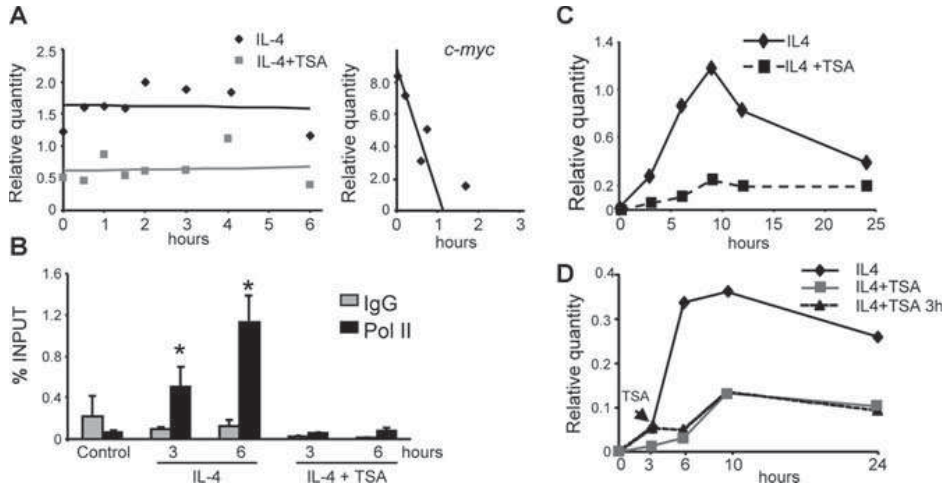

Figure 3. TSA inhibits *arginase-1* expression at the transcriptional level. (A) Macrophages previously stimulated with IL-4 or IL-4 plus TSA for 6 h were treated with DRB (20 μg/mL) and actinomycin D (5 μg/mL) and *arginase-1* and *c-myc* was measured after the indicated times by quantitative RT-PCR. The figures show one representative result of three independent experiments. (B) ChiP assays were performed using an antibody against Pol II. (C) Macrophages were cultured in the presence or absence of TSA for 1 h and then stimulated with IL-4 for the indicated times and *arginase-1* expression was determined by real-time PCR. (D) Macrophages were stimulated with IL-4, and TSA was added at the same time, or 3 h after the IL-4 stimulus and *arginase-1* expression was determined. Each point was performed in triplicate and the results are shown as mean ± SD. All assays are representative of at least four independent experiments. $^*p < 0.01$ in relation to the controls when all the independent experiments had been compared; nonparametric Wilcoxon test.

thereby confirming previous observations [40] (Fig. 5C and E). The addition of TSA did not have any effect on the 8-Br-cAMP-induced protein expression, as determined by LAP (Fig. 5E) or LIP (data not shown). This observation reinforces the notion that TSA does not regulate C/EBPβ activity through a modulation of LAP/LIP expression.

TSA abolished the DNA binding of C/EBPβ without modifying the amounts of the protein. This finding prompted us to look for post-transcriptional modifications of this transcription factor in response to TSA treatment that allow us to explain the results observed. C/EBPβ has many lysine residues that are potential substrates of acetylation. Using an antibody against C/EBPβ acetyl-lysine residues 215 and 216, we observed that TSA treatment of macrophages induced C/EBPβ acetylation (Fig. 5F). The acetylation of 215 and 216 residues, placed in the DNA-binding domain of the protein, have been implicated in the regulation of the DNA interaction with C/EBPβ [41]. In conclusion, these data suggest that C/EBPβ acetylation on lysine residues 215–216, which inhibits DNA binding, is at the basis of *arginase-1* inhibition.

Discussion

Chromatin remodeling is an essential mechanism that regulates gene transcription. Acetylation and deacetylation play pivotal roles in modifying not only histone but also the activity of several transcription factors [10, 11, 42]. Here, we provide evidence that deacetylase activity is required for IL-4- and cAMP-dependent *arginase-1* induction. The requirement of deacetylase activity is related to *arginase-1*, as well as other IL-4-induced genes such as *fizz1* or *mrc1,* but is not a common feature since TSA does not inhibit the expression of *ym1, mgl1*, or *mgl2*. We have shown that IL-4 and cAMP induce *arginase-1* expression in macrophages at transcriptional level and that TSA does not exert its function through an increase in mRNA degradation. These results were confirmed by the observation that TSA abolishes the IL-4- or cAMP-dependent binding of the polymerase II to the *arginase-1* promoter. Also, these results suggest that the acetylation of transcription factors or co-activators is involved in the inhibition of transcription.

The transcription of *arginase-1* induced by IL-4 or cAMP in macrophages is regulated by a composed element, placed about 3 kb upstream of the transcription start site, which binds STAT6 and C/EBPβ [30, 40]. These elements are also present in other genes whose expression was abolished by TSA. A 3 bp mutation in the CCAAT box of the *arginase-1* enhancer element abolishes the response to IL-4, thereby confirming the involvement of C/EBPβ in *arginase-1* induction [30]. Using EMSA assays, it was found that C/EBPβ binds to the *arginase-1* enhancer [30]. Using chromatin immunoprecipitation assays, we found that a small amount of C/EBPβ was associated with the promoter prior to the IL-4 stimulus. However, the recruitment of C/EBPβ increased 5–6-fold after IL-4 exposure.

STAT6 recruits many HATs, which act as co-activators. Initially, CBP/p300, like SRC1, bind directly to STAT6 [43, 44] and later p/CIP join the complex [45] to form an enhanceosome that contacts the general transcriptional machinery at the start site. However, the inhibition of HATs by TSA does not prevent the

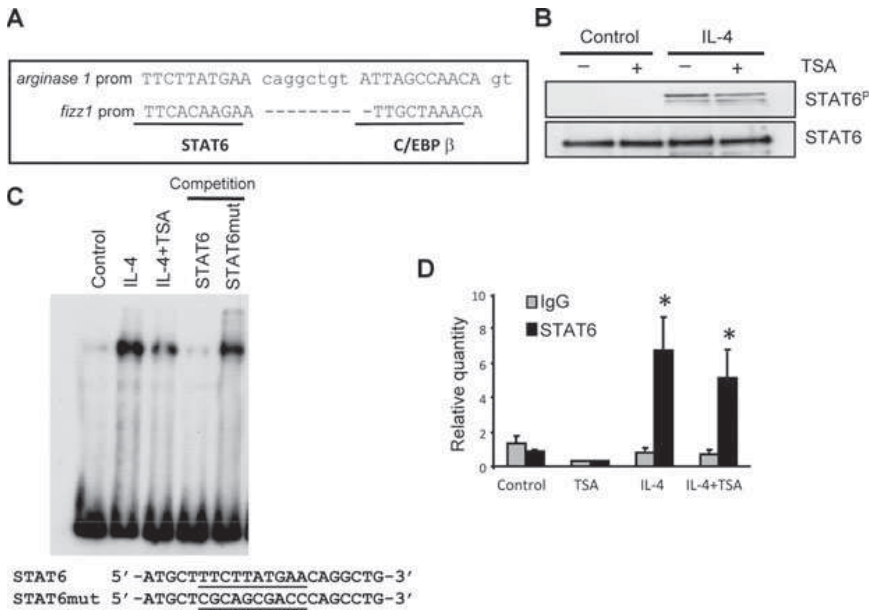

Figure 4. TSA does not inhibit the STAT6 binding to the *arginase-1* promoter in IL-4-activated macrophages. (A) Sequences of STAT6 and C/EBPβ-binding sites in the promoters of *arginase-1* and *fizz1*. (B). Macrophages were treated with TSA for 1 h and then stimulated with IL-4 for 6 h. Phosphorylation of STAT-6 was determined by western blot in total protein extracts. (C) In vitro binding of STAT6 to the *arginase-1* promoter by EMSA. Competition experiments were performed by adding a 100-fold excess of the cold oligonucleotides to the nuclear extracts before addition of the radiolabeled probe. The sequences of the oligonucleotides used are shown at the bottom of the figure. (D) In vivo binding of STAT6 to the *arginase-1* promoter using ChiP assays with an antibody against STAT6 and a fragment of DNA corresponding to *arginase-1* enhancer element that was amplified by real-time PCR. Data were normalized using the amplification of an irrelevant fragment of DNA, and finally expressed as relative quantity. Each point was performed in triplicate and the results are shown as mean + SD. All the experiments are representative of at least four independent experiments. $^*p < 0.01$ in relation to the controls when all the independent experiments had been compared; nonparametric Wilcoxon test.

binding of STAT6 to the *arginase-1* promoter. In addition to the modification of histone proteins, acetylation has been shown to affect the activities of transcription factors [46]. Here, we show that the induced binding of C/EBPβ to the *arginase-1* promoter by IL-4 or cAMP is lost under the effect of TSA. This inhibition of DNA binding correlates with the C/EBPβ acetylation and could be explained by the suppressive effect of TSA on HDAC. We cannot exclude that STAT6 is required for the recruitment of HDACs. Recently, it has been shown in macrophages that STAT6 is a facilitator of the nuclear receptor PPARγ, which promotes DNA binding and consequently increases the number of regulated genes as well as the magnitude of the response [47]. However, in the absence of PPARγ, no reduction of IL-4-dependent induction of *arginase-1* was observed.

The regulation of C/EBPβ activity by the acetylation/deacetylation mechanism has been described previously. In concordance with our results, it has been demonstrated that the acetylation of C/EBPβ lysine residues 215–216 represses the transcription of the *inhibitor of DNA binding 1* (*Id-1)* gene by diminishing its DNA-binding activity [41]. It will be interesting to mutate lysine residues 215–216 in C/EBPβ and examine whether this affects alternative activation. However, technically, this experiment is very difficult to perform in nontransformed macrophages due to the inefficient transfection capacity (maximum 5–10% of the cells).

In contrast to the acetylation of C/EBPβ in lysine residues 215–216, acetylation of lysine 39 seems to be required to allow C/EBPβ to act as a transactivator in several genes, these related mainly to adipogenesis and growth hormone response [48, 49]. Thus, acetylation on different lysine residues in the same transcription factor can result in opposite effects on the transcription of specific target genes. We propose that acetylation of C/EBPβ on lysine residues 215–216 determines the outcome of *arginase-1* expression during IL-4 stimulation. In this scenario, by early hyperacetylation of these residues, TSA impedes the recruitment of C/EBPβ and RNA poll II to the *arginase-1* promoter and limits *arginase-1* expression without modifying STAT6 activity.

The induction of arginase by IL-4 requires SHIP degradation [50]. However, a complete understanding of *arginase-1* regulation may be relevant because it may provide new targets to control the expression of this gene. Arginase-1 is receiving increasing attention because arginine consumption at inflammatory loci could be a limiting factor of the immune response as a result of the requirement of this essential amino acid for the growth

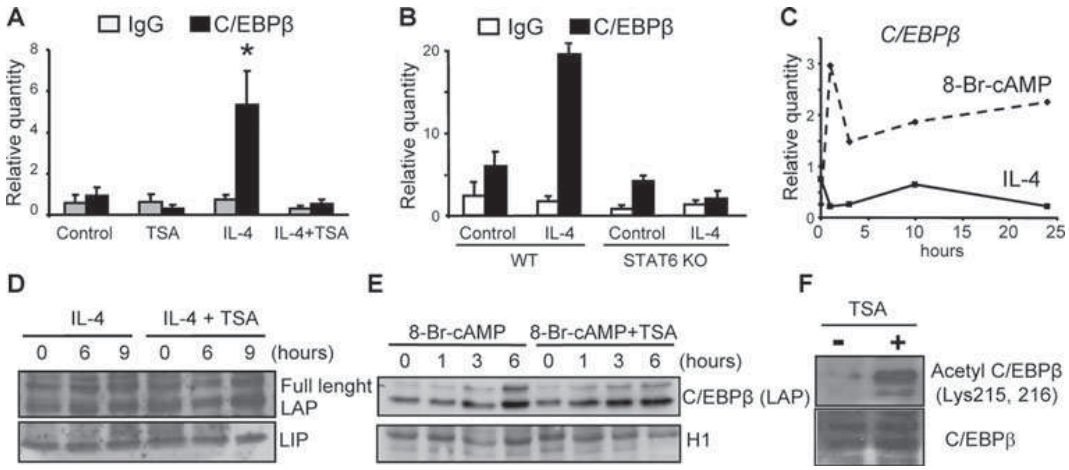

Figure 5. Acetylation of C/EBPβ blocks *arginase-1* expression in response to IL-4. (A) Macrophages were stimulated with IL-4 for 4 h with or without previous treatment with TSA. ChiP assays were then performed to evaluate the in vivo binding of C/EBPβ to the *arginase-1* promoter. (B) Similar conditions as in (A) but in this case we used macrophages from STAT6 KO mice. Each point was performed in triplicate and the results are shown as mean + SD. $^*p < 0.01$ in relation to the controls when all the independent experiments had been compared; nonparametric Wilcoxon test. (C) Macrophages were treated with IL-4, 8-Br-cAMP with or without TSA for the indicated times and the levels of mRNA were determined. (D) Macrophages were stimulated with IL-4 for the indicated times with or without previous TSA treatment. C/EBPβ proteins were evaluated by western blot using specific antibodies. (E) Macrophages were cultured as in (C). C/EBPβ proteins were evaluated by western blot using specific antibodies. As control, we used histone 1 (H1). (F) Acetylated-CEBP/β was evaluated by western blot. All the experiments are representative of at least four independent experiments with similar results.

of many cells involved in the immune system [5–7]. Thus, a better understanding of the molecular/signaling mechanism(s) regulating *arginase-1* may provide an attractive opportunity to manipulate macrophage activation in diseases in which these cells contribute to the pathology. In line with this hypothesis, and on the bases of the results presented here, deacetylase inhibitors (e.g. TSA) prevent the induction of several genes involved in pathologies such as allergy, asthma, and fibrosis [4].

Materials and methods

Reagents

Recombinant murine IL-4 was purchased from R&D systems, 8-Br-cAMP, Actynomicin D and 5, 6-dichlorobenzimidazole riboside (DRB) were from Sigma, and TSA was from Tocris Bioscience. The Abs used were as follows: anti-phospho-STAT6 (Cell Signaling); anti-β-actin (Sigma-Aldrich); anti-acetyl C/EBPβ (Lys215, Lys216) (Millipore); anti-RNA Pol II (N20); anti-C/EBPβ (C-19); anti-histone H1 (N-19); and anti-STAT6 (M20) (Santa Cruz Biotechnology). Peroxidase-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories) or anti-mouse (Sigma-Aldrich) was used as a secondary Ab. All other chemicals were of the highest purity grade available and were purchased from Sigma-Aldrich. Deionized water was further purified with Millipore Milli-Q System A10.

Cell culture

Bone marrow-derived macrophages were isolated from 6-week-old male BALB/c mice (Charles River Laboratories, Wilmington, MA, USA), as described [51]. After 7 days of culture, a homogeneous population of adherent macrophages was obtained (>99% Mac-1^+). To synchronize the cells, at 80% confluence, they were deprived of M-CSF for 16–18 h before being subjected to the treatments. Animal use was approved by the Animal Research Committee of the Government of Catalonia (number 2523).

RNA extraction and real-time RT-PCR

RNA was extracted with Tri Reagent (Sigma), following the manufacturer's instructions. One microgram of RNA was retrotranscribed using Moloney murine leukemia virus reverse transcriptase RNase H Minus (Promega) and real-time PCR was performed as described [52]. Data were expressed relative to the expression in each sample of β-actin. The primer sequences are described in Table 1.

Arginase activity

Arginase activity was measured as described [24]. In brief, cells were lysed and arginine hydrolysis was conducted by incubating the lysate with L-arginine at 37°C for 15–120 min. The urea concentration was measured and one unit of enzyme activity was

Table 1. Primer sequences by real-time PCR

Gene	Primer	Sequence
β-*actin*	Forward	ACTATTGGCAACGAGCGGTTC
	Reverse	AAGGAAGGCTGGAAAAGAGC
Arginase-1	Forward	TTGCGAGACGTAGACCCTGG
	Reverse	CAAAGCTCAGGTGAATCGGC
Fizz1	Forward	CCTTCTCATCTGCATCTCCCTG
	Reverse	GCTGGATTGGCAAGAAGTTCC
Ym1	Forward	TGTTCTGGTGAAGGAAATGCG
	Reverse	CGTCAATGATTCCTGCTCCTGT
Mrc1	Forward	AATGAAGATCACAAGCGCTGC
	Reverse	TGACACCCAGCGGAATTTCT
Arginase-2	Forward	TGAGAGATGTGGAGCCTCCTGA
	Reverse	CCATCACCTTCTGGATCCCAA
Mgl2	Forward	CCAGAACTTGGAGCGGGAAGAGAA
	Reverse	CTCAAGTCTCGGCCTGCCTGC
C-myc	Forward	AACAGCTTCGAAACTCTGGTGC
	Reverse	CGCATCAGTTCTGTCAGAAGGA

defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per minute.

EMSA

Cells were lysed and nuclear extracts obtained as described [53]. EMSA assay was used as described [27]. The probes were synthesized by Sigma and correspond to a STAT6-binding element 2.86 Kb upstream of the *arginase-1* transcription start site [30] and the mutant, as indicated in Figure 4C.

Chromatin immunoprecipitation (ChIP) assay

This assay was performed as described previously [27]. The primers used correspond to the *arginase-1* promoter transcription start site: 5′GGCCGTAACCCTAAAAGACA3′ 5′CGGAGCCAGTTGTTGGATA3′ or to the enhancer element placed at −2.88 Kb: 5′GGCACAACTCACGTACAGACA3′, 5′TGAGGCATTGTTCAGACTTCC3′.

Statistical analysis

The nonparametric Wilcoxon test for paired differences was used in all calculations [54].

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References

1 **Brancato, S. K. and Albina, J. E.**, Wound macrophages as key regulators of repair: origin, phenotype, and function. *Am. J. Pathol.* 2011. **178**: 19–25.

2 **Kreider, T., Anthony, R. M., Urban, J. F., Jr. and Gause, W. C.**, Alternatively activated macrophages in helminth infections. *Curr. Opin. Immunol.* 2007. **19**: 448–453.

3 **Shearer, J. D., Richards, J. R., Mills, C. D. and Caldwell, M. D.**, Differential regulation of macrophage arginine metabolism: a proposed role in wound healing. *Am. J. Physiol.* 1997. **272**: E181–E190.

4 **Wynn, T. A. and Barron, L.**, Macrophages: master regulators of inflammation and fibrosis. *Semin. Liver Dis.* 2010. **30**: 245–257.

5 **Hesse, M., Modolell, M., La Flamme, A. C., Schito, M., Fuentes, J. M., Cheever, A. W., Pearce, E. J. et al.**, Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J. Immunol.* 2001. **167**: 6533–6544.

6 **Vincendeau, P., Gobert, A. P., Daulouede, S., Moynet, D. and Mossalayi, M. D.**, Arginases in parasitic diseases. *Trends Parasitol.* 2003. **19**: 9–12.

7 **Kropf, P., Fuentes, J. M., Fahnrich, E., Arpa, L., Herath, S., Weber, V., Soler, G. et al.**, Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. *Faseb. J.* 2005. **19**: 1000–1002.

8 **Munder, M.**, Arginase: an emerging key player in the mammalian immune system. *Br. J. Pharmacol.* 2009. **158**: 638–651.

9 **Takeuch, O. and Akira, S.**, Epigenetic control of macrophage polarization. *Eur. J. Immunol.* 2011. **41**: 2490–2493.

10 **Wang, Z., Zang, C., Cui, K., Schones, D. E., Barski, A., Peng, W. and Zhao, K.**, Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell* 2009. **138**: 1019–1031.

11 **Haberland, M., Montgomery, R. L. and Olson, E. N.**, The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat. Rev. Genet.* 2009. **10**: 32–42.

12 **Spange, S., Wagner, T., Heinzel, T. and Kramer, O. H.**, Acetylation of non-histone proteins modulates cellular signalling at multiple levels. *Int. J. Biochem. Cell Biol.* 2009. **41**: 185–198.

13 **Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V. et al.**, Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 2009. **325**: 834–840.

14 **Adcock, I. M.**, HDAC inhibitors as anti-inflammatory agents. *Br. J. Pharmacol.* 2007. **150**: 829–831.

15 **Grabiec, A. M., Tak, P. P. and Reedquist, K. A.**, Targeting histone deacetylase activity in rheumatoid arthritis and asthma as prototypes of inflammatory disease: should we keep our HATs on? *Arthritis Res. Ther.* 2008. **10**: 226.

16 **Brogdon, J. L., Xu, Y., Szabo, S. J., An, S., Buxton, F., Cohen, D. and Huang, Q.**, Histone deacetylase activities are required for innate immune cell control of Th1 but not Th2 effector cell function. *Blood* 2007. **109**: 1123–1130.

17 **Roger, T., Lugrin, J., Le Roy, D., Goy, G., Mombelli, M., Koessler, T., Ding, X. C. et al.**, Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. *Blood* 2011. **117**: 1205–1217.

18 **Mullican, S. E., Gaddis, C. A., Alenghat, T., Nair, M. G., Giacomin, P. R., Everett, L. J., Feng, D. et al.**, Histone deacetylase 3 is an epigenomic brake in macrophage alternative activation. *Genes. Dev.* 2011. **25**: 2480–2488.

19 **Haffner, I., Teupser, D., Holdt, L. M., Ernst, J., Burkhardt, R. and Thiery, J.**, Regulation of arginase-1 expression in macrophages by a protein kinase A type I and histone deacetylase dependent pathway. *J. Cell Biochem.* 2008. **103**: 520–527.

20 **Xaus, J., Cardo, M., Valledor, A. F., Soler, C., Lloberas, J. and Celada, A.**, Interferon gamma induces the expression of p21waf-1 and arrests macrophage cell cycle, preventing induction of apoptosis. *Immunity* 1999. **11**: 103–113.

21 **Sebastian, C., Serra, M., Yeramian, A., Serrat, N., Lloberas, J. and Celada, A.**, Deacetylase activity is required for STAT5-dependent GM-CSF functional activity in macrophages and differentiation to dendritic cells. *J. Immunol.* 2008. **180**: 5898–5906.

22 **Arpa, L., Valledor, A. F., Lloberas, J. and Celada, A.**, IL-4 blocks M-CSF-dependent macrophage proliferation by inducing p21(Waf1) in a STAT6-dependent way. *Eur. J. Immunol.* 2009. **39**: 514–526.

23 **Yeramian, A., Martin, L., Serrat, N., Arpa, L., Soler, C., Bertran, J., McLeod, C. et al.**, Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. *J. Immunol.* 2006. **176**: 5918–5924.

24 **Classen, A., Lloberas, J. and Celada, A.**, Macrophage activation: classical versus alternative. *Methods Mol. Biol.* 2009. **531**: 29–43.

25 **te Poele, R. H., Okorokov, A. L. and Joel, S. P.**, RNA synthesis block by 5, 6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) triggers p53-dependent apoptosis in human colon carcinoma cells. *Oncogene* 1999. **18**: 5765–5772.

26 **Celada, A., Klemsz, M. J. and Maki, R. A.**, Interferon-gamma activates multiple pathways to regulate the expression of the genes for major histocompatibility class II I-A beta, tumor necrosis factor and complement component C3 in mouse macrophages. *Eur. J. Immunol.* 1989. **19**: 1103–1109.

27 **Casals, C., Barrachina, M., Serra, M., Lloberas, J. and Celada, A.**, Lipopolysaccharide up-regulates MHC class II expression on dendritic cells through an AP-1 enhancer without affecting the levels of CIITA. *J. Immunol.* 2007. **178**: 6307–6315.

28 **Brewer, G.**, An A + U-rich element RNA-binding factor regulates c-myc mRNA stability in vitro. *Mol. Cell Biol.* 1991. **11**: 2460–2466.

29 **Pauleau, A. L., Rutschman, R., Lang, R., Pernis, A., Watowich, S. S. and Murray, P. J.**, Enhancer-mediated control of macrophage-specific arginase I expression. *J. Immunol.* 2004. **172**: 7565–7573.

30 **Gray, M. J., Poljakovic, M., Kepka-Lenhart, D. and Morris, S. M., Jr.**, Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. *Gene* 2005. **353**: 98–106.

31 **Stutz, A. M., Pickart, L. A., Trifilieff, A., Baumruker, T., Prieschl-Strassmayr, E. and Woisetschlager, M.**, The Th2 cell cytokines IL-4 and IL-13 regulate found in inflammatory zone 1/resistin-like molecule alpha gene expression by a STAT6 and CCAAT/enhancer-binding protein-dependent mechanism. *J. Immunol.* 2003. **170**: 1789–1796.

32 **Welch, J. S., Escoubet-Lozach, L., Sykes, D. B., Liddiard, K., Greaves, D. R. and Glass, C. K.**, TH2 cytokines and allergic challenge induce Ym1 expression in macrophages by a STAT6-dependent mechanism. *J. Biol. Chem.* 2002. **277**: 42821–42829.

33 **Hou, J., Schindler, U., Henzel, W. J., Ho, T. C., Brasseur, M. and McKnight, S. L.**, An interleukin-4-induced transcription factor: IL-4 stat. *Science* 1994. **265**: 1701–1706.

34 **Mikita, T., Campbell, D., Wu, P., Williamson, K. and Schindler, U.**, Requirements for interleukin-4-induced gene expression and functional characterization of Stat6. *Mol. Cell Biol.* 1996. **16**: 5811–5820.

35 **Alam, T., An, M. R. and Papaconstantinou, J.**, Differential expression of three C/EBP isoforms in multiple tissues during the acute phase response. *J. Biol. Chem.* 1992. **267**: 5021–5024.

36 **Xu, G., Zhang, Y., Zhang, L., Roberts, A. I. and Shi, Y.**, C/EBPbeta mediates synergistic upregulation of gene expression by interferon-gamma and tumor necrosis factor-alpha in bone marrow-derived mesenchymal stem cells. *Stem Cells* 2009. **27**: 942–948.

37 **Cardinaux, J. R., Allaman, I. and Magistretti, P. J.**, Pro-inflammatory cytokines induce the transcription factors C/EBPbeta and C/EBPdelta in astrocytes. *Glia.* 2000. **29**: 91–97.

38 **Descombes, P. and Schibler, U.**, A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* 1991. **67**: 569–579.

39 **Welm, A. L., Mackey, S. L., Timchenko, L. T., Darlington, G. J. and Timchenko, N. A.**, Translational induction of liver-enriched transcriptional inhibitory protein during acute phase response leads to repression of CCAAT/enhancer binding protein alpha mRNA. *J. Biol. Chem.* 2000. **275**: 27406–27413.

40 **Albina, J. E., Mahoney, E. J., Daley, J. M., Wesche, D. E., Morris, S. M., Jr. and Reichner, J. S.**, Macrophage arginase regulation by CCAAT/enhancer-binding protein beta. *Shock* 2005. **23**: 168–172.

41 **Xu, M., Nie, L., Kim, S. H. and Sun, X. H.**, STAT5-induced Id-1 transcription involves recruitment of HDAC1 and deacetylation of C/EBPbeta. *EMBO J.* 2003. **22**: 893–904.

42 **Gregory, P. D., Wagner, K. and Horz, W.**, Histone acetylation and chromatin remodeling. *Exp. Cell Res.* 2001. **265**: 195–202.

43 **Razeto, A., Ramakrishnan, V., Litterst, C. M., Giller, K., Griesinger, C., Carlomagno, T., Lakomek, N. et al.**, Structure of the NCoA-1/SRC-1 PAS-B domain bound to the LXXLL motif of the STAT6 transactivation domain. *J. Mol. Biol.* 2004. **336**: 319–329.

44 **Gingras, S., Simard, J., Groner, B. and Pfitzner, E.**, P300/CBP is required for transcriptional induction by interleukin-4 and interacts with Stat6. *Nucleic Acids Res.* 1999. **27**: 2722–2729.

45 **Arimura, A., vn Peer, M., Schroder, A. J. and Rothman, P. B.**, The transcriptional co-activator p/CIP (NCoA-3) is up-regulated by STAT6 and serves as a positive regulator of transcriptional activation by STAT6. *J. Biol. Chem.* 2004. **279**: 31105–31112.

46 **Kouzarides, T.**, Acetylation: a regulatory modification to rival phosphorylation? *EMBO J.* 2000. **19**: 1176–1179.

47 **Szanto, A., Balint, B. L., Nagy, Z. S., Barta, E., Dezso, B., Pap, A., Szeles, L. et al.**, STAT6 transcription factor is a facilitator of the nuclear receptor PPARgamma-regulated gene expression in macrophages and dendritic cells. *Immunity* 2010. **33**: 699–712.

48 **Ruffell, D., Mourkioti, F., Gambardella, A., Kirstetter, P., Lopez, R. G., Rosenthal, N. and Nerlov, C.**, A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair. *Proc. Natl. Acad. Sci. USA* 2009. **106**: 17475–17480.

49 **Hasselgren, P. O.**, Ubiquitination, phosphorylation, and acetylation–triple threat in muscle wasting. *J. Cell Physiol.* 2007. **213**: 679–689.

50 **Weisser, S. B., McLarren, K. W., Voglmaier, N., van Netten-Thomas, C. J., Antov, A., Flavell, R. A. and Sly, L. M.**, Alternative activation of macrophages by IL-4 requires SHIP degradation. *Eur. J. Immunol.* 2011. **41**: 1742–1753.

51 **Celada, A., Gray, P. W., Rinderknecht, E. and Schreiber, R. D.**, Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. *J. Exp. Med.* 1984. **160**: 55–74.

52 **Sanchez-Tillo, E., Comalada, M., Farrera, C., Valledor, A. F., Lloberas, J. and Celada, A.**, Macrophage-colony-stimulating factor-induced proliferation and lipopolysaccharide-dependent activation of macrophages requires raf-1 phosphorylation to induce mitogen kinase phosphatase-1 expression. *J. Immunol.* 2006. **176**: 6594–6602.

53 **Valledor, A. F., Xaus, J., Marques, L. and Celada, A.**, Macrophage colony-stimulating factor induces the expression of mitogen-activated protein kinase phosphatase-1 through a protein kinase C-dependent pathway. *J. Immunol.* 1999. **163**: 2452–2462.

54 **Snedecor, G. and Cochran, W.**, *Statistical methods*. Iowa State University Press, Ames, Iowa, 1967.

Abbreviations: **DRB**: 5,6-dichlorobenzimidazole riboside · **HAT**: histone acetyl-transferase · **HDAC**: histone deacetylase · **Id-1**: inhibitor of DNA-binding 1 · **LAP**: liver activator protein · **LIP**: liver inhibitor protein · **TSA**: Trichostatin A

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MAJOR ARTICLE

Arginine Transport Is Impaired in C57Bl/6 Mouse Macrophages as a Result of a Deletion in the Promoter of *Slc7a2* (CAT2), and Susceptibility to *Leishmania* Infection Is Reduced

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Host genetic factors play a crucial role in immune response. To determine whether the differences between C57Bl/6 and BALB-C mice are due only to the production of cytokines by T-helper 1 cells or T-helper 2 cells, we obtained bone marrow–derived macrophages from both strains and incubated them with these cytokines. Although the induction of *Nos2* and *Arg1* was similar in the 2 strains, infectivity to *Leishmania major* differed, as did macrophage uptake of arginine, which was higher in BALB-C macrophages. The levels of interferon γ– and interleukin 4–dependent induction of the cationic amino acid transporter *SLC7A2* (also known as "cationic amino acid transporter 2," or "CAT2") were decreased in macrophages from C57Bl/6 mice. This reduction was a result of a deletion in the promoter of one of the 4 AGGG repeats. These results demonstrate that the availability of arginine controls critical aspects of macrophage activation and reveal a factor for susceptibility to *Leishmania* infection.

Keywords. macrophage; *Leishmania*; arginine; amino acid transporter; immune response; susceptibility.

Through epidemiological and population studies, it has been established that, for many pathogens, host genetic factors play an important role in the onset and progression of infection, the type of disease that develops, and the ultimate outcome of infection [1]. In some murine models of human pathogens, the pathogenesis of the infection and the immune response are extremely well reproduced [2, 3].

Host genetics modulate the clinical manifestations of patients with diverse infections, such as leishmaniasis [4]. Human leishmaniasis has been mimicked in the laboratory by infection of mice with *Leishmania major*. Most mouse strains control *L. major* infection, but some, such as BALB-C, develop progressive lesions and systemic disease [5]. The genetic predisposition to this infection in mice correlates with the production of cytokines (mostly interleukin 4 [IL-4]) by T-helper 2 (Th2) cells, while resistance corresponds to cytokines (predominantly interferon γ [IFN-γ]) produced by T-helper 1 (Th1) cells [3]. However, recent data have challenged the simplicity of this model and have revealed a much greater complexity in the mechanisms of acquired resistance [5, 6].

Treatment of macrophages with Th2 cytokines induces alternative activation of macrophages, or M2, as opposed to Th1, which is termed classical activation, or M1 [7]. The way in which arginine is catabolized is crucial. Th2 cytokines induce arginase 1, which degrades arginine in polyamines and proline that are

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used for cellular proliferation and collagen production. In contrast, one of the hallmarks of M1 activation is the generation of nitric oxide (NO) by an inducible nitric oxide synthase (NOS2) [8].

The cationic amino acid transporter (CAT) family is composed of CAT-1, -2A, -2B, and -3. The substrate transported is almost identical for cationic amino acids. The major difference between these transporters is at the level of tissue specificity and regulation of their expression. In mouse macrophages, *Slc7a1* is constitutively expressed and is not modified by activating agents, while *Slc7a3* is not detected, and *Slc7a2a* is induced during M1 and M2 activation [9].

To determine whether the differences between C57Bl/6 and BALB-C mice are due only to the production of Th1 or Th2 cytokines, we obtained bone marrow–derived macrophages from both strains and incubated them with these cytokines. Infectivity to *L. major* differed, as did macrophage uptake of arginine. The decreased arginine transport in C57Bl/6 mice was due to a reduction in the expression of the transport system gene *Slc7a2* (CAT2) [9] as a result of a deletion in the promoter.

METHODS

Reagents

Recombinant IL-4, interleukin 10 [IL-10], and IFN-γ were purchased from R&D (Minneapolis, MN). All the other products were of the highest grade available and were purchased from Sigma.

Mice and Cell Culture

BALB-C and C57Bl/6 mice were purchased from Charles River Laboratories (Wilmington, MA), and 6–8-week-old females were used in accordance with a protocol that was approved by the Animal Research Committee of the University of Barcelona (number 2523). Bone marrow–derived macrophages were isolated as described elsewhere [9].

Determination of *L. Major* Growth In Vitro

L. major LV39 (MRHO/SU/59/P-strain) was kindly provided by Dr I. Muller (Imperial College London, United Kingdom). In vitro studies with *L. major* were carried out as described elsewhere [10]. Macrophages were activated in the presence or in the absence of 100 μM of nor-NOHA (Bachem, Switzerland). After 4 hours the cultures were infected with *L. major* parasites. After 96 hours, the macrophages were washed and lysed, and a limiting dilution assay was performed to determine the number of viable parasites.

Determination of Arginase Activity and NO Production

Arginase activity was measured in macrophage lysates as described elsewhere [11]. Cells were lysed and arginine hydrolysis was conducted by incubating the lysate with L-arginine at 37°C. NO was measured as nitrite using the Griess reagent [11].

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Real-time PCR was performed as described elsewhere [12]. Data were expressed relative to β-actin. The primer sequences are described in the Supplementary Materials.

Catabolism of L-Arginine

Catabolism of arginine was determined as described [13] by incubating macrophages with L-[U-^{14}C]arginine. The catabolic products were evaluated by thin-layer chromatography.

Arginine Transport

Transport of L-[3^{H}]arginine (Amersham) was measured as described elsewhere, using radioactive arginine [9, 14].

Transfection of Small-Interfering RNA (siRNA)

siRNA was obtained from Dharmacon and transfected by electroporation as described elsewhere [15].

Northern Blot

Total RNA was extracted and separated by electrophoreses in an agarose/formaldehyde gel as described elsewhere [16]. Samples were transferred by capillarity to a nylon membrane. RNA was then fixed in the membrane by UV irradiation. The probe was prepared as indicated in the Supplementary Materials.

Transient Transfection and Dual-Reporter *Renilla* Luciferase Assays

The construction of reported plasmids is described in the Supplementary Materials. For *Renilla* luciferase assays, RAW264.7 cells were used as described elsewhere [15].

Electrophoretic Mobility Shift Assay (EMSA)

Cells were lysed and nuclear extracts obtained, and an EMSA was performed as described [15]. The probes were synthesized by Sigma and correspond to a STAT6 binding element 2.86 kb upstream of the *Arg1* transcription start site [17].

Promoter Analysis

To determine the promoter(s) used to transcribe *CTN-RNA*, total RNA was reverse transcribed using a RT primer specific designed from the unique 3′ untranslated region (UTR). These complementary DNAs were further amplified by PCR, using forward PCR primers from each of the exon 1 variants representing promoters A–E [18] and a reverse primer from exon 3.

Statistical Analyses

Experimental results were analyzed using a 2-tailed Mann–Whitney *U* test and the Wilcoxon paired test [19].

RESULTS

Distinct Responses in Macrophages of BALB-C and C57Bl/6 Mice

To determine whether the distinct susceptibility of BALB-C and C57Bl/6 mice to leishmaniasis is attributable exclusively to the cytokines produced, we incubated bone marrow–derived macrophages produced in vitro with Th1 or Th2 cytokines. These primary cultures of macrophages represent a homogeneous population of cells that respond in vitro to activating stimuli. Using this approach, we circumvented the distinct cytokine production profiles of the 2 strains by comparing only the response of macrophages. Macrophages were stimulated with IFN-γ, and 4 hours later the cultures were infected with *L. major*. After 96 hours, these cells were washed and lysed, and a limiting dilution assay was performed to determine the number of viable parasites. Interestingly, C57Bl/6 macrophages showed *L. major* proliferation similar to that of BALB-C macrophages (Figure 1*A*). However, under IL-4 incubation, parasites showed a higher proliferation in BALB-C than in C57Bl/6 macrophages (Figure 1*A*). We have shown [10] that treatment of macrophages with N^{ω}-hydroxy-nor-L-arginine (nor-NOHA) inhibits the activity of arginase and, therefore, the conversion of L-arginine into ornithine and spermine, both of which are required for *L. major* growth. In our conditions, nor-NOHA abolished the effect of IL-4 on parasite growth in the macrophages of both strains, thereby suggesting that arginase is critical for this growth.

The differences in parasite growth observed in the macrophages could be attributed to the distinct expression of arginase in BALB-C and C57Bl/6 mice. However, no differences in the induction of messenger RNA (mRNA) between these 2 phenotypes were found when macrophages were incubated with IL-4 alone or with IL-4 plus IL-10 (Figure 1*B*). To explore the extent of arginase activity, we determined the production of urea [20]. After activating macrophages, we lysed them and then added arginine. Again, no differences were found between macrophages from the 2 mouse strains (Figure 1*B*).

Next, we determined the catabolism of arginine when macrophages were induced by IL-4. After activation, these cells were incubated with radiolabeled arginine for 2 and 6 hours. The products of degradation were then resolved using thin-layer chromatography. Although arginase activity was similar in macrophages from both strains, the consumption of arginine was lower in the C57Bl/6 strain, while the production of ornithine, citrulline, spermine, and proline was higher in BALB-C mice (Figure 1*C*). As macrophages do not accumulate putrescine, spermidine, or glutamate, no differences were detected between the 2 strains.

Given that NO plays a major role in killing *L. major* [8], we examined the expression of *Nos2* in C57Bl/6 and BALB-C macrophages after activation with IFN-γ or after addition of lipopolysaccharide (LPS). No differences were found in the expression of *Nos2* (Figure 1*D*) or its protein (data not shown). Interestingly, a significant difference was observed in NO production. BALB-C macrophages produced more NO than C57Bl/6 macrophages (Figure 1*D*).

Distinct Arginine Transport in Macrophages From BALB-C and C57Bl/6 Mice

So far, the data showed that although the amounts of NOS2 and arginase 1 were similar in macrophages from the C57Bl/6 and BALB-C mouse strains, there was a significant difference in the catabolism of arginine as NO or as proline and spermine. This finding implies that if these strains show similar amounts of these enzymes and fewer products of the catabolism of the substrate, the amount of arginine inside the macrophages of these strains differs. This notion led us to further explore the transport of this amino acid in macrophages. For this purpose, we determined the uptake of radiolabeled arginine by these cells in the 2 mouse phenotypes [9]. Several transporters handle arginine in macrophages. We previously showed that in basal conditions, >75% of the total transport rate corresponds to system y^+L. There is a second component of arginine transport into macrophages that is insensitive to L-leucine, even in the presence of Na^+, which is inhibited by treatment with the sulfhydryl-specific reagent N-ethyl maleimide (NEM). This NEM-sensitive component corresponds to system y^+. The participation of the B^{o+} and b^{o+} systems was excluded by measuring transport in medium with or without sodium [9, 14].

BALB-C macrophages treated with Th1 or Th2 cytokines showed a drastic increase in arginine transport. This effect was not inhibited by treatment with NEM, thereby indicating that the increase was due to system y^+L (Supplementary Figure 1). Given that the treatment with cytokines did not modify the amount of arginine transported by system y^+L, we calculated the difference that corresponds to the inducible y^+ system (Figure 2*A*). In C57Bl/6 macrophages, the cytokines also induced an increase in arginine transport through system y^+, although it was significantly less (Figure 2*A*). These results explain why parasite growth decreased in BALB-C macrophages stimulated with Th1 cytokines and showing an increased production of NO. In contrast, in the presence of Th2 cytokines and, thus, an increased production of polyamines, which are required for the growth of the parasite [21], the number of parasites increased.

Distinct Induction of *Slc7a2* in Activated Macrophages From BALB-C and C57Bl/6 Mice

We showed that the increase in arginine transport induced in the macrophages by the 2 types of cytokines was mediated by the y^+ system and that the gene induced was *Slc7a2* [9, 22]. Using quantitative PCR, we determined the induction of this gene in macrophages from the 2 mouse phenotypes. Th1 and

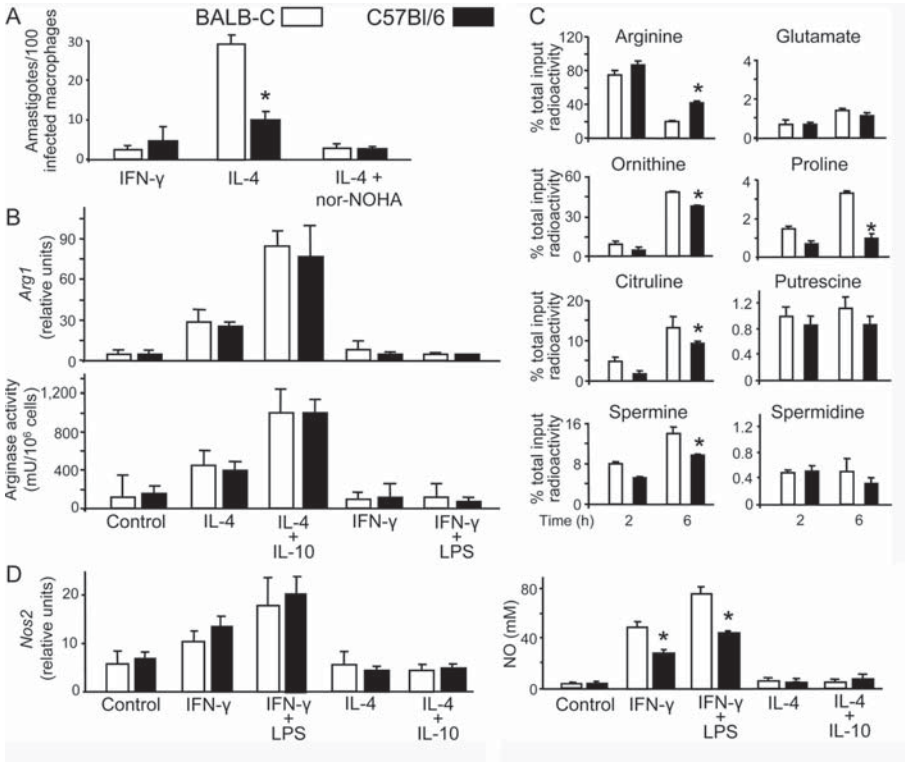

Figure 1. Distinct infectivity to *Leishmania major* in activated macrophages from BALB-C and C57Bl/6 mice. *A*, Macrophages from BALB-C or C57Bl/6 mice were stimulated with interleukin 4 (IL-4; 10 ng/mL), with or without nor-NOHA, or interferon γ (IFN-γ; 10 ng/mL) and infected with *L. major* parasites. After 96 hours, the number of viable parasites was determined by limiting dilution analysis. *B*, Expression of *Arg1* and arginase activity was measured in macrophages cultured for 24 hours in the absence or presence of IL-4 alone or IL-4 plus IL-10 (10 ng/mL) or in the presence of IFN-γ alone or IFN-γ plus lipopolysaccharide (LPS; 10 ng/mL). *C*, Degradation of arginine was determined in macrophages stimulated with IL-4. *D*, Expression of *Nos2* and NO production was measured in macrophages under the same conditions as in panel *B*. In all figures, data are representative of at least 4 experiments. Each determination was made in triplicate, and the values are mean + SD. **P* < .01 when the results of the 4 experiments were compared.

Th2 cytokines induced the expression of *Slc7a2* in both strains; however, in BALB-C mice this induction was greater (Figure 2*B*).

To determine the contribution of *Slc7a2* to the functional activities of macrophages, we inhibited its expression, using siRNA (Figure 2*C*). The macrophages of both animal models showed a significant decrease in *Slc7a2* expression, as well as in the amount of arginine taken up (Figure 2*C*). As a functional consequence of *Slc7a2* inhibition, the amount of NO produced in response to treatment with IFN-γ, with or without LPS, was drastically reduced (Figure 2*C*), without modifications in the amount of *Nos2* induced or *Arg1* (Figure 3). This observation confirmed our previous results obtained with the *Slc7a2* knockout model [9]. These findings demonstrate that the differences in the functional activity of macrophages of these 2 strains of mice are due to the differential expression of the *Slc7a2* arginine transporter.

Slc7a2 has various isoforms, the expression of which depends on the use of 5′ and 3′ untranslated regions. However, the translated region is the same for all transcripts. In macrophages, the 5′ untranslated region used is 1A. Independently of the 5′ region transcribed, the 3′ region differs in length as a result of the presence of 2 distinct polyadenylation sites. These sites are separated by almost 4 kb, and they determine the length of the 2 isoforms expressed in macrophages, which have been identified as *CTN-RNA* and *mCAT-2* (8 and 4.4 kb, respectively) [23]. The isoform *CTN-RNA* is diffusely distributed in nuclei and is also localized in paraspeckles [23]. Under stress, *CTN-RNA* is posttranscriptionally cleaved to produce protein-coding *mCAT-2* mRNA. In our experiments, we used an exon probe hybridizing both *CTN-RNA* and *mCAT-2* [23]. As described in several cell lines, including macrophages [23], no detectable mRNA was found in the nontransformed macrophages before activation, as shown by Northern blot findings.

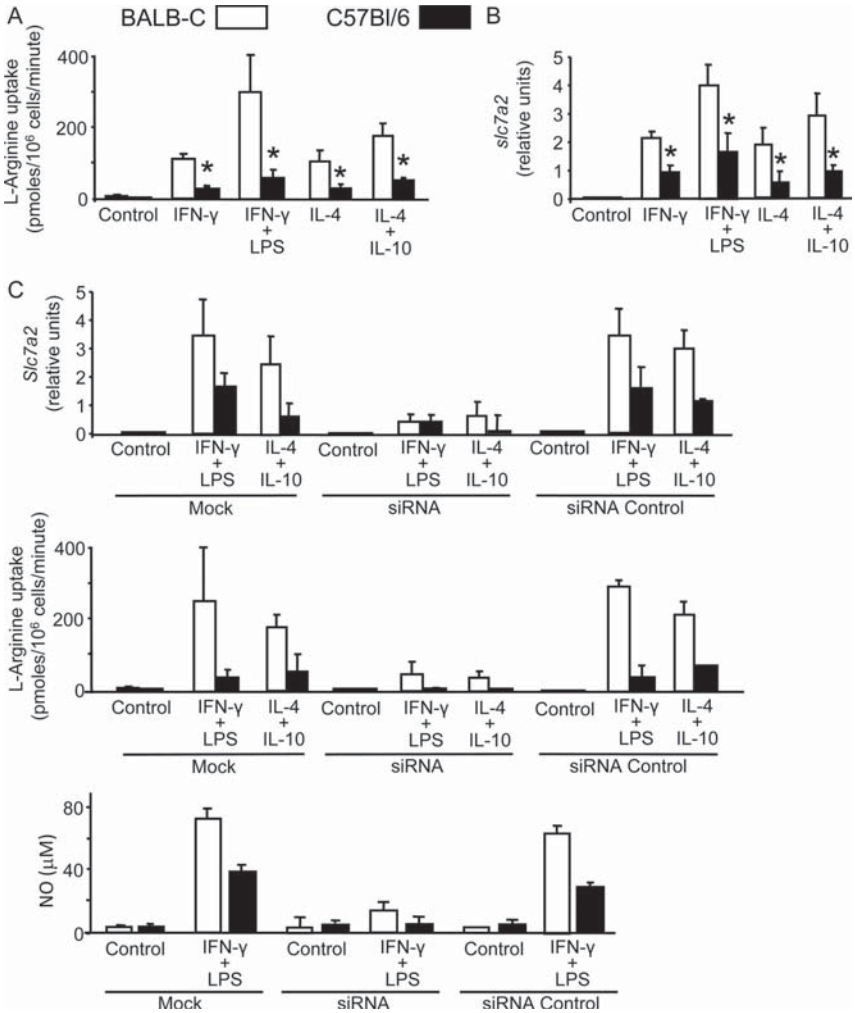

Figure 2. BALB-C macrophages show higher arginine transport than those of the C57Bl/6 strain. *A*, Arginine transport mediated by the y^+ system was measured in macrophages cultured for 24 hours in the absence or presence of interferon γ (IFN-γ), with or without lipopolysaccharide (LPS), or in the presence of interleukin 4 (IL-4), with or without interleukin 10 (IL-10). *B*, The expression of *Slc7a2* was measured by quantitative reverse transcription polymerase chain reaction, using macrophages treated in the same conditions as in panel *A*. *C*, Macrophages were electroporated with small-interfering RNA (siRNA) to *Slc7a2*, scrambled control siRNA, or medium (Mock). They were then activated as in panel *A*, and the levels of *Slc7a2*, arginine uptake, and NO production were measured.

As a control, we used mRNA from the liver, which expressed both species of mRNA (Figure 4*A*). Incubation of macrophages from both strains with Th1 or Th2 cytokines induced mRNA, *CTN-RNA*, and *mCAT-2* (Figure 4*A*). However, BALB-C cells showed greater amounts of these products than C57Bl/6 macrophages. To exclude different kinetics of *CTN-RNA* and *mCAT-2* induction in the 2 strains, we performed time-course experiments. After 3 hours of incubation with IL-4, macrophages simultaneously expressed both species of mRNA, reaching maximum expression at 6 hours (Figure 4*B*). On the basis of these results, we conclude that there is a quantitative difference in the expression of *CTN-RNA* and *mCAT-2* between C57Bl/6 and BALB-C mice, thereby confirming the results involving arginine transport and quantitative PCR. In the absence of available antibody, the distinct amounts of mRNA shown by the 2 mouse strains could explain the differences in the functional capacities of macrophages in these phenotypes.

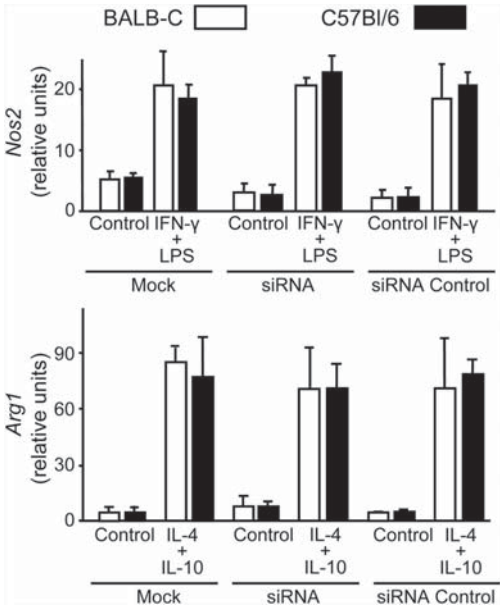

Figure 3. Inhibition of *Slc7a2* does not inhibit the expression of *Nos2* or *arginase 1*. Macrophages were electroporated with small-interfering RNA (siRNA) to *Slc7a2,* scrambled control siRNA, or medium (Mock). They were then cultured for 24 hours in the absence or presence of interferon γ (IFN-γ) plus lipopolysaccharide (LPS) or in the presence of interleukin 4 (IL-4) plus interleukin 10 (IL-10). *Nos2* and *Arg1* expression was then measured using quantitative PCR. Data are representative of at least 4 experiments. Each determination was made in triplicate, and values are mean ± SD.

To confirm the role of the SLC7A2 arginine transporter in the infectivity of macrophages to *Leishmania*, we inhibited *Slc7a2* expression, using siRNA. While infectivity was not reduced in macrophages from C57Bl/6 mice treated with IL-4, it was drastically diminished in macrophages from BALB-C mice (Figure 4*C*).

A Deletion in the *Slc7a2* Promoter of C57Bl/6 Mice Impairs the Cytokine-Based Induction of *Slc7a2*

To examine whether the increase in *Slc7a2* expression induced by cytokine treatment occurred at the transcriptional level or was due to mRNA stabilization, we determined the half-life of *Slc7a2* transcripts in cells treated with IFN-γ and LPS. Macrophages were treated with IFN-γ and LPS for 9 hours, thereby inducing *Slc7a2*. Actinomycin D was then added at a concentration sufficient to block all further mRNA synthesis, as determined by [3H]UTP incorporation [15]. We then isolated mRNA from aliquots of cells at a range of intervals. Northern blot measurement of *Slc7a2* expression allowed us to estimate that the half-life of *CTN-RNA* and *mCAT-2* in resting cells was very stable (Figure 5*A*). Treatment with IFN-γ and LPS did not modify the stability of *CTN-RNA* or *mCAT-2*, thus indicating that the induction of *Slc7a2* in response to cytokines was at the transcriptional level. Similar results were found when macrophages were activated with IL-4 and IL-10 (Figure 5*A*).

For the transcription of *Slc7a2,* multiple promoters (A–E, each with a unique exon variant, exons 1A–1E, respectively, composing the 5′ UTRs) are used in a tissue-specific manner [18]. PCR analysis showed that *CTN-RNA* was exclusively transcribed by the distal promoter A in macrophages from both strains of mice (Figure 5*B*), as described in the macrophage-like cell line RAW264.7 [23].

To determine the presence of mutations in the regulatory region next to exon 1A, we sequenced 1300 nucleotides of the *Slc7a2* promoter from both mouse strains. Alignment of these sequences allowed us to observe that, 352–348 bp upstream of exon A, the sequence AGGG was absent in C57Bl/6 mice but present in BALB-C mice (Figure 6*A*). Interestingly, these 4 bases in BALB-C mice were repeated 4 times while the C57Bl/6 strain had only 3 repeats. These sequences are binding sites for a number of transcription factors, such as SP1, LYF1, and MZF1, and the AGGG deletion abrogates the binding of these factors [24].

Next, we analyzed the functional activity of the A promoters in BALB-C and C57B1/6 mice. For this purpose, a fragment from their A promoters was linked to the luciferase reporter gene. Because of the difficulty in transfecting nontransformed macrophages, we used the macrophage-like cell line RAW264.7. The vectors were transfected, and luciferase activity was measured. Each construct was cotransfected with the *Renilla* expression vector. All luciferase activity values were normalized to the level of *Renilla* expression to correct for any differences in transfection efficiency. In unstimulated macrophages, the construct comprising 1193 bp of the A promoter of BALB-C and C57Bl/6 mice showed little activity (Figure 6*B*). However, macrophage stimulation with Th1 or Th2 cytokines induced high expression of the promoter of BALB-C mice. In contrast, induction was low when the same treatments were made with the promoter of the C57Bl/6 strain. To confirm that the deletion of AGGG was responsible for the decreased activity of *Slc7a2* in the macrophages of C57Bl/6 mice, we deleted the AGGG motif in the promoter of BALB-C mice. The promoter with this mutation was not induced when macrophages were treated with either IFN-γ or IL-4 (Supplementary Figure 2). To determine the areas of the promoter that are important for induction by Th1 or Th2 cytokines, we performed several deletions. An area between −773 and −473 bp was observed to be critical to elicit induction (Figure 6*B*). However, the 4 AGGG repeats are also required, because mutation of one of these repeats abolished the induction. Therefore, we conclude that the deletion of the AGGG motif is responsible for the distinct expression of *CTN-RNA* and *mCAT-2* shown by BALB-C and C57Bl/6 macrophages.

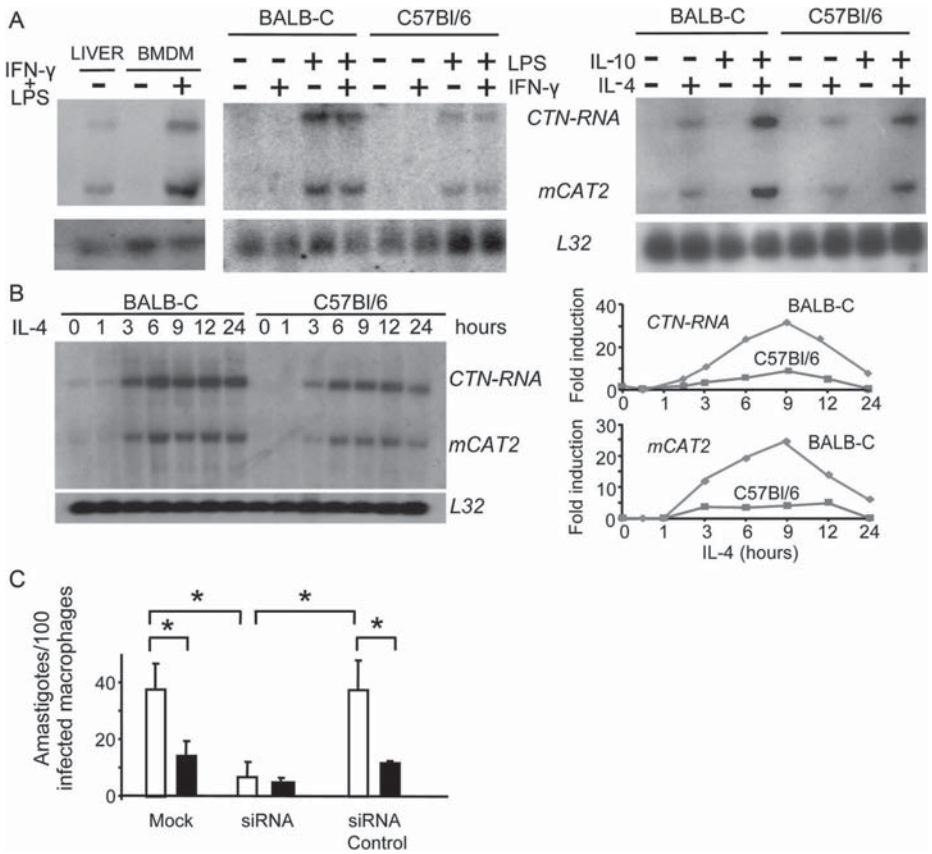

Figure 4. Increased expression of *Slc7a2* by macrophages from BALB-C mice, compared with macrophages from C57Bl/6 mice. *A*, Northern blot analysis of total RNA, using an exon 11 probe that detects both the 8-kb *CTN-RNA* and the 4.2-kb *mCAT-2* isoforms in liver and activated macrophages. Macrophages were cultured for 12 hours in the absence or presence of interferon γ (IFN-γ), with or without lipopolysaccharide (LPS), or in the presence of interleukin 4 (IL-4), with or without interleukin 10 (IL-10). *B*, Time-course determination of *Slc7a2* in macrophages treated with IL-4. The quantification of *CTN-mRNA* and *mCAT-2* is shown at the bottom. *C*, Infectivity of macrophages with *Leishmania major* parasites depends on SLC7A2 expression. Macrophages were treated with IL-4, and the experiment proceeded as described in Figures 1*A* and 2*C*.

To establish whether distinct protein complexes were associated with the promoters of BALB-C and C57Bl/6 mice, we performed gel electrophoresis DNA binding assays. Nuclear extracts were prepared from IFN-γ–treated macrophages from the 2 mouse strains. When the extracts of these cells were incubated with a probe corresponding to the BALB-C mice, 2 types of DNA-protein complexes were obtained, one weak and the other strong. These complexes were obtained when we used either the nuclear extracts from macrophages from BALB-C or C57Bl/6 mice (Figure 6*C*). When we used a probe composed of oligonucleotide corresponding to the region where the AGGG motif was deleted, only the weak band was detected, while the stronger one was absent. Given that no differences were found using proteins of either of the 2 mouse strains, we conclude that the DNA-binding proteins are present in the nuclear extracts of macrophages from BALB-C and C57B1/6 mice and that the defect of the binding proteins that bound to the C57B1/6 promoter is due to the deletion in the AGGG motif.

DISCUSSION

Using cultures in vitro of macrophages, we reveal that arginine transport is a critical factor for genetic predisposition to *L. major* infection in animal models and that this transport system could partly explain the different susceptibility of BALB-C and C57Bl/6 strains of mice to this infection. Our results demonstrate that arginine transport in activated BALB-C and C57Bl/6 macrophages differs. These differences were due to differentially transcribed *Slc7a2*, which encodes a cationic amino acid transporter called CAT2. The decreased expression

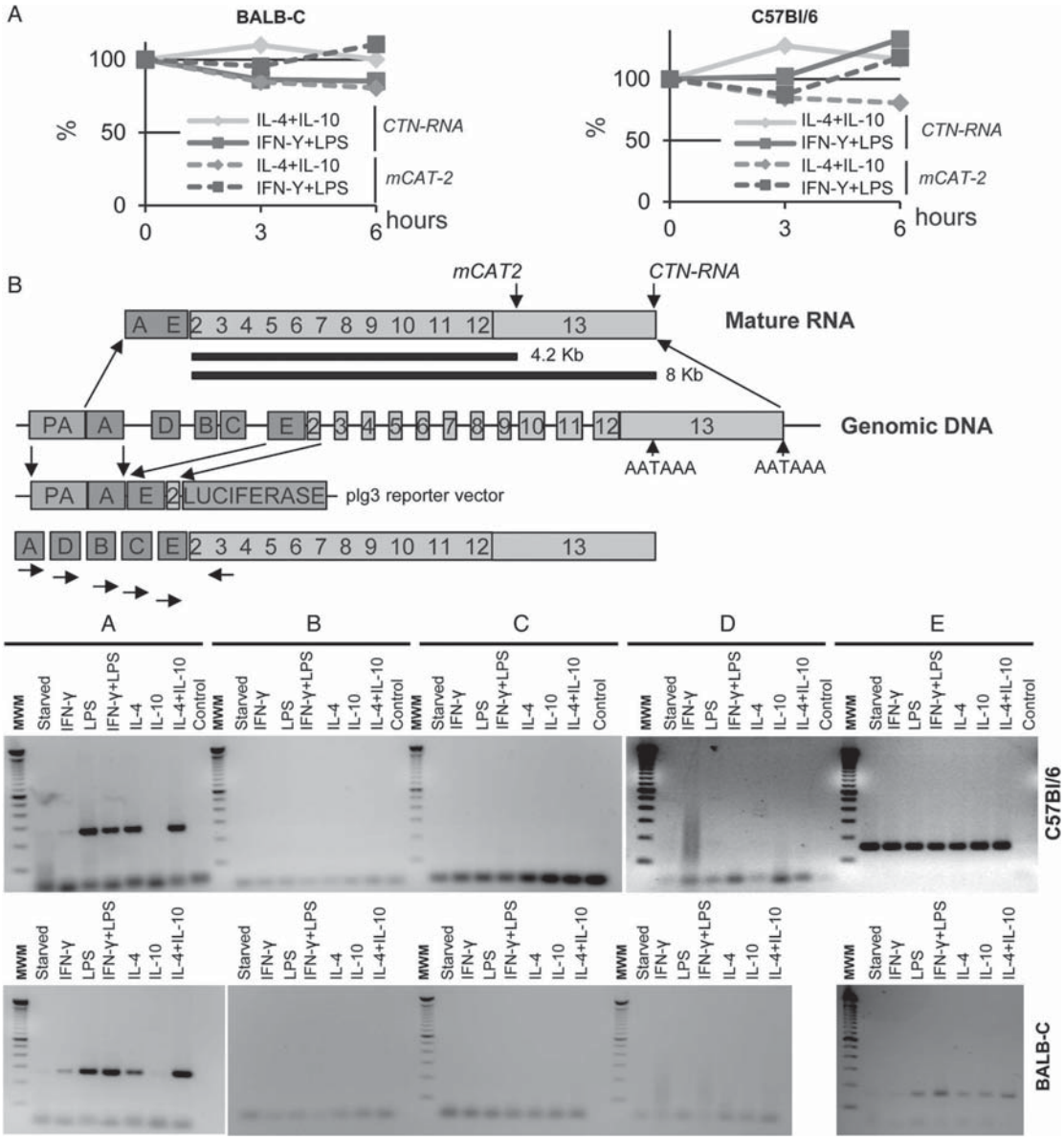

Figure 5. *Slc7a2* is induced transcriptionally in macrophages through promoter A. *A,* Macrophages from BALB-C and C57Bl/6 mouse strains were treated with the indicated activators for 6 hours, and then DRB (20 μg/mL) and actinomycin D (5 μg/mL) were added. CTN-RNA and mCAT-2 were measured by Northern blot after the indicated times. Cell viability was >95% for all culture conditions. The figure shows 1 representative result of 3 independent experiments. *B,* Promoter analysis of CTN-RNA was performed using reverse transcription (RT) primers from a CTN-RNA–specific region, followed by polymerase chain reaction (PCR) with all of the exon 1 variant–specific primer pairs. On top, a map of *Slc7a2* is shown. Macrophages from both strains of mice were treated with interferon γ (IFN-γ) or interleukin 4 (IL-4) for 9 hours. "Starved" denotes macrophages cultured in the absence of cytokines. "Control" represents the PCR result without complementary DNA. The schematic representation of mCAT-2 and CTN-RNA is shown at the top. The arrows represent the primer pairs used to amplify the RT products, with specific forward primers for each exon 1 variant (representing promoters A–E with unique exons 1A–E, respectively) and a common reverse primer (from exon 3). Abbreviations: IL-10, interleukin 10; LPS, lipopolysaccharide.

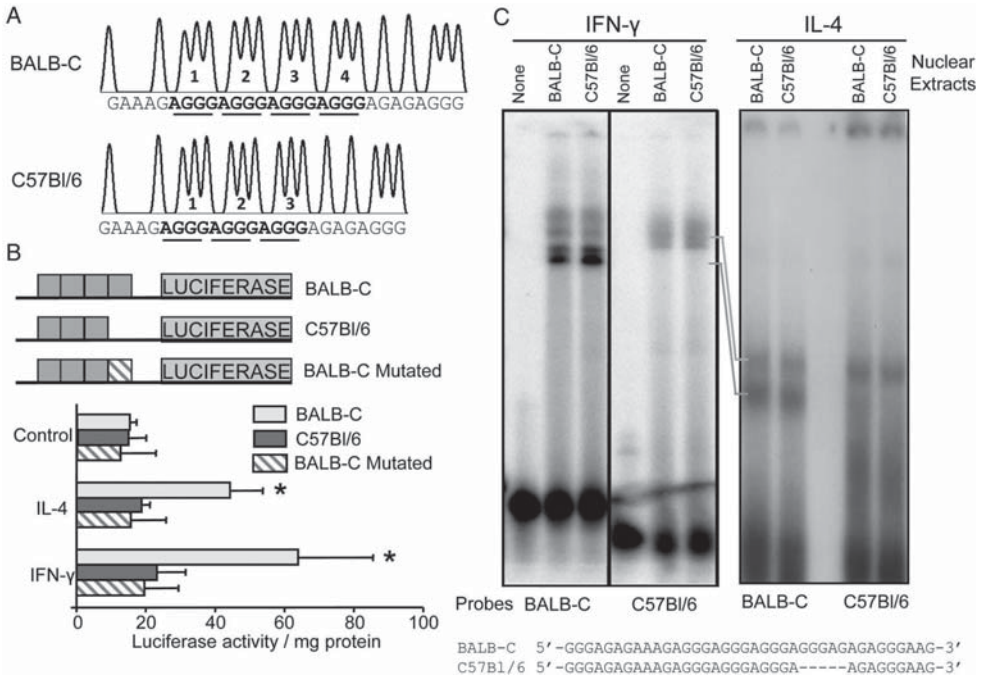

Figure 6. The decreased expression of *Slc7a2* by macrophages from C57Bl/6 mice is due to a deletion in the promoter. *A,* Sequencing analysis of the A promoter of *Slc7a2* in macrophages from BALB-C and C57Bl/6 strains. *B,* The expression of *Slc7a2* A promoter was determined in the macrophage-like cell line RAW264.7 transiently transfected with the reporter plasmids containing the A promoter from BALB-C and C57Bl/6 strains, as well as from BALB-C mice with the deletion found in the C57Bl/6 strain. *C,* For the DNA-binding assays, we used fragments of the A promoter of the BALB-C and C57Bl/6 strains as probes. The sequences of the oligonucleotides are indicated in the figure. Nuclear extracts were obtained from macrophages from both strains of mice treated for 9 hours with interferon γ (IFN-γ) or interleukin 4 (IL-4).

of *Slc7a2* in C57Bl/6 cells was caused by a deletion in the promoter in an area with 4 AGGG repeats. This deletion abrogates the formation of a palindromic sequence where several transcription factors, such as SP1, LYF1, or MZF1, can bind [25].

Arginine is critical for the innate immune response. This essential amino acid is required for macrophage growth [26] and for M1 and M2 activation [9]. When macrophages are alternatively activated, arginase 1 is produced, and then arginine is degraded to proline and polyamines, both types of molecules being required for *L. major* growth [10]. Our experiments point to a link between decreased arginine uptake and reduced *L. major* growth. Nevertheless, to be certain of this relationship, we should compare the growth of *L. major* in identical macrophages with 4 or 3 AGGG repeats in the promoter of *Slc7a2*. However, this experiment is technically impossible. The data presented here provide a new explanation for the susceptibility of BALB-C mice to intracellular parasite replication in nonhealing *L. major* infections. In this mouse model, the amount of arginine that entered the macrophages was much higher than that in C57Bl/6 mice. This increased uptake may favor the growth of *Leishmania* organisms, thereby serving as another factor that influences susceptibility to the disease.

To evade immune responses, some pathogens generate their own arginases [10, 27, 28] or induce arginase expression in the host [29, 30]. Arginine then becomes limited for the production of NO by NOS2, an essential mechanism for host defense against many pathogens [8]. Production of a high level of arginase 1 blocks the immune response locally at the site of pathology, causing local depletion of arginine, which impairs the capacity of T cells in the lesion to proliferate and produce IFN-γ [6].

The catabolism of arginine by macrophages has emerged as a critical mechanism for the regulation of the immune response, not only in *L. major* infection but also in several other parasitic diseases [31, 32]. If the amount of arginine available is important, then the system through which this amino acid is introduced into macrophages is also critical. After interaction with cytokines, these cells show a considerable increase in arginine cellular uptake as a result of the induction of the SLC7A2 transport system, which is the limiting factor for NO production

and for arginine catabolized by arginase 1 [9, 22]. Our results demonstrate that the availability of arginine is a factor in the susceptibility to *Leishmania* infection.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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G. S., A. Y., S. P. L., and L. S. B. performed experiments; M. M. performed experiments and supervised the research; and J. L. and A. C. designed and supervised the research and wrote the manuscript.

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Potential conflict of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References

1. Hill AV. Aspects of genetic susceptibility to human infectious diseases. Annu Rev Genet **2006**; 40:469–86.
2. Lipoldova M, Demant P. Genetic susceptibility to infectious disease: lessons from mouse models of leishmaniasis. Nat Rev Genet **2006**; 7:294–305.
3. Solbach W, Laskay T. The host response to *Leishmania* infection. Adv Immunol **2000**; 74:275–317.
4. Blackwell JM, Fakiola M, Ibrahim ME, et al. Genetics and visceral leishmaniasis: of mice and man. Parasite Immunol **2009**; 31:254–66.
5. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat Rev Immunol **2002**; 2:845–58.
6. Modolell M, Choi BS, Ryan RO, et al. Local suppression of T cell responses by arginase-induced L-arginine depletion in nonhealing leishmaniasis. PLoS Negl Trop Dis **2009**; 3:e480.
7. Gordon S. Alternative activation of macrophages. Nat Rev Immunol **2003**; 3:23–35.
8. Bogdan C. Oxidative burst without phagocytes: the role of respiratory proteins. Nat Immunol **2007**; 8:1029–31.
9. Yeramian A, Martin L, Serrat N, et al. Arginine transport via cationic amino acid transporter 2 plays a critical regulatory role in classical or alternative activation of macrophages. J Immunol **2006**; 176:5918–24.
10. Kropf P, Fuentes JM, Fahnrich E, et al. Arginase and polyamine synthesis are key factors in the regulation of experimental leishmaniasis in vivo. Faseb J **2005**; 19:1000–2.
11. Classen A, Lloberas J, Celada A. Macrophage activation: classical versus alternative. Methods Mol Biol **2009**; 531:29–43.
12. Marques L, Brucet M, Lloberas J, Celada A. STAT1 regulates lipopolysaccharide- and TNF-alpha-dependent expression of transporter associated with antigen processing 1 and low molecular mass polypeptide 2 genes in macrophages by distinct mechanisms. J Immunol **2004**; 173: 1103–10.
13. Comalada M, Yeramian A, Modolell M, Lloberas J, Celada A. Arginine and macrophage activation. Methods Mol Biol **2012**; 844:223–35.
14. Martin L, Comalada M, Marti L, et al. Granulocyte-macrophage colony-stimulating factor increases L-arginine transport through the induction of CAT2 in bone marrow-derived macrophages. Am J Physiol Cell Physiol **2006**; 290:C1364–72.
15. Serra M, Forcales SV, Pereira-Lopes S, Lloberas J, Celada A. Characterization of Trex1 induction by IFN-gamma in murine macrophages. J Immunol **2011**; 186:2299–308.
16. Valledor AF, Xaus J, Marques L, Celada A. Macrophage colony-stimulating factor induces the expression of mitogen-activated protein kinase phosphatase-1 through a protein kinase C-dependent pathway. J Immunol **1999**; 163:2452–62.
17. Gray MJ, Poljakovic M, Kepka-Lenhart D, Morris SM, Jr. Induction of arginase I transcription by IL-4 requires a composite DNA response element for STAT6 and C/EBPbeta. Gene **2005**; 353:98–106.
18. Finley KD, Kakuda DK, Barrieux A, Kleeman J, Huynh PD, MacLeod CL. A mammalian arginine/lysine transporter uses multiple promoters. Proc Natl Acad Sci U S A **1995**; 92:9378–82.
19. Snedecor G, Cochran W. Statistical methods. Ames, Iowa: Iowa State University Press, **1967** (edition t, ed.).
20. Munder M, Eichmann K, Modolell M. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4+ T cells correlates with Th1/Th2 phenotype. J Immunol **1998**; 160:5347–54.
21. Roberts SC, Tancer MJ, Polinsky MR, Gibson KM, Heby O, Ullman B. Arginase plays a pivotal role in polyamine precursor metabolism in Leishmania. Characterization of gene deletion mutants. J Biol Chem **2004**; 279:23668–78.
22. Nicholson B, Manner CK, Kleeman J, MacLeod CL. Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. J Biol Chem **2001**; 276:15881–5.
23. Prasanth KV, Prasanth SG, Xuan Z, et al. Regulating gene expression through RNA nuclear retention. Cell **2005**; 123:249–63.
24. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res **1995**; 23:4878–84.
25. Ihn H, LeRoy EC, Trojanowska M. Oncostatin M stimulates transcription of the human alpha2(I) collagen gene via the Sp1/Sp3-binding site. J Biol Chem **1997**; 272:24666–72.
26. Yeramian A, Martin L, Arpa L, et al. Macrophages require distinct arginine catabolism and transport systems for proliferation and for activation. Eur J Immunol **2006**; 36:1516–26.
27. Das P, Lahiri A, Chakravortty D. Modulation of the arginase pathway in the context of microbial pathogenesis: a metabolic enzyme moonlighting as an immune modulator. PLoS Pathog **2010**; 6:e1000899.
28. Fitzpatrick JM, Fuentes JM, Chalmers IW, et al. *Schistosoma mansoni* arginase shares functional similarities with human orthologs but depends upon disulphide bridges for enzymatic activity. Int J Parasitol **2009**; 39:267–79.
29. El Kasmi KC, Qualls JE, Pesce JT, et al. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol **2008**; 9:1399–406.
30. Qualls JE, Neale G, Smith AM, et al. Arginine usage in mycobacteria-infected macrophages depends on autocrine-paracrine cytokine signaling. Sci Signal **2010**; 3:ra62.
31. Hesse M, Modolell M, La Flamme AC, et al. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. J Immunol **2001**; 167:6533–44.
32. Vincendeau P, Gobert AP, Daulouede S, Moynet D, Mossalayi MD. Arginases in parasitic diseases. Trends Parasitol **2003**; 19:9–12.