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Functional Studies of the SLE-Risk Genes BANK1 and BLK in B-cell Pathways

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El doctorando, Alejandro Díaz Barreiro, y la directora de la tesis, Marta Alarcón Riquelme, garantizamos al firmar esta tesis doctoral que el trabajo ha sido realizado por el doctorando bajo la dirección de la directora de la tesis y, hasta donde nuestro conocimiento alcanza, en la realización del trabajo se han respetado los derechos de otros autores a ser citados cuando se han utilizado sus resultados o publicaciones.

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TABLE OF CONTENTS

I. ABSTRACT 13

II. INTRODUCTION 23

1. An overview to systemic lupus erythematosus 23

1.1. Clinical features 23

1.2. Epidemiology 27

1.3. Treatment 27

2. Etiology of SLE 28

3. Immunity in health 32

3.1. Emergence of pattern recognition receptors. A link between innate and adaptive immunities 33

3.2. Importance of nucleic acid-sensing Toll-like receptors in immunity 34

4. PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS 37

4.1. Type I interferon and plasmacytoid dendritic cells 37

4.2. The B cell compartment 39

4.2.1. B cell signaling in autoimmunity 40

The B lymphocyte kinase, BLK 43

The B-cell scaffold protein with ankyrin repeats, BANK1 45

The Myeloid differentiation primary response protein 88, MyD88 48

The TNF receptor-associated factor 6, TRAF6 49

III. OBJECTIVES 54

IV. MATERIALS AND METHODS 58

V. RESULTS 72

VI. DISCUSSION 96

Effects of the SLE risk variant BLK 71Thr 96

BANK1 role in TLR pathways 100

VII. CONCLUSIONS 108

VIII. REFERENCES 112

ABBREVIATIONS 132

ABSTRACT/RESUMEN

I. ABSTRACT

Systemic lupus erythematosus (SLE) is a multisystemic and chronic disorder considered as the prototype autoimmune disease. It is characterized by the production of autoantibodies against nuclear self-antigens. Subsequent deposition of immune complexes in end-organs such as kidneys, joints or skin causes inflammation and tissue damage. The factors that lead to the onset of SLE are several and of different nature. The disease develops when environmental factors, such as infections or ultraviolet light, affect individuals genetically predisposed to encourage the breakdown of tolerance to ubiquitous self-antigens. In this sense, the study of the genetic basis of lupus has been of enormous importance to describe the processes that underlie its pathophysiology.

During the last decade, the development of genome wide association studies (GWAS) and the concomitant technological advances have led to the identification of more than 60 loci associated with the risk of developing lupus. Most of them are genes encoding proteins with a role in the innate and/or the adaptive immune responses, and as a whole, this information displays which functional pathways are altered in the disease. However, much of this knowledge will not have a medical application unless we accurately figure out in what molecular networks and how these proteins contribute to the development of autoimmunity. Biological therapies targeting B lymphocytes for their modulation or depletion are a clear example of this. Their development would not have been possible without prior basic research such as the one presented here.

B lymphocyte kinase (BLK) and the B-cell scaffold protein with ankyrin repeats BANK1 are two examples of genes consistently associated with SLE through GWAS. In addition, both proteins show an expression almost restricted to B lymphocytes and have been shown to act together upon B cell receptor (BCR) signaling.

In 2012 our group identified a rare variant associated with lupus in the *BLK* gene. A substitution of an alanine for a threonine at position 71 (A71T), within the SH3 domain of the protein, generated a kinase with increased susceptibility to degradation. In order to describe in depth the effects of this non-synonymous variant over BLK homeostasis and function, we have compared mRNA expression, protein degradation, ubiquitination, subcellular localization and traffic toghetehr with coinmunoprecipitation experiments. The variant increases the *in vitro* ubiquitination of the kinase and

degradation rate in B cells. In addition, it enhances the overall threonine- and tyrosine-phosphorylation of the protein. We hypothesize this is caused by the disruption of an intramolecular interaction between the SH3 domain and the linker segment that joins the kinase and SH2 domains of BLK, which has been shown to regulate the activity of this type of kinases. In addition, it has been described that BLK is ubiquitinated after its activation as a signal for degradation, which supports our hypothesis. On the other hand, the A71T substitution also affects the interaction of BLK with BANK1 by significantly reducing it. In contrast, neither an effect on localization nor subcellular trafficking were observed.

Low levels of BLK have been repeatedly proposed as a risk factor in the development of SLE. The effect of variant A71T, in this sense, would **represent** an additive risk effect.

BANK1 contains a putative TIR domain sequence. Our group has intensively investigated its involvement in the Toll-like receptor pathway. We have previously reported BANK1 effects as a positive regulator of TLR7 and TLR9 signaling in mouse models, however little is known about its exact functions. Two isoforms of BANK1 have been described: the Full Length (FL) and the so-called Delta 2 (D2), which lacks the TIR domain-encoding exon 2. TIR domains are key players in TLRs signaling pathways, being found for example in the master regulator MyD88. In addition, we have also identified several TRAF6 putative binding sites within BANK1 sequence. In order to test the functionality of all these domains, we performed coinmunoprecipitation assays with both proteins, MyD88 and TRAF6. The D2 variant displayed a decreased ability to bind both proteins, suggesting that the TIR domain is functional, and that some TRAF6 binding site/s may be affected by alternative splicing of the isoform. On the other hand, we found that FL BANK1 enhances TRAF6 Lys-63-linked ubiquitination, whereas D2 fails to induce it probably because of the altered binding to MyD88 and TRAF6.

Taken together, these data implicate BANK1 in the TLR pathway and suggest its role as a signaling enhancer mediated through the interaction with MyD88 and TRAF6, particularly by promoting TRAF6 Lys-63-linked ubiquitination.

The description of pathological pathways such as those studied in this work together with other findings achieved daily in the world's laboratories will allow the development of new personalized and effective therapies for SLE.

I. RESUMEN

El lupus eritematoso sistémico (LES) es un trastorno multisistémico y crónico considerado el prototipo de enfermedad autoinmune. Se caracteriza por la producción de autoanticuerpos contra antígenos nucleares, y la consiguiente deposición de complejos inmunes en órganos diana tales como riñones, articulaciones o piel, causa inflamación y daño tisular. Los factores que llevan a desencadenar el LES son varios y de distinta naturaleza. La enfermedad se desarrolla cuando factores ambientales, tales como infecciones o la luz ultravioleta, inciden en individuos genéticamente predispuestos para propiciar la ruptura de la tolerancia a antígenos propios ubicuos. En este sentido, el estudio de las bases genéticas del lupus ha sido de enorme importancia para describir los procesos que subyacen a su fisiopatología.

Durante la última década, el desarrollo de estudios de asociación de genoma completo y el avance de las capacidades científico-técnicas ha llevado a la identificación de más de 60 *loci* asociados con el riesgo a desarrollar lupus. Muchos de ellos son genes que codifican proteínas con un papel en la respuesta inmunológica, tanto innata como adaptativa, y en su conjunto, esta información evidencia las rutas funcionales alteradas en la enfermedad. Sin embargo, mucho de este conocimiento no adquirirá un carácter traslacional a menos que descifremos exactamente en qué redes moleculares y cómo contribuyen estas proteínas al desarrollo de autoinmunidad. Un claro ejemplo de esto son las terapias biológicas dirigidas a la modulación y depleción de linfocitos B, las cuales no hubiesen sido posibles sin una investigación básica previa.

La quinasa *BLK (*del inglés, *B lymphocyte kinase)* y el adaptador *BANK1 (*del inglés, *B-cell scaffold protein with ankyrin repeats)* son dos ejemplos de genes asociados repetidamente con el LES a través de estudios de asociación de genoma completo. Ambos, además, tienen una expresión casi restringida a linfocitos B y se ha demostrado que actúan conjuntamente como señalizadores del receptor de células B (BCR).

En 2012 nuestro grupo identificó una variante rara asociada a lupus en el gen *BLK*. Una sustitución de un alanina por una treonina en la posición 71 (A71T), dentro del dominio SH3 de la proteina, generaba una quinasa con mayor susceptibilidad a la degradación. Con el objeto de describir con profundidad los efectos de esta variante no

sinónima sobre BLK y su función, hemos llevado a cabo ensayos de expresión de RNA mensajero, degradación de proteína, ubiquitinación, localización y tráfico subcelular y coinmunoprecipitación. La variante incrementa la ubiquitinación *in vitro* de la quinasa y su velocidad de degradación en células B. Además, aumenta la fosforilación global de tirosinas y treoninas de la proteína. Proponemos que todo esto se debe a la disrupción de una interacción intramolecular entre el dominio SH3 y el segmento enlazador de los dominios quinasa y SH2 de BLK, la cual se ha demostrado que regula la actividad de este tipo de quinasas. Además, se ha descrito que BLK es ubiquitinada tras su activación como señal para su degradación, lo cual apoyaría esta hipótesis. Por otro lado, la sustitución A71T también afecta a la interacción de BLK con BANK1, disminuyéndola significativamente. Por el contrario, no se observó ningún efecto sobre la localización, ni el tráfico subcelular.

Bajos niveles de BLK han sido propuestos en numerosas ocasiones como un factor de riesgo en el desarrollo del LES. El efecto de la variante A71T, en este sentido, tendría un valor de riesgo aditivo.

BANK1 contiene un dominio TIR putativo. Nuestro grupo ha investigado intensamente su participación en la ruta de los receptores tipo Toll. Hemos reportado previamente sus efectos como potenciador de la señalización del TLR7 y TLR9 en modelos de ratón, sin embargo poco se sabe sobre que funciones exactas. Se han descrito dos isoformas de BANK1: la forma completa (*Full length*) y la llamada Delta 2 (D2), que carece del exón 2, el cual codifica el dominio TIR. Este dominio es clave en estas vías de señalización, encontrándose por ejemplo en el regulador maestro MyD88. Además, hemos identificado también que BANK1 posee dominios putativos de unión a TRAF6. Con objeto de testar la funcionalidad de todos estos dominios, hemos llevado a cabo ensayos de coinmunoprecipitación con ambas proteínas, MyD88 y TRAF6. La variante D2 tiene una capacidad disminuida de unión a ambas proteínas, lo que apunta a que el dominio TIR es funcional, y que los dominios de unión a TRAF6 podrían verse afectados debido al *splicing* alternativo de la isoforma. Por otro lado, hemos encontrado que BANK1 ejerce un efecto potenciador sobre la ubiquitinación de TRAF6 ligada por lisinas 63, el cual se ve afectado en la variante D2, probablemente debido a la alteración de la unión a MyD88 y TRAF6.

En su conjunto, estos datos implican a BANK1 en la ruta de los TLRs y sugieren que su papel potenciador de la señalización podría ejercerse a través de la interacción con las proteínas MyD88 y TRAF6, especialmente mediante la promoción de la ubiquitinación de la última.

La descripción de rutas patológicas como las que hemos estudiado en este trabajo, sumada al resto de hallazgos que se llevan a cabo diariamente en los laboratorios del mundo nos permitirá el desarrollo de nuevas terapias personalizadas y efectivas para el LES.

INTRODUCTION

II. INTRODUCTION

1. An overview to systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multisystemic and chronic disorder considered as the prototype autoimmune disease. It is characterized by the production of autoantibodies against nuclear self-antigens, and the subsequent deposition of immune complexes in end-organs, e.g. kidneys or skin [1, 2]. It has become clear that SLE is caused by a complex combination of genetic and environmental factors. More than 60 genes and loci have been identified until now by genome-wide association studies (GWAS) to be associated to SLE and can lead to altered gene expression and/or protein function that eventually result in abnormal immune responses and the development of the disease [3-7]. Given the importance of the humoral aspect of the disease, B cells are key components contributing to the complex pathophysiology of SLE and are therefore considered a target for current biological therapies [8].

1.1. Clinical features

Given the systemic nature of the disease a number of organs and systems can be affected in SLE (**Figure 1**). The most general symptoms are anorexy, fatigue, fever and weight loss, and normally course together with more specific manifestations that take place especially during flares. The most common ones are briefly described below [9-13]:

Most of SLE patients (50-100%) display **musculoskeletal manifestations** such as arthralgias and/or myalgias. Non-erosive, simetric arthritis is also very frequent, affecting predominantly proximal interphalangeal, etacarpophalangeal, carpal and knee joints. Flares can also course with miositis/myopathy, either as a consequence of the generalized inflammation or because of the treatment.

Cutaneous affectations are present in many lupus patients during some period of the disease. They can be very diverse from SLE specific to non-specific lesions, and they are further divided into acute, subacute and chronic. The most characteristic manifestation is the typical “butterfly” or malar rash (**figure 1**). Photosensitivity occurs in 60-100 % of SLE patients and it is a major mechanism that triggers skin lesions in

sun-exposed areas as well as other kind of manifestations in SLE patients.

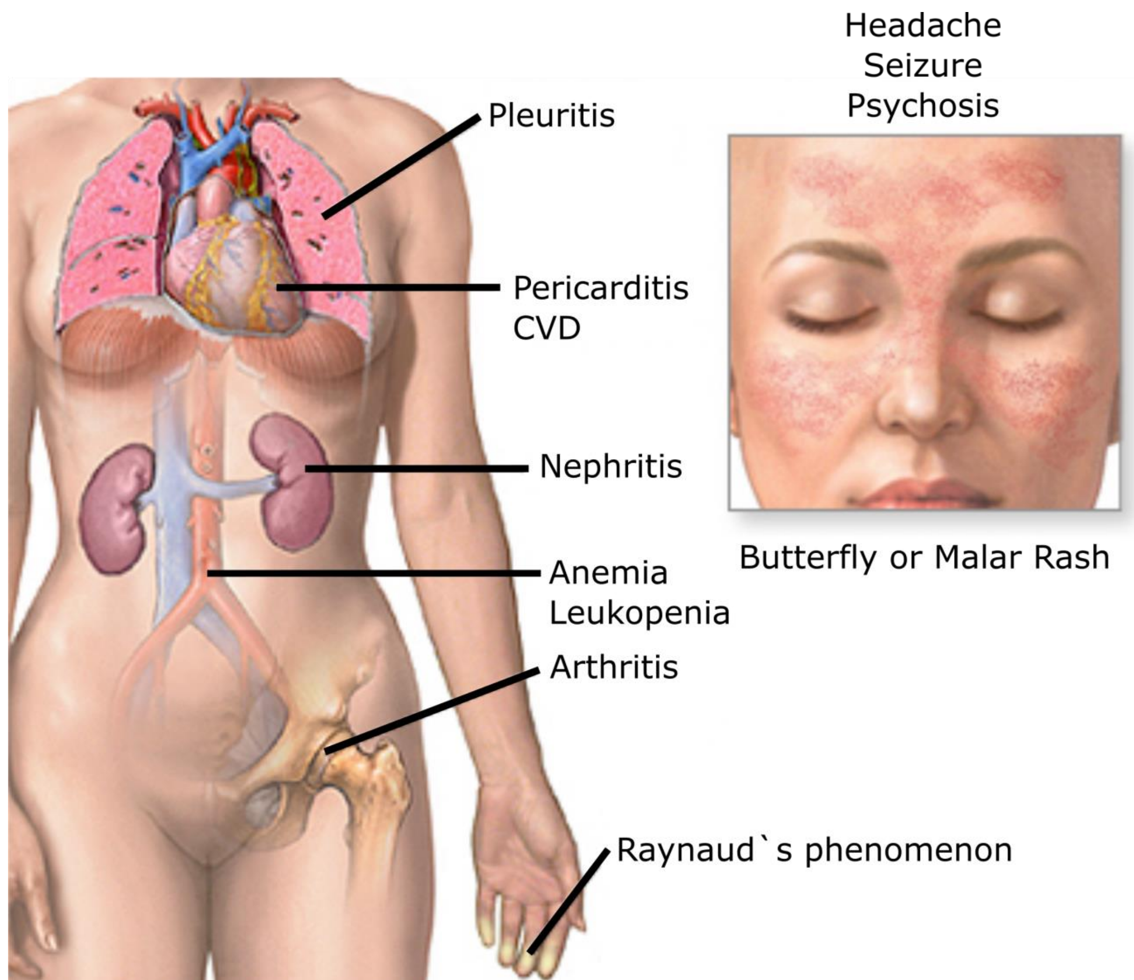


Figure 1. General clinical manifestations in SLE. Systemic inflammation leads to damage of the tissues most involved in disease pathology. Immune complexes deposition and the associated complement activation have been pointed as the main mechanism responsible. Adapted from A.D.A.M. (http://www.adam.com)

Renal disease affects 30-80 % of the patients depending on their ethnicity, although many of them develop a type of subclinical disease. Individuals that undergo lupus nephritis have a significantly worse prognosis than SLE patients without nephritis (88 % v.s. 94 % 10-years survival rate). One of the reasons for this is that it impacts the clinical picture both through kidney damage itself and also indirectly by affecting side-effects of the therapies.

Neuropsychiatric involvement is the least understood manifestation in SLE

and one of the major causes of morbidity and mortality. It can affect both the CNS, producing syndromes as aseptic meningitis, headache (including migraine), cerebrovascular disease, seizure, anxiety or mood disorders, and psychosis; or the PNS causing for example acute inflammatory demyelinating, myasthenia gravis or polyneuropathy. Some of these symptoms, however, are sometimes undistinguishable from other diseases which make the diagnosis of lupus-related neuropsychiatric manifestations a very complicated task.

Some **cardiovascular manifestations** such as pericarditis may occur in around 25 % of lupus patients. It has been also reported an increased risk for myocardial infarction in SLE patients that cannot be explained exclusively by the traditional CVD risk factors. This is probably because an accelerated, premature atherosclerosis that is presented in many individuals with SLE, a disease that it is actually considered itself a risk factor for CVD. Other complications in decreasing order of frequency are: diffuse thickening of the mitral and aortic valves, vegetations, valvular regurgitation and stenosis.

Haematological abnormalities are very often found in lupus patients. Anaemia and leukopenia are the most frequent ones, followed by thrombocytopenia, and normally correlate with periods of disease activity. They are usually the presenting factors of the disease.

Other tissues and organs can be affected generating a number of manifestations such as pleuritis, Raynauds phenomenon dyspepsia, peptic ulcers, abdominal pain, hepatomegaly, lymphadenopathy, splenomegaly, conjunctivitis, and those related with the antiphospholipid syndrome.

Although some of the listed manifestations such as lupus nephritis are clinically the most life threatening complications for patients, in a lupus awareness survey developed for the Lupus Foundation of America in 2012, participants pointed to pain (65%), lifestyle changes (61%), and emotional problems associated with lupus (50%) as the most difficult aspects of coping with lupus [14].

The complexity of disease features required the confection of a set of criteria for classification in 1982 [15] which had to be updated in 1997 [16] and it is still used as a guide for physicians to diagnose SLE (**Table 1**). The combination of clinical signs

with laboratory tests is the basis for this classification. The concurrence of at least 4 of the 11 criteria, presented simultaneously or serially during a given period of observation, determines the positive diagnosis of SLE.

Table 1. 1997 Update of the 1982 American College of Rheumatology Revised Criteria for Classification of systemic lupus erythematosus.

CRITERIA	FEATURES
1.Malar Rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4. Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by physician
5. Nonerosive arthritis	Involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Pleuritis or pericarditis	**1.** Pleuritis -convincing history of pleuritic pain or rubbing heard by a physician or evidence of pleural effusion, ***OR;*** **2.** Pericarditis -documented by electrocardigram or rub or evidence of pericardial effusion
7. Renal disorder	**1.** Persistent proteinuria > 0.5 g per day or > than 3+ if quantitation not performed, ***OR;*** **2.** Cellular casts -may be red cell, hemoglobin, granular, tubular, or mixed
8. Neurologic disorder	**1.** Seizures -in the absence of offending drugs or known metabolic derangements; e.g., uremia, ketoacidosis, or electrolyte imbalance, ***OR;*** **2.** Psychosis -in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9. Hematologic disorder	**1.** Hemolytic anemia -with reticulocytosis, ***OR;*** **2.** Leukopenia- < 4,000/mm^3 on ≥ 2 occasions, ***OR;*** **3.** Lyphopenia- < 1,500/ mm^3 on ≥ 2 occasions, ***OR;*** **4.** Thrombocytopenia- <100,000/ mm^3 in the absence of offending drugs
10. Immunologic disorder	**1.** Anti-DNA: antibody to native DNA in abnormal titer, ***OR;*** **2.** Anti-Sm: presence of antibody to Sm nuclear antigen; ***OR;*** **3.** Positive finding of antiphospholipid antibodies: **3.1.** An abnormal serum level of IgG or IgM anticardiolipin antibodies, **3.2.** A positive test result for lupus anticoagulant using a standard method, or;

	3.3. A false-positive test result for at least 6 months confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
11. Positive antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs

Adapted from American College of Rheumatology web page (https://www.rheumatology.org).

1.2. Epidemiology

Improvement of mild disease diagnosis by immunological techniques such as detection of antinuclear antibodies anti-DNA and anti-ENA, and the longer survival time of patients because of new treatments and a better comprehension of the disease, have severely increased the estimates of incidence and prevalence in the last decades. It is a complicated estimation and values vary in a wide range even in countries where it would be expected to be accurate because of the good quality of data collection systems. Overall, calculations suggest an incidence of 0.3–31.5 cases per 100,000 individuals per year and a prevalence of 3.2–517.5 cases per 100,000 individuals [17]. Despite this huge variation, nowadays some trends are very clear regarding the distribution of the disease across populations. It has an obvious gender-bias expression with a 9:1 ratio of female to male patients, affecting predominantly 15 to 45 years old women. Different genetic analyses developed in multiple populations have evidenced that ethnicity is another key factor predisposing to SLE [18]. Individuals of African-American, Asian, and admixed Native American-European (primarily Spanish) ancestries display a much more sever lupus, with more cases of renal inflammation and periods of disease activity. In turn, prevalence does not necessarily correlate with these observations [7, 19-23].

1.3. Treatment

The current therapies for lupus are mainly directed to reduce systemic inflammation, as well as to prevent flares and avoid irreversible organ and tissue damage. Beside the general recommendations on the use of high protection sun creams, the maintenance of a balanced diet, making physical exercise, avoiding smoking, stress, and controlling CVDs risk factors [24, 25], several drugs are being used against different targets with relative effectiveness.

Drugs for SLE treatment may be classified in four main groups regarding their mechanisms of action: systemic inflammation directed treatment, immune cell targeted therapies, those targeting co-stimulatory signaling pathways, and anti-cytokine therapies [26]. They are of diverse nature including nonsteroidal antimmflamatory drugs, antimalarial drugs, oral glucocorticoids, immunosuppressive and cytotoxic agents (generally for organ involvement), and biological treatments [27] (**Table 2**).

Table 2. Biological treatments available or potentially available for the treatment of SLE

TARGET/MECHANISM	DRUGS
B cells	B-cell depleting therapy: **Rituximab** B-cell modulating therapy: **Epratuzumab** Inhibition of B-cell survival: **Belimumab, Atacicept** Other potential B-cell (plasma cell) targeting strategies: **Bortezomib**
T cells	Inhibition of T-cell function: **Abatacept, Ruplizumab, Toralizumab, Lupuzor**
Interleukin 6	**Tocilizumab**
Tumour necrosis factor α inhibitors	**Infliximab, Etanercept**
Type I interferon inhibitors	**Sifalimumab, Rontalizumab**
Complement inhibitors	**Eculizumab**

Adapted from [11]

As depicted in **Table 2**, B cells are a key target in the immunotherapy of SLE because of the production of auto-antibodies, a major mediator of inflammation in lupus. However, the efficiency of B cell targeted therapies has not reached the expectations and the most important ones will be discussed in the section of SLE pathogenesis.

2. Etiology of SLE

Systemic lupus erythematosus is a multifactorial disease in which individuals genetically predisposed under certain environmental cues, experience a break of self-tolerance. Consequently, an immune response against ubiquitous self-antigens is triggered. There are factors of both genetic and epigenetic nature behind these processes, as well as environmental influences such as infections, sex hormones and

diet.

Genetic factors

Genetic involvement in SLE is supported by many observations. For instance, the grade of disease concordance is much higher in monozygotic twins (24%-35%) than in their dizygotic counterparts (2%-5%), and familial aggregation is very high in lupus [28, 29].

The development of candidate gene studies in the past, led us to the determination of several genetic associations in SLE such as the MHC class II region [30], Fc gamma receptors genes, *PTPN22* or *STAT4* [7]. However, this type of analysis required a previous knowledge or assumption which enabled the design of a hypothesis-based study. In the last decade, the great advances in high-throughput genotyping technologies and computational data analysis have allowed us to take different approaches. Genome wide association studies (GWAS) are powerful tools that have permitted the confirmation and identification of a large number of risk genes and loci in a hypothesis-free fashion. Several SLE GWAS [3, 4, 31-36] (mostly in European and Asian populations) have helped to describe more than 60 risk genes and loci for SLE (**Table 3**), many of them replicated in meta-analyses across multiple populations [7]. Notably, and somehow expected at the same time, majority of the genes identified encoded products which are highly relevant within immune signaling.

Table 3. Genes/loci associated with risk to SLE

Gene	Region	OR	Other immune pathways	Population
NFkB signaling				
TNIP1	5q33.1	1.27		AS, EU, LA
TNFAIP3	6q23	1.71		EU, AS
SLC15A4	12q24.32	1.26	TLR and type I IFN signaling	AS, EU
PRKCB	16p11.2	1.15	B-cell function and signaling	AS, EU
UBE2L3	22q11.21	1.22		EU, AS, AS, AA, HS
IRAK1/MECP2	Xq28	1.31	B-cell function and signaling	EU, AS, AA
TLR and type I IFN signaling				
IFIH1	2q24	1.15		EU, AS, AA
miR146a	5q34	1.26		EU, AS
IRF5/TNPO3	7q32	1.61		EU, AS, HS, LA
IRF7/PHRF1	11p15.5	1.28		EU, AA, AS
IRF8	16q24.1	1.25	Neutrophil and monocyte function and signaling, B-cell function and signaling	EU
TYK2	19p13.2	1.20		EU
TLR7	Xp22.3	1.25		AS, EU, HS
DNA degradation, apoptosis and clearance of cellular debris				
TREX1	3p21.31		TLR and type I IFN signaling	EU

ATG5	6q21	1.19		EU
RAD51B	14q23-q24.2	1.14		EU, LA
DNASE1	16p13.3			AS
Immune complex processing and phagocytosis				
C1Q	1p36	CD		
FCGR2A/B	1q23	1.35		EU, AS, AA
CR2	1q32.2	1.54		EU, AS
C1R/C1S	12p13	CD		
C4A/B	6p21.3	6.50		EU
Neutrophil and monocyte function and signaling				
ITGAM	16p11.2	1.70		EU,AA, HS, LA, AS
ICAMs	19p13.2	1.67		EU, AA, HS, AS
NCF2	1q25	1.19	Neutrophil and monocyte function and signaling	EU, LA
IL10	1q31-q32	1.19		EU
RASGRP3	2p25.1-p24.1	1.43		AS, LA, EU
BANK1	4q24	1.38		EU, AS, AA
MSH5	6p21.3	1.79		EU
PRDM1	6q21	1.20	B and T-cells function and signaling	EU
BLK	8p23-p22	1.22		EU, AS, AA
LYN	8q12	1.3		EU
ETS1	11q23.3	1.37		AS, EU
ELF1	13q13	1.26		AS
CIITA/SOCS1	16p13.13	1.21	B-cell function and signaling	EU
B and T-cell function and signaling				
STAT4	2q32	1.50	B-cell function and signaling	EU, AS, AA, HS, LA
IKZF2	2q34	1.24		EU
AFF1	4q21	1.21		AS, EU
IKZF1	7p12.2	1.39		AS, EU
CSK	15q24.1	1.32		EU
IKZF3	17q21	1.40		EU, AA
T-cell function and signaling				
PTPN22	1p13.2	1.35		EU, HS
TNFSF4	1q25	1.22		EU, AS, AA, HS
CD80	3q13.3-q21	1.27		AS
IL12A	3q25.33	1.14		EU
TCF7	5q31.1	1.45		EU
PPP2CA	5q31.1	1.30		EU, HS, AS, AA
PDHX/CD44	11p13	1.23		EU, AS, AA, HS, LA
Other genes				
SMG7	1q25	1.23		EU
PXK/ABHD6	3p14.3	1.25		EU, AS, AA
NMNAT2	1q25.3	1.72		EU, LA
UHRF1BP1	6p21	1.17		EU, AS
JAZF1	7p15	1.19		EU, LA
XKR6	8p23.1	1.23		EU
WDFY4	10q11.22	1.24		EU, AS, LA
ARID5B	10q21	1.18		AS, EU
DHCR7|NADSYN1	11q13.4	1.23		EU
SH2B3	12q24	1.13		EU
ATG16L2|FCHSD2|P2RY2	11q13.4	1.59		AS
PLD2	17p13.1	1.25		EU
TET3	2p13	1.33		AS, EU

GPR19	12p13	1.26	AS
DRAM1	12q23	2.27	AS
CXorf21	Xp21.2	1.14	EU
PRPS2	Xp22.3	1.19	AS, LA

Adapted from [7]. References for genetic studies are shown in the source article. OD, odd ratio of the risk allele; CD, complete deficiency; EU, European ancestry; AS, Eastern Asian ancestry; AA, African American; HS, Hispanic (Latin American in the USA); LA, Latin American ancestry.

Participation of genes from both innate and adaptive immunity suggests the importance of these two responses in maintaining self-tolerance, which will be discussed during the introduction. The most interesting conclusions inferred from all GWAS information are the biological pathways most likely involved in SLE pathophysiology (shown in bold in Table 2 and only for an easier interpretation) [7, 37]. In addition, the role of these pathways has been repeatedly suggested by their associated clinical findings in SLE patients [38] as well as through functional studies of causal variants [39]. Despite the numerous risk genes found, odd ratios are generally low and do not explain the whole heritability of the disease [40]. In these sense, the role of rare variants [41-43], which are not covered by GWAS because of extremely low frequency, or epigenetics [44-46], could be important to fill the gap of missing heritability in SLE [40].

There are different mechanisms proposed on how the environment interacts with epigenetics and genetics to trigger the onset of SLE. For instance, it has been proposed that environmental pressure could induce the DNA hypomethylation observed usually in many lupus patients [47, 48]. The process, which would be induced by the increment of oxidative stress in CD4+ T cells, was suggested to be mediated by decreased activity of protein kinase C, the extracellular signal-regulated kinase ERK and the DNA methyltransferase I. Thus, factors such as viral infections, cigarette smoking, medications, UV light or chemicals could eventually release the epigenetically-repressed expression of potentially harmful genes to trigger autoimmunity. Among them, it has been reported the abnormal expression of IL-4, -5 and -13, interferon and CD40L[49].

If in addition to this, we add the effect of the risk variants identified by GWAS, which can participate both at the translational level or regulating the expression of genes, we are faced with a set of mechanisms that can potentially explain the onset of

disease pathogenesis.

3. Immunity in health

Human body shows three different levels of defense against pathogens. First, we find a surface layer with components such as enzymes or mucus, which can have their own anti-micorbial or anti-attachment functions. These barriers are part of the innate immunity and neither skin nor mucus cavities are the optimal environment for microorganisms' survival, which need to cross them in order to develop within the host. If they finally succeed, the second line of innate immunity is activated with the participation of different cell types (**Figure 2**) and the complement system. Tissue resident-macrophages and immature dendritic cells engulf foreign microorganisms to initiate a cascade of mechanisms that ultimately orchestrate the third barrier of defense, the adaptive immune response (**Figure 2**). The extreme importance of this connection between the innate and adaptive immune responses and the role of PRRs will be discussed below in this section. Non-phagocytic mature dendritic cells which have processed antigens in the focus of infection migrate then to secondary lymphoid organs to meet and stimulate circulating naive T lymphocytes through antigen presentation. After clonal expansion of those CD4 T cells with the specific TCR and their development into effector helper lymphocytes, these stimulate –among others– BCR-specific B cells to form germinal centers and to become antibody producing-plasma cells. On the other hand, CD8 effector T cells take care mostly of virus infections by its direct cytotoxic action. Some of the activated B and T cells will remain after infection is already under control to confer a lasting protection to the host [50].

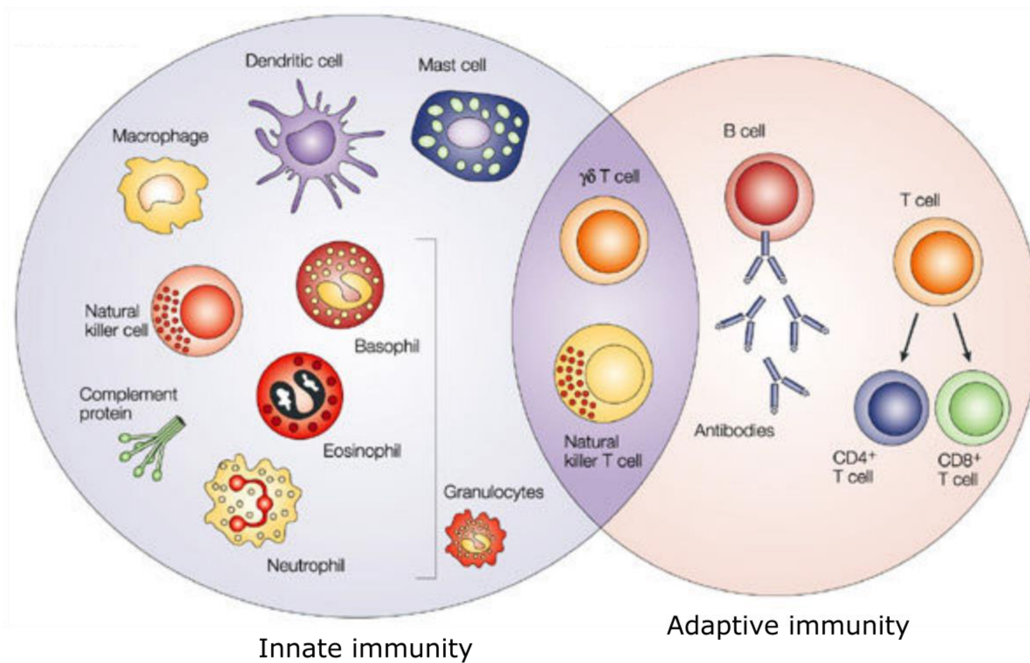


Figure 2. Cellular components of the innate and adaptive immunities. Most of the cells of innate immunity belongs to the myeloid lineage, whereas adaptive immunity central players come from lymphoid precursors. Natural killer cells are from lymphoid origin but since they respond to infections with no antigen specificity they are encompassed in the innate immunity. γδ T cells are the only lymphoid cells that exert professional phagocytic activity [51] and natural killer T cells are a very rare group of T lymphocytes that share properties of both T cells and natural killer cells. Adapted from [52].

3.1. Emergence of pattern recognition receptors. A link between innate and adaptive immunities

It was premonitory when Charles Janeway proposed in 1989 that immune system had evolved to specifically respond to infectious organisms by recognizing some patterns shared by different pathogens. He referred for the first time to **pattern recognition receptors** as the germline non-clonal receptors in charge of this function of the innate immunity. These interactions would deliver activated APCs with the necessary co-stimulatory molecules on their surface to stimulate specific naïve T lymphocytes and thus initiate the immune response. In addition, since the recognized patterns (now known as **PAMPs**) were postulated to exist only in foreign organisms, these PRRs were the perfect mechanism to distinguish non-self from self [50]. However, a couple of questions were still unanswered by Janeways´s model such as the role of the immune

response in graft rejection and tumors, or the dysfunctions observed in autoimmunity [53]. During the following decade many researchers provided substantial findings supporting Janeway´s ideas, including himself with the identification of the first mammalian TLR (now known as TLR4) together with Medzhitov and Preston-Hurlburt [54]. Some new postulations made by other researchers even complemented his ideas as is the case of the “danger theory” by Polly Matzinger in 1994 [55]. The latest concept actually helped to solve Janeway theory´s lack of consistence in the above mentioned observations. **Danger-associated molecular patterns** are signals such as DNA or RNA that are released from injured tissues and - similarly to PAMPs – are able to start a noninfectious inflammatory response. Now we know these are important mechanisms meditating, for example, the trigger of autoimmunity. Discovery of **Toll-like receptors** and the identification of their associated PAMPs during the nineties were of formidable relevance since they supported all these theories with an experimental basis [56]. It is a fact that stimulation of PRRs is not only a prerequisite for virtually all adaptive immune responses, but also shapes the decisions made in each of their steps. A clear proof of this is the historical necessity of adding certain TLR ligands (e.g. killed bacteria) as vaccine adjuvants to raise antibodies against innocuous antigens [57]. Thus, pathogen sensors such as TLRs control the expression of co-stimulatory molecules like CD80 and CD86 in APCs, particularly DCs and macrophages. These complement the signal from the antigen displayed by MHC class II molecules and promote naive T cells to become effector helper lymphocytes. In turn, most B cells need co-stimulation from these expanded T helper cells together with the antigen-specific BCR engagement for their activation. However, these are not the only requisites for an optimal antibody response by B cells, whose activation details will be discussed in the next sections [50].

3.2. Importance of nucleic acid-sensing Toll-like receptors in immunity

TLRs are expressed in a broad range of cells and tissues. Among all the existing TLRs, TLR7 and TLR9 are sensors located in early endosomes that recognize single-stranded RNA and hypomethylated DNA oligonucleotides, respectively [58]. Therefore, they constitute two of the main barriers against viruses and bacteria, and their engagement leads to the secretion of pro-inflammatory cytokines and type I interferons (IFN) [59, 60]. At the same time, their intra-cytoplasmic location prevents them from concurring

with host DNA which is normally abundant in the extracellular space [61].

Plasmacytoid dendritic cells are professional type I IFN-producing cells that selectively express TLR7 and TLR9, in contrast to cDCs and monocytes (**Table 4**). Almost every cell type is able to produce type I IFNs but the amount released by pDCs against viral infections is up to 1000-fold higher than the other of the cells [62]. Type I IFNs not only directly interrupt viral replication but also induce the antiviral action of NK cells, cDCs, B cells, and T cells [63], hence triggering and orchestrating innate and adaptive antiviral responses. TLR9 deficiency in mouse leads to the complete abrogation of IFN alpha production by pDCs against the TLR9 ligand ODNs CpG [64]. It was also shown to be required for their response against DNA viruses such as herpes simplex virus 1 and 2, and murine cytomegalovirus [65-68]. TLR7, in turn, is required for pDCs response to ssRNA viruses such as influenza virus, sendai virus, respiratory syncytial virus and vesicular stomatitis virus [69-72]. pDCs also possess cytoplasmic RLRs, but unlike fibroblasts and cDCs [73-75], neither the deficiency of RIG-1 nor of the RLR main adapter IPS1 prevented pDCs from producing IFN alpha against Newcastle disease and sendai RNA viruses [75, 76]. This highlights the exclusive participation of endosomal TLRs in nucleic-acid sensing of pDCs and suggests this cell type has a unique role in immunity [63].

Table 4. TLRs differential expression in human blood cell subsets.

	Monocyte	**cDC**	**pDC**	**B cells**
TLR1	**++**	**+**	**+/-**	**+**
TLR2	**++**	**++**	**-**	**+/-**
TLR3	**-**	**++**	**-**	**-**
TLR4	**++**	**+**	**-**	**+/-**
TLR5	**+**	**++**	**-**	**-**
TLR6	**+/-**	**+**	**+/-**	**++**
TLR7	**-**	**-**	**++**	**++**

TLR8	++	+	-	+/-
TLR9	-	-	++	++

Adapted from [77].

Although **B cell** subsets exhibit some differences in TLR expression patterns, B cells express almost all TLRs, including the nucleic-acid sensing TLR7 and TLR9 (**Table 4**). TLR signaling has been traditionally considered an exclusive innate immune response, however now we know it intrinsically exerts important effects over B cell functions such as antibody production, antigen presentation and cytokine secretion [78-80]. It has been shown that TLR-dependent signaling is required not only in DCs (to induce a T-cell response), but also in B cells for a proper antibody production. Responses based on IgM and IgG1 antibodies production were almost completely dependent on TLRs, closely followed by IgG2c and finally IgE antibodies showing to be independent of TLR signaling [78]. These results were similar in a work with human cells [81]. Authors proposed TLRs as the "third signal" necessary to activate naïve B cells in a model combining BCR triggering by a specific antigen, the cognate interaction with a T helper cell, and the stimulation of any of the TLRs upregulated upon BCR engagement. Induction of TLRs expression by BCR triggering is indeed an interesting system that provides immune responses with high specificity since it concentrates innate signals predominantly in antigen-engaged B cells [81].

4. PATHOGENESIS OF SYSTEMIC LUPUS ERYTHEMATOSUS

Release of nuclear material by injured tissues in lupus patients has been repeatedly postulated as a hallmark in the triggering of disease immunopathogenesis. As reviewed in the etiology section, UV light and/or virus or bacterial infections could potentially mediate this tissue damage in genetically predisposed individuals to trigger autoimmunity. Self DNA and RNA are strong candidates in this matter, and consequently the central role of B cells and pDCs in pathologic mechanisms is expected because of their selective expression of TLR7 and TLR9 (**Table 4**). In addition, their respective involvement in antibody secretion and type I IFN production – both highly associated with disease phenotype-, makes them of extreme interest in the study of SLE (**Figure 3**) [82].

4.1. Type I interferon and plasmacytoid dendritic cells

Type I interferon was firstly associated with autoimmune phenotypes in 1979 [83]. IFNα and serum from lupus patients were reported to induce expression of chemokine receptor CCR7 by healthy monocytes, rendering "primed" cells prone to acquire a migrating DC phenotype. This differentiation, however, required a complementary signal from TLRs, suggesting a mechanism through which microbial infections can exacerbate lupus [84]. Furthermore, it was found that IFN-I serum levels correlate with disease activity and anti-dsDNA antibodies. One common characteristic of peripheral blood mononuclear cells from patients with active lupus is the upregulation of interferon-stimulated genes, and this expression pattern –known as *interferon signature*- was shown to correlate with disease severity. Thus, clinical consequences such as skin rush, fever or leucopenia are associated with IFNα levels. Interestingly, the expression of IFN-inducible genes can be inhibited by 90 % by targeting IFNα, which is not possible by means of anti-IFNβ or anti-IFNγ treatments. These data point to IFNα as the causal agent of the so-called *interferon signature* in SLE [85, 86]. Recent findings in an autosomal recessive syndrome also revealed the causal involvement of IFN-I in disease pathogenesis. Dysfunction of a pathway that avoids accumulation of endogenous retroelements produced a SLE-like phenotype and a similar pattern of ISGs expression [87-89].

Genetic evidence is also overwhelming since a considerable percentage of the identified SLE susceptibility loci are directly or indirectly related to IFN-I signaling or synthesis [90]. Transcription factor *IRF5* displays one of the most evident and consistent association with SLE besides the MHC region [91], and some of its risk variants correlates with elevated serum IFNα [92]. *IRF7*, which interacts with the adaptor MyD88 to promote IFNα expression after TLR activation showed a similar pathologic association [93]. *IRAK1*, *IFIH1* and *TYK2* are other examples of IFN-related loci associated with SLE risk [90]. The importance and efficacy of type I IFNs during anti-viral immunity is a double-edged sword. When considered in the context of autoimmunity, their functions have the potential to promote and perpetuate self-responses causing tissue damage and the associated clinical manifestations [94-98].

pDCs continuously secrete IFN-I in response to TLR7 and TLR9 recognition of host nuclear material. Internalization of self-nucleic acids-containing ICs is mainly mediated by FcγRIIA with the subsequent translocation to early endosomes where TLR signaling is triggered. As a consequence, IFN-I promote cDCs maturation and activation, which in turn induce autoreactive T-cells to become effectors [63]. pDCs accumulate in skin lesions of lupus patients [62] and they were described to upregulate IRF3 and IRF5 expression compared to healthy individuals. This upregulation, in addition, was correlated with IFNα serum levels [99]. pDCs showed elevated numbers in SLE patients bloodstream, whereas cDCs were instead diminished [100].

A number of studies observed an expansion of IL-17-producing T cells in SLE patients [101, 102]. Increased amounts of IL-17 were also observed in serum and kidneys, suggesting an involvement of this cytokine in inflammation and tissue damage [103, 104]. Interestingly, human pDCs were reported to promote Th17 differentiation as a consequence of IL-1β and IL-23 secretion after TLR7 ligation [105]. In addition, in response to TLR9 stimulation with CpG, pDCs inhibited T regulatory cell function, expressed inflammatory cytokines such as IL-6 or IFNα, and promoted Th17 polarization [106, 107].

4.2. The B cell compartment

A model with two main aspects of B cell-mediated autoimmunity initiation has been recently supported [108]. On the one hand, B cell signaling could increase the autoreactive pool of naïve BCRs during immature and transitional B cell development; on the other hand, signaling through different receptors in B cells may promote peripheral activation of self-reactive B cell clones [108].

B cells subpopulations

The majority of the knowledge gained so far about aberrancies within different B cell subsets in SLE derives from studies in rodents. In many cases the intrinsic characteristics of cell populations can differ substantially to those in humans, and therefore conclusions must be addressed with great care. Being that clarified, the following are some of the insights provided by different studies, most of them carried out in mice.

B1 B cells are a good example of a population with features described mainly by studies in mice. They are divided into B1-a and B1-b subsets regarding their expression, or the absence, of the CD5 marker, respectively. B1 B cells produce natural antibodies important for responses against encapsulated bacteria. Although CD5+ B cells are increased in both SLE and pSS, these cells could rather be an expanded population of pre-naïve conventional B2 cells instead of the human homologue of B1-a B cells [109, 110]. In addition, there is not sufficient evidence to consider CD5 as a lineage marker and instead, it was suggested that it could be regarded as an activation marker [110].

Conventional B2 B cells represent the adaptive fraction of humoral immunity. After being selected through central tolerance checkpoints in the bone marrow, surviving precursors are released to circulation in an immature form so-called transitional, and migrate to the spleen. In mice, they largely mature in this organ while being subjected to tightly regulated peripheral checkpoints. Finally, they differentiate into mature naïve B cells that recirculate mainly to B cell follicles in secondary lymphoid organs (**follicular B cells**), or into **marginal zone B cells**, which remain in the spleen. In humans, however, these steps are much less clear [111]. B2 B cells are preferentially involved in T cell-dependent responses [112]. Follicular B cells represent

the biggest fraction of peripheral B cells and are widely considered the conventional B cells. Once in the secondary lymphoid organs they receive T cell help in the boundaries of the follicles to initiate GC reactions in the presence of their specific antigen [113]. Here, they will undergo SHM and CSR to finally differentiate into memory cells and long-lived plasma cells [110]. Interestingly, GC-derived antigen-specific memory B cells and Ig-secreting plasma cells are both increased in SLE patients [114, 115]. T helper cell-dependent over-activation of GCs was pointed out as the most likely cause of this increment [111]. On the contrary, MZ and B1 B cells both display innate-like functions [116, 117]. Studies in mice suggested they exert their responses principally in a T cell-independent manner and are thought not to participate in undergoing GC reactions [110].

Of note, an antibody-independent role for B cells was proposed in the development of autoimmunity. Autoimmune-prone mice developed a mild lupus-like phenotype when they were reconstituted with B cells lacking the ability to secrete immunoglobulins [118]. Antigen presentation or cytokine expression by B cells was suggested as the mechanisms responsible of disease triggering in this model [119].

Several studies have described different abnormalities in peripheral B cell populations in SLE [120-123], however, it is not clear whether they are intrinsic or rather a consequence of other immunological alterations. Also, these alterations are very variable and patients can display a strong lymphopenia, or instead, normal or even increased lymphocyte counts, showing no correlation with treatment or disease activity [119]. Additional studies will be required to extrapolate all the knowledge derived from rodents to a human context. Thus, it will be possible to delineate the quantitative and qualitative contributions of every B cell subset participating in SLE pathogenesis.

4.2.1. B cell signaling in autoimmunity

B-cell receptor signaling

The whole signaling network of B cell receptor is highly dependent on interactions mediated by Src homology 2 (SH2), Src homology 3 (SH3) and phospholipid-binding pleckstrin homology (PH) domains. They are largely represented across the proteins of the pathway and bind to phosphorylated tyrosines, proline rich and hydrophobic

regions, and phosphatidylinositol lipids, respectively [124].

The BCR is a multimeric protein structure comprising an antigen-binding membrane immunoglobulin (mIg), generated by rearrangement of immunoglobulin heavy and light chain genes, which is non-covalently bound with disulfide-linked signal transducing components Ig-α (CD79a) and Ig-β (CD79b). mIg engagement by an antigen induces receptor aggregation and signaling cascade initiation by phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) within Ig-α and Ig-β cytoplasmic tails. Each of the two mIg-associated components harbor a single ITAM motif comprised of two tyrosine residues surrounded by specific consensus sequences. Subsequent tyrosine-phosphorylation of these ITAMs by the Src family of protein kinases LYN, FYN, or BLK promotes the recruitment of SYK kinase through SH2 domain-mediated interactions. SYK recruitment to Ig-α and Ig-β components triggers its activation through tyrosine-phosphorylation, and the initiation of downstream signaling pathways [125].

One of these activated signaling cascades starts with the Syk-mediated phosphorylation of the B cell linker protein BLNK, which serves as a platform for the induction of several other signaling pathways. Among them, the phosphorylation and activation of phospolypase C-gamma-2 by SYK and BTK kinases is an important event in BCR signaling ultimately leading to Ca^{2+} release from intracellular stores. Indeed, deficiency in the activity of these two kinases has been reported to generate severe B cell dysfunctions [125]. PLCG2 is phosphorylated also by Src kinases LYN and BLK [126]. Together, all these processes are needed for the B cell to enter activation programs involving cell survival, proliferation and migration [127].

Alterations of BCR signaling have been reported in SLE patients [128, 129], however, more studies are needed to reach a better understanding of the pathologic mechanisms underlying disease pathogenesis. In the last decade, new evidence is arising from functional studies carried out with risk genes derived from the GWAS era [39, 130, 131]. As mentioned before, many of the genes associated with risk to SLE are key players in immune signaling, such as BLK, BANK1 or TRAF6. The most important findings on these proteins related to autoimmunity will be discussed in the next sections.

Src family of tyrosine kinases

The Src family of non-receptor protein kinases comprise eight members which are divided into two subsets: SRC, YES1, FYN and FGR (subfamily A), and BLK, HCK, LCK and LYN (subfamily B). They display a high level of homology and a conserved organization of domains [132]. SFKs modular structure is composed of an N-terminal SH4 domain containing an acylation site (**Table 5**) important for subcellular distribution and trafficking [133], a unique segment of 50-70 aminoacids, SH3 and SH2 domains, a linker segment, a kinase domain harboring a positive regulatory tyrosine, and a C-terminal tail with a negative regulatory tyrosine [134, 135]. Upon SFK auto-inhibition by phosphorylation of the C-terminal tyrosine, the SH3 and SH2 domains turn inward and interact intra-molecularly, locking and stabilizing the KD in an inactive conformation. The SH3 domain binds the linker segment that connects the SH2 and KD, and the SH2 domain binds the phosphorylated C-terminal tail. It is noteworthy that the intra-molecular sequences bound by the SH3 and SH2 domains are not 'high-affinity' binding sites, e.g. the linker segment of Src does not contain the canonical PxxP motif from SH3 ligands, but the linkers of both Src and Hck do adopt a PPII helical conformation in association with their respective SH3 domains. The fact that these domains use their ligand-binding surfaces to maintain the autoinhibited conformation and that these interactions are, in general, weak has important implications for controlling the activation state of Src kinases [136-138].

Table 5. Src family of protein kinases

NAME	Acylation (postition)	Tissue expression
SRC SUBFAMILY A		
SRC (536 aa)	N-myr-Gly (2)	Ubiquitous
YES1 (543 aa)	N-myr-Gly (2) S-pal-Cys (3)	Ubiquitous
FYN (537 aa)	N-myr-Gly (2) S-pal-Cys (3) S-pal-Cys (6)	Ubiquitous, higher expression in hematopoietic cells
FGR (529 aa)	N-myr-Gly (2) S-pal-Cys (3) S-pal-Cys (6)	Myeloid cells (high) and B cells (middle)
SRC SUBFAMILY B		
BLK (505 aa)	N-myr-Gly (2)	B cells, γδ T cells
HCK (526 aa)	N-myr-Gly (2)	Hematopoietic cells (mainly myeloid)
LCK (509 aa)	N-myr-Gly (1) S-pal-Cys (3) S-pal-Cys (5)	T cells
LYN (512 aa)	N-myr-gly (2) S-pal-cys (3)	Myeloid cells and B cells

Although the molecular mechanisms of SFKs activation are complex and may vary depending upon cell types and extracellular signals, it is generally believed that full activation of SFKs is achieved by the following order: 1) Substrates such as receptors, adaptors, or effectors interact with the SH2/3 domains of inactive SFKs to open up the assembled conformation, and direct SFKs to appropriate intracellular locations; and 2) tyrosine phosphatases dephosphorylate the exposed c-terminal inhibitory pTyr to stabilize the active conformation; and 3) the activated SFKs undergo trans/auto-phosphorylation on a tyrosine residue within the activation loop to lock the catalytic pocket into the fully active conformation [139].

SFKs are involved in many key cellular processes such as proliferation, survival, migration and differentiation. They participate in a number of cellular pathways involving receptors of cytokines, growth factors, adhesion proteins, G-coupled receptors or steroid hormones. Immune receptors such as the BCR and some of its co-inhibitory receptors like CD22 also promote the phosphorylation and activation of SFKs. Playing such important roles in cell signaling, their activity must be tightly regulated in order to avoid potentially dangerous dysfunctions. Indeed, alterations in SFKs have been consistently associated with risk of cancer and autoimmunity [140, 141]. Particularly, both LYN [31] and BLK [3] kinases have been genetically associated with risk to SLE.

The B lymphocyte kinase, BLK

BLK is involved in B-cell receptor signaling and B-cell development [142-146]. It was first described to be specifically expressed in human B cells [147] (http://biogps.org). Additionally, the onset of its expression was pointed to early stages in B cell development and increased until B cells became mature displaying high expression levels [148]. Supporting these findings, transgenic mice expressing a constitutively active form of BLK generated an expanded pre-B cell population [142, 149] by mechanisms that were dependent on pre-BCR signaling [142]. Also, *BLK*, *FYN* and *LYN* triple KO mice displayed strong B cell lymphopenia [150]. However, BLK KO mice did not show any significant phenotype regarding B cell development, *in vitro*

activation, or antibody production against both T cell-dependent and independent antigens [148]. All these studies underline the redundant nature of some of the signaling roles of Src kinases.

More recently it has been shown that BLK is also expressed in γδ T cells subtypes, but not in αβ T cells or in other lymphoid precursors. Development of IL-17-producing γδ T cells in mice was shown to depend specifically on BLK expression [151]. Another study in mouse delineated with more detail the differential expression of BLK in mature B cell subsets. MZ B cells displayed high expression levels, B1 B cells intermediate to high levels, and FO B cells low levels. The study also suggested that MZ B cells are decreased in BLK-deficient mice but at the same time they are hyper-responsive to both *in vitro* and *in vivo* BCR stimulation [146].

Unlike LYN association, polymorphisms in BLK have consistently been associated with SLE [3, 152]. Several other autoimmune diseases such as rheumatoid arthritis [153-157]; systemic sclerosis [158]; primary Sjögren's syndrome [159]; dermatomyositis [160] and Kawasaki disease [161] also sowed association with BLK haplotypes. Most of the disease-associated BLK haplotypes significantly correlate with lower expression of BLK mRNA in immortalized B cell lines [3, 162]. Further, a rare genetic variant producing an alanine to threonine substitution in position 71 was found to potentially render lower BLK protein stability and also correlated with susceptibility to SLE [163], suggesting once again that low BLK levels confer risk for autoimmunity. More recently, we found a correlation between risk *BLK* genotypes and increased B1-cell subset and lupus-related phenotypes in mice and humans [164].

BLK phosphorylates in vivo the Ig α and β subunits of the B cell receptor and is able to phosphorylate *in vitro* the receptors of the Fc portion of IgG, FCGR2A, FCGR2B and FCGR2C [165, 166]. BLK kinase is known to bind the phosphatidylinositol lipase C-gamma-2 [167] and our group has previously shown that BLK participates in the formation of a complex between PLCG2 and the B-cell adaptor protein with ankyrin repeats BANK1 during BCR signaling. This complex is transiently induced by IgM stimulation in B-cell lines and its constitution is largely dependent on the BLK level of activation, as well as on its lipidation pattern [126]. Following these BCR engagement-induced events, it has been shown that activated murine BLK is preferentially degraded by the ubiquitin-proteasome pathway and that its ubiquitination

is mediated by the E3 ubiquitin ligase E6AP [168].

The B-cell scaffold protein with ankyrin repeats, BANK1

Yokoyama and colleagues described BANK1 for the first time in 2002 as a B cell scaffold protein with ankyrin repeats that is tyrosine phosphorylated by Syk upon BCR stimulation. At that time it was hypothesized that BANK1 functioned in calcium mobilization specific to foreign antigen-induced immune responses because of its confined expression to functional BCR-expressing B cells [169]. Among other predicted functional residues and domains, it contains a number of sites of tyrosine phosphorylation and proline-rich motifs which serve to interact with proteins harboring SH2 and SH3 domains, such as BLK and LYN [126] (**Figure 3**). A previous study based on mutational analyses suggested that LYN SH3 domain integrity is not determinant for this interaction and rather supported a mechanism of binding dependent on tyrosine phosphorylation and LYN SH2 domain [170]. Nevertheless, the exact domains and mechanisms orchestrating the binding between BANK1 and its Src-kinases partners are yet far from being well understood.

Two isoforms of BANK1 were described in 2008 by Kozyrev and colleagues. The first one was considered the full-length isoform whereas the second one, lacking the exon 2 because of alternative splicing, was called Delta 2 (D2) [4]. Four years later, Troutman et al. found that exon 2 encoded a cryptic putative TIR domain, a motif essential in TLRs and IL-R signaling [171], but no specific experiments to prove its functionality were performed. Expression of the shorter D2 isoform was initially associated with protection to develop SLE in a GWAS [4], but this association could not be replicated in a more recent study [172]. BANK1 has also been genetically associated through GWASs with other inflammatory diseases such as systemic sclerosis [173] and RA [174, 175].

Bank1 knockout mice presented enhanced germinal center formation and IgM production in response to T-dependent antigens [176], suggesting, in contrast to previous studies, a regulatory role for BANK1 in B cell signaling. Further analyses in *Bank1* and *Cd40* double knockout mice suggested that BANK1 exerts its effects specifically through CD40 signaling and prevents B cells from becoming hyperactive by

negatively regulating Akt activation [176]. More recently, a study also investigating BANK1 role in BCR/CD40 signaling, but this time in human peripheral B cells, apparently suggested opposite findings [130]. Compared with non-risk cells, BANK1 non-synonymous risk variants correlated with decreased BCR and CD40 signaling, displaying lower levels of Akt activation and consistent decreased levels of the Akt-regulated transcription factor FOXO1. Subjects with risk haplotypes also presented an expanded memory B cell compartment, whose percentages within the whole set of individuals (risk and non-risk) were in turn correlated with FOXO1 levels. Differences between a mouse model and humans, or between non-synonymous variants and a knockout system may account for these inconsistencies across studies.

On the other hand, it was described a role for BANK1 in TLR9 pathway. Experiments performed in mouse *Bank1*$^{-/-}$ splenic B cells revealed a reduction in TLR9-induced p38 phosphorylation. IL-6 expression was decreased in BANK1-deficient B cells upon CpG stimulation. Affectation of IL-6 translation initiation was suggested as the mechanism responsible because of the observed reduced phosphorylation of kinases MNK1/2 and the translation initiation factor eIF4E in this cells [177]. Finally, the effects of BANK1 deficiency in a lupus *Sle1.Yaa* mouse model [178], in which TLR7 duplication plays a key role in disease development were described. Purified splenic B cells from B6.Sle1.yaa.Bank1-/- mice showed reduced *Ifnb*, *Ifna4*, *Irf7*, *Aicda* and *Stat1* expression, impaired STAT1-Tyr701 phosphorylation and nuclear translocation of IRF7 after TLR7 agonist stimulation. Importantly, all of this was accompanied by the significant reduction of both serum IgG anti-dsDNA autoantibodies and the pathogenic-associated IgG2c antibodies. The B cell activating factor BAFF was also reduced in BANK1 deficient mice sera [179].

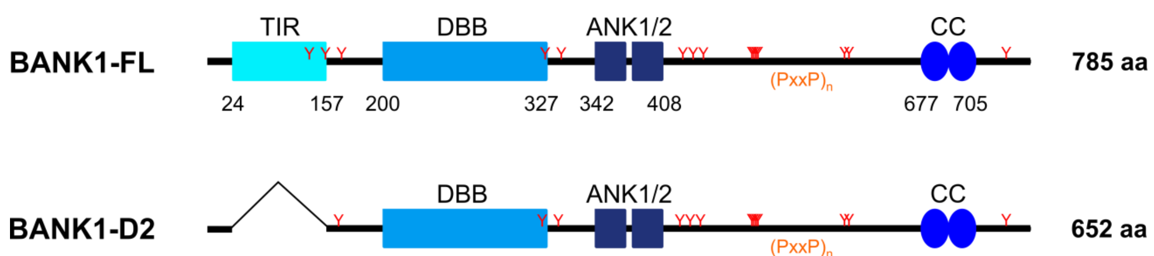


Figure 3. Structure of BANK1 Full length and D2 isoforms. D2 isoform lacks the cryptic TIR domain encoded by exon 2. **TIR,** Toll/interleukin-1 receptor domain, found as a putative

domain [171]; **Y**, tyrosines predicted to bind SH2 domains; **DBB** (aa 200-327), Dof/BCAP/BANK motif, mediating dimerization; **ANK**, ankyrin repeats: ANK1 (aa 342-371), ANK2 (aa 378-408), involved in protein interactions; **PxxP**, proline rich and hydrophobic regions predicted to bind SH3 domains; CC: coiled-coil domain.

4.2.2. TLR signaling

In contrast to pDCs, in B cells the BCR can deliver DNA-associated autoantigens directly to early endosomes, leading to strong B cell activation [180]. It has been recently shown that BCR internalization promoted by DNA-containing antigens is a requirement for TLR9 translocation to autophagosome-like compartments, where TLR9 and BCR co-localized [181].

All TLRs, except TLR3, initiate signaling via the common adaptor myeloid differentiation factor 88 (MyD88) [182]. Upon TLR activation, MyD88 alone, or together with the Toll/interleukin-1 receptor (TIR)-domain-containing adaptor protein (TIRAP), is recruited forming a complex via their TIR domains. MyD88 interacts then with IL-1R-associated kinase (IRAK) 4 through its death domain and thereby activates IRAK1 and IRAK2 [183, 184], which then directly interact with tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6. As a result, TRAF6 is Lys63-ubiquitinated, activated, and further propagates the signal leading to the activation of interferon-regulatory factor 7 (IRF7), nuclear factor-kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs). These signaling pathways potentially provide the framework to produce high amounts of type I IFNs, pro-inflammatory cytokines, and expression of co-stimulatory molecules for the host inflammatory response [63].

Tlr7 transgenic mice spontaneously develop an SLE-like disease as a result of TLR7 overexpression. Notably, mDCs and pDCs in this model were unable to cause an autoimmune phenotype in the absence of B cells. On the contrary, disease development was dependent on the concomitant action of innate signals and T-cell-mediated stimulation upon B cells [185]. BXSB.*Yaa* male mice harbor a duplication of the *TLR7* gene and show a lethal autoimmune phenotype with 50% mortality within 30 weeks. It was recently suggested that high IL-6 secretion by B cells in this mouse model promotes differentiation of IL-21-secreting T FO helper cells, which in combination trigger disease manifestations [186]. Elevated serum IL-6 is an indicator of disease

activity in lupus patients [187-189], and interestingly, its increased secretion in the BXSB.*Yaa* mice was orchestrated by TLR7, BCR and IFN-I receptor signaling integration in B-cells [186]. In addition, TLR7 deficiency in several mouse models leads to the amelioration of renal affectation and mortality [190]. Although more controversial, the role of TLR9 in autoimmunity has been also highlighted by a number of studies [191-194]. Production of anti-dsDNA and anti-chromatin autoantibodies in the MRL/Mplpr/lpr murine lupus model was specifically diminished by TLR9 deficiency, although strikingly, no effect was observed on the clinical outcome or the development of nephritis [195]. In addition, a study analyzing C57BL/6-lpr/lpr mice showed that anti-nucleosome antibody production was completely abrogated in the absence of TLR9, whereas no changes were observed in anti-dsDNA autoantibody titers [196]. In turn, other studies rather support a protective role for TLR9 against self-tolerance breakdown. In 2006, Christensen and colleagues further studied the effects of TLR9 in MRL/Mp$^{lpr/lpr}$ mice and showed that its genetic deficiency leads to increased B cell activation markers and pathogenic IgG antibody production, as well as an acceleration of nephritis and mortality [197].

The Myeloid differentiation primary response protein 88, MyD88

MyD88 is a key link between TLRs and IRAK kinases in the initiation of most TLR pathways, except for TLR3 which signals through the TRIF adaptor. Among the rest of TLR pathways in which MyD88 is involved, TLR7 and TLR9 are strictly dependent on MyD88 for signal propagation [63]. Although it has not been genetically associated with autoimmune diseases, some mutations in the gene were found in lymphomas and some others correlated with increased susceptibility to certain infections [56]. An implication in innate autoimmune processes is therefore not surprising because of its strategic position in the transduction cascade [198-200]. For instance, FcγRIIB−/−.B6 mouse is a strain-specific SLE model that develops glomerulonephritis and premature mortality as a consequence of pathogenic autoantibody deposition in the kidneys [201]. Myd88-deficiency in this model specifically prevented self-reactive IgM-B cells from class switching to pathogenic IgG subclasses, thereby reducing disease manifestations and mortality [202]. Also, MyD88 deficient mice were not able to produce IL-6 or TNF when exposed to ligands for TLR2, TLR4, TLR5, TLR7 or TLR9 [63].

The TNF receptor-associated factor 6, TRAF6

TRAF6 was firstly reported by GWAS analyses to be associated with rheumatoid arthritis [203] and subsequently with SLE [204]. TRAF6 is a member of the TRAF family and acts as an adaptor protein downstream of multiple receptor families such as the TNFR superfamily [205], the tumor growth factor-β receptor (TGF-βR), the T-cell receptor as well as TLRs/interleukin-1 receptor (IL-1R) [206, 207] being critical for numerous immune cell subsets in development, homeostasis and activation. Thereby, it is involved in the direct activation of NFκB, MAPK, phosphoinositide 3-kinase (PI3K), Akt/PKB pathway, and the interferon regulatory factors (IRFs) 5 and 7. TRAF6 has also been shown to perform signaling cross-talk with the JAK-STAT non-canonical pathway by recruiting STAT1 into the TLR signalosome via phosphorylation of its serine 727 [208]. TRAF family members consist of an N-terminal Zn RING finger domain, a series of five Zn finger domains, a coiled-coil TRAF N-domain and a C-terminal TRAF-C domain which is specific for TRAF6 as it provides it with signaling specificity towards consensus P-X-E-X-X-aromatic/acidic motifs and P-X-E-X-X-X-aromatic/acidic motifs, respectively [209, 210]. These motifs are found in interacting proteins such as IRAK1 and IRAK2 [211, 212], TNFR family members like CD40 [213], and receptor activator of NF-κB (RANK) [214]. It is believed that TRAF6 is recruited to signaling platforms interacting partly with proteins that contain TRAF6 binding motifs but also with proteins already present in the platform. Activation of TRAF6 protein requires its Zn RING finger domain which holds E3 ubiquitin ligase activity [215], and together with the ubiquitin conjugating enzyme Ubc13 and the Ubc-like protein Uev1A catalyzes its own site-specific ubiquitination through the synthesis of unique lysine-63 (K63) polyubiquitin chains rather than through the conventional K48-linked chains typically associated with proteasomal degradation [216, 217]. TRAF6 K63-linked autoubiquitination is often used as read-out for TRAF6 activation [218].

Traf6-deficient mice revealed that TRAF6 is required for proper B cell maturation, baseline IgM production as well as T cell-independent antigen-specific IgM production, and finally for generation of long-lived Ig-secreting bone marrow plasma cells. However, TRAF6 exhibits redundancy with TRAF2/3 in GC formation. In addition, B cells from TRAF6-ΔB mice failed to activate MAPK and NF-κB and to proliferate in response to LPS, CpG-DNA, and anti-CD40 antibody stimuli *in vitro* [219, 220].

B cell-specific biological treatments

Rituximab is a chimeric monoclonal IgG1 anti-CD20 antibody that effectively depletes B cells for 6-12 months and has been assessed in two clinical trials: EXPLORER and LUNAR [221, 222]. However, none of them have met their endpoints and only one trial showed promising effects of Rituximab as a steroid-sparing drug in the treatment of lupus nephritis [223].

Belimumab is a monoclonal human antibody that blocks the TNF family-related cytokine BLys (*aka* B-cell activating factor, BAFF). BAFF binds three receptors on the surface of B cells: BR3, TACI and BCMA, and inactivation of BAFF binding to BR3 by *Belimumab* promoted apoptosis and decreased maturation of B cells [224]. In fact, it was the first biological treatment approved in the USA and Canada for those cases where standard drugs failed to control disease activity [225].

Finally, *Epratuzumab* is a humanised anti-CD22 IgG1 monoclonal antibody that inhibits BCR signaling by engaging the inhibitory co-receptor CD22 on the surface of mature IgM+/IgD+ B cells [226]. It reduces peripheral B cell count in individuals with SLE, and during a phase IIb trial (EMBLEM) of patients with moderate to severe lupus, it decreased symptoms and disease activity (especially in the cardiorespiratory and nervous systems) [227, 228].

Cytotoxic/immunosuppressive agents are the basis of SLE treatment. They are not specific and have many undesired, significant side-effects which need to be avoided in order to keep patients safety. Despite all the efforts made to develop new biological targeted treatments for SLE, there is still much to be done to control disease impact on patients. The great heterogeneity of lupus makes this goal a very complicated and ambitious task. A deeper comprehension of all the intracellular and intercellular immune signaling molecules -especially those genetically associated with SLE- will allow us to develop new specific and personalized treatments [229, 230].

Objectives

III. OBJECTIVES

1. BLK Ala71Thr SLE risk variant

A) To corroborate the effect of the Ala71Thr variant on the stability of endogenous BLK protein in a B lymphocyte model.

B) To analyze the influence of variant rs55758736 (A71T) on the BLK mRNA and protein expression levels.

C) To determine whether the newly formed threonine is susceptible to phosphorylation, and if so, what are the consequences of such phosphorylation.

D) To investigate the effect of the BLK variant over the functionality of the SH3 interaction domain where it lies:

D.1) On the alteration of subcellular localization and trafficking of BLK.

D.2) Effects on the interaction with protein partners of BLK.

2. BANK1 in TLR pathways

A) To search for BANK1 partners in TLR pathways based on its protein motifs:

A.1) To determine whether the cryptic TIR domain of BANK1 is functional.

B) To study BANK1 activity within TLRs pathways.

Materials and Methods

IV. MATERIALS AND METHODS

Cloning, fluorescent tagging and targeted mutagenesis of expression vectors

Cloning PCR reactions were developed with the high fidelity Kapa Taq Polymerase (Kapa Biosystems) following manufacturer recommendations. PCR products were separated by electrophoresis in an agarose gel, discriminated by length and purified by an agarose PCR clean-up kit (NucleoSpin Gel and PCR Clean-up, Machery-Nagel). Genes fused to a V5 epitope sequence in this work were cloned into the pcDNA3.1D/V5-His-TOPO expression vector (Thermo Scientific) following instructions of the manufacturer. In this system, a CACC tail is added to the forward cloning primer before the ATG initiation codon. A complementary, overhanging GTGG tail in the vector ensures the correct insertion of the amplicon through a process driven by the enzyme topoisomerase I. The overhang in the cloning vector (GTGG) invades the 5′ end of the PCR product, anneals to the added bases, and stabilizes the PCR product in the correct orientation. After ligation, 1 µl of ligation product was added to 25 µl of One Shot™ TOP10 Chemically Competent E. coli (Thermo Fisher Scientific). The mix was incubated on ice for 45 min and then for 30 s at 42°C in a waterbath. 125 µl of commercial SOC medium (Thermo Scientific) were added and bacteria incubated for 30 min at 37 °C and shacking. Next, 100 ul of bacteria were seeded in LB-agarose selective plates with ampicillin and incubated overnight at 37°C.

The day after, bacteria colonies were picked and quickly soaked in PCR reaction mix to immediately incubate them in 3 ml of selective LB medium with ampicillin. Colony-PCRs were developed to check the correct insertion of the gene by adding a specific primer pair matching at both the insert and the vector backbone sequences. PCRs were run in a 1 % agarose gel and positive clones identified by the presence of bands with the expected length. 24 h after, plasmid DNAs were recovered from positive colonies by Sigma-Aldrich GenElute™ Plasmid Miniprep Kit and sent for sequencing at Genyo´s Genomics unit. Sequences were checked by Sequencher software [231]. Expression of clones was assessed by WB against V5 tag after trasnfecting and extracting the protein from HEK293 cells (see section Cell Lysis and Western Blotting).

For fluorescent tagging, GFP, YFP, CFP or RFP were excised from vectors

already available in the lab by using restriction enzymes *NotI*, *XbaI* and *ApaI*, alternatively when a target sites were found within the gene sequence. Subsequently they were added in frame to the C-terminus of the expression vectores, between the gene sequence and the V5 epitope.

Mutations in codifiying sequences of the cloned genes were generated by site-directed mutagenesis. After developing a PCR with the original expression vector as template and primers harboring the necessary mismatches (**Table 6**), PCR products were digested with DpnI enzyme and transformed into competent E. coli bacteria. A threonine was introduced at position 71 of *BLK-YF-V5* plasmid and a glutamic acid at the same position in the *BLK-WT-V5* plasmid.

BLK was amplified using a pool of cDNAs from five LCLs as template. The rest of the plasmids used were already available in the lab at the moment of starting the thesis project or kindly provided by the Cellular Signal Integration Lab, Institute of Molecular Toxicology and Pharmacology, German Research Center for Health and Environment (GmbH), Helmholtz Centre, Munich (specifically pRK5-*Myc-TRAF6*, pRK5-*RFP-TRAF6,* pRK5-*HA-Ubiquitin-K63* and pRK5 empty vectors). Myc tag and RFP were fused at the N-terminus. All plasmids sequences were checked by sequencing.

Table 6. Primers used for cloning of the expression constructs, directed mutagenesis and cDNA amplification for BLK mRNA degradation assay or RT-qPCRs

Constructs/template	Name	Sequence
pcDNA-BLK-WT	BLKf	5´-cacccATGGGGCTGGTAAGTAGC-3´
(cloning)	BLKr	5´-CTAGGGCTGCAGCTCGTACTG-3´
pcDNA-BLK-71T	BLKA71Ef	5´- TATGACTACACCACTATGAATG -3´
(mutagenesis)	BLKA71Er	5´- CGATCATTCATAGTGGTGTAGTC -3´
BLK cDNA	BLK71f	5´- CCGATGAACACCTGGATG -3´
(mRNA degradation)	BLK71r	5´- GCCACCAGTCTCCAGTT -3´

Overhanging sequence CACC for directional clonning is shown in lower case.

Genotyping and Immortalized B cell lines

Genotyping of samples from control individuals at the Oklahoma Medical Research Foundation was done using the Illumina ImmunoChip (Illumina) that contained the BLK rs55758736 A71T genetic variation. Confirmation of heterozygous samples was carried out using Sanger sequencing.

Permanent B cell lines were established by EBV transformation of peripheral blood lymphocytes from donors previously genotyped for rs55758736.

Cell culture and transfections

Transformed B cells and lymphoma B *cell lines BJAB and Namalwa* were maintained in RPMI 1640 medium supplemented with Glutamax (Thermo Fisher Scientific) and 10% fetal bovine serum (Biowest). Embryonic kidney HEK293T and U2OS cell lines were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific), supplemented with Glutamax (Thermo Fisher Scientific) and 10% fetal bovine serum (Biowest). Embryonic kidney HEK293T cells and Lymphoma *cell line BJAB* were obtained from The American Type Culture Collection (ATCC). U2OS cell line was kindly provided by Dr. Daniel Krappmann (Helmholtz Zentrum, Munich). All cell lines were authenticated by the supplier and negative for mycoplasma contamination test.

Transient cell transfections for exogenous co-immunoprecipitation (CoIP) experiments across the thesis were carried out with jetPRIME reagent (Polyplus Transfection). The amounts of reagent, buffer and plasmid DNA (pDNA) were scaled depending on the number of cells to be transfected following manufacturer´s instructions. Cells were seeded in 6-well plates and transfected when reached 60-80 % confluency, normally 24 h after seeding. 24h post-transfection, cells were washed in ice-cold DPBS (Thermo Fisher Scientific), harvested and lysed.

For interaction assay in the BJAB B cell line between recombinant BLK71A/T and endogenous BANK1, tranfections of 45 x 10^6 cells per condition were done with 100 µl tips (7.5 x 10^6 cells/nucleofection) from Neon® Transfection System (Thermo Fisher Scientific). For nucleofections, 80 µg of each pDNA from highly clean and

concentrated preparations (~ 3-4 µg/µl) were used with the following conditions: 1100 v, 20 ms, 2 pulses. 48 h later, cells where stimulated, washed with 1 mM orthovandate in cold DPBS (Thermo Fisher Scientific) before lysis with 0.5% NP-40 lysis buffer (see recipe in Cell lysis and Western Blotting section).

Cell treatment and stimulation

In case of HEK293 cells treatment with 25 µM pervanadate or 10 µM MG132: 24 h after transfection cell media was removed and replaced with fresh media containing the compound at the desired concentration for the indicated times. Pervanadate was freshly prepared by mixing 10 mM of sodium orthovanadate (Sigma-Aldrich) solution with 10 mM H_2O_2 for 20 min at room temperature. Next, catalase (Sigma-Aldrich) was added at a final concentration of 200 mg/ml to remove residual H_2O_2.

B cell lymphoma cell lines were centrifuged at 200 x g at room temperature a day prior stimulation, washed with 1x DPBS (Thermo Fisher Scientific) and medium was replaced with fresh RPMI. On the day of stimulation, cells were centrifuged, counted and left unstimulated or stimulated with 5 µg/mL Imiquimod (Invivogen) and 2 µM CpG (ODN2006) (Invivogen), for endogenous BANK1 CoIPs. Cells were harvested together after times of stimulation. In case of recombinant BLK71A/T interaction with endogenous BANK1 in the BJAB B cell line, cells were washed after 48 h of transfection and stimulated for 5 min with 10 µg/ml F(ab´)2 anti-IgM (Southern Biotech) 0.5 µM and CpG (ODN2006) (Invivogen).

Cell lysis and Western Blotting

In general, cell pellets were lysed with NP-40 lysis buffer containing 0.5% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM Na_3VO_4, 1 mM PMSF and protease and phosphatases inhibitor cocktails (Roche) for 20 minutes on ice and centrifuged at 20000 x g for 10 minutes at 4°C. For immunoprecipitations, a fraction of the lysate was kept for input loading. When needed, sample protein concentrations were quantified with a Micro BCA Protein Assay Kit (Thermo Fisher Scientific).

For BANK1-TRAF6-MyD88 endogenous CoIP reactions cells were specifically lysed in 2.5 mL RIPA buffer (150 mM NaCl, 5 mM EDTA, pH8.0, 50 mM Tris-HCl, pH8.0, 1% NP-40, 0.5% $C_{24}H_{39}NaO_4$, 1mM sodium fluoride and 1 mM sodium vanadate and protease inhibitor cocktail) dissolving using a 20G needle. Further incubation followed for 20 minutes in a cold room on a rotor. Afterwards, the lysates were centrifuged at 20.000 x g for 40 minutes at 4°C. Cleared lysates were transferred to fresh 15 mL Falcon tubes from which 30 μL were used to test expression levels of endogenous proteins and the rest of the lysates were used to perform endogenous IP reactions.

For ubiquitination assays lysis, please see their section.

All lysates and immunoprecpitations were resolved by SDS-PAGE electrophoresis and transferred to PDVF membranes using Trans-Blot Turbo (Biorad). Membranes were blocked either in 5% non-fat dried milk or bovine serum albumin (BSA) (US Biological, A1310, USA) in buffer supplemented with 0.1% Tween-20 (Sigma-Aldrich, USA) on a rocking shaker for 1 hour at room temperature.

Antidobies

The primary antibodies used were: anti-BLK clone 7A12 (Abnova; #H00000640-M02); anti-V5 (Thermo Fisher Scientific; #R96025); anti-HA antibody (clone 3F10, Roche); anti-BANKu1 (#HPA037002), anti-GAPDH (#SAB3500247), Anti-β-Actin clone AC-15 (#A5441), anti-FLAG(M2) (#F3165) (all from Sigma-Aldrich); anti-MyD88 (#3699S), anti-Phospho-Threonine (#9381), anti-P-Tyr 100 (#9411) (all from Cell Signaling); anti-TRAF6 (H-274, sc-7221), anti-TRAF6 (D-10, sc-8409), anti-TLR7 (V-20, sc-16245), anti-TLR9 (N-15, sc-13215), anti-Myc (9E10, sc-40), anti-ubiquitin clone P4D1 (sc-8017) and rabbit IgG (sc-2027) and mouse IgG (sc-2025) as non-specific control immunoglobulins (all from Santa Cruz). The secondary antibodies used were: anti-Mouse IgG-HRP (Santa Cruz; #sc-2005) anti-Rabbit-HRP (Santa Cruz; #sc-2004) and anti-Chicken–HRP (Sigma-Aldrich; #A9046).

Cytokine production

Human IL-6 and TNFα (both BD Biosciences) were measured by ELISA according to the manufacturer's instructions to check whether Namalwa cells were properly stimulated with either IMQ or CpG.

Ubiquitination assays

For BLK project, after treatment with pervanadate and MG132, cells were centrifuged, washed with ice-cold DPBS (Thermo Fisher Scientific) and lysed in 400 µl Co-IP lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 0.5% NP-40, 10% glycerol, 1mM DTT, 10 mM sodium fluoride, 8 mM β-glycerophosphate, 300 µM sodium vanadate and protease inhibitor cocktail) containing 1 % SDS. Precipitated genomic DNA was removed from lysates by first passing samples through 21G needles and secondly through 26G needles. Next, centrifugation was done at maximum speed for 45 minutes at 4°C. Subsequently, 30 µl of the lysate was kept for immunoblotting analyses and the rest was diluted 10-fold with Co-IP buffer to perform Co-IP reactions preserving the antibodies integrity with a final concentration of 0.1% SDS. 10-fold diluted extracts were incubated overnight at 4ºC with gentle rotation with 1 µg anti-V5 antibody (Thermo Fisher Scientific). 24 h later, Protein G Sepharose beads (Thermo Fisher Scientific) were added to extracts followed by incubation on a rotor at 4°C for 1h. Next, extracts were washed three times with 0.1% NP-40 Co-IP buffer containing protease inhibitors (Roche) and finally boiled in 20 µl of 2x LDS sample buffer (Thermo Fisher Scientific) at 95°C for 8 minutes. Samples were analyzed by Western blotting.

For TRAF6 ubiquitination, 24 h post-transfection cells were harvested, washed with ice-cold DPBS (Thermo Fisher Scientific) and lysed in 400 µl Triton-X buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Roche)) containing 1 % SDS. To remove precipitated genomic DNA from lysates, samples were firstly passed through 21G needles and secondly through 26G needles. Afterwards, centrifugation followed at maximum speed and 4°C for 45 minutes. Thereafter, 30 µl of the lysate was used for immunoblotting and the rest to perform Co-IP reactions. Extracts were diluted 10-fold with Triton-X buffer to have a final concentration of 0.1% SDS and anti-HA antibody was added for IP reactions. The next day, Protein G Sepharose (Thermo Fisher Scientific) was added to the extracts and

incubated on a rotor followed for 1h at 4°C. Afterwards, extracts were washed three times in Triton-X buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail (Roche)), boiled in 40 µl 2x LDS sample buffer for 8 minutes at 95°C and analyzed by Western blotting.

Co-immunoprecipitations

CoIPs in this thesis were done with Dynabeads® Protein G (Thermo Fisher Scientific) adapting the manufacturer´s protocol as follows: Ab-Ag complexes were washed with 0.1 % NP-40 (recipe in Cell lysis and Western Blotting section). Co-immunoprecipitation against targeted protein was carried out with 1 µg of antibody per reaction. Proteins were resolved as described in Cell lysis and Western Blotting section. The following paragraph explains the only exception (beside ubiquitination assays: see their section) because of the specifically required interaction conditions, which are specified:

For BANK1-TRAF6-MyD88 endogenous CoIP in Namalwa B cell line, lysates were combined with TRAF6 antibody (D-10) (Santa Cruz) or mouse-IgG as a non-specific control for immunoglobulins (Santa Cruz) in a total volume of 4 ml (filled in with fresh lysis buffer) overnight on a rotor at 4ºC. The next day, Protein G Sepharose (Thermo Scientific) was added for 1-2 hours at 4°C. IPs were washed three times in CoIP buffer (150 mM NaCl, 25 mM HEPES, pH7.5, 0.2% NP40 (w/v), 1 mM glycerol, 10 mM NaF, 8 mM β-glycerophosphate, 300 µM Na_3VO_4 and protease inhibitors) and boiled in 40 µl 2x LDS sample buffer for 8 minutes at 95°C and analyzed by Western blotting.

Protein degradation assay

Cells were seeded in 24 well plates and treated with 20 µg/ml cycloheximide (Sigma) in DMSO or DMSO alone (time 0) for different times. Cellular extracts were resolved on SDS-PAGE and blotted as described above. Relative quantification of the bands was carried out against GAPDH loading control using ImageJ software [232].

RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Total RNA was purified using the High Pure RNA isolation kit (Roche) and 1 μg total RNA was used as template to synthesize cDNA with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). The quantity and integrity of RNA were evaluated using Nanodrop 2000 device (Thermo Fisher Scientific) and a 2100 Bioanalizer (Agilent Technologies), respectively. Only samples with a RIN above 9.0 were used.

BLK expression was measured by relative quantification using QuantStudio™ 3D Digital PCR System with probes Hs01017452_m1 for BLK (FAM), and Hs001003268 for HPRT1 (VIC) normalizer using cDNA equivalent to 10 ng and 20 ng of RNA. Fluorescence in QuantStudio™ 3D Digital PCR 20K Chips (Thermo Fisher Scientific) was measured by QuantStudio™ 3D Digital PCR Instrument (Thermo Fisher Scientific) and data were analyzed by QuantStudio™ 3D AnalysisSuite™ Cloud Software (Thermo Fisher Scientific). Quality thresholds were set at 0.5 for every chip.

For BANK1 project, qRT-PCR was performed in 384-well microplates using an ABI Prism 7500 detection system (Applied Biosystems) with the iTaq™ universal SYBR® Green one-step kit (Biorad). Each sample was measured in duplicate in two independent experiments. C_T values of samples were normalized to the corresponding C_T values of *HPRT*, and relative expression levels were calculated by the $2^{-\Delta\Delta CT}$ method described by Livak and Schmittgen (74). Primer pairs utilized are listed in **Table 1 (Cloning, fluorescent tagging and targeted mutagenesis of expression vectors section)**

Allelic mRNA Degradation Assay

A71T variant (rs55758736) heterozygotes were treated with 1 μg/ml actinomycin D (Sigma) for 0, 1, 3, 6 hours. RNA was isolated by a High Pure RNA Isolation Kit (Roche) and cDNA was synthesized using Transcriptor First Strand cDNA Kit (Roche). Restriction Fragment Length Polymorphism technique was adapted to cDNA instead of DNA in order to compare the differential degradation of transcripts in BLK Ala71Thr heterozygous cell lines (**Figure 4**). cDNAs were amplified using primes targeting the SNP rs55758736 (**Table 6**). PCR product was digested with restriction enzyme AciI and loaded into an agarose gel for electrophoretic separation.

Quantification of bands intensity was carried out with ImageJ software and normalized to the length of the corresponding restriction fragment.

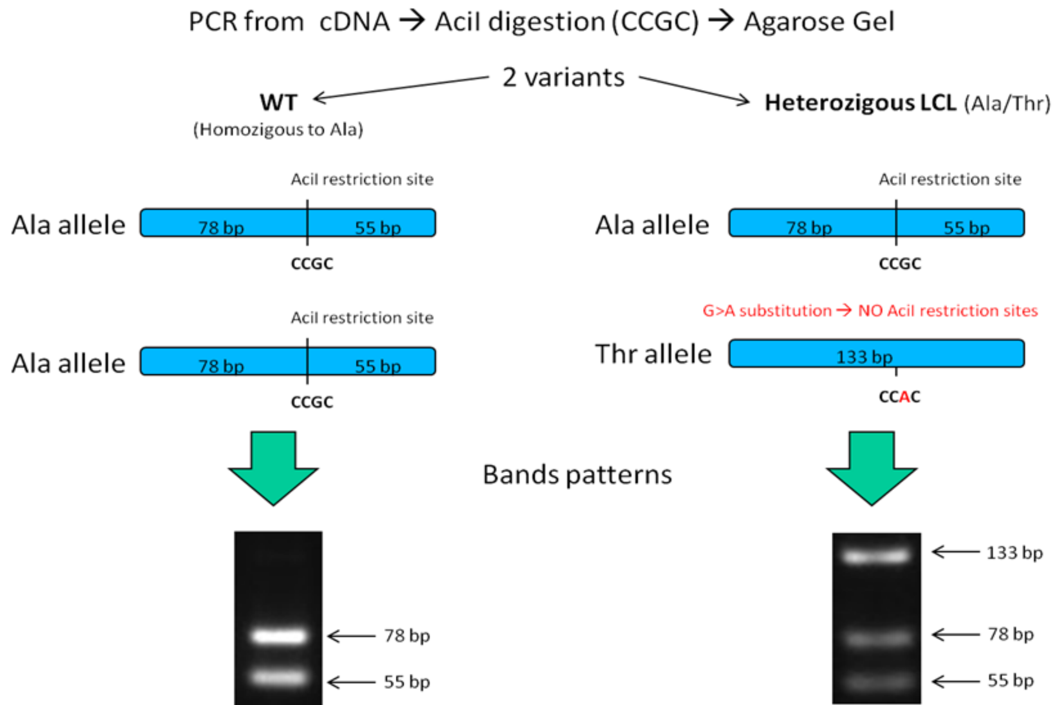


Figure 4. RFLP adaptation diagram. Measurement of bands intensities can give information about allele specific degradation. Examples of the different band patterns obtained from Ala-Ala and Ala-Thr genotypes are shown. We selected the uncut, highest band for 71Thr allele quantification and the lower of the two bands below for the 71Ala counterpart. However, both bands coming from the 71Alanine product were quantified and gave equal relative values.

Microscopy

For BLK 71Thr project, HEK293 cells were grown and transfected for 24 h on Lab-Tek chamber slides coated with poly-D-lysine (Beckton Dickinson). For co-localization analysis, cells were fixed and mounted with SlowFade Gold Antifade Reagent containing DAPI (Thermo Fisher Scientific). Confocal microscopy was performed using a Zeiss 710 confocal scanning microscope with a Zeiss Plan-Apochromat 63x1.40NA oil-immersion objective.

In FRAP experiments we used the 488-nm line argon laser at maximum power (25 mW) with a pinhole adjustment resulting in a 28.6 µm optical slice. The region of

interest containing the fusion protein was recorded as follows: (1) pre-bleach recording (scanning 5 images with 488nm laser line 5% power and time interval 1.94 sec/image), (2) bleaching and scanning of Region of Interests (30-35 iterations scans with 488 nm laser line 100% power for 4.65 sec) and (3) post-bleaching recording (scanning 35 images with 488 nm laser line 5% power and time interval 1.94 sec/image). An additional ROI in a similar sub-cellular location was monitored in parallel to detect fluorescence fluctuations independent of bleaching. A third ROI was placed outside the cell to measure background fluorescence. Average fluorescence intensities within ROIs were measured under the same conditions for each data set. For each construct this procedure was repeated ten times. The relative recovery of fluorescence was calculated at the FRAP region after background and photobleaching subtraction using the normalization according to Siggia[2]. Half-time of fluorescence recovery was calculated using the normalization of Axelrod[3].

For the BANK1 project, co-localization studies with recombinant proteins were carried out in U2OS cells which were grown and transfected on Lab-Tek chamber slides coated with poly-D-lysine (Beckton Dickinson). After 24 hours of transfection, cells were fixed and mounted with ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific) containing, where CFP tag was not present, Hoechst 33342 dye. Confocal microscopy was performed on a Zeiss 710 confocal scanning microscope with a Zeiss Plan-Apochromat 63× oil-immersion objective.

Statistics

Every P value in this work was determined by the Mann Whitney non-parametric *U*-test.

RESULTS

BLK ALA71THR RISK VARIANT

V. RESULTS

BLK mRNA is a short-lived transcript and the 71Thr variant does not affect transcript abundance or mRNA degradation rate

As our group described in 2012, the 71Thr increased the degradation rate of the protein *in vitro* [163]. However, the uncommon G>A variant could be also altering mRNA levels either pre- or post-transcriptionally. To address this issue we investigated a large local DNA sample population to identify individual carriers of the polymorphism. We genotyped 1560 DNA samples for the SNP rs55758736 and detected two homozygous individuals for the minor allele (Thr) and 43 heterozygotes, all of them confirmed by Sanger sequencing. Immortalization of LCLs of the three different genotypes (one Thr-Thr and several Ala-Thr and Ala-Ala) by EBV infection allowed us to assess differences in transcript abundance between the 71Ala and 71Thr alleles. Since the risk allele of SNP rs132771136 in BLK is correlated with low levels of BLK mRNA, we selected those cell lines with a non-risk promoter in order to measure mRNA expression in the most independent manner. Thus, we quantified mRNA amounts in relation only to genotypes of SNP rs55758736 (**Figure 5**). There was no correlation between the presence of the 71Thr allele and transcript abundance.

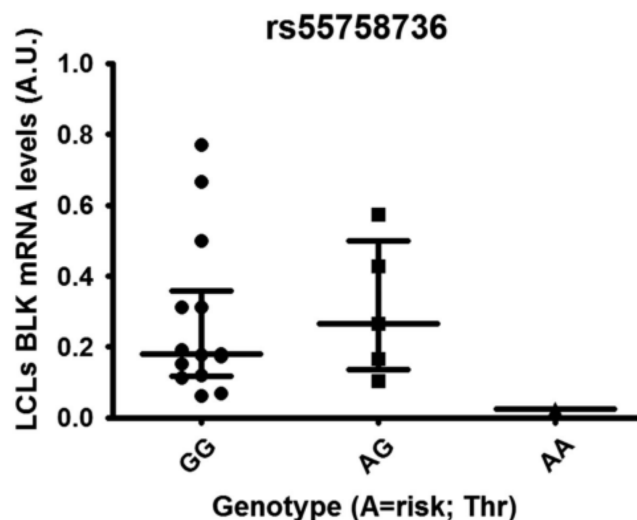


Figure 5. The minor allele of SNP rs55758736 does not alter transcript abundance of BLK. BLK mRNA expression in relation to the different genotypes in transformed B-cell lines from unrelated individuals was measured using QuantStudio™ 3D Digital PCR System (Thermo Fisher Scientific). Fourteen cell lines homozygous for the Alanine allele (GG), five heterozygous cell lines (GA) and the cell line homozygous for the Threonine allele (AA) were

analyzed. The Thr-Thr cell line was, in addition, analyzed in three independent experiments and the mean is represented. The TaqMan® probes (Thermo Fisher Scientific) selected for relative quantification were Hs01017452_m1 for BLK (FAM), and Hs001003268 for HPRT1 (VIC) normalizer. All cell lines were homozygous for the major allele of the SNP rs13277113 in the promoter region of BLK that has been shown to be a major determinant of BLK mRNA abundance [3, 163]. Error bars represent the median with interquartile range.

In order to extend the study on BLK mRNA, we further tested differences in transcript stability between alleles. Modeling of the mRNA structure of both variants at http://mfold.rna.albany.edu did not suggest any difference that could alter transcript stability (data not shown). We also tested the degradation rate of the two mRNA isoforms experimentally using transcription inhibition followed by allelic specific quantification in heterozygotes. The method we developed is a variation of the previously described RFLP technique [163] using total cDNA instead of genomic DNA (**Figure 4 in materials and methods section**). It allowed us to compare decay of each allele in equal cellular conditions given that both transcripts are produced simultaneously in the same cell. Using the heterozygous LCL Ala-Thr 1, we observed an acute decrease in total BLK mRNA after 1 hour of actinomycin D treatment (**Figure 6A-B**). According to the classification described by Friedel et al. [233], BLK mRNA would be a short-lived transcript. However, both isoforms followed exactly the same kinetics of decay suggesting that the G>A substitution does not alter the mRNA degradation rate (**Figure 6B**). The evaluation of two additional heterozygous LCLs (Ala-Thr 3 and 4) displayed similar results showing no differences on the transcript degradation rate between alleles (**Figure 6C**).

These results show that the SNP rs55758736 has no effect over BLK mRNA abundance or stability, and point to other mechanisms as the responsible for the increment of BLK-71Thr protein degradation rate.

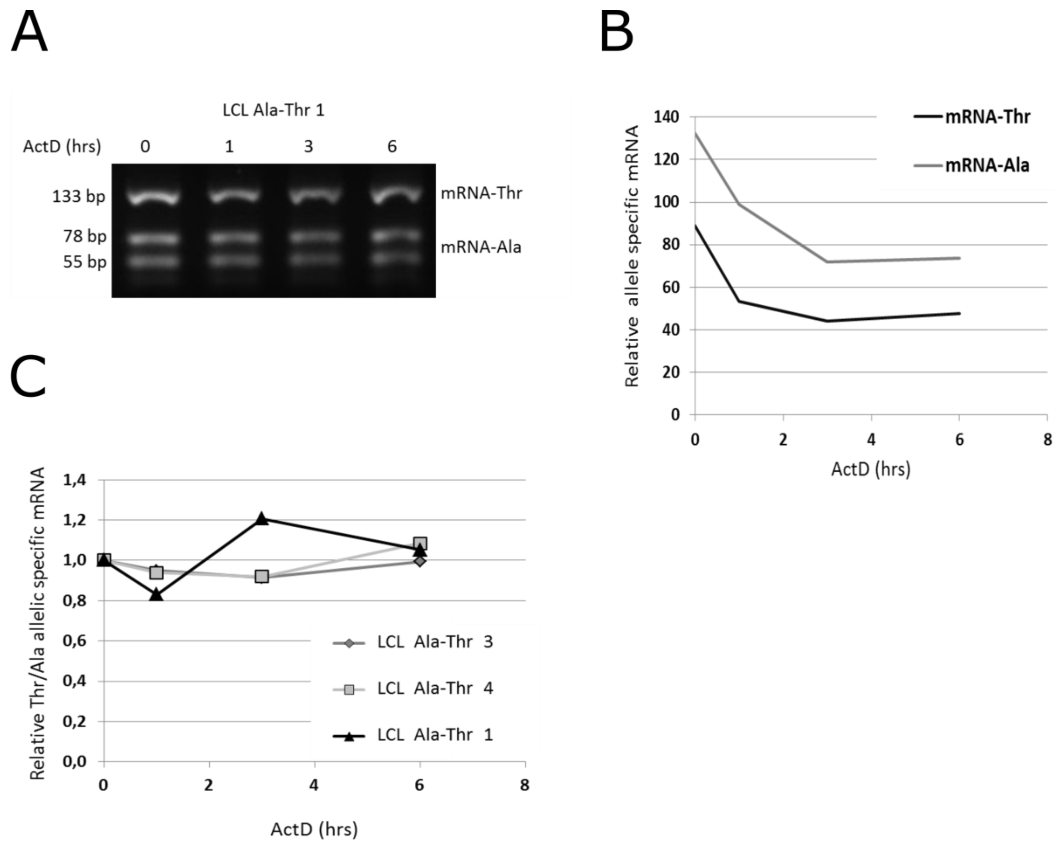


Figure 6. Differences in transcript stability between the allelic variants of rs55758736. (A) Agarose gel showing the AciI digestion of the amplified cDNA from the heterozygous LCL Ala-Thr 1. Each lane corresponds to an increasing time of transcription inhibition, in hours. The uncut 133 bp band correspond to the minor allele (Thr) and the 78 bp and 55 bp bands to the major allele (Ala). (B) Quantification of the bands of the gel in A. The intensity of the bands was normalized with the length of the corresponding restriction fragment. The amounts of each transcript were plotted against time of treatment. (C) Extended analysis using two more heterozygous lines. The variation of the ratio Thr/Ala after treatment is maintained indicating similar degradation rate of each allelic transcript variants. The ratio was normalized to 1 at time 0. LCL, lymphoblastoid cell line; ActD, actinomycin D.

Cells carrying the minor allele (71Thr) of rs55758736 show reduced BLK protein and increased protein degradation rate.

We then investigated whether the protein degradation rate was also accelerated in the case of endogenous BLK-Thr isoform as it was suggested by our results with recombinant protein in HEK293T cells [163]. For this, we used our derived homozygous LCLs to compare the kinetics of protein degradation between BLK-Thr

and BLK-Ala following cycloheximide treatment, an inhibitor of protein synthesis. As shown in **Figure 7A-B**, the endogenous Thr isoform degrades at a higher rate in LCLs. Of note, the protein half-life was reduced 50 %, decreasing from 12 h to 6 h due to the Ala71Thr substitution. The differences were less obvious at the end of the treatment likely because estimation of protein was less accurate because of the extremely low levels of protein. Note that the western blot of LCL Thr-Thr in **Figure 7A** was over-exposed in order to appreciate the kinetics. The original difference of expression between both isoforms in LCLs is depicted in **Figure 7C**.

The Thr-Thr LCL showed a very low level of BLK protein when compared to the average level of a cell line homozygous for the WT allele (**Figure 7C**). In adition, LCLs derived from heterozygous (G/A-Ala/Thr) subjects displayed significantly ($p < 0.001$) reduced protein levels as measured by Western Blot density quantification compared to cells from G/G-Ala/Ala subjects (**Figure 7D**).

Together, these data demonstrate for the first time that SNP rs55758736 affects BLK endogenous protein degradation and it is highly correlated with the amount of protein in B cells.

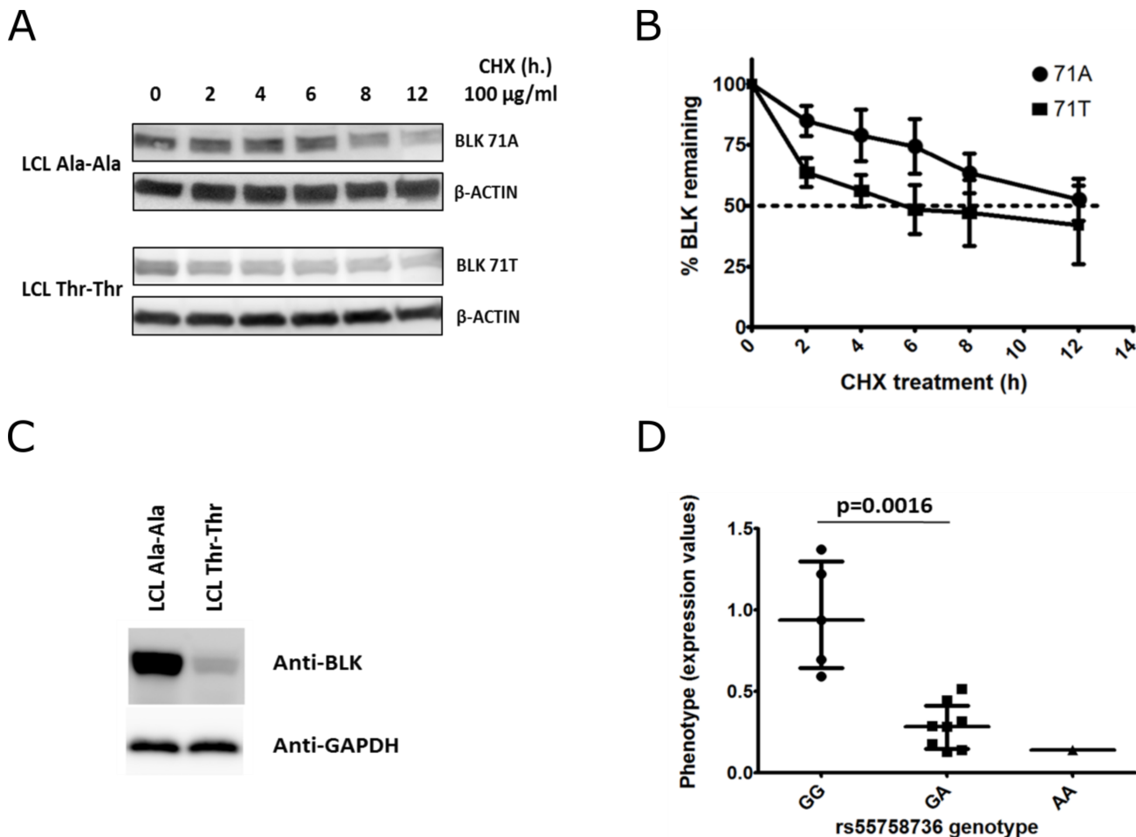


Figure 7. B-cells lines carrying the BLK 71thr variant have reduced protein level due to faster degradation rate. (A) Kinetics of protein degradation. Comparison of endogenous BLK between a 71Ala-Ala cell line and the line homozygous to Thr. Western blot representative of three independent experiments. Cells were grown in the same conditions and treated with 100 μg/ml cycloheximide for various times as indicated. The western blot of LCL Thr-Thr is over-exposed to appreciate the kinetics of degradation between both cell lines (note the original difference of expression in figure 3C). (B) Kinetic of BLK degradation after cycloheximide treatment (as in (A)) using three immortalized cell lines homozygous to Ala and the cell line 71Thr-Thr, which was assessed in two independent experiments. Symbols show mean and standard error. (C) Western blot of homozygous immortalized cell lines for the SNP rs55758736. The blot shows whole cell lysates from a representative line expressing the BLK 71Ala isoform and the line expressing the BLK 71Thr isoform. The membrane was developed with mouse anti-BLK clone 7A12 (Abnova) and re-probed with chicken anti-GAPDH (Sigma-Aldrich). (D) Quantification of BLK protein by western blot in five cell lines homozygous to Ala (GG-genotype), eight cell lines heterozygous (GA-genotype) and the cell line homozygous to Thr (AA-genotype). P value was determined by the Mann Whitney non-parametric *U*-test. Error bars represent the median with interquartile range. Quantification of the bands was done using ImageJ. LCL, lymphoblastoid cell line; CHX, cycloheximide.

The 71Thr variant enhances BLK ubiquitination in vitro

It has been shown that murine BLK is regulated through the ubiquitin-proteasome pathway after kinase phosphorylation and activation [168]. Since the BLK-71Thr variant is more susceptible to degradation, an increased regulatory ubiquitination might be expected in comparison to the WT form. In order to test this hypothesis, we transfected HEK293T cells with the 71Ala and 71Thr isoforms. As described previously, cells were treated with pervanadate to induce activation of the kinase and MG132 to prevent proteasomal degradation. As predicted, the BLK-71Thr became considerably more polyubiquitinated than the 71Ala isoform (**Figure 8A; IB: anti-Ub lanes 5-8**). Oda et al. also showed that after pervanadate treatment, two different forms of BLK are distinguished by electrophoresis migration probably because of phosphorylation events associated with kinase activation. These forms correspond to the activated and inactivated kinase (an upper and a lower band, respectively) [168]. **Figure 8A** shows the same phenomena and interestingly, the observed ratio between the upper and lower bands was increased in the 71Thr isoform compared to 71Ala (**IBs: anti-V5;**

lanes 5-8). Accordingly, such imbalance was accompanied by an enhanced signal of tyrosine-phosphorylation (**Figure 8A; IB: anti-pTyr, lanes 5-8**).

The alanine-to-threonine substitution at position 71 takes place within the RT-Src loop (**Figure 8B and 8D**), a segment shared by all the members of the SFKs (**Figure 8D, only kinases from Subfamily B are shown**). The residue is not conserved across the family, but a 3D model of the SH3 domain structure shows that the *de novo* threonine is located on the surface of the protein (**Figure 8C**), which expose it to the action of endogenous kinases. To test whether the residue was indeed susceptible to phosphorylation we performed an experiment under the same conditions shown on figure 4A. To some extent, the 71Thr variant seemed to be more threonine phosphorylated than its alanine counterpart, especially after 4 h of treatment (**Figure 8E; IB: anti-pThr, lanes 5-8**)

Overall, our findings point to ubiquitination and proteasomal degradation as the mechanisms responsible for the faster degradation of the BLK-71Thr variant.

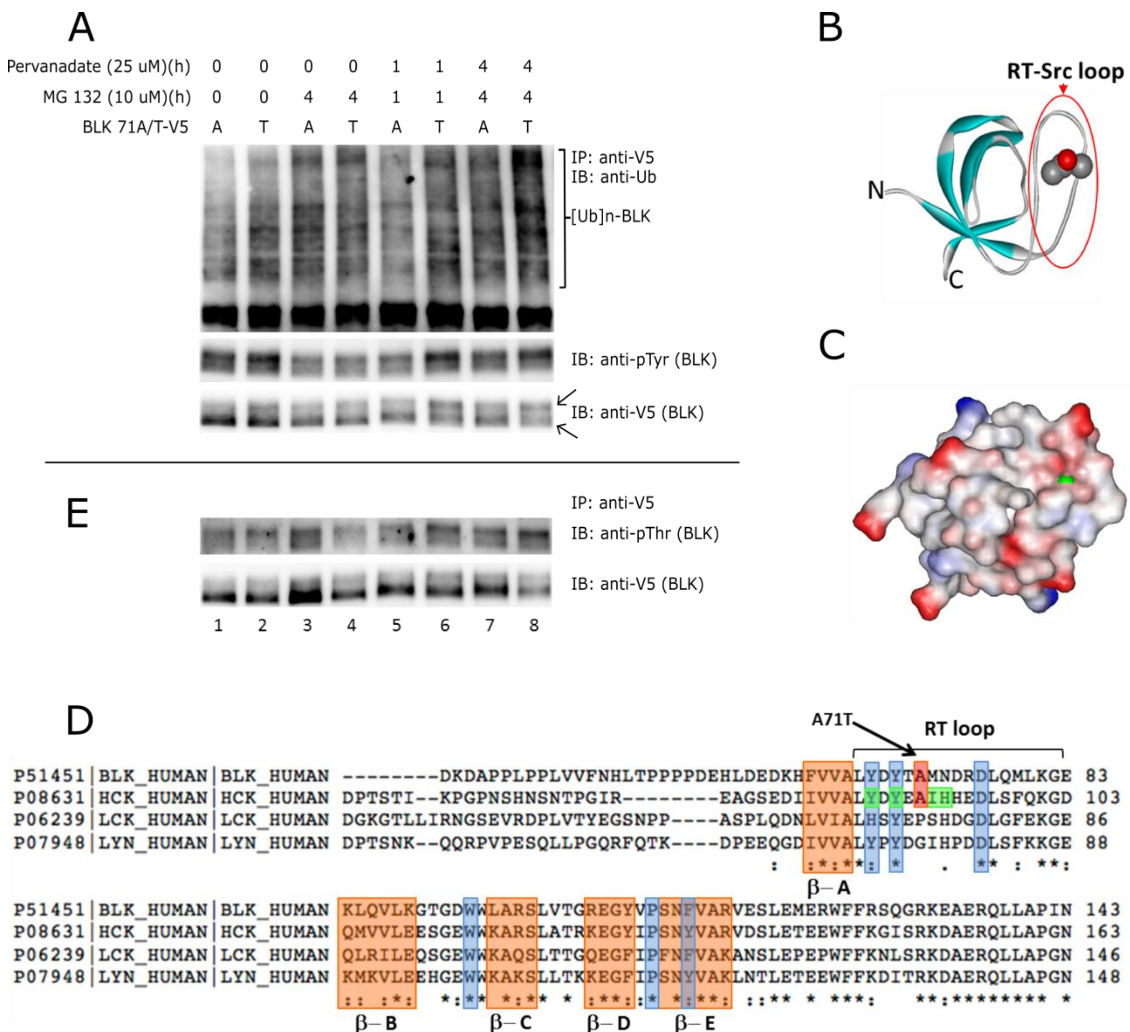
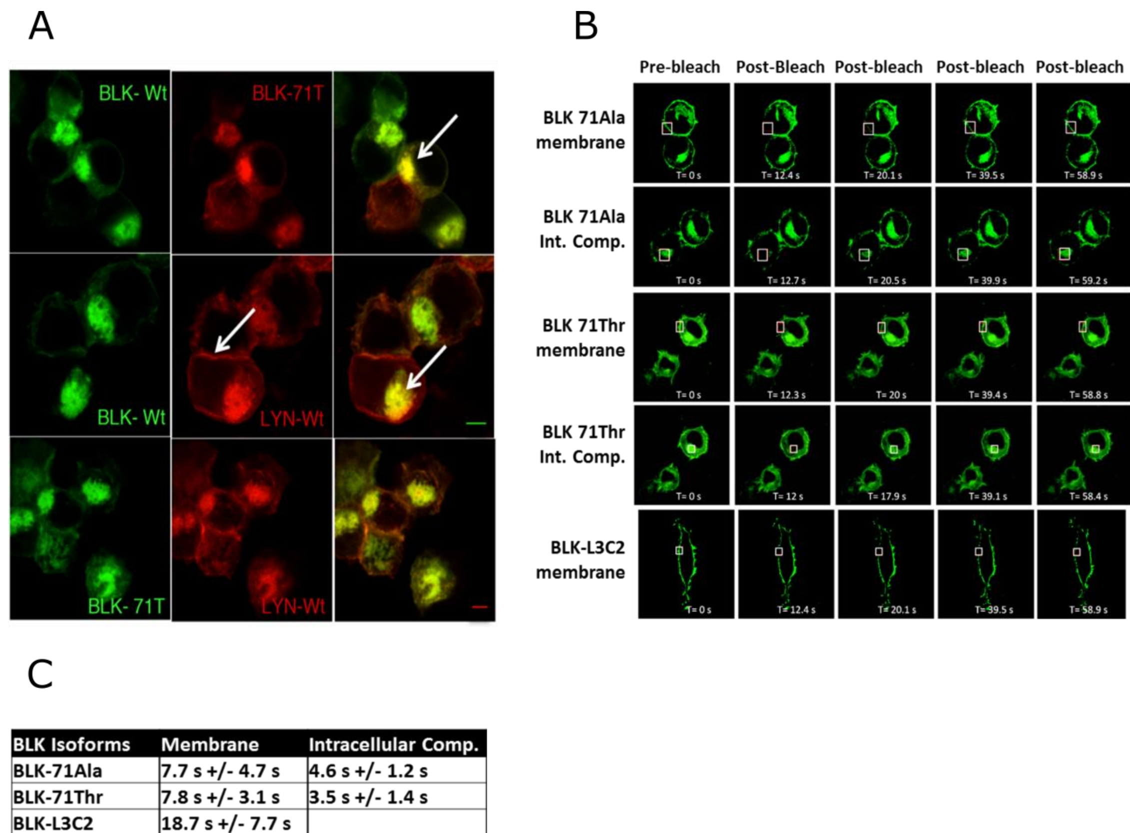


Figure 8. The 71Thr substitution renders a protein that is highly ubiquitinated **(A)** Western blot of BLK 71Ala/Thr isoforms fused to the V5 tag and immunoprecipitated from HEK293T cell lysates. Cells were treated at the indicated times with pervanadate to induce kinase activation and MG132 to inhibit proteasomal degradation. Two BLK bands are distinguished by PAGE migration (IB: anti-V5) and marked by arrows: the upper and lower bands strongly resemble those described for the activated and inactivated forms of the kinase, respectively. In the last four lanes, the upper/lower band ratio is increased by the 71Thr variant. The upper band of 71Thr isoform also shows increased signal from Tyr-phosphorylation (IB: anti-pTyr) and remarkably, poly-ubiquitination of the variant is highly up-regulated (IB: anti-Ub). **(B)** Model showing the flexible RT-Src loop of the SH3 domain where the Ala71Thr substitution takes place. **(C)** Model of the 3D structure built with SWISS MODEL [234] showing that the Thr residue is located on the surface of the protein (the reactive oxygen marked in green). **(D)** Partial sequences alignment of the human subfamily B of Src kinase. The Ala71Thr site is marked in red and beta-strands in orange. Residues within the RT-Src loop involved in the HCK regulatory, intramolecular interaction between SH3 domain and SH2-KD linker are shown in green. 91Ala of HCK (homologue of 71Ala in BLK) is highlighted in red. The substrate binding residues most conserved along known human SH3 domains are marked in light blue. An * (asterisk) indicates positions which have a single, fully conserved residue; a : (colon) indicates conservation between groups of strongly similar properties; a . (period) indicates conservation between groups of weakly similar properties. **(E)** As in figure 3A, imbalance in BLK bands ratios between 71Ala and 71Thr is also appreciated by protein migration and Thr-phosphorylation signal (IB: anti-V5 and anti-pThr, respectively; last four lanes). Experimental conditions are the same as in figure 3A. KD, kinase domain.

The BLK A71T substitution does not affect sub-cellular localization or trafficking

BLK is known to traffic between the cytoplasmic face of the plasma membrane and perinuclear compartments [133]. We hence tested whether the A71T substitution affects the trafficking or the subcellular localization of the protein by FRAP assays and co-localization experiments, respectively. Because the intracellular distribution of the Src-kinases is determined by posttranslational lipid modification at the N-terminus [235], we fused the fluorescent tag at the C-terminus to avoid interference. Cells co–expressing both isoforms showed perfect co-localization (**Figure 9A, top**). For comparison, we co-expressed each variant with LYN, a closely related kinase whose localization has been extensively studied [135, 236, 237]. Both isoforms of BLK co-

localized with LYN at the perinuclear area although in both cases LYN accumulated more at the plasma membrane (**Figure 9A, middle and lowest row**). Next, we analyzed the molecular trafficking between the two isoforms. FRAP assay is a technique widely spread in the study of molecular dynamics within and between subcellular compartments in living cells [238, 239]. Data were acquired 20 h after transfection, when expressed proteins were readily visible. We measured the time of recovery both at the plasma membrane and the intracellular compartments. We did not observe any significant difference between the two variants, although we noticed a general faster trafficking of BLK in the intracellular compartments than at the plasma membrane (**Figure 9B**). In order to validate our approach, we introduced a mutation in the BLK protein generating an additional palmitoylated site by substituting Leu3 for Cys. The mutation mimics the natural lipidation of LYN [240]. Trafficking of this BLK chimera was significantly reduced (**Figure 9C**) which indicates that small differences in trafficking between isoforms are negligible when comparing with the effect of a mutation in the lipidation site.



BLK Isoforms	Membrane	Intracellular Comp.
BLK-71Ala	7.7 s +/- 4.7 s	4.6 s +/- 1.2 s
BLK-71Thr	7.8 s +/- 3.1 s	3.5 s +/- 1.4 s
BLK-L3C2	18.7 s +/- 7.7 s	

Figure 9. Sub-cellular distribution and trafficking of BLK isoforms. (A) The isoforms 71Ala (BLK-wt) and 71Thr (BLK-71Thr) share the same sub-cellular location when co-expressed in HEK 293 cells (marked with an arrow in the upper right panel). The structurally similar kinase LYN co-localizes with

BLK and accumulates more at the plasma membrane (marked with an arrow in the central panel). (B) Quantification of *in vivo* trafficking by FRAP. The time to recovery was measured at the plasma membrane and in the intracellular compartments for both isoforms. (C) Summary of the FRAP quantification indicating the half-time to recovery (in seconds) after photo-bleaching. The two BLK isoforms have similar recovery time, showing faster recovery in the intracellular compartments than at the plasma membrane. To validate the approach a mutation introducing an additional palmitoylation site to BLK was generated by the Leu3Cys substitution. This mutation changed the pattern of distribution of the protein, making it localizes to the plasma membrane. The L3C2 mutant showed significantly slower trafficking than the two BLK isoforms. Images were taken with a 63x objective using a ZEISS confocal microscope. Bars = 5 µm. Cross-talk between light channels was ruled out by assessing the emission on cells expressing only one fluoresce protein and excited sequentially with both lasers.

The BLK 71Thr isoform shows impaired binding to the adaptor BANK1 after activation of B cells

BLK interacts physically with the adaptor molecule BANK1 in B cell signaling36. The SH3 domain of BLK has a number of proline-rich potential ligands within the BANK1 sequence [126] thus we aimed to analyze the effect of the 71Thr variation on this interaction. Many studies have provided insights on the amino acids that determine the affinity and specificity of SH3 domains [241-244]. **Figure 8D** shows, in light blue, the ligand binding residues most conserved among human known SH3 domains [245] three of which lie within the highly flexible RT-Src loop. Given their proximity to the 71Thr site, we hypothesized that the residue substitution could modify locally the amino acids disposition and therefore alter BLK interactions. For this we modeled the structures of the SH3 domain of BLK harboring either the 71Ala or 71Thr residues through Phyre2 web service [246] and analyzed them with Ramachandran Plot Explorer 1.0 (http://boscoh.com/ramaplot/) (data not shown). As predicted, the 71Thr created potential H-bonds and steric clashes with at least three of the surrounding residues: Y69 and D76, involved in ligand binding through the formation of one “xP” and one “specificity” pocket, respectively; and residue N73 (**Figure 8D**). To test experimentally the consequences of these observations upon the binding to BANK1, we transfected it into HEK293T cells along with both BLK isoforms and developed immunoprecipitation experiments. The BLK 71Thr isoform displayed a greatly reduced binding to BANK1 (**Figure 10A**). The constitutive active form of BLK (Y501F; YF) increased the binding capacity of BANK1 likely through the phosphorylation of BANK1 tyrosine residues and subsequent binding to the SH2 domain, as it was proposed previously [126, 170].

The phosphorylation of BANK1 was significantly reduced when co-expressed with the 71Thr isoform (**Figure 10B**), which suggests that the binding to BANK1 through the SH3 domain is required for subsequent phosphorylation of the adaptor protein. Our in vitro data suggests a two-step binding reaction, first an SH3 binding to the proline rich region of BANK1 followed by phosphorylation of BANK1 and further binding of the SH2 domain of BLK to the newly phosphorylated sites. A similar model of binding has been proposed for the interaction of the LYN kinase and its adaptor Cbp [247].

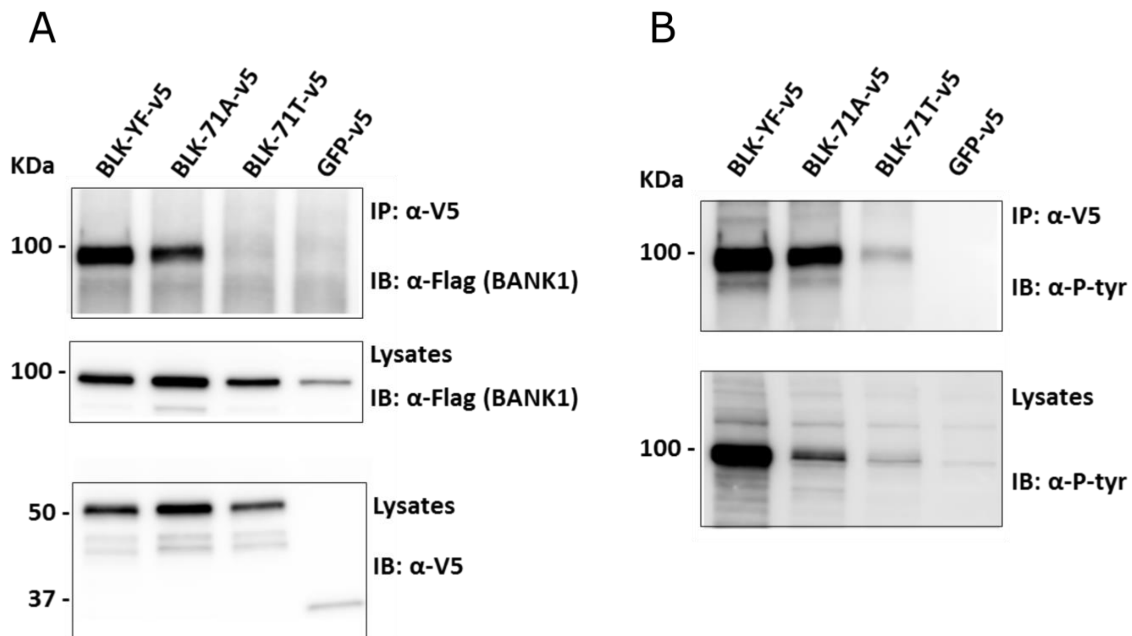


Figure 10. The BLK Ala71Thr variation leads to reduction of the binding to the adaptor molecule BANK1. (A) Western blot showing the immunoprecipitation (IP) of HEK 293 cell extracts following co-transfection with a plasmid expressing the adaptor protein BANK1 tagged with the Flag epitope and the two BLK isoforms. The construct coding for the constitutive active form of BLK (BLK-YF-v5) was used as positive control and the construct coding for GFP as negative control. The BANK1 protein was detected using the anti–Flag antibody (upper panel). The total lysate used for the IP was interrogated sequentially with anti-Flag (middle panel) and anti-V5 (bottom panel). (B) Tyrosine phosphorylation of BANK1 was detected with an anti-phosphotyrosine antibody. The BLK 71Ala wild type isoform phosphorylates BANK1 more extensively than the 71Thr isoform. Western blots in figures A and B are representative from three independent experiments.

To test the disruption of the interaction in a more physiological model, we transfected both BLK isoforms and a GFP negative control into the BJAB B-cell line

and analyzed the binding to endogenous BANK1 after BCR and TLR9 co-stimulation. Following immunoprecipitation against V5 tag, the 71Thr showed decreased binding to BANK1 (**Figure 11**), likely by altering the spatial disposition of one or more key ligand-interacting residues (**Figure 8D, marked in blue**). However, the reduction in the interaction was less drastic than in HEK293T co-transfection experiments. Dissimilar expression between recombinant and endogenous proteins possibly accounted for this difference. Also, and consistent with our findings in HEK293 cells (**Figure 8A**), the 71Thr variant was more Tyr-phosphorylated than the 71Ala isoform (**Figure 11**). These findings demonstrate that the ligand binding function of BLK SH3 domain is disrupted by the A71T substitution after B cell stimulation.

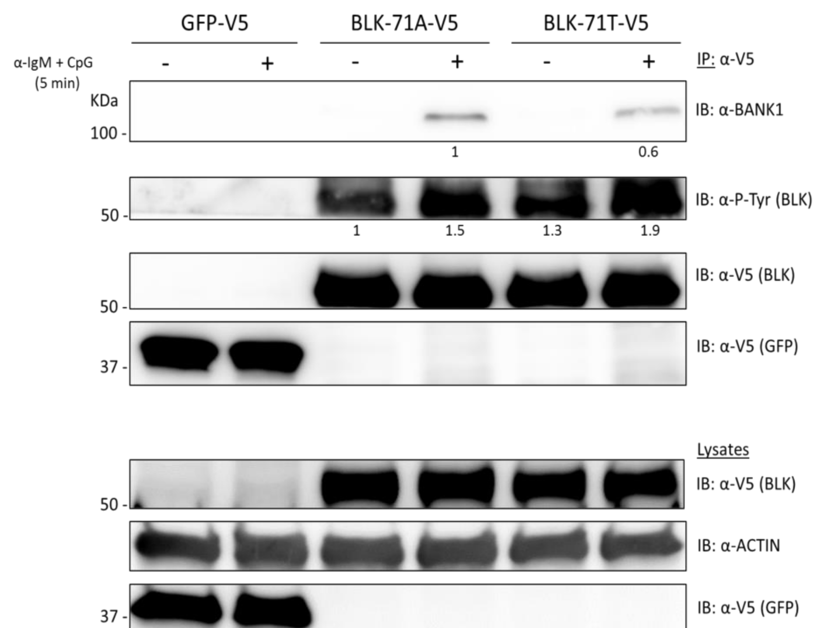


Figure 11. The BLK 71Thr variant shows impaired binding to the endogenous adaptor BANK1 in BJAB B-cell line and is more Tyr-phosphorylated. Cells were transfected with GFP, BLK-71Ala and -71Thr V5-fused constructs and stimulated with anti-IgM (10 µg/ml) and CpG (0,5 µM) for 5 min. Immunoprecipitations were carried out with anti-V5 antibody and membranes developed with the indicated antibodies. The interaction with the endogenous adaptor protein BANK1 is reduced, possibly because of an alteration of the SH3 domain ligand binding function. Global Tyr-phosphorylation of BLK-71Thr isoform is enhanced both before and after stimulation (IB: α-P-Tyr). Quantification of the bands is shown below the blots and normalization was carried out against the respective BLK amount in the immunoprecipitation. The bands with a value of 1 were used as reference for comparison. Whole cell lysates show similar protein expression for each construct.

BANK1 IN TLR PATHWAYS

BANK1 contains TRAF6 binding motifs

Mouse BANK1 has been implicated in the signaling of TLR receptors 7 and 9 in two previous studies [177, 179]. A role was somehow expected since BANK1 exon 2 was found to encode a cryptic TIR domain [171], a motif mediating homotipic interactions between key signalling proteins of TLR pathways such as MyD88 or TIRAP. We first aimed to determine the position BANK1 might play in the MyD88-TRAF6 pathway. TRAF6 protein needs a specific consensus motif in its interacting proteins represented either as motif P-X-E-X-X-aromatic/acidic residue [209] or as motif P-X-E-X-X-X-aromatic/acidic residue [210]. We therefore searched the BANK1 protein sequences of both BANK1 full-length (FL) and Delta 2 (D2) for this motif. We considered the natural occuring BANK1 D2 isoform in our experiments because it was a useful tool to also test the functionality of its TIR domain. Indeed, we found four putative TRAF6 binding motives in the FL isoform (**Figure 12A, underlined**) and five in the D2 short isoform of BANK1 (**Figure 12B, underlined**). The first two binding motives in BANK1-FL (the second and third ones in D2) are fused, sharing some of the residues. The fifth binding motif is formed by the absence of exon 2 in the D2 isoform (**Figure 12B, highlighted in red**). It is not a full consensus TRAF6 binding motif as it contains an aspartate instead of glutamate in the third position (Glu24Asp) (**Figure 12B**). However, it has been described that a similar mutation in CD40, a well-known target of TRAF6, enhanced its affinity for TRAF6 [248].

To sum up, BANK1 domains and binding sites strongly implicates the b cell adaptor in TLRs singnaling and suggests a possible binding to TRAF6 and MyD88.

A

>NP_060405.4 B-cell scaffold protein with ankyrin repeats isoform 1 [Homo sapiens]

MLPAAPGKGLGSPDPAPCGPAPPGNTKDIIMIYEEDAEEWALYLTEVFLHVVKREAILLYRLENFS
FRHLELLNLTSYKCKLLILSNSLLRDLTPKKCQFLEKILHSPKSVVTLLCGVKSSDQLYELLNISQ
SRWEISTEQEPEDYISVIQSIIFKDSEDYFEVNIPTDLRAKHSGEISERKEIEELSEASRNTIPLA
VVL**P**T**E**I**P**C**E**NPG**E**IFIILRDEVIGDTVEVEFTSSNKRIRTRPALWNKKVWCMKALEFPAGSVHVN
VYCDGIVKATTKIKYYPTAKAKECLFRMADSGESLCQNSIEELDGVLTSIFKHEIPYYEFQSLQTE
ICSQNKYTHFKELPTLLHCAAKFGLKNLAIHLLQCSGATWASKMKNMEGSDPAHIAERHGHKELKK
IFEDFSIQEIDINNEQENDYEEDIASFSTYIPSTQNPAFHHESRKTYGQSADGAEANEMEGEGKQN
GSGMETKHS**P**L**E**VGS**E**SSEDQYDDLYVFIPGADPENNSQEPLMSSRPPLPPPRPVANAFQLERPHF
TLPGTMVEGQMERSQNWGHPGVRQETGDEPKGEKEKKEEEKEQEEEEDPYTFAEIDDSEYDMILAN
LSIKKKTGSRSFIINRPPAPTPRPTSIPPKEETTPYIAQVFQQKTARRQSDDDKFRGLPKKQDRAR
IESPAFSTLRGCLTDGQEELILLQEKVKNGKMSMDEALEKFKHWQMGKSGLEMIQQEKLRQLRDCI
IGKR**P**E**E**ENV**Y**NKLTIVHHPGGKETAHNENKFYNVHFSNKLPARPQVEKEFGFCCKKDH

B

>NP_001120979.2 B-cell scaffold protein with ankyrin repeats isoform 3 [Homo sapiens]

MLPAAPGKGLGSPDPAPCGPA**P**P**D**SED**Y**FEVNIPTDLRAKHSGEISERKEIEELSEASRNTIPLAV
VL**P**T**E**I**P**C**E**NPG**E**IFIILRDEVIGDTVEVEFTSSNKRIRTRPALWNKKVWCMKALEFPAGSVHVNV
YCDGIVKATTKIKYYPTAKAKECLFRMADSGESLCQNSIEELDGVLTSIFKHEIPYYEFQSLQTEI
CSQNKYTHFKELPTLLHCAAKFGLKNLAIHLLQCSGATWASKMKNMEGSDPAHIAERHGHKELKKI
FEDFSIQEIDINNEQENDYEEDIASFSTYIPSTQNPAFHHESRKTYGQSADGAEANEMEGEGKQNG
SGMETKHS**P**L**E**VGS**E**SSEDQYDDLYVFIPGADPENNSQEPLMSSRPPLPPPRPVANAFQLERPHFT
LPGTMVEGQMERSQNWGHPGVRQETGDEPKGEKEKKEEEKEQEEEEDPYTFAEIDDSEYDMILANL
SIKKKTGSRSFIINRPPAPTPRPTSIPPKEETTPYIAQVFQQKTARRQSDDDKFRGLPKKQDRARI
ESPAFSTLRGCLTDGQEELILLQEKVKNGKMSMDEALEKFKHWQMGKSGLEMIQQEKLRQLRDCII
GKR**P**E**E**ENV**Y**NKLTIVHHPGGKETAHNENKFYNVHFSNKLPARPQVEKEFGFCCKKDH

FIGURE 12. BANK1-FL and -D2 contain several TRAF6 consensus binding motif P-X-E-X-X-X-aromatic/acidic residue. (**A**) Amino acid sequence of BANK1-FL protein is shown with exon 2 highlighted in blue and the four TRAF6 binding motifs present in BANK1 are underlined. In addition, the core binding amino acids proline (P), glutamate (E) or tyrosine (Y) in the consensus binding motif are shown in bold. (**B**) Amino acid sequence of BANK1-D2 is shown with the four TRAF6 binding motifs already present in BANK1-FL. The motif that was formed due to the lack of exon 2 is shown in red. Core amino acids of binding motifs are shown in bold.

BANK1 coimmunoprecipitates and co-localizes with MyD88 and TRAF6 in vitro

To determine whether TRAF6 can interact with both isoforms of BANK1, we performed transient transfections of Myc-TRAF6 together with BANK1-FL-V5 or BANK1-D2-V5 in HEK293 cells to subsequently carry out immunoprecipitation reactions using an anti-MYC antibody. We found that TRAF6 interacts with both isoforms of BANK1 (**Figure 13A**) although we noticed a slight decrease in the binding

to BANK1-D2. After Western blot with an anti-BANK1 antibody, we quantified the bands of both isoforms and determined an average reduction of around 40%. We then performed co-localization analysis of RFP-TRAF6 with the two BANK1 isoforms (GFP). Both proteins showed a homogeneous distribution in the cytoplasm with some accumulations, but also formed small punctate structures in which both isoforms co-localized with TRAF6 (**Figure 13B, arrows**).

The TLR signaling adaptor MyD88 contains two interaction motifs: a death domain and a TIR domain, both involved in homotypic interactions and linked by a interdomain [249]. We therefore investigated by coimmunoprecipitation and co-localization studies the possible binding to BANK1 through its putative, conformational TIR domain. Western blot analysis of the immunoprecipitations confirmed the interaction (**Figure 13C**). Of note, the D2 isoform lacking the TIR domain exhibited a sharp decrease in the binding to MyD88 of around 70 %, strongly suggesting this protein motif as the main mediator of the interaction. Additional studies by confocal microscopy confirmed the spatial co-localization of both proteins in an overexpression system in the U2OS cell line (**Figure 13D**). Almost all the ectopically expressed MyD88 showed a perfect co-localization with a fraction of both BANK1 isoforms, especially in the perinuclear area. Inversely, the FL and D2 forms presented two clearly defined fractions: the one mentioned previously, overlapping MyD88 around the nucleus; and a second one showing no co-localization with MyD88 and homogeneous distribution throughout the cytoplasm (**Figure 13D, arrows**).

TRAF6 and MyD88 form a complex with IRF7 after TLR7, TLR8 and TLR9 stimulation, triggering the production of IFN alpha [93]. Since we found that BANK1 interacts and shares subcellular regions with TRAF6 and MyD88 separately, we tested their co-localization pattern when expressed together. Microscopy experiments displayed high overlapping of the three proteins in the U2OS cell line (**Figure 13E**). Analysis and comparison of a number of images for all the combinations, including single transfections (data not shown), showed that BANK1 was nearly always homogenously distributed in the cell without displaying any accumulation (**e.g. Figure 13E; Delta 2 panel, cell at the bottom**). Interestingly, MyD88 co-expression normally induced a fraction of BANK1 to accumulate in the perinuclear area. Instead, TRAF6 tends to shift BANK1 localization pattern to punctuate-like structures dispersed across the cytoplasm. The pattern observed repeatedly when combining the three proteins

resembles more to that of BANK1 and MyD88 alone, suggesting that BANK1 and TRAF6 concentrates more in the presence of MyD88 (**Figure 13E**). Finally, no appreciable differences were found in any of the localization experiments between Full-Length and Delta 2 isoforms.

We show for the first time the interaction of BANK1 with two of the major regulators of TLR pathways, MyD88 and TRAF6. Additionally, we demonstrate that BANK1 cryptic TIR domain is indeed functional and seems to be crucial for the binding to MyD88. In turn, disruption of this domain also affects partly the interaction with TRAF6 by mechanisms that are a matter of investigation in our group and probably involve modification of TRAF6 binding sites.

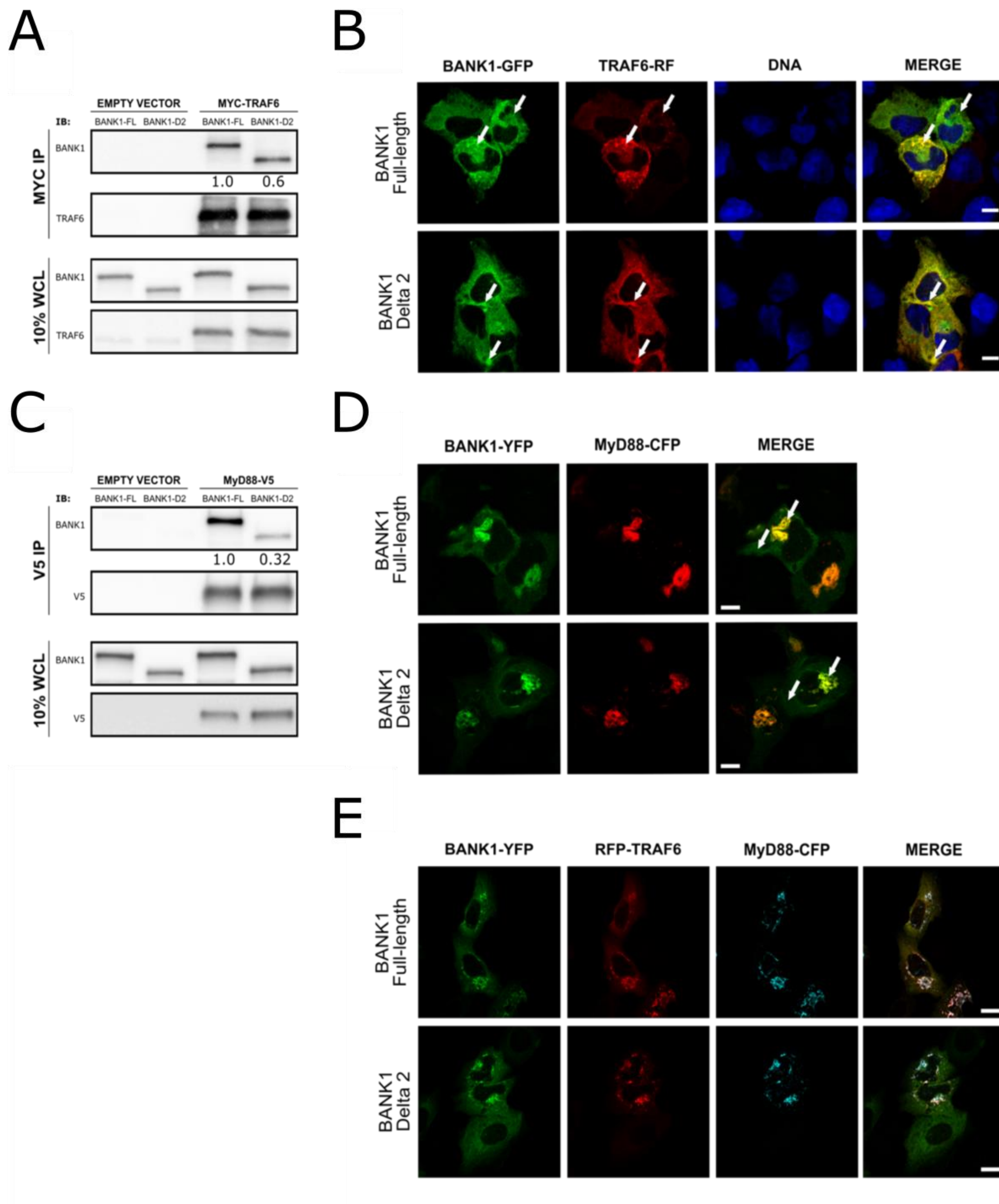


FIGURE 13. BANK1-FL and –D2 isoforms co-localize and differentially interacts with TRAF6. (**A**) Overexpression of Myc-TRAF6 in combination with BANK1-FL or BANK1-D2 in HEK293 cells and immunoprecipitation with anti-MYC antibody. Immunoblotting with the indicated antibodies shows TRAF6 interaction with both isoforms of BANK1. Numbers represent BANK1 bands intensity relative to the amount of TRAF6 immunoprecipitated. A value of one was assigned to BANK1-FL as a reference. In average, D2 isoform binds 40% less to TRAF6 than FL. Quantification of bands was performed with ImageJ. (**B**) RFP-TRAF6 and BANK1-GFP vectors for both isoforms were transfected into U2OS cells to study their subcellular localization and co-localization. BANK1 FL and D2 showed similar subcellular localization pattern, namely situated homogenously in the cytoplasm forming sometimes accumulations and also defined small punctate structures in which both isoforms co-localized with TRAF6 (arrows, MERGE). (**C**) HEK293 cells transfected with V5-tagged MyD88 or empty vector (EV) together with Flag-tagged BANK1 constructs. Co-immunoprecipitations were carried out against V5 epitope and membranes developed with the indicated antibodies. Numbers represent BANK1 bands intensity relative to the amount of MyD88 immunoprecipitated. A value of one was assigned to BANK1-FL as a reference. In average, D2 isoform binds 70% less to MyD88 than FL. Quantification of bands was performed with ImageJ. (**D**) MyD88-CFP and BANK1-YFP vectors for both isoforms were transfected into U2OS cells to study their subcellular localization and co-localization. Note that YFP-BANK1 isoforms are shown in green and MyD88-CFP in red for a better appreciation of merged images. (**E**) TRAF6-RFP, MyD88-CFP and BANK1-YFP vectors for both isoforms were transfected into U2OS cells. All three proteins highly co-localized in both punctuate structures throughout the cytoplasm and big perinuclear accumulations. There were no appreciable differences between BANK1 isoforms in any combination, however, TRAF6 pattern shifted more to perinuclear accumulations in the presence of MyD88 compared to transfection only with BANK1 (Figure 9B) or when expressed alone (data not showen). DNA of cells in figures B, D and E was stained with Hoechst 33528 and images were taken with a 63x objective using a ZEISS confocal microscope. Bars = 10 µm. Cross-talk between light channels was ruled out by assessing the emission on cells expressing only one fluoresce protein and excited sequentially with both lasers.

BANK1 forms a complex with MyD88 and TRAF6 in B cells

To study the interaction of BANK1 with MyD88 and TRAF6 under more natural conditions and to investigate any influences on protein binding upon stimulation of endosomal TLRs, we moved our focus to the human B cell line Namalwa. We first determined protein expression of BANK1, TRAF6 and MyD88 in Namalwa cells upon stimulation with the TLR7 and TLR9 agonists imiquimod and CpG, respectively (**Figure 14A**). Cells were stimulated with 5 µg/mL IMQ and protein kinetics was determined over a time frame of 48h. All three proteins showed an increase of their levels within a period of 0.5-1 hour upon IMQ stimulation (**Figure 14A**). After that

time, protein expression decreased and did not change until a second wave at around 12h when protein levels increased again. With CpG stimulation (1 µM), only a very slight increase in the first 30 min was observed for all three proteins (**Figure 14A**). However, protein levels increased at later stages of stimulation starting at around 8h (**Figure 14A**).

In order to confirm our findings in an over-expression system we next aimed to determine whether BANK1, TRAF6 and MyD88 could also interact endogenously. In unstimulated conditions, a complex comprising the three proteins was already detected (**Figure 14B**). However, at this point we cannot determine the correct order of binding of the proteins. We then stimulated cells with the TLR7 agonist IMQ and the TLR9 agonist CpG for 30 minutes to study any influences on protein interaction. Surprisingly, we could not detect significant changes in the binding of BANK1 to TRAF6 upon either stimulation. The binding to MYD88 remained also stable (**Figure 14B**).

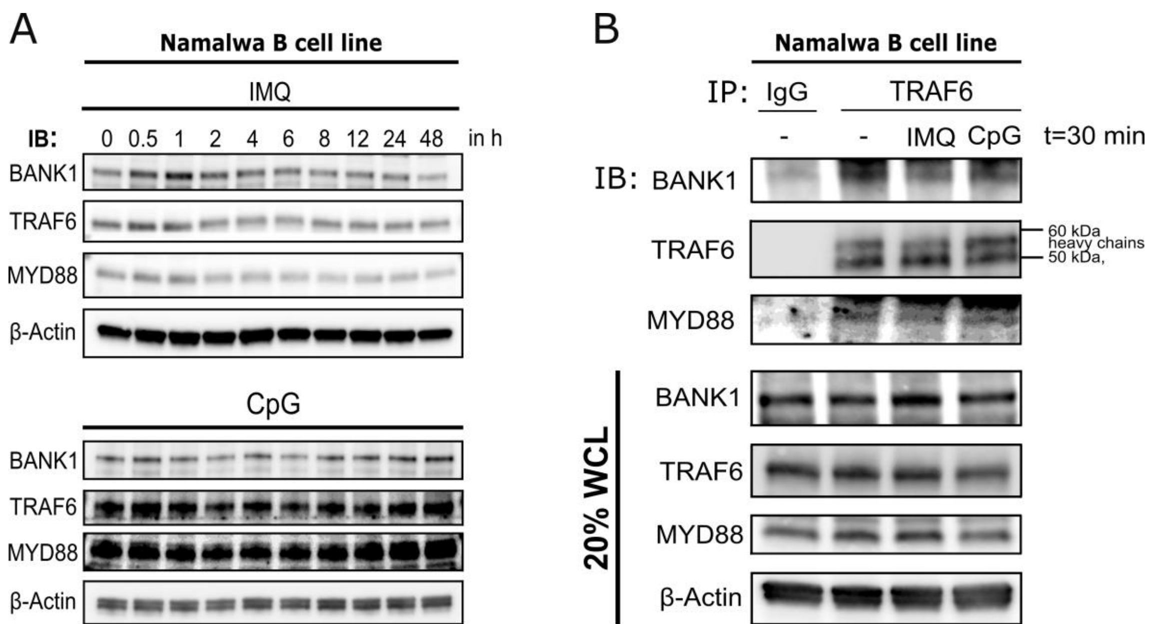


Figure 14. BANK1, TRAF6 and MyD88 interact in human B cells (A) A time course experiment of protein expression was performed in unstimulated and stimulated Namalwa B cells with IMQ and CpG over 48h for BANK1, TRAF6 and MyD88. Protein levels showed for all three proteins an increase around 0.5-1 hour of stimulation with IMQ. Although less obvious, the same effect was observed for cells stimulated with CpG. (B) Endogenous co-immunoprecipitation was performed in the B cell line Namalwa using an anti-TRAF6 antibody. Immunoblotting with anti-BANK1 and anti-MyD88 showed interactions that were already visible in unstimulated conditions. The intensity of the bindings did not change significantly upon TLRs stimulation.

BANK1 enhances TRAF6 K63-linked ubiquitination in vitro

We next aimed to explain the functional role underlying BANK1 participation in the well-known MyD88-TRAF6 pathway. As explained in the introduction, BANK1 was suggested to act in TLR7 signaling by inducing type I IFN expression to subsequently stimulate the autocrine IFNAR-STAT1/JNK axis [179]. Upon stimulation of different TLRs, TRAF6 is known to be recruited to TIR signaling complexes and to be activated to further transduce the signals [218]. It has been shown to interact with MyD88 and IRF7 to induce IFN alpha expression in a process involving TRAF6-mediated ubiquitination of IRF7 [93]. Although not completely understood, TRAF6 K63-linked ubiquitination and oligomerization are generally assumed as a sign of its activation, which eventually leads to ubiquitination of targeted proteins. After demonstrating their interaction, we tested experimentally the influence of BANK1 over TRAF6 K63-linked ubiquitination. **Figure 15** shows ubiquitination assays carried out between TRAF6 and both BANK1-FL and –D2 isoforms in HEK293 cells after transfection and immunoprecipitation with an anti-HA antibody. Importantly, BANK1-FL increased TRAF6 K63-linked ubiquitination in a dose-dependent manner (**Figure 15**), whereas remarkably, and according to our interaction assays, BANK1-D2 isoform almost completely failed inducing polyubiquitination of TRAF6 (**Figure 15**). On the other hand, the effect exerted by the FL form seemed to be saturated by excessive amounts of the protein (**Figure 15, left panel, last two lanes**).

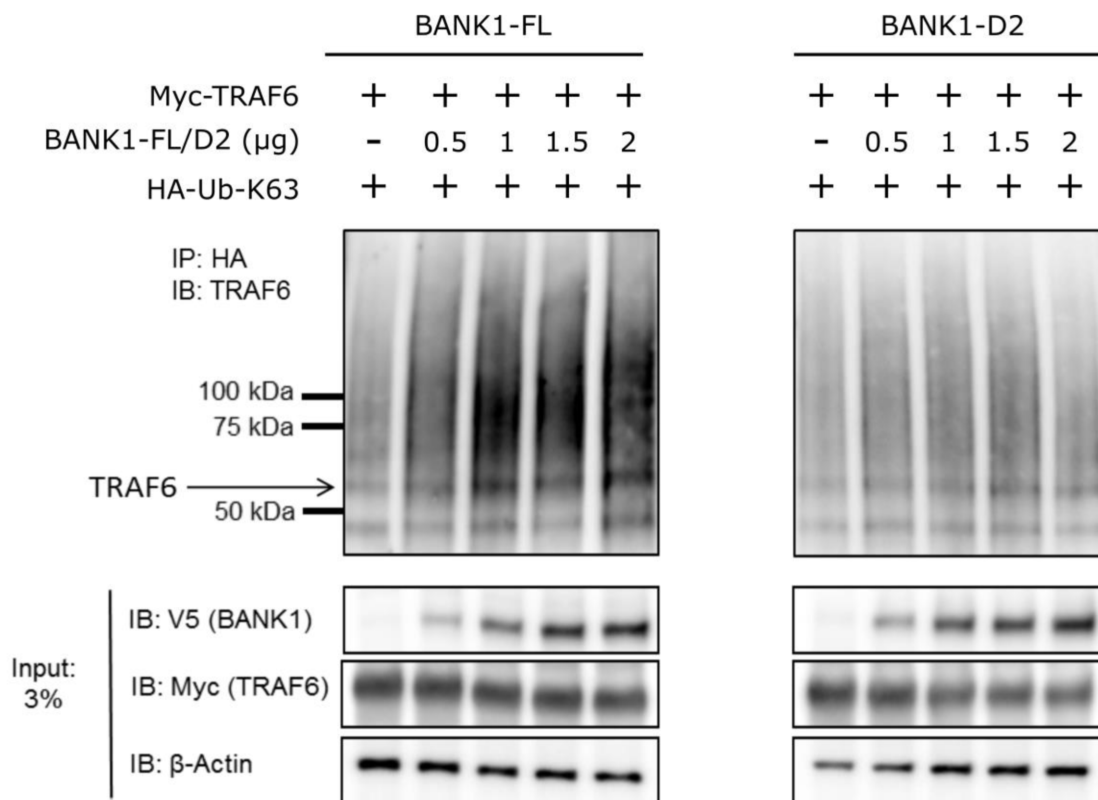


Figure 15. BANK1 increases TRAF6 K63-linked ubiquitination *in vitro*. HEK293 cells were transfected with TRAF6 and both BANK1 isoforms, together with recombinant K63-ubiquitin. Immunoprecipitations were directed against ubiquitin with an anti-HA antibody and membranes developed with anti-TRAF6 to test the effect of FL and D2 over TRAF6 K63-linked ubiquitination. Exposure of the membranes was done at the same time. TRAF6 was gradually more K63-ubiquitinated with increasing amounts of BANK1-FL, which seemed to reach its maximum effect at 1.5 µg of pDNA (IB: anti-TRAF6, left panel). BANK1-Inversely, D2 isoform co-expression did not show any significant effect over TRAF6 K63-linked ubiquitination (IB: anti-TRAF6, right panel).

DISCUSSION

VI. DISCUSSION

Effects of the SLE risk variant BLK 71Thr

We have shown that the SNP rs55758736 (A71T) in BLK, which produces a substitution of an alanine for a threonine within the SH3 domain of the kinase, has major effects on BLK protein levels in B cells. The 71Thr variant is more threonine- and tyrosine-phosphorylated and shows enhanced ubiquitinatination, which renders a protein prone for degradation. In addition, the binding and phosphorylation of the B-cell signaling adaptor BANK1 is also affected by the 71Thr. Overall, we have highlighted both the importance of the SH3 domain of BLK in recognizing substrates and controlling its degradation, and the potential effects of a missense variant over signaling networks in immune cells.

Model of a SH3-mediated activity regulation for BLK

Since the Ala71Thr substitution takes place on the surface of the SH3 domain and is therefore accessible to serine/threonine kinases, we expected a difference in phosphorylation between the BLK 71Ala and 71Thr isoforms. Unexpectedly, together with the increased phosphorylation of the isoform carrying the *de novo* threonine we noticed a retarded PAGE migration that largely resembles that observed by Oda *et al* for the activated form of BLK [168]. The slower migration could be also promoted by conformational changes associated to the Thr-phoshorylation itself, however, the increased Tyr-phosphorylation and degradation of the 71Thr isoform strongly links this variant to enhanced activation of the kinase, as shown previously. On the other hand, the concurrence of both phenomena cannot be ruled out. According to the activation hypothesis, there is significant evidence pointing to a key role of SH3 domains in controlling SFK activity [132, 138]. Therefore, we investigated the potential consequences of this missense variant over BLK intrinsic regulatory mechanisms. The SH3 RT-Src loop, which is shared by all the members of the SFKs (**Figure 8D**; only SrcB subfamily is shown) plays a crucial role in maintaining the assembled, auto-inhibited conformation of some of the Src-kinases. For instance in HCK, the member with highest sequence homology to BLK. The crystal structure of near full-length, down-regulated Hck (PDB code 1QCF) showed the interaction between the SH3 and SH2-KD linker, where some of the interacting residues were in close proximity to the

homologue 91Ala in HCK (**Figure 8D**; Alanine shown in red and interacting residues in green). It is interesting to hypothesize that the Ala71Thr substitution in BLK could alter the intramollecular interactions involved in its down-regulation. For this purpose, we modeled the structures of the SH3 domain of both BLK isoforms and analyzed them with Ramachandran Plot Explorer 1.0 (http://boscoh.com/ramaplot/) (as pointed in results section for BLK-BANK1 interaction). As predicted, the 71Thr created potential H-bonds and steric clashes with at least three of the surrounding residues: Y69, N73 and D76 (**Figure 8D**; partially marked in blue). Interestingly, Y69 homologue in HCK (Y89) was shown to be a key residue involved in the SH3-mediated regulation of kinase activity (**Figure 8D**; marked in green). The dominant role of SH3 domain controlling HCK activity [137] suggests our hypothesis derived from BLK phosphorylation and ubiquitination findings might be correct.

Also, as proposed for the SFKs member Lck, displacement of this interaction by SH3 high affinity ligands may open up the assembled state even without requiring dephosphorylation of its inhibitory c-terminal Y505. Subsequent trans/auto-phosphorylation of the activating Y394 within the kinase domain will poise the Lck protein into a double phosphorylated state, called “DPho Active”, where both regulatory tyrosines (Y394 and Y505) are concurrently phosphorylated and the protein retains kinase activity [250, 251]. In the case of the BLK 71Thr variant the situation could be similar since the Ala to Thr substitution might also “displace” the SH3-linker interaction by altering the conformation of the highly flexible RT-Src loop. This would make the Y389 in the activation loop more accessible to trans/auto-phosphorylation without the need to dephosphorylate the c-tail inhibitory Y501, rendering a BLK protein in the “Dpho Active” state. In addition, a previous study demonstrated that unlike c-Src, a recombinant form of Hck with an intact SH2-tail inhibitory interaction was still able to display kinase activity when both kinases were exposed to an SH3 ligand with equivalent affinity [137]. Differences in the strength of the SH3-linker segment regulatory interaction between Src-kinases likely accounts for this observation. Whereas in the model proposed for Lck the SH2-tail binding is released by displacement of the SH3-KD intramollecular interaction , in the case of Hck this seems not to be a requirement for kinase activation. In contrast, c-Src activation appears to be predominantly dependent on the concomitant engagement of SH2 and SH3 domains by ligands [137, 252]. As proposed by Alvarado et al., Src family members seem to have

different sensitivities to SH3 and SH2 ligands even though they adopt highly similar conformations upon the closed, inhibited state. Such distinctions could explain their ability to respond differently to specific cellular environments and display non-redundant activities even when concurring in the same pathways [137].

Our phosphorylation findings in the BJAB B-cell line might place BLK close to either the Lck or Hck suggested models (**Figure 11**). In the steady state, BLK needs to be predominantly phosphorylated on its inhibitory Y501 to maintain a close, down-regulated conformation. We speculate that the 71Thr might be disrupting the SH3-linker segment interaction in some of the BLK proteins to trigger the observed substantial up-regulation of the global Tyr-phosphorylation. This could be mediated by the exposition of Y389 to trans/auto-phosphorylation and subsequent activation, as described above.

Enhanced degradation of BLK 71Thr

The endogenous BLK 71Thr isoform showed enhanced degradation, which explains lower protein levels in individuals carrying this allele. However, the penetrance of the genetic variation is determined by the specific abundance of the transcript, which is likely dictated by polymorphisms in the promoter region as we did not find any correlation between the 71Thr allele and transcript levels. Since it is not possible to assess the degradation rate of the Thr variant in the heterozygous form, we were very fortunate to find a homozygous donor for the 71Thr minor allele, especially considering the low minor allele frequency of 0.015 in our population, which was almost identical to that annotated in dbSNP on NCBI (0.016).

Although the degradation effect of the variant was probably overcome by a strong promoter activity in our transfection experiments, in the context of endogenous proteins the proposed continuous activation of the kinase by the Ala71Thr substitution might drastically decrease BLK protein levels because of permanent proteosomal degradation. These findings may explain the strong correlation we found between risk genotypes and reduced BLK protein abundance in LCLs. In this sense, we have demonstrated that the 71Thr variant is more poly-ubiquitinated than its major allele counterpart following inhibition of the proteasome, a pathway known to degrade BLK activated forms through the ubiquitin ligase E6AP [168].

Altered binding to BANK1 and B cell signaling

In fact, the proximity of the 71Thr to highly conserved ligand-binding residues could explain the inefficiency of this enhanced activation in forcing BANK1 interaction. 3D modeling analysis of the 71Thr variation showed potential *de novo* interactions with loop amino acids Y69 and D76 (data not shown), which determine affinity and specificity to the ligand, respectively. Given the flexible nature of the RT-loop, the consequences of the variant on domain conformation are practically unpredictable. Taken together, these data suggest that the triggering of BLK binding to BANK1 depends to a large extent on its SH3 domain.

Increased expression levels of BANK1 and reduced levels of BLK are, each, associated with increased risk for SLE. The findings discussed above point the 71Thr variant in the same direction, as demonstrated by its detrimental effect on BLK protein levels. However, a key question still to be answered is how the reduction of BLK leads to the characteristic hyper-activation of B cells in lupus patients and in mice [146]. Early reports indicate that BLK acts as a BCR activation factor [166, 253]. More recently the published data suggest that BLK may function as either a positive or negative regulator of BCR activation [131, 142, 146]. Furthermore, our group has recently shown that aged female *BLK*$^{+/-}$ and *BLK*$^{-/-}$ mice produced higher anti-dsDNA IgG antibodies and developed immune complex–mediated glomerulonephritis compared with *BLK*$^{+/+}$ mice. Starting at young age, in addition, *BLK*$^{+/-}$ and *BLK*$^{-/-}$ mice accumulated increased numbers of peritoneal cavity B1a cells, and as the mice aged, they differentiated into class-switched CD138^{+} IgG-secreting cells in the spleen. Aged *BLK*$^{+/-}$ and *BLK*$^{-/-}$ mice also showed increased infiltration of B1a cells into the kidneys. In humans, healthy individuals had BLK genotype-dependent levels of anti-dsDNA IgG antibodies as well as increased numbers of a B1-like cell population in peripheral blood. Finally, the presence of B1-like cells in the tubulointerstitial space of human lupus kidney biopsies was described [164]. Although the specific nature and functions of B-1 cells is not yet completely understood, B-1-like cells have been reported to be increased in SLE and pSS [110].

In agreement with the most recently published data on the role of BLK in B-cells and autoimmunity, the reduction of BLK abundance could lead to lowered thresholds for BCR signaling [131] by insufficient propagation of inhibitory signals,

and consequently the loss of self-tolerance. High levels of BANK1 could result in the sequestration of the kinase away from its substrates (as shown in reference [254]) with the subsequent reduction of the inhibitory activity of BLK on B-cell activation at the plasma membrane. In the case of the BLK-71Thr variant this effect would be compensated by decreased binding to BANK1, but the excessive proteasomal degradation of the kinase may still reduce the inhibitory signals of BLK. BLK could then be an example of how the lack of inhibition of activation and lack of control of tolerance checkpoints is a major mechanism behind the development of autoimmunity.

BANK1 role in TLR pathways

Our group started to study intensely the role of BANK1 in TLR pathways a few years ago. Since the publication of BANK1 effects over TLR9 signaling in 2013 [177], we have gained some important insights onto the pathological consequences of BANK1 expression in mouse models regarding SLE phenotypes [179]. Although some experiments were done in the context of TLR signaling, at the molecular level our research had been traditionally focus on the B cell receptor signaling [126]. It was not until recently when we began to investigate more deeply the niche BANK1 might occupy within innate pathways. In this sense, experiments carried out previously by my former colleague Dr Bernal Quirós, showed that although BANK1 did display enhanced PLA signal with TLR9 after CpG stimulation, there was no effect over NFkB activity, as suggested by luciferase reporter assays [255]. Now, for the first time we are able to hypothesize with some degree of certainty about one of the specific sites where BANK1 exerts its function in TLR signaling. Thus, the main achievements of this work are: 1) the identification of the *in vitro* and *in vivo* interactions of BANK1 with two of the key TLR signaling molecules: the master adaptor MyD88 and the E3 ubiquitin ligase TRAF6; 2) the experimental evidence suggesting BANK1 TIR domain is functional and its implications; and 3) the influence found of BANK1 over TRAF6 K63-linked polyubiquitination.

The molecular processes leading to the characteristic hyperactivity of B lymphocytes in lupus are unknown. BCR signaling alterations have been reported [108], and this is further supported by the identification of numerous SLE-risk variants within genes involved in such pathway, for instance *BANK1, BLK, LYN or PTPN22* [7]. The

same applies for TLR and type I IFN signaling [108], where risk associations are found in genes such as *TLR7, IRF5, IRF7* or *TYK2* [7]. In this regard, our group has previously shown that the risk allele (G) of the SNP rs10516487 in BANK1 correlates with increased levels of its mRNA in lymphoblastoid cell lines [172]. This is further supported by eQTL analyses made by our group in lymphoblastoid cell lines showing that BANK1 risk alleles correlate with its increased expression (data not shown). In general, these findings suggest that elevated levels of BANK1 might contribute to a higher risk of developing SLE. On the other hand, Kozyrev and colleagues suggested that, in turn, the D2 isoform of BANK1 had the potential to protect against SLE trigger [256].

TRAF6 binding and induction of its K63-linked ubiquitination by BANK1

Interestingly, our results show that BANK1 D2 isoform has a profound inability to induce K63-linked ubiquitination of TRAF6 *in vitro*. Since this type of ubiquitination on TRAF6 is associated with increased TLR pathway activity, and this in turn suggests enhanced cell activation [218], our results could be interpreted as a mechanism by which the D2 isoform confers this protection to SLE. One could hypothesize that B cells would be then less activated in individuals with higher expression of the D2 isoform with respect to the FL form. Although this correlation between D2 BANK1 and protection could not be replicated in a more recent work [172], additional studies are needed in larger cohorts of B cells from patients and controls to determine the specific relationship between BANK1 isoforms and disease risk.

Moreover, the self-association capacity of the TIR domain-lacking BANK1 D2 form was shown to be highly impaired [172], suggesting a role for BANK1's cryptic TIR domain in protein aggregation. In fact, TIR domains have been reported to mediate protein homodimerization [257, 258]. For example, a synthetic compound modeled after MyD88 TIR domain structure was able to block its dimerization without affecting the association capacity of its death domain [258]. On the light of this, it is tempting to speculate that BANK1 could utilize its TIR domain in conjunction to the DBB domain to self-associate. Whether this impaired dimerization is related to the decreased TRAF6 K63-linked ubiquitination observed in our experiments, or alternatively, the

functionality of some of the TRAF6 binding sites in BANK1 is altered in the shorter D2 protein is not known.

BANK1 binding to MyD88

La interacción de BANK1 con proteinas conteniendo dominios TIR era esperada desde que en 2012 se descubrió un dominio críptico dentro de la secuencia de la proteína. Teniendo en cuenta además los hallazgos de nuestro grupo apoyando su papel funcional en la ruta de los TLRs, decidimos investigar su posible interacción con el regulador maestro MyD88. Experimentos de coinmunoprecipitación sugieren fuertemente que el dominio TIR de BANK1 es de hecho funcional y media dicha interacción. Of note, el análogo de BANK1 BCAP, fue descrito como un adaptador con acción dual en la señalización del BCR y los TLRs. Recientemente además se ha descrito la estructura de su dominio TIR, confirmando las inferencias de Troutman et al. en 2012. Por un lado, es un regulador negativo de la respuesta inflamatoria vía TLRs 4, 7 y 9, comunicando su señalización con la ruta PI3K. Y por el otro es fosforilado por SYK y BTK tras la estimulaión del BCR. Se demonstró que BCAP es capaz de unir los TLRs directamente, pero también puede unir adaptadores TIR como MyD88. Recientemente, se ha propuesto un mecanismo para su papel regulador de TLRs a través de su intereacción con la fosfolipasa C gamma y PI3K. Se propuso que promueve la depleción de los fosfolípidos de membrana requeridos por el adaptador TIRAP para su interacción con los TLRs y posterior transducción de la señal. Sin embargo, muchos TLRs señalizan a través de MyD88, y no de TIRAP, y esto no explicaría el bloqueo de su señalización en el ratón deficiente para BCAP. BANK1 posee un dominio TIR con una alta homología al de BCAP (40%). También es capaz de unir PLCG2 para catalizar la metabolización de fosfolipidos de membrana, sin embargo, su acción parece ser más bien antagónica a la reguladora de BCAP tras ligación de TLRs. Además, a diferencia de BCAP, su interacción descrita con PLCG2 es transciente y menos estable, lo que podría explicar algunas de estas diferencias. Aunque mucho trabajo hay por delante aún, nuestros hallazgos sobre la interacción TIR-dependiente de BANK1 con MyD88 podrían sugerir que el rol de BANK1 está directamente relacionado con el MyDosome y la activación de TRAF6.

Todo esto pone de manifiesto que adaptadores del BCR juegan un papel en la señalización de TLRs, sin embargo más investigaciones son necesarias para elucidar completamente la red de moléculas e interacciones implicadas en este cross-talk, y especialmente en cómo puede contribuir al desarrollo de autoinmunidad.

The interaction of BANK1 with TIR domain-containing proteins was expected since in 2012 a cryptic domain was discovered within its protein sequence [171]. Taking also into account our findings supporting BANK1´s functional role in TLR pathways [177, 179] we decided to investigate a possible interaction with the master regulator MyD88. Our coinmunoprecipitation experiments strongly suggest that the TIR domain of BANK1 is indeed functional and mediates such interaction. Of note, the analog of BANK1, BCAP, was described as an adaptor with dual action in BCR and TLR signaling [171, 259]. Very recently the molecular structure of its TIR domain was experimentally corroborated [260], confirming the inferences of Troutman et al. in 2012 [171]. On the one hand, it is a negative regulator of the inflammatory response via TLRs 4, 7 and 9, communicating its signaling with the PI3K pathway [171, 261, 262]. On the other hand, it is phosphorylated by SYK and BTK after stimulation of the BCR [259]. It was demonstrated that BCAP is able to bind TLR adapters like MyD88 or TRAM [260]. Authors proposed a mechanism for its regulatory role for TLRs through its interaction with phospholipase C gamma and PI3K. It was proposed that it promotes depletion of membrane phospholipids required by the TIRAP adaptor for its interaction with TLRs and subsequent signal transduction. However, many TLRs signaling depend on MyD88, and not on TIRAP, and this hypotesis would not explain the blockade of their transduction in the BCAP-deficient mouse [260]. BANK1 has a TIR domain with a high homology to that of BCAP (40%). It is also able to bind PLCG2 to catalyze the metabolism of membrane phospholipids, however, its action seems to be rather antagonistic to the BCAP regulator after ligation of TLRs. In addition, unlike BCAP, the interaction described with PLCG2 is transient and less stable, which could explain some of these differences. Although much work is still ahead, our findings on the TIR-dependent interaction of BANK1 with MyD88 might suggest that the role of BANK1 is directly related to the MyDosome and the activation of TRAF6.

Together, these data show that BCR adaptors play a role in TLR signaling, however more research is needed to fully elucidate the network of molecules and

interactions involved in this cross-talk, and especially how it can contribute to the development of autoimmunity.

CONCLUSIONS/CONCLUSIONES

VII. CONCLUSIONS

1. The BLK Ala71Thr substitution increases endogenous BLK protein degradation. The Thr variant is more threonine- and tyrosine-phosphorylated and polyubiquitinated *in vitro*, suggesting an enhanced regulation by ubiquitination.

2. Ectopically expressed BLK 71Thr is more tyrosine-phosphorylated upon IgM and CpG stimulation of B cells.

3. LCLs from individuals carrying the 71Thr risk variant display decreased protein amounts of the BLK kinase.

4. The BLK 71Thr substitution within the SH3 domain impairs the binding to the endogenous BANK1 adaptor in B cells.

5. The Ala71Thr substitution does not affect BLK subcellular localization or trafficking.

6. The SNP rs55758736 of BLK does not alter mRNA degradation rate or transcript abundance in B cells.

7. Full length BANK1 contains four putative TRAF6 binding sites and the splicing of the D2 isoform creates an extra binding site.

8. BANK1 interacts constitutively with the key TLR signaling adaptor MyD88 and the E3 ubiquitin ligase TRAF6 in B cells. The three proteins highly co-localize in an overexpression system.

9. The BANK1 cryptic TIR domain is functional and the D2 isoform shows impaired binding to TRAF6, particularly to the TIR domain-containing adaptor MyD88.

10. BANK1 promotes TRAF6 K63-linked ubiquitination *in vitro* in a TIR domain-dependent fashion.

VII. CONCLUSIONES

1. La sustitución de una alanina por una treonina en posición 71 aumenta la degradación de la proteína BLK endógena. La variante de BLK con la treonina es globalmente más fosforilada en treoninas y tirosinas, y más poliubiquitinada en un sistema de sobreexpresión, lo que sugiere que su regulación por ubiquitinación está aumentada.

2. La variante de la proteina BLK 71Thr expresada ectópicamente se ve más fosforilada en sus residuos tirosina tras la estimulación con IgM y CpG en células B.

3. Líneas linfoblastoides de individuos portadores de la variante de riesgo de BLK 71Thr poseen cantidades disminuidas de la quinasa.

4. La sustitución de la alanina en posición 71 de la proteína BLK por una treonina, dentro del dominio SH3, perturba su interacción con el adaptador BANK1 endógeno en células B.

5. La misma sustitución en posición 71 no afecta a la localización o tráfico subcelular de la quinasa BLK.

6. El SNP rs55758736 de BLK no altera la tasa de degradación de su RNA mensajero o la abundancia del transcripto en células B.

7. La forma completa de BANK1 (*Full length*) contiene cuatro sitios putativos de unión a TRAF6 y el *splicing* alternativo de la isoforma Delta 2 crea un sitio de unión adicional.

8. BANK1 interacciona constitutivamente con el principal adaptador de señalización de receptores tipo Toll, MyD88, y la ubiquitina ligasa E3, TRAF6, en células B. Las tres proteínas co-localizan altamente en un sistema de sobreexpresión.

9. El dominio TIR críptico de BANK1 es funcional y la isoforma Delta 2 presenta una unión alterada a TRAF6, y en particular, al adaptador MyD88 que a su vez también posee un dominio TIR.

10. BANK1 promueve la ubiquitinación de TRAF6 en lisinas 63 en un sistema de sobreexpresión. Este efecto además es dependiente del dominio TIR de BANK1.

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ABBREVIATIONS

ALA: alanine

APCs: antigen presenting cells

BCR: B cell receptor

cDCs: conventional dendritic cells

CFP: cyam fluorescent protein

CNS: central nervous system

CoIPs: coimmunoprecipitations

CVD: cardiovascular disease

DCs: dendritic cells

DPBS: Dulbeco´s phosphate-buffered saline

eQTL: expression quantitative trait loci

FRAP : fluorescence recovery after photobleaching

GFP : green fluorescent protein

HEK293 : human embrionic kidney 293

IFN : interferon

IFN-I : type I interferons

IMQ : imiquimod

KD : kinase domain

LCLs : lymphoblastoid cell lines

PAMPs : pathogen-associated molecular patterns

PCR : polymerase chain reaction

pDCs : plasmacytoid dendritic cells

pDNA : plasmid DNA

PLA : proximity ligation assay

PNS: peripheral nervous system

pSS: primary Sjögren syndrome

RFP: red flurescent protein

SFKs: Src-family of protein kinases

SH2: Src homology 2

SH3: Src homology 3

ssRNA: single-stranded RNA

TCR: T cell receptor

THR: threonine

TLR: Toll-like receptor

TNF: Tumor necrosis factor

WB: western blot

WT: wild type

YFP: yellow fluorescent protein