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## New roles of the Lyn tyrosine kinase in psoriasis development and cell death

Lazaro Emilio Aira Diaz

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# THÈSE DE DOCTORAT

## Nouveaux rôles de la protéine kinase Lyn dans le développement du psoriasis et dans la mort cellulaire

**Lazaro Emilio AIRA DIAZ**

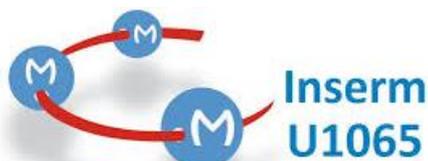
Equipe 2 : Mort cellulaire, différenciation and cancer

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moléculaires et cellulaires  
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Dirigée par : Dr. Sandrine MARCHETTI  
Soutenue le : 25 avril 2018, à Nice, C3M,  
Bâtiment Archimède, Hôpital de l'Archet 2

Devant le jury, composé de :

Dr. Chloé FERAL, Présidente du Jury, IRCAN  
Dr. Nicolas CHARLES, Rapporteur, CRI  
Dr. Serge ROCHE, Rapporteur, CRBM  
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# RESUME



La famille des kinases Src, dont Lyn fait partie, joue un rôle clé dans le contrôle de nombreux processus biologiques. Lyn a une fonction bien établie dans les cellules hématopoïétiques, jouant un rôle important dans la régulation des désordres hématopoïétiques. Lyn est notamment impliqué dans le maintien de différentes leucémies et son expression protéique est altérée dans les tumeurs solides. Plusieurs études ont mis en évidence qu'elle avait un rôle anti-apoptotique. Lyn peut être clivée par les caspases, protéases à cystéine impliquées dans l'apoptose et l'inflammation, ce qui donne une protéine tronquée avec une localisation subcellulaire, cytosolique, différente de la forme entière membranaire. Ainsi, elle pourra avoir accès à de nouveaux substrats qui expliqueraient son rôle de suppresseur de la mort cellulaire par apoptose. Nous avons ainsi montré que Lyn cytosolique (cLyn) régulait Bim, un membre pro-apoptotique de la famille Bcl-2, impliqué dans le contrôle de l'apoptose mitochondriale en le phosphorylant sur les tyrosines 92 et 161. Cette modification post-traductionnelle entraîne une inhibition de la fonction pro-apoptotique de Bim, en augmentant son interaction avec les membres anti-apoptotiques tels que Bcl-XL, limitant ainsi la perméabilisation de la membrane externe mitochondriale et l'apoptose des cellules.

Lyn possède également un rôle pro-inflammatoire. Nous avons préalablement montré que la surexpression de cLyn, chez la souris, conduit à un syndrome inflammatoire de la peau, ressemblant au psoriasis humain. Sur la base de ce résultat, nous avons voulu savoir si la tyrosine kinase Lyn jouait un rôle dans cette maladie chronique de la peau, qui est caractérisée par une différenciation anormale des kératinocytes et une augmentation de l'infiltrat immunitaire, conduisant à la formation de plaques de psoriasis, la principale caractéristique clinique de la maladie. L'analyse de l'expression de Lyn chez des patients souffrant de psoriasis a montré que Lyn était surexprimée dans la peau lésionnelle par rapport à la peau non lésionnelle ou saine, résultats confirmés dans deux modèles de psoriasis chez la souris. De façon intéressante, nous avons montré que l'augmentation de l'expression de Lyn se situe à la fois dans le derme et dans l'épiderme chez l'homme et chez la souris, indiquant que le recrutement de cellules immunitaires dans la peau lésionnelle mais aussi la modulation de Lyn dans les kératinocytes sont impliqués. Par ailleurs, une augmentation de l'expression de Lyn a été observée dans les kératinocytes humains stimulés par le TNF- $\alpha$  et l'IL-17A, deux cytokines importantes dans le psoriasis. Afin de déterminer le rôle de Lyn dans cette maladie cutanée nous l'avons induit chez des souris déficientes pour Lyn. Une réduction significative du phénotype cutané a été observée dans les souris LynKO par rapport aux souris WT, identifiant Lyn comme un nouvel acteur dans la pathogénie du psoriasis. De plus, nos résultats ont établi que l'expression de Lyn dans les kératinocytes semblait cruciale et suffisante pour le maintien du phénotype psoriasique, indiquant un nouveau rôle de Lyn dans la régulation des kératinocytes.

Au cours de ce travail, nous avons observé que les caspases inflammatoires étaient activées dans la peau lésionnelle de patients atteints de psoriasis. Les caspases inflammatoires, suite à leur activation au sein de l'inflammasome, vont cliver l'IL-1 $\beta$  et l'IL-18, ce qui conduit à leur maturation. Elles jouent donc un rôle important dans le contrôle de l'inflammation en réponse à un agent pathogène mais participent également à la pathogénie de nombreuses maladies inflammatoires, comme le diabète et l'obésité. Nous avons alors voulu savoir si les caspases participaient au développement du psoriasis. Nous avons pu montrer que lorsque nous induisons une maladie semblable au psoriasis chez des souris, l'inactivation des caspases inflammatoires ou son inhibition pharmacologique réduisait de façon significative le développement de la maladie par rapport aux souris WT. Bien que les cellules immunitaires et les kératinocytes soient capables de sécréter de l'IL-1 $\beta$  via l'activation de l'inflammasome, nos données ont établi que seule l'activation des caspases inflammatoires dans le système immunitaire semblait nécessaire pour une réponse inflammatoire complète.

En résumé, l'ensemble de mon travail de thèse a permis de montrer un mécanisme moléculaire par lequel la kinase Lyn régule négativement la voie apoptotique mitochondriale, ce qui peut contribuer à la transformation et/ou la résistance des cellules cancéreuses. D'autre part, nos résultats montrent que Lyn pourrait être un régulateur important du psoriasis, et notre étude indique que les caspases inflammatoires activées dans les cellules immunitaires sont impliquées dans la pathogénie du psoriasis. A ce jour, bien que plusieurs traitements aient été développés pour le psoriasis, la maladie reste non résolue, donc le développement de cibles thérapeutiques contre Lyn et les caspases inflammatoires pourraient être intéressants pour le traitement de la maladie.

Mots clés : Src-family kinase, Lyn, Bcl-2 family, Bim, Inflammatory caspases, Psoriasis



***A LA FAMILIA.***



***NOSOTROS SÓLO PODEMOS SABER QUE NO SABEMOS NADA.***

***Y ESE ES EL MÁS ALTO GRADO DE LA SABIDURÍA HUMANA.***

***LEÓN TOLSTOI,***

***LA GUERRA Y LA PAZ***



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# TABLE OF CONTENTS

LIST OF ABBREVIATIONS .....	4
LIST OF FIGURES.....	8
LIST OF TABLES .....	9
FOREWORD.....	11
INTRODUCTION .....	15
I. Src family of non-receptor tyrosine kinase.....	17
I.I. Definition, subfamilies .....	17
I.II. Structure.....	19
I.III. Localization of SFK.....	24
a) Within the tissues.....	24
b) Within the cells .....	25
I.IV. SFK activity regulation.....	26
a) Inactive and active forms.....	26
b) Activation mechanisms of SFK.....	27
(i) Regulation by protein-protein interactions .....	27
(ii) Regulation by phosphorylation and dephosphorylation .....	29
c) Inactivation mechanisms of SFK .....	31
d) Redox-dependent regulation of SFK .....	34
I.V. Regulation of SFK expression.....	34
a) At the mRNA level.....	34
b) At the protein level .....	35
I.VI. Physiological roles of SFK .....	36
a) Physiological functions of SFK in tissues.....	39
(i) In bones.....	39
(ii) In the nervous system .....	40
(iii) In the hematopoietic system.....	41
b) Physiological functions of SFK in biological processes .....	41
(i) In cell differentiation.....	41
(ii) In mitochondrial functions .....	42
II. Lyn tyrosine kinase .....	43

II.I. General presentation of Lyn tyrosine kinase .....	43
II.II. Localization of Lyn tyrosine kinase .....	44
a) Within the tissues .....	44
b) Within the cells .....	45
II.III. Role of Lyn tyrosine kinase .....	46
a) In signaling .....	46
b) In cells .....	48
(i) B cells .....	48
(ii) Myeloid cells .....	51
(iii) Osteoclast .....	52
c) In biological processes .....	53
(i) Implication of Lyn tyrosine kinase in cell death .....	53
(ii) Implication of Lyn tyrosine kinase in inflammation .....	58
• Role of Lyn in allergy .....	58
• Controversial roles of Lyn in asthma .....	59
• Roles of Lyn in other inflammatory diseases .....	60
III. Apoptosis .....	62
III.I. Bcl-2 family members .....	63
III.II. Bim .....	64
III.III. Caspases .....	67
a) Caspases and inflammation .....	68
IV. Psoriasis disease .....	73
IV.I. Is it psoriasis an autoimmune disease or an autoinflammatory syndrome? ...	73
IV.II. General characteristics of psoriasis disease .....	74
IV.III. Genetic susceptibility of psoriasis disease .....	75
IV.IV. Environmental triggers of psoriasis disease .....	78
IV.V. Clinical classification of psoriasis .....	79
IV.VI. Histological features of psoriasis .....	80
IV.VII. Clinical disease assessment .....	81
IV.VIII. Psoriasis pathogenesis .....	82
a) Principal cell types involved in psoriasis development .....	83
b) Principal cytokines implicated in psoriasis pathogenesis .....	85
IV.IX. Keratinocytes and psoriasis .....	88
IV.X. Psoriasis treatment .....	89

IV.XI. Mouse models of psoriasis disease.....	91
RESULTS .....	95
RESUME ARTICLE 1: .....	97
<i>The oncogenic tyrosine kinase Lyn impairs the pro-apoptotic function of Bim</i> .....	97
RESUME ARTICLE 2: .....	99
<i>Caspase 1/11 deficiency or pharmacological inhibition mitigates the psoriasis-like phenotype in mice</i> .....	99
RESUME ARTICLE 3: .....	101
<i>Lyn tyrosine kinase: a pivotal factor in psoriasis pathogenesis</i> .....	101
DISCUSSION AND PERSPECTIVES.....	103
Role of the Lyn tyrosine kinase in cell death.....	105
Role of pro-inflammatory caspases in Psoriasis.....	109
Role of Lyn tyrosine kinase in Psoriasis development .....	116
CONCLUSION .....	123
REFERENCES .....	127
ANNEXES.....	177
ARTICLE 4.....	179
ARTICLE 5.....	181
ARTICLE 6.....	183
CURRICULUM VITAE .....	185
LISTE DES PUBLICATIONS .....	190
SUMMARY .....	193
RESUME.....	197

# LIST OF ABBREVIATIONS

<b>3-IB-PP1:</b>	3-iodobenzyl Analog of the kinase inhibitor PP1
<b>Abl:</b>	Abelson murine leukemia viral oncogene homolog 1
<b>AIM2:</b>	Absent in melanoma 2
<b>ALL:</b>	Acute lymphoblastic leukemia
<b>ALR:</b>	AIM-2- like receptors
<b>AML:</b>	Acute myeloid leukemia
<b>ASC:</b>	Apoptosis-associated speck-like protein containing C-terminal CARD
<b>ATP:</b>	Adenine triphosphate
<b>BCL-2:</b>	B-cell lymphoma -2 family
<b>BCR:</b>	B cell receptor
<b>BFK:</b>	Brk family kinases
<b>BH:</b>	Bcl-2 homology
<b>Bim:</b>	Bcl-2 interacting mediator of cell death
<b>Blk:</b>	B lymphocytic kinase
<b>BMDM:</b>	Bone marrow-derived macrophages
<b>Brk:</b>	Breast tumor kinase
<b>BSA:</b>	Body surface area
<b>Btk:</b>	Brutus tyrosine kinase
<b>CARD:</b>	Caspase recruitment domain-containing protein
<b>Cbp-PAG1:</b>	Csk-binding protein/phosphoprotein associated with glycosphingolipid-enriched microdomains
<b>Cbl:</b>	Casitas B-cell lymphoma
<b>CCL:</b>	Chemokine ligand
<b>CD:</b>	Cluster of differentiation
<b>CDK:</b>	Cyclin-dependent kinase
<b>Chk:</b>	Csk-homology kinase
<b>CLL:</b>	Chronic lymphocytic leukemia
<b>CML:</b>	Chronic myeloid leukemia
<b>CRC:</b>	Colorectal cancer
<b>CRH:</b>	Corticotrophin-release hormone
<b>Csk:</b>	C-terminal kinase
<b>CTLA-4:</b>	Cytotoxic T lymphocyte antigen 4
<b>Cyr61/CCN1:</b>	Cysteine-rich protein angiogenic inducer 61
<b>DAMP:</b>	Damage-associated molecular patterns
<b>DISC:</b>	Death-inducing signaling complex

<b>DNA:</b>	Deoxyribonucleic acid
<b>DSS:</b>	Dextran sulfate sodium
<b>EGF:</b>	Epidermal growth factor
<b>ER:</b>	Endoplasmic reticulum
<b>ERK:</b>	Extracellular signal-regulated kinase
<b>EMT:</b>	Epithelial mesenchymal transition
<b>Fak:</b>	Focal adhesion kinase
<b>FcγR:</b>	Fc gamma receptor
<b>FcεR:</b>	Fc epsilon receptor
<b>Fgr:</b>	Gadner-Rasheed feline sarcoma virus
<b>FOxO:</b>	Forhead-box class O
<b>Frk:</b>	Fyn-related kinase
<b>Fyn:</b>	Proto-oncogene tyrosine kinase
<b>Gab2:</b>	Grb2-associated binder 2
<b>G-CSF:</b>	Granulocyte colony-stimulator factor
<b>GM-CSF:</b>	Granulocyte macrophage colony-stimulator factor
<b>GWAS:</b>	Genome-wide association studies
<b>Hck:</b>	Hematopoietic cell kinase
<b>HDM:</b>	House dust mite
<b>HIF-1α:</b>	Hypoxia-inducible factor 1α
<b>HLA:</b>	Human leucocyte antigen
<b>HS1:</b>	Hematopoietic cell specific Lyn substrate 1
<b>HSP:</b>	Heat shock protein
<b>IBD:</b>	Inflammatory bowel disease
<b>Ig:</b>	Immunoglobulin
<b>IL:</b>	Interleukin
<b>IL-2Rα+:</b>	IL-2 plus diphtheria toxin
<b>ITAM:</b>	Immunoreceptor tyrosine-based activation motifs
<b>ITIM:</b>	Immunoreceptor tyrosine-based inhibitory motifs
<b>IFN:</b>	Interferon
<b>IRF5:</b>	Interferon regulatory factor 5
<b>IRS-1:</b>	Insulin receptor substrate 1
<b>JNK:</b>	c-Jun N-terminal kinase
<b>Lck:</b>	Lymphocytic-specific protein tyrosine kinase
<b>LPS:</b>	Lipopolysaccharides
<b>Lyn:</b>	Lck/Yes novel tyrosine kinase
<b>MAPK:</b>	Mitogen-activated protein kinase

<b>M-CSF:</b>	Macrophage colony-stimulator factor
<b>MHC:</b>	Major histocompatibility complex
<b>MMP:</b>	Matrix metalloproteinase
<b>MOMP:</b>	Mitochondrial outer membrane permeabilization
<b>MP2A:</b>	Membrane protein 2A
<b>NFATc1:</b>	Nuclear factor of activated T cells c1
<b>NF-κB:</b>	Nuclear factor kappa B
<b>NOD:</b>	Nucleotide-binding oligomerization domain
<b>NLR:</b>	NOD-like receptors
<b>OVA:</b>	Ovalalbumin
<b>OXPHOS:</b>	Oxidative phosphorylation
<b>PAMP:</b>	Pathogen-associated molecular patterns
<b>PASI</b>	Psoriasis Area Severity Index
<b>PBMC:</b>	Peripheral blood mononuclear cells
<b>PDGF:</b>	Platelet-derived growth factor
<b>PGA:</b>	Physician's global assessment
<b>Ph+:</b>	Philadelphia chromosome
<b>PI3K:</b>	Phosphatidylinositol 3 kinase
<b>PK:</b>	Protein kinase
<b>PLCγ1:</b>	Phospholipase C gamma 1
<b>PP:</b>	Protein phosphatase
<b>PSORS:</b>	Psoriasis susceptibility
<b>PTP1B:</b>	Protein-tyrosine phosphatase non-receptor type 1B
<b>PTP:</b>	Protein-tyrosine phosphatase
<b>PTPN:</b>	Protein-tyrosine phosphatase non-receptor type
<b>RANK:</b>	Receptor activator of NF-κB
<b>PUVA:</b>	8-methoxypsoralen ultraviolet light A
<b>RAS:</b>	Rat sarcoma virus oncogene
<b>Ras-GAP:</b>	ras-GTPase activating protein
<b>RNA:</b>	Ribonucleic acid
<b>ROS:</b>	Reactive oxygen species
<b>Runx3:</b>	Run-related transcription factor 3
<b>S100:</b>	S100 calcium binding proteins
<b>SAPK:</b>	Stress-activated protein kinase
<b>SFK:</b>	Src family of non-receptor proteins kinases
<b>SLE:</b>	Systemic lupus erythematosus
<b>SH:</b>	Src homology

<b>SHIP1:</b>	SH2-containing inositol phosphatase
<b>SHP:</b>	Src homology 2 domain-containing tyrosine phosphatase
<b>SNP:</b>	Single nucleotide polymorphism
<b>Src:</b>	Short of sarcoma virus oncogene
<b>SOCS1:</b>	Suppressor of cytokine signaling 1
<b>Srm:</b>	Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites
<b>SPT:</b>	Serine palmitoyltransferase
<b>STAT:</b>	Signal transducer and activator of transcription
<b>Syk:</b>	Spleen tyrosine kinase
<b>TCR:</b>	T cell receptor
<b>TLR:</b>	Toll like receptor
<b>Th:</b>	T-helper
<b>TNF:</b>	Tumor necrosis factor
<b>Tom1L1:</b>	Target of myb1-like 1
<b>UD:</b>	Unique domain
<b>ULBR:</b>	Unique lipid binding regions
<b>VEGF:</b>	Vascular endothelial growth factor
<b>WT:</b>	Wild type
<b>Yes:</b>	Yamaguchi sarcoma virus oncogene
<b>Yrk:</b>	Yes-related tyrosine kinase

# LIST OF FIGURES

<b>Figure 1:</b>	Families of tyrosine kinase proteins	18
<b>Figure 2:</b>	General structure of SFK members	20
<b>Figure 3:</b>	Inactive and active conformations of SFK	27
<b>Figure 4:</b>	Activation and inactivation mechanisms of SFK	31
<b>Figure 5:</b>	Related-receptors to SFK activation	36
<b>Figure 6:</b>	Implication of SFK in different signaling pathways	39
<b>Figure 7:</b>	Schematic representation of Lyn tyrosine kinase	44
<b>Figure 8:</b>	Lyn regulation of signaling pathways	47
<b>Figure 9:</b>	BCR signaling	48
<b>Figure 10:</b>	Positive and negative regulatory effect of Lyn	49
<b>Figure 11:</b>	Lyn mutations in genetically engineered mice	51
<b>Figure 12:</b>	Subcellular localization of cLyn	55
<b>Figure 13:</b>	Principal phenotypical characteristic of cLyn mice	61
<b>Figure 14:</b>	Two major apoptosis pathways	62
<b>Figure 15:</b>	Bcl-2 family members	64
<b>Figure 16:</b>	Bcl-2 family members control cell life and death	65
<b>Figure 17:</b>	Bim isoforms	66
<b>Figure 18:</b>	Domain organization and classification of human caspases	67
<b>Figure 19:</b>	Canonical and non-canonical inflammasomes	70
<b>Figure 20:</b>	Histological features of psoriasis disease	81
<b>Figure 21:</b>	Inflammatory pathways in both phases of psoriasis	84
<b>Figure 22:</b>	Inflammatory triangle in psoriasis pathogenesis	86
<b>Figure 23:</b>	Recapitulative schema of the anti-apoptotic role of Lyn	108
<b>Figure 24:</b>	Recapitulative schema of the role of pro-inflammatory caspases in psoriasis	115
<b>Figure 25:</b>	Recapitulative schema of the role of Lyn tyrosine kinase in psoriasis	121

# LIST OF TABLES

<b>Table 1:</b>	Members and molecular weights of the SFK	19
<b>Table 2:</b>	Amino acid alignments of SFK	22
<b>Table 3:</b>	Expression of the SFK proteins	25
<b>Table 4:</b>	Phosphorylation sites of SFK	29
<b>Table 5:</b>	Principal substrates of SFK	38
<b>Table 6:</b>	Phenotype of mice deficient for SFK	40
<b>Table 7:</b>	Dual role of Lyn modulating myeloid-cell signaling	52
<b>Table 8:</b>	Roles of Lyn in inflammation	58
<b>Table 9:</b>	Classification of psoriasis comorbidities	75
<b>Table 10:</b>	Genetic-associated loci related with psoriasis development	77
<b>Table 11:</b>	Clinical classification of psoriasis disease	79
<b>Table 12:</b>	Targeted therapies in psoriasis treatment	91



# FOREWORD



Members of the Src family are non-receptor protein tyrosine kinases, which have been implicated in several cellular signaling processes regulating cell growth, differentiation, apoptosis, migration, immune response, adhesion and metabolism. Specifically, Lyn is a member of these intracellular-associated kinases, which shares structure and regulation mechanisms with the other members of the family.

While Lyn was originally identified as a hematopoietic kinase due to its crucial role in B cell receptor and myeloid cells presenting a positive and a negative role, nowadays; it is well known that Lyn is expressed in many tissues. In fact, Lyn is involved in the transmission of signal coming from different cellular receptors and is implicated in the phosphorylation of several substrates, mediating the activation or inhibition of different cellular processes.

Deregulation of Lyn expression results in antibody-mediated autoimmune disease and an increase in its expression and/or activity has been associated to malignant diseases and resistance to treatment. Indeed, a caspase-dependent cleaved form of Lyn was associated to negative control of apoptosis, which could be related with chemoresistance in hematological cancer.

Thus, during the first part of my thesis, we were highly interested in understanding the mechanism, by which, this particular form of Lyn acted, in vitro, as an apoptosis suppressor.

Moreover, and quite the contrary to its in vitro role, the overexpression in mice of this caspase-cleaved form of Lyn induces a psoriasis-like phenotype, suggesting a possible function of this protein in this skin pathology. Psoriasis is a chronic immune-mediated disorder with a strong genetic basis affecting around 125 million people worldwide, whose incidence is increasing. It is a disease with negative effects on the quality of life of the patient and the patient's family, which has been unfortunately related to social stigmatization and discomfort, impairing the interpersonal relationships as well as impacting sexual well-being and capacity for intimacy. In fact, psoriasis patients usually express feeling shame, guilt, and they manifest discrimination and difficulty to obtain an employment; which has contributed to anxiety and depression; thus conferring significantly physical and psychological distress. Although no cure exists today for psoriasis, several treatments have been developed, which are prescribed depending on the severity of the symptoms. However, this common skin disease is a complex multifactorial disease, which has been linked to several comorbidities, thus it requires a multidisciplinary approach to understand its genetic and immunological bases, because albeit the pathogenicity of this disease is fairly well

understood, the etiology remains uncertain. Therefore, identification of differentially expressed genes in psoriasis lesions could lead to the development of new specific biological treatments.

Thus, during the second and the third part of my thesis, we were focused in comprehending the role of pro-inflammatory caspases and Lyn tyrosine kinase in the development of psoriasis.

# **INTRODUCTION**



Tyrosine phosphorylation is one of the most important covalent modifications carried out in multicellular organisms as consequence of intracellular communications during embryogenesis and maintenance of adult tissues. This process is produced by enzymes called protein tyrosine kinases which catalyze the transfer of gamma-phosphate group from one molecule of adenine triphosphate (ATP) to hydroxyl groups located in tyrosine residues from protein substrates ([Hubbard and Till, 2000](#)). In general, tyrosine kinases are a huge group of proteins presenting in all cellular types, which have been classified into two big families according to their cellular localization. The first family is named transmembrane receptor protein tyrosine kinase that includes 58 members organized in 20 sub-families ([Lemmon and Schlessinger, 2010](#)) and the second family is called non-receptor protein tyrosine kinase, with a cytosolic localization and composed by 32 members that fall into 10 subfamilies ([Tsygankov, 2003](#)) (**Figure 1**). The largest subfamily of non-receptor protein kinases and principal focus of this thesis is the Src family, which has been implicated in several cellular processes such as cell growth, differentiation, adhesion, migration and survival ([Roskoski, 2015](#); [Ingley, 2012](#); [Ingley, 2008](#); [Tsygankov, 2003](#); [Hubbard and Till, 2000](#)).

## I. Src family of non-receptor tyrosine kinase

### I.I. Definition, subfamilies

The Src family proteins of non-receptor tyrosine kinases (SFK) play a key role in the regulation of signal transduction through a diverse set of cell surface receptors due to several stimuli ([Parsons and Parsons, 2004](#)). Although the protein which gives the name to the family, Src, was discovered one hundred years ago; the understanding of the activity of its members, in terms of both mediators of biological signal and kinases, is quite recent ([Martin, 2001](#)). In fact, through the research of Dr Peyton Rous in 1911 on a chicken tumor produced by the Rous sarcoma virus ([Weiss and Vogt, 2011](#)), the protein v-Src was discovered. First associated to a viral oncogene, several years after it was demonstrated that c-Src (hereafter referred as Src) is the normal cellular homologue, which is encoded by a physiological gene ([Stehelin et al, 1976](#)), being the first proto-oncogene to be described and characterized.

This family of proto-oncogene proteins is composed by eight members (Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk) with similar structure and molecular weight among 51 and 62 kDa, which are all specific to vertebrates and have been classified in two different subfamilies according to their amino acid similarities: the SrcA or related to Src (Src,

Fyn, Yes, Fgr) and the SrcB or related to Lyn (Lyn, Lck, Hck and Blk) (**Table 1**) ([Ingleby, 2008](#); [Parsons and Parsons, 2004](#); [Manning et al, 2002](#); [Thomas and Brugge, 1997](#)).



**Figure 1:** Families of tyrosine kinase proteins

The family of tyrosine kinase proteins is classified in two principal groups according to their cellular localization. First: receptor tyrosine kinase composed by 58 members grouped in 20 subfamilies. Second: non-receptor tyrosine kinases integrated by 32 members grouped in 10 subfamilies. Src family kinases from part of the non-receptor tyrosine kinase group ([Lemmon and Schlessinger, 2010](#)).

It is worth noting that Yrk (**Yes-related tyrosine kinase**) can be considered as another member of the SFK family, but this protein is only found in chicken ([Thomas and Brugge, 1997](#)). Further, other three proteins have been considered as SFK related proteins, which are Frk (**Fyn-related kinase**), Brk (**Breast tumor kinase**) and Srm (**Src-related kinase lacking C-terminal regulatory tyrosine and N-terminal myristoylation sites**); being Frk considered as a sub-family of SFK ([Ingleby, 2008](#); [Manning et al, 2002](#); [Thomas and Brugge, 1997](#)). However, currently these three proteins have been grouped inside a new family, the BFK (**Brk family kinases**) family ([Goel and Lukong, 2016](#); [Goel and Lukong, 2015](#)). Although both families are evolutionarily related, the differences found concerning the lack of acylation in the BFK family and the presence of a highly conserved exon structure between the three members of BFK ([Serfas and](#)

[Tyner, 2003](#)), but distinct from those belonging to the SFK, led to separate these three proteins in a different group from SFK.

Src family kinases (SFK): Members	
SrcA (Src related proteins)	SrcB (Lyn related proteins)
<b>Src:</b> short of <b>Sar</b> coma virus oncogene	<b>Lyn:</b> Lyn/yes novel tyrosine kinase
<b>Fyn:</b> proto-oncogene tyrosine-protein kinase	<b>Lck:</b> Lymphocytic-specific protein tyrosine kinase
<b>Yes:</b> Yamaguchi sarcoma virus oncogene	<b>Hck:</b> Hematopoietic cell kinase
<b>Fgr:</b> Gardner-Rasheed feline sarcoma virus	<b>Blk:</b> B lymphocytic kinase

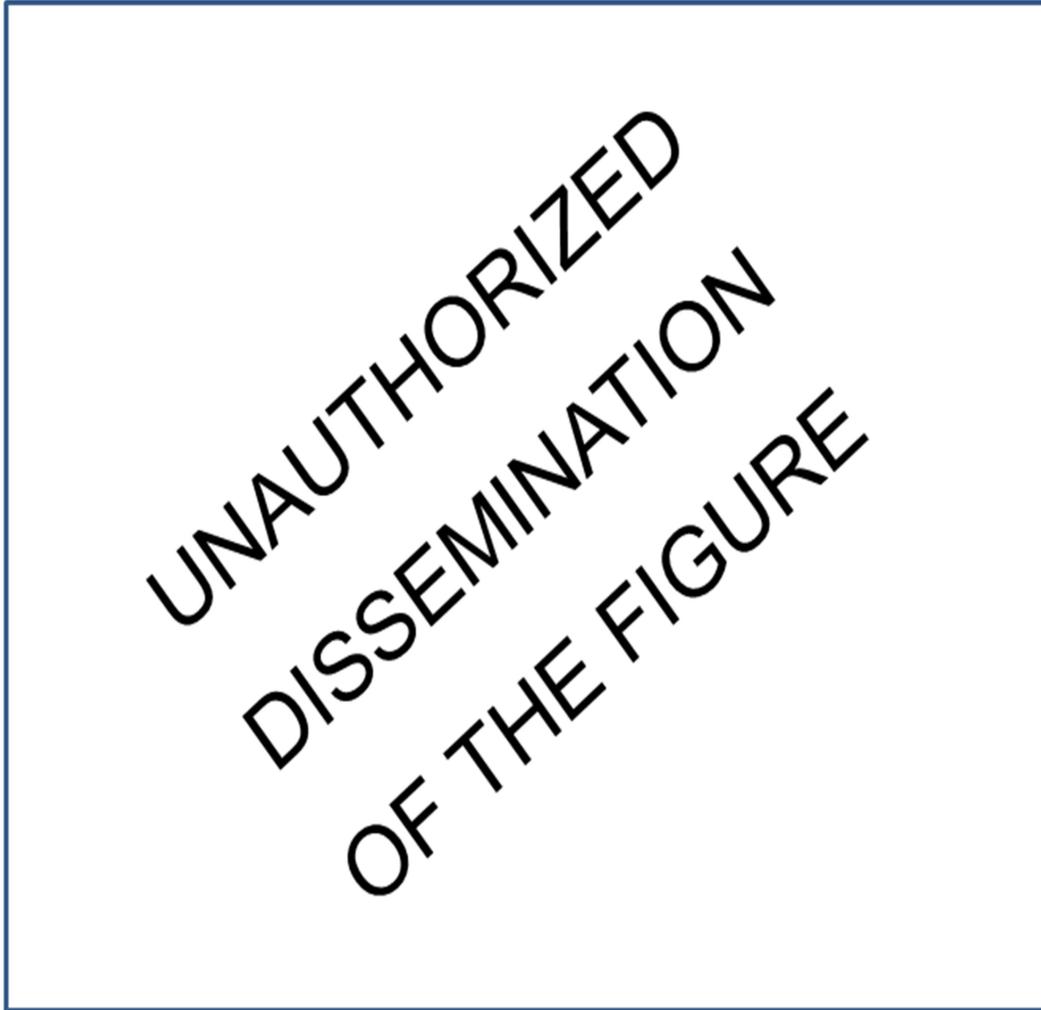
Src family kinases (SFK): Isoforms and molecular weights	
SrcA (Src related proteins)	SrcB (Lyn related proteins)
<b>Src:</b> p60: c-Src, N1-Src; p62: N2-Src	<b>Lyn:</b> LynA (p56), LynB (p53); cleaved p54, p51
<b>Fyn:</b> p59: FynT and FynB	<b>Lck:</b> p56
<b>Yes:</b> p61	<b>Hck:</b> p59, p61
<b>Fgr:</b> p55, p58	<b>Blk:</b> p55

**Table 1:** Members and molecular weights of the SFK

Two subfamilies have been described inside the SFK: SrcA (Src related proteins) and SrcB (Lyn related proteins).

## I.II. Structure

All members of the SFK share a common structure, which can be organized in six different regions: (1) N-terminal region, where the Src homology (SH) 4 domain is found containing sites for lipid modifications; (2) the poorly-conserved unique domain (UD); (3) the SH3 domain, necessary to bind to specific proline-rich sequences; (4) the SH2 domain, which can bind to specific sites of tyrosine phosphorylation; (5) the SH1 domain, where the catalytic function of the protein is localized and (6) a C-terminal region at the end of the protein, which carries the negative regulatory sequence (**Figure 2A**) ([Roskoski, 2015](#); [Boggon and Eck, 2004](#); [Roskoski, 2004](#)).



**Figure 2:** General structure of all members of SFK

- A. All SFK share a common structure composed from N to C terminal by the SH4 domain, the unique region, the domains SH3 and SH2, the catalytic domain SH1 and at the end of the protein the regulatory sequence ([Roskoski, 2015](#); [Boggon and Eck, 2004](#)).
- B. Ribbon diagram illustrating the tri-dimensional structure of human Src (Taken from [Roskoski, 2004](#)).

The SH4 domain is a very short sequence, around 15 amino acids, present in all SFK in the N-terminal region. In this domain, SFK experience lipid modifications called acylation processes characterized by the binding of fatty acids myristate and/or palmitate. Both lipid modifications are necessary for membrane association of SFK. The binding of myristate to the Glycine in position 2 is an irreversible co-translational/post-translational modification and can take place in all SFK. However the binding of palmitate to Cysteine residues is also present in all SFK, except in Src and Blk. Palmitoylation is a reversible post-translational modification and is possible to find monopalmitoylated SFK (Lyn, Yes) and dually palmitoylated (Fyn, Fgr and Lck) (**Table**

**2A)** ([Okada, 2012](#)). It is worth noting that palmitoylation has been involved in the stabilization of SFK in the lipid rafts ([Oneyama et al, 2009](#)), playing an important role in the localization and function of SFK. On the other hand, it has been demonstrated that myristoylation is sufficient to exclude localization of palmitoylated SFK proteins in the nucleus ([McCabe and Berthiaume, 1999](#)). Further, if Gly2 is mutated by alanine it is not possible to insert the palmitate in Cys3 or Cys6, indicating that N-myristoylation is required to palmitoylation takes place ([Alland et al, 1994](#); [Koegl et al, 1994](#)). However, dual palmitoylations have been observed in absence of myristoylation ([McCabe and Berthiaume, 1999](#)). Moreover, the palmitoylation state specifies the trafficking of SFK inside cells. In fact a monopalmitoylated protein, like Lyn, is secreted to the plasma membrane by Golgi region along the secretory pathway ([Kasahara et al, 2004](#)), but a dually palmitoylated protein, like Fyn, is directly secreted to the plasma membrane ([Sato et al, 2009](#)), showing that a mutated dually palmitoylated SFK lacking the second palmitate is transported to the plasma membrane like a monopalmitoylated SFK. A non-palmitoylated protein like Src is moved quickly between the plasma membrane and late endosomes or lysosomes, and a mutation in the palmitoylation site of Lyn produces its intracellular traffic in a similar way to Src ([Kasahara et al, 2007](#)); suggesting that palmitoylation is necessary to distinguish traffic between Src and Lyn. Generally, non-palmitoylated but myristoylated SFK are unable to efficiently associate to plasma membrane leading to a reduction in signaling pathways ([Resh, 2006b](#); [Resh, 2006a](#)). Furthermore, palmitoylation might be involved in malignant transformation. For example, deletion of the palmitoylation site in Yes protein drives in a reduction of the Yes transforming activity in colorectal cancer (CRC) cells ([Dubois et al, 2015](#)).

The Unique domain (UD) is a poorly-conserved region inside all SFK that is involved in their specific interactions and their regulation, which is constituted by 50-70 amino acids ([Boggon and Eck, 2004](#)). Thus, this region confers the diversity to the family. For example, the UD of Lck is directly involved in the interactions with co-receptors such as cluster of differentiation (CD)4 and CD8 in T cells via a zinc complex ([Kim et al, 2003](#)). Further, through the UD, Fyn and Lyn could interact with non-phosphorylate motifs from the B cell receptor (BCR) ([Pleiman et al, 1994](#)). Phosphorylation sites have been showed in this domain for Hck that significantly contributes to their activation ([Johnson et al, 2000](#)) and in general phosphorylation processes in this domain play a key role in the regulation of SFK ([Amata et al, 2014](#)). Moreover, the UD of Fyn, Lyn ([Luciano et al, 2001](#)) and Src ([Hossain et al, 2013](#)) have been implicated in the induction of apoptosis with important consequences for their intracellular localization and activity. In fact, in an ischemic stroke rat model, Src is

cleaved by calpains in the UD generating a truncated form of this protein that is located at the cytosol inducing neuronal cell death ([Hossain et al., 2013](#)). Further, the interaction of Src with the N-methyl-D-aspartate receptors can be lost in the presence of a peptide consisting of amino-acids 40-49 of the UD of Src ([Liu et al., 2008](#)), demonstrating that the link between inflammatory and neuropathic pain behavior is dependent upon Src. Furthermore, lipid binding regions have been identified in the UD emphasizing the versatility of this region and mutations in these lipid binding regions of Src lead to strong phenotypes in the maturation of *Xenopus laevis* oocytes ([Perez et al., 2013](#)). However, it has been recently reported that features from a well-conserved sequence involving aromatic residues have been found in the UD of SFK ([Arbesu et al., 2017](#)).



**Table 2:** Amino acid alignments of SFK

- A. N-terminal domain. All SFK are modified post-translationally by the binding of fatty acids to the SH4 domain. Myristoylation (the red one in Glycine residues) is found in all members of the family while palmitoylation (the yellow one in Cysteine residues) is observed in all members, except in Src and Blk.
- B. Activation loop and C-terminal regulatory tail. All SFK present a highly conserved sequence in the activation segment for the autophosphorylation (Tyr 416) and a highly negative short sequence of around 20 amino acids to produce the inactivation of these proteins. ([Okada, 2012](#)).

Although SH3 and SH2 domains do not have a catalytic function, they allow protein-protein interaction and play a crucial role in the SFK activity. SH3 domain is composed by around 60 residues distributed in five anti-parallel  $\beta$ -strands, which are

packed at almost right angles; thus, constituting a  $\beta$ -barrel (**Figure 2B**). The five  $\beta$ -strands are connected by two prominent loops called the RT and N-Src loops ([Roskoski, 2015](#); [Bauer et al., 2005](#)). The main function of this domain is to bind proline-rich ligands (xPxxP, being “x” any amino acid), which are recognized by the aromatic and hydrophobic residues present in the two prominent loops ([Lim et al., 1994](#)), although recent studies have shown that the ligand-binding surface of an SH3 domain can be molded to interact with a variety of ligands ([Kaneko et al., 2008](#)). The xPxxP motifs form a canonical type II polyproline helix, which has two possible binding orientations, the first one, called class I, in direction N-ter toward C-ter and the second one, and called class II, in the opposite direction ([Lim et al., 1994](#)). Therefore, this domain has a key role in the interaction among the target protein and the other domains of SFK ([Boggon and Eck, 2004](#)). Indeed, Fyn SH3 domain can link to the proline motif in the immune cell adaptor SKAP55 ([Kang et al., 2000](#)) and also interact with the SH2 domain of the adaptor protein SAP ([Chen et al., 2006](#); [Latour et al., 2003](#)), regulating in both cases the receptor signaling in immune response.

SH2 domain (**Figure 2**) is constituted by around 100 amino acids and allows the binding of SFK to distinct C-terminal amino acid sequences to the phosphotyrosine ([Roskoski, 2004](#)). This domain consists of a central-three stranded  $\beta$ -sheet with a single helix packed against each side forming two pockets. The first pocket, highly conserved, is implicated in the recognition of phosphotyrosine residues and contains a conserved arginine residue (Arg178 in human Src), which is important in electrostatic interactions with the phosphorylated tyrosine. The second pocket, highly variable, binds one or more (normally three or five) C-terminal hydrophobic residues of the phosphorylated tyrosine ([Roskoski, 2015](#)). X-ray crystallographic studies concluded that SH2 domains from Fgr, Fyn, Lck and Src preferred pY-E-E-I motifs in their substrates comparing to other sequences ([Waksman and Kuriyan, 2004](#)); nevertheless this domain can bind to other huge group of sequences that do not belong to this typical motif (pY-E-E-I). For example, the SH2 domain binds intramolecularly to the C-terminal phosphotyrosine resulting in the inhibition of the protein activity. The sequence of this intramolecular site is pY-Q-P-G, then as this sequence is a non-optimal SH2-binding sequence, its binding can be displaced for optimal sequence leading to the activation of the protein ([Roskoski, 2015](#)).

After the SH2 domain, an organized type II helix region, called SH2-kinase linker, is found. Although this region is a proline-rich sequence, it does not contain the xPxxP motif, which is the favorite binding site for the SH3 domain of the SFK. Nonetheless, this region is involved in the regulation of SFK activity. For example in the case of Hck,

SH2-kinase linker acts as an activator switch representing a suboptimal ligand for the SH3 domain in the context of downregulation of the protein ([Trible et al, 2006](#)).

SH1 domain (**Figure 2**) is composed by around 300 amino acids and carries the catalytic activity of the SFK. Two main residues, highly conserved in the SFK family, are observed inside this domain; the first one for the ATP binding and the second one for the transfer of a phosphate group, which lead to the protein activation. The motif GxGxxG and the lysine K275 (in the Src protein) are involved in the ATP binding. Moreover, the tyrosine 416 (in the Src protein), presents in the region named activation segment, is implicated in the autophosphorylation process; necessary for the fully activation of the SFK. Therefore the activation segment is found highly conserved also among all SFK (**Table 2B**) ([Boggon and Eck, 2004](#)). In general, this domain has a bi-lobe structure (**Figure 2B**). The small lobe, in its N-terminal region, is composed by a five-stranded antiparallel  $\beta$ -sheets and an important  $\alpha$ C-helix. The large lobe is mainly  $\alpha$ -helical with six conserved segments containing the activation segment. Nucleotide binding as well as the transfer of a phosphate group is done in the space between these two lobes ([Roskoski, 2015](#)).

Finally, at the end of the protein in the C-terminal region, is located a short sequence of around 20 amino acids (QYQ), which has the negative regulatory domain of the SFK family and is highly conserved among all SFK (**Table 2B**). Within this domain, another highly conserved tyrosine residue is found, the tyrosine 527 (in the Src protein), which is critical for the inactivation of SFK activity, once this tyrosine residue is phosphorylated ([Roskoski, 2015](#); [Boggon and Eck, 2004](#)).

### I.III. Localization of SFK

#### a) Within the tissues

Two principal localizations, according to their expression, we can find through the SFK (**Table 3**). Firstly, an ubiquitous expression is seen for Src, Fyn and Yes. However, whether each one is analyzed separately is possible to see that expression level is different according to the analyzed tissue. For example, Src has a strong expression in the brain, osteoclasts and platelets; while Yes is strongly found in the brain, epithelial tissues, lungs and platelets ([Senis et al, 2014](#); [Brown and Cooper, 1996](#)). Moreover, Src, Yes and Fyn are well expressed in the skin ([Rotzer et al, 2015](#); [Szalmas et al, 2013](#)). The second group is composed Fgr, Blk, Hck, Lck and Lyn, which have a main expression in hematopoietic cells ([Poh et al, 2015](#); [Thomas and Brugge, 1997](#)). However, recent published data have shown that particularly Hck ([Wang et al, 2017a](#)) and Lyn ([Hernandez-Rapp et al, 2014](#)) can be observed in other

cellular tissues, like the brain. Additionally, it has been demonstrated that Lyn is activated in many solid cancers ([Liu et al, 2016a](#); [Ingley, 2012](#)); demonstrating that Lyn expression is, in fact, less restricted than what was originally considered.

SFK protein	Expression
Src	Ubiquitous
Fyn	Ubiquitous
Yes	Ubiquitous
Fgr	B cells and myeloid cells
Blk	B cells
Hck	Myeloid cells
Lck	T cells, Natural killer T cells, but also in the brain
Lyn	B cells and myeloid cells, but also in brain, keratinocytes, fibroblasts, kidney, prostate and lung

**Table 3:** Expression of the SFK proteins

Two major groups within SFK have been established according to their tissue localization: (1) Ubiquitous expression. In this group Src, Yes and Fyn are found. (2) Hematopoietic system expression. In this group the rest of SFK members such as Fgr, Blk, Hck, Lck and Lyn are found. However, it is noteworthy that members of this group are also observed in others tissues ([Thomas and Brugge, 1997](#)).

### **b) Within the cells**

Generally, SFK proteins are present inside all cell types and after myristoylation and palmitoylation processes, they are located in the cytoplasmic face of plasma membrane, although all SFK can be also found in the Golgi complex, phagosomes, endosomes and secretory granules ([McCabe and Berthiaume, 1999](#)). Moreover, it has been recently described that Src is present and activated on exosomes, specifically in prostate cancer exosomes ([DeRita et al, 2017](#)), enhancing proliferation and migration. In fact, Src might stimulate exosome production with pro-migratory activity on endothelial cells, being important in the cell-to-cell communication ([Imjeti et al, 2017](#)). Further, different isoforms have been described for SFK members, leading to distinct subcellular localizations. For example, two isoforms (p59 and p61) have been reported for Hck ([Lock et al, 1991](#)), differing in the acylation process. In fact, the larger one is located into the lysosomes and the shorter one is found in the plasma membrane, because it is the only isoform where palmitoylation modification can occur ([Poh et al,](#)

[2015](#); [Thomas and Brugge, 1997](#)). Normally, in plasma membrane and according to lipid modifications, SFK are observed in the lipid rafts ([Rege et al, 2006](#)), which has been associated with the regulation of their activity. Indeed, Lyn recovered from lipid rafts has a markedly higher specific activity comparing to Lyn from the rest of plasma membrane ([Young et al, 2003](#)). Additionally, reduced levels of Src in lipid rafts have been found, when one of its substrates, target of myb1-like 1 (Tom1L1), is associated to clathrin heavy chain; leading to a decrease in the Src transforming activity. However, the relocation of Tom1L1 in lipid rafts promotes deoxyribonucleic acid (DNA) synthesis induced by Src activation ([Collin et al, 2007](#)). Moreover, SFK can be also observed in the perinuclear endosomes ([Lowell and Soriano, 1996](#)), as well as in the nucleus, the mitochondria and in the microtubules ([Sulimenko et al, 2006](#)). Furthermore, in the particular case of Lyn and Fyn, a caspase-dependent cleaving process can occur during the apoptosis in their N-terminal region. This cleavage leads to the loss of the part involved in the anchoring to the membrane, conducting to their localization into the cytosol ([Luciano et al, 2001](#); [Ricci et al, 1999](#)). Therefore, different subcellular localizations are found in SFK, which has an incidence in their functions and can change their accessibility to distinct substrates ([discussed in I.VI. Physiological roles of SFK](#)).

## I.IV. SFK activity regulation

### a) Inactive and active forms

Crystallography studies have evidenced that SFK can exist in two conformations ([Pucheta-Martinez et al, 2016](#); [Bernado et al, 2008](#)), which has allowed a better understanding of different mechanisms involved in the activity regulation of this family.

The first conformation is the inactive form, which is principally found under basal conditions and is defined as a restrained closed conformation. In this state the Tyr 527 (referred to Src protein) is observed phosphorylated, by the action of other tyrosine kinase proteins such as Csk (C-terminal Src kinase) ([Okada, 2012](#)) and Chk (Csk homology kinase) ([Advani et al, 2017](#)) ([discussed in I.IV. c\) Inactivation mechanisms of SFK](#)). Indeed, Tyr527 phosphorylation keeps SH3 and SH2 domains attached to N-lobe and C-lobe from the kinase domain, respectively. This structure is called the clamp ([Harrison, 2003](#)) (**Figure 3A**) and it has been recently demonstrated that the active or the inactive state of the catalytic domain is close dependent from the SH2 and SH3 bound orientation, suggesting the crucial function of these domains in SFK conformations ([Fajer et al, 2017](#)). To stabilize this inactive conformation, two intramolecular interactions are needed: firstly the binding of the SH3 domain to the

SH2-kinase linker domain and second, the binding of the negative regulatory sequence to the SH2 domain, forming a structure called the latch ([Xu et al, 1999](#); [Xu et al, 1997](#)). Therefore, in this closed form the catalytic site of the SFK is blocked and the enzymatic activity of these proteins is completely switched off.

The second conformation of the SFK is the active form, which is produced after the binding of ligands, leading to an opened conformation (**Figure 3B**). In general, three mechanisms have been described to explain the activation of SFK. The first one includes the binding of proteins to the SH3 and SH2 domains, destabilizing the clamp (unclamping) ([Lerner et al, 2005](#); [Lerner and Smithgall, 2002](#)). The second one involves the dephosphorylation of the C-terminal tail by phosphatases, breaking the binding between Tyr527 and SH2 domain (unlatching) ([Huang et al, 2011](#); [Somani et al, 1997](#)). Finally, the third mechanism to produce SFK activation is characterized by the autophosphorylation of the high-conserved tyrosine residue in the activating loop of SH1 domain (switching) ([Roskoski, 2015](#)).



**Figure 3:** Inactive and active conformations of SFK

- A. Inactive form of SFK. Restrained closed conformation due to intramolecular interactions.
- B. Active form of SFK. Opened conformation due to autophosphorylation ([Harrison, 2003](#)).

## **b) Activation mechanisms of SFK**

### **(i) Regulation by protein-protein interactions**

Intramolecular interactions keep SFK in the inactive state, but external interactions encourage the active state of these proteins because all proteins that are able to interact with SH2 and SH3 domains of SFK could interrupt the clamp (**Figure 4**) and promote the activation of this family. Therefore, Src structure allows its regulation by competition between intramolecular and external binding partners.

It was previously described that in the inactive form of SFK, intramolecular interactions in domains SH3 and SH2 are needed to stabilize this conformation. However, these interactions are not from high affinity ([Roskoski, 2015](#)); reason through which they can be easily displaced by a ligand of greater affinity. Thus, adaptor molecules with either specific xPxxP motifs or phosphorylated pY-E-E-I related sequences are required to initiate SFK signaling processes through the dismantling of SH3 and SH2 intramolecular associations ([Ingleby, 2008](#)). In general, SFK are controlled by receptor protein-tyrosine kinases, integrin receptors, G proteins and Fc-coupled receptors as well as cytokine and steroid hormone receptors. Thus, all these proteins are able to interact with SH3 and SH2 or both domains leading to the activation of the SFK. Nevertheless, it is worth noting that these receptors not only regulate SFK activity, but SFK can also regulate the functional activity of receptors; being this dual function of SFK as both effectors and regulators which allows them to facilitate the cross-talk between different receptors ([Thomas and Brugge, 1997](#)).

The protein Nef from human immunodeficiency virus, which has a polyproline motif, can interact with the SH3 domain of Hck, leading to a decrease in CD1a lipid antigen presentation ([Shinya et al, 2016](#)). This interaction, which has an unusual high affinity, does not require a displacement in the tail dephosphorylation and SH2 domain ([Lerner and Smithgall, 2002](#)). Fc-gamma receptor (FcγR)IIa is a member of the immunoglobulin gene superfamily, which is comprised by an extracellular domain that binds the Fc region of an IgG, a single pass transmembrane domain, and a cytoplasmic tail that contains two YxxL immune receptor tyrosine-based activation motifs (ITAM) ([Van den Herik-Oudijk et al, 1995](#)). In human platelet, FcγRIIa is the only Fc receptor and its antigen-binding leads to an activation of SFK, through interaction with SH2 domain, producing ITAM phosphorylation ([Zhi et al, 2015](#)). Indeed, the focal adhesion kinase (Fak) Pyk2 is able to activate Lyn and Fyn in thrombin-stimulated platelets ([Canobbio et al, 2015](#)). Moreover, the adaptor-like molecule, SAP, expressed in immune cells promotes the selective recruitment and activation of FynT by binding to its SH3 domain ([Latour et al, 2003](#)). Further, other proteins like Fak and p130Cas can interact with SH2 and SH3 domains of Src, producing its activation ([Matsui et al, 2012](#)). In fact, SFK/Fak interaction and signaling mitigates drug effects in models of Epidermal Growth Factor (EGF) receptor-mutated lung cancer ([Ichiara et al, 2017](#)). Moreover, interaction between SH2 domain and the activation segment of the EGF receptor are needed for the Src activation of this receptor ([Chen et al, 2017](#)).

Protein-protein interactions leading to the activation of SFK have been not only demonstrated with SH3 and SH2 domains, but also with the catalytic domain. Indeed,

G protein-coupled receptors can bind to the SH1 domain of Src and Hck, producing a conformational switch giving access to the catalytic site ([Ma et al, 2000](#)). Moreover, intramolecular association of SH3 domain and SH2-kinase linker regions has a key role in down-regulation of the kinase activity. For example, in a truncated form of Hck SH3 domain and SH2-kinase linker have structure and relative orientations similar to those observed in normal Hck, but SH2-kinase linker failed in engaging to SH3 domains, supporting the idea that these non-catalytic regions work together as a “conformational switch” that modulates kinase activity in a manner unique ([Alvarado et al, 2010](#)).

Thus, three principal functions have been attributed to SH2 and SH3 domains inside SFK structure and regulation: i) impairing enzyme activity by intramolecular contacts, ii) proteins-containing SH2 and SH3 domains interact with SFK, displacing intramolecular association of SH2 and SH3 domains driving in SFK activation, and iii) proteins-containing SH2 and SH3 domains serves as substrates of SFK.

(ii) Regulation by phosphorylation and dephosphorylation

Several sites of phosphorylation have been described for SFK, which correspond to Tyr and Ser/Thr residues and can be phosphorylated and dephosphorylated by several proteins (**Table 4**).

Residues	Protein kinases
Ser12	PKC
Ser17	PKA
Thr34, Thr46, Ser72	CDK/Cdc2
Tyr138	PDGF receptor
Tyr213	PDGF receptor, Her2
Tyr416	Autophosphorylation
Tyr527	Csk, Chk

**Table 4:** Phosphorylation sites of SFK

SFK can be phosphorylated in Tyr and Ser/Thr residues leading to the inactivation or activation to these proteins. ([Roskoski, 2005](#)).

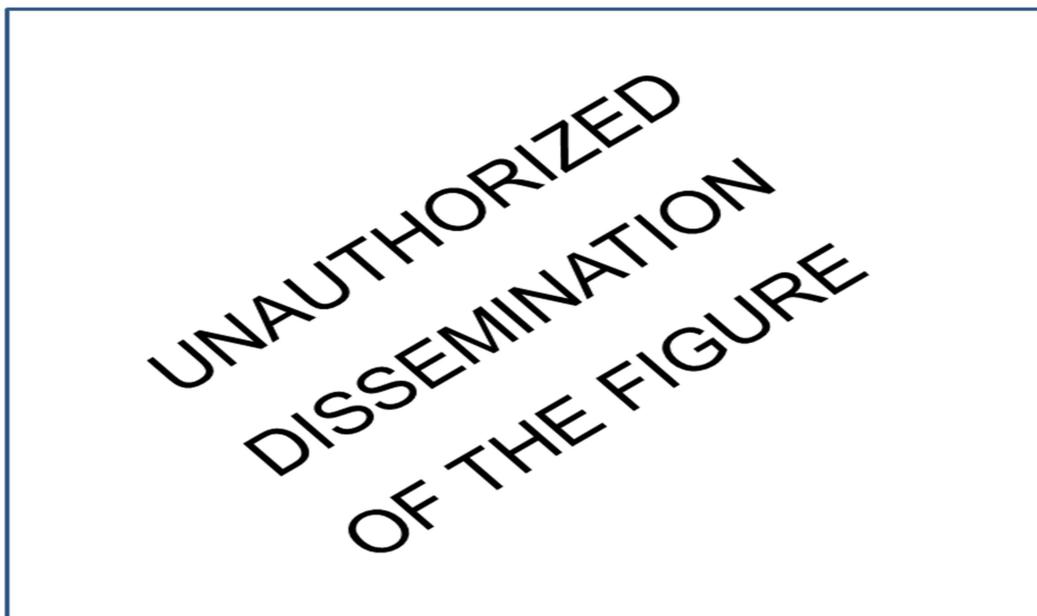
Dephosphorylation of the inhibitory tyrosine 527 (Tyr527 referred to Src) and phosphorylation of the activator tyrosine 416 (Tyr416 referred to Src protein) are both processes required to complete the activation of SFK.

The unlatching, name of the dephosphorylation process on the Tyr527, is produced after removing of this tyrosine from the SH2-binding pocket. Once unlatched, Tyr527 reverts accessible to several protein tyrosine phosphatases. These phosphatases can be cytoplasmic or transmembrane. Among the cytoplasmic enzymes, the protein-tyrosine phosphatase (PTP) non-receptor type 1 (PTP1)B and the Src homology 2 domain-containing tyrosine phosphatase (SHP)-1 and SHP-2 have been described. Moreover, among the transmembrane enzymes CD45, PTP $\alpha$ , PTP $\epsilon$  and PTP $\lambda$  have been shown ([Roskoski, 2005](#)) (**Figure 4**).

The switching, name of the phosphorylation process on the Tyr416, is one of the most important processes to achieve the maximal kinase activity ([Schindler et al. 1999](#)) (**Figure 4**). In this case the  $\alpha$ -helix, which is present in the activation segment and that impairs access to substrates, changes its conformation; being the N-terminal lobe from kinase domain re-orientated and allowing to C-terminal lobe to adopt an active conformation ([Thomas and Brugge, 1997](#)). Autophosphorylation of Tyr416 is an intermolecular process ([Barker et al. 1995](#)) and is consistent with the ability of SFK to dimerize. It is a process that is mediated by trans-autophosphorylation and not by cis-autophosphorylation, and further, can be achieved by other tyrosine kinases ([Sun et al. 2002](#)). The trans-autophosphorylation finding correlates with the fact that exogenous substrates can partially inhibit SFK *in vitro*, because if this process occurred in a cis-autophosphorylation way, it would be less inhibited by competition with exogenous substrates ([Sun et al. 2002](#)). It is worth noting that mutation on Tyr416 to Phe leads to a significant decrease in the transforming potential of SFK ([Ferracini and Brugge, 1990](#)), indicating that transforming potential of SFK is directly correlated with Tyr416 phosphorylation level ([Woods and Verderame, 1994](#)). Moreover, it is worth noting that the highly conserved amino acid Gly449, in the particular case of Src protein, was recently described as a crucial residue mediating autophosphorylation-dependent maximal kinase activity in Src activation process ([Tong et al. 2017](#)).

Beside the well-known of tyrosine phosphorylation in SFK activity regulation, phosphorylation of Ser and Thr residues has also been implicated in this aspect. Although Ser/Thr phosphorylation mechanism is not well elucidated, several studies have shown it leads to the activation of SFK through molecular changes as result of weak interactions between SH2 and SH3 domains ([Shenoy et al. 1992](#)). Several enzymes have been described to phosphorylate SFK in Ser/Thr residues such as protein kinase (PK)-A, PKC and cyclin-dependent kinase (CDK1/CdC2) ([Roskoski, 2005](#)). Moreover, it has been demonstrated that involvement of SFK in mouse sperm capacitation is through Ser/Thr phosphorylation by PKA and inhibitors of this protein

impair all capacitation parameters including *in vitro* fertilization ([Krapf et al, 2010](#)). Additionally, in human sperm capacitation Ser/Thr phosphorylation by PKA is not only required, but the inactivation of Ser/Thr phosphatases, which remove phosphorylation in these residues, as protein phosphatase (PP)-1 $\gamma$  and PP2A are also needed ([Battistone et al, 2013](#)).



**Figure 4:** Activation and inactivation mechanisms of SFK

The clamp, latch and switch keep SFK in the inactive state. The unclamping, unlatching and switching processes lead to the activation of SFK. Then inactivation is produced by PTP-BL recruitment, Tyr419 dephosphorylation and Tyr527 Csk- and Chk-dependent phosphorylation ([Roskoski, 2015](#)).

### **c) Inactivation mechanisms of SFK**

To inactivate SFK, these proteins have to become in their close restrained conformation, in which Tyr527 has to be phosphorylated and interacting with SH2 domain and, SH3 domain has to be connected to the SH2-kinase linker; rendering these proteins in an enzymatically inactive form. Thus, the latch and the clamp are formed again, leading to a swift off in the Tyr416.

For that, it has been demonstrated that the ubiquitously expressed human phosphatase PTP-BAS (BAS is referred to basophils) and its homologous in mice PTP-BL (BL for basophils-like) can interact with active SFK dephosphorylating Tyr416 ([Palmer et al, 2002](#)) (**Figure 4**). Further, other phosphatases can also dephosphorylate Tyr416. For example, protein-tyrosine phosphatase non-receptor type (PTPN)-2 has as major substrates Fyn and Lck; and deficiency in this phosphatase in human breast

cancer cell lines has been associated with high SFK signaling ([Nunes-Xavier et al., 2013](#)). Moreover, it has been described that PTPN-18 dephosphorylates Fyn in the activation segment ([Wang et al., 2001](#)). However, dephosphorylation of Tyr416 is not enough to inactivate SFK, so phosphorylation of Tyr527 is necessary to achieve the inactive form of this family leading to the formation of the latch. Indeed, it is largely known that mutations in this site of phosphorylation lead to an overactivation of Src in colon cancer ([Martinez-Perez et al., 2017](#); [Irby et al., 1999](#)). Two kinases are the major responsible for the phosphorylation of Tyr527. The first one is Csk, which is expressed in all mammalian cells and the second one is Chk, whose expression is restricted to breast, hemopoietic cells, neurons and testes ([Brown and Cooper, 1996](#)); although Chk has been found in other cell types such as colon tissue ([Zhu et al., 2008](#)) and smooth muscle ([Radhakrishnan et al., 2011](#)), but at lower levels. Both proteins, share with SFK the SH3, SH2 and kinase domains, but they do not have the acylation motif in the N-terminal region, the autophosphorylation site in the kinase domain and the regulator tail in the C-terminal ([Okada, 2012](#); [Roskoski, 2004](#)) (**Figure 4**).

The interaction of Chk with Tyr416 renders SFK inactive, but also Chk might interact with SFK forming a noncovalent inhibitory complex that impairs their activation in an independent-phosphorylation way ([Chong et al., 2004](#)). However, Chk could also bind to an unphosphorylated state in SFK, which prevents the activation segment phosphorylation. In fact, the lack of this non-catalytic inhibitory mechanism produces an over activation of SFK in Chk-deficient colorectal cancer cells ([Advani et al., 2017](#)).

Being Csk a cytoplasmic protein ([Okada et al., 1991](#)), it needs the intervention of other proteins to have access to the subcellular compartments where SFK might be found. One of these proteins is Cbp/PAG1 (Csk-binding protein/Phosphoprotein Associated with Glycosphingolipid-enriched microdomains) ([Kawabuchi et al., 2000](#)). Cbp/PAG1 is restricted in its inactive state to the lipid rafts in the plasma membrane, but after activation of SFK, Cbp/PAG1 is phosphorylated by SFK through the interaction of SH3 domain from SFK with one proline-rich region in Cbp/PAG1 protein. Once activated Cbp/PAG1 recruits Csk and the activated SFK to the cholesterol-enriched microdomains forming a Cbp-Csk-SFK complex, where Csk can phosphorylate SFK on the Tyr527 producing its inactivation ([Espada and Martin-Perez, 2017](#)). Therefore, Cbp/PAG1 plays an important role controlling cell transformation induced by Src activation ([Kanou et al., 2011](#)) and in Cbp deficient mice an alteration to late erythroid development related with an increase in Lyn activity was found ([Plani-Lam et al., 2017](#)). Additionally, Csk can also interact with other scaffolding proteins like paxillin, insulin receptor substrate (IRS)-1 and caveolin-1 ([Zhang et al., 2016b](#); [Nuche-](#)

[Berenguer et al, 2015](#); [Vang et al, 2012](#)). Indeed, a strong association between the SH3 domain from Csk and the proline-rich motif present in the C-terminal region of the non-receptor protein-tyrosine phosphatase PEP, present in hemopoietic cells, has been found ([Cloutier and Veillette, 1996](#)). This association suggested a more efficient environment to inhibit signal transductions by SFK *in vivo*, but recent studies have probed that dissociation of this complex is necessary to the recruitment of this phosphatase to plasma membrane to start its down-regulation process, specifically in T cells ([Vang et al, 2012](#)).

It is worth noting that while the interaction of Csk with SFK leads to the phosphorylation of Tyr527, the autophosphorylation of Tyr416 can override the inhibition produced by Tyr527 phosphorylation ([Sun et al, 1998](#)). In fact, it was demonstrated in Src and Fyn proteins that another tyrosine residue can be phosphorylated by the platelet-derived growth factor (PDGF) receptor. The phosphorylation of Tyr213 (referred to Src protein) nullifies the effect of Tyr527 phosphorylation due to a decrease in the ability of SH2 domain to form intramolecular binding with Tyr527. So, activation of Tyr213 produces a twofold activation of these SFK ([Stover et al, 1996](#)).

Phenotypes of deficient mice for Csk (Csk<sup>-/-</sup>) or Chk (Chk<sup>-/-</sup>) reveal functional redundancy of these proteins in cells where both proteins are co-expressed, indicating that Csk and Chk should have overlapping functions ([Lee et al, 2006](#); [Samokhvalov et al, 1997](#)). Nevertheless, specific functions have been also observed. In colorectal cancer cells and in brain tumor cells when Chk expression is abolished, but not Csk expression, an overactivation of SFK is still found ([Kim et al, 2004](#)). Thus, the presence of Csk in these cells fails to suppress the aberrant activation of SFK, maybe because in these cells Chk is necessary to control SFK activation.

In general, it has been evidenced that the overactivation of SFK in cancer cells can be due to a decrease in the expression of Chk ([Zhu et al, 2008](#)) as result of a reduced expression in the Cbp/PAG1 ([Huang et al, 2011](#); [Oneyama et al, 2008](#)) and by an upregulation of phosphatases that target the Tyr527 ([Bjorge et al, 2000](#)). Moreover, although reduced levels of Csk have been found in some cancer ([Yang et al, 2015](#)), generally Csk is expressed at normal levels in many types of cancer, but a downregulation in Cbp/PAG1 interferes with Csk translocation to the membrane leading to the overactivation of SFK in these kinds of cancer ([Okada, 2012](#)). Furthermore, it has been reported that deletion of Csk in granulocytes produces hyper-responsiveness to pathogens ([Thomas et al, 2004](#)), indicating a function of this protein in the regulation of immune system; and a crucial role was also appreciated in the

maintaining of squamous epithelia because of its conditional inactivation induced defects in the skin leading to chronic inflammation and epidermal hyperplasia ([Yagi et al. 2007](#)).

#### **d) Redox-dependent regulation of SFK**

Recently, it has been proposed that Reactive Oxygen Species (ROS) can regulate the activity of SFK ([Corcoran and Cotter, 2013](#)). Transient ROS production triggered by the integrin-dependent linking of adherent cells to the extracellular matrix induces a redox-dependent activation of Src associated to cell proliferation ([Giannoni et al. 2005](#)). Moreover, it is worth to note that switching on a moderate and transient production of endogenous ROS, *in situ*, in human immortalized keratinocytes leads to a strong proliferative signal that relies on Src activity and is associated with a transient induction of Cyclin D1 expression ([Blazquez-Castro et al. 2012](#)). Additionally, Lyn can act as a redox sensor in the recruitment of neutrophils to the wound site and the hydrogen peroxide produced leads to the autophosphorylation and activation of Lyn through direct oxidation of its Cys466 ([Yoo et al. 2011](#)).

### **I.V. Regulation of SFK expression**

#### **a) At the mRNA level**

There are not too many examples about the role of transcription factors in the transcriptional regulation of SFK. It has been demonstrated that the ets transcription factor/oncogene family is implicated in B-cell gene regulation and such as those involved in differentiation and proliferation processes. In fact, one member of this family, ELF-1, has been linked to transcriptional regulation of Blk, Lck and Lyn tyrosine kinase proteins and another one, NERF, was able to transactivate the Lyn and Blk promoters from B cells ([Oettgen et al. 1996](#)).

Small, non-coding, single stranded ribonucleic acid (RNA), known as microRNA (miR) have been involved in cancer development and metastasis ([Lin et al. 2010](#); [Calin and Croce, 2006](#)), suppressing the expression of the targeted gene at post-transcriptional level. It has been shown that miR-203 can suppress Src protein expression. In fact, lower levels of miR-203 have been found in lung cancer patients, which have been correlated with an increase expression in Src protein levels ([Wang et al. 2014](#)), resulting in an over-activation of the Ras-extracellular signal-regulated kinase (ERK) pathway promoting resistance to cell death in lung cancer cells. Moreover, it was recently demonstrated that Src expression was up-regulated in esophageal cancer tissues, resulting in a proliferation of cancer cells and a resistance to cell death; by downregulation expression of the direct Src repressor miR-1 ([Liao et al. 2017](#)).

DNA methylation is an epigenetic mechanism that occurs in CpG islands playing a crucial role in the regulation of gene expression ([Deaton and Bird, 2011](#)). In fact, reduced methylation patterns for Lyn and Src proteins have been observed in gastric cancer patients leading to increased levels of mRNA and protein expression ([Mello et al, 2015](#)), enhancing invasiveness and metastasis of this kind of cancer. Thus, DNA methylation can be involved in regulating of the expression of these protein kinases. In fact, DNA methylation of porcine Lyn promoter was correlated with an increase in its expression levels ([Xiao et al, 2012](#)).

### **b) At the protein level**

Another mechanism involved in the regulation of SFK is the stability of these proteins. In a general way, regulation of a protein is not only related with the phosphorylation/dephosphorylation levels, but also with the expression level inside the cells. One of the mechanisms that control the expression level is the ubiquitination process leading to protein degradation by the proteasome pathway. Harris et al ([Harris et al, 1999](#)) showed that the active form of SFK is targeted for degradation and therefore fewer stables than the inactive conformation of the protein. In fact, in mice with a gain-of-function of Lyn (mice with a constitutively overactivation of Lyn) an inverse correlation between activity and protein level expression is observed: the more active the protein is, the less it is expressed ([Harder et al, 2001](#)).

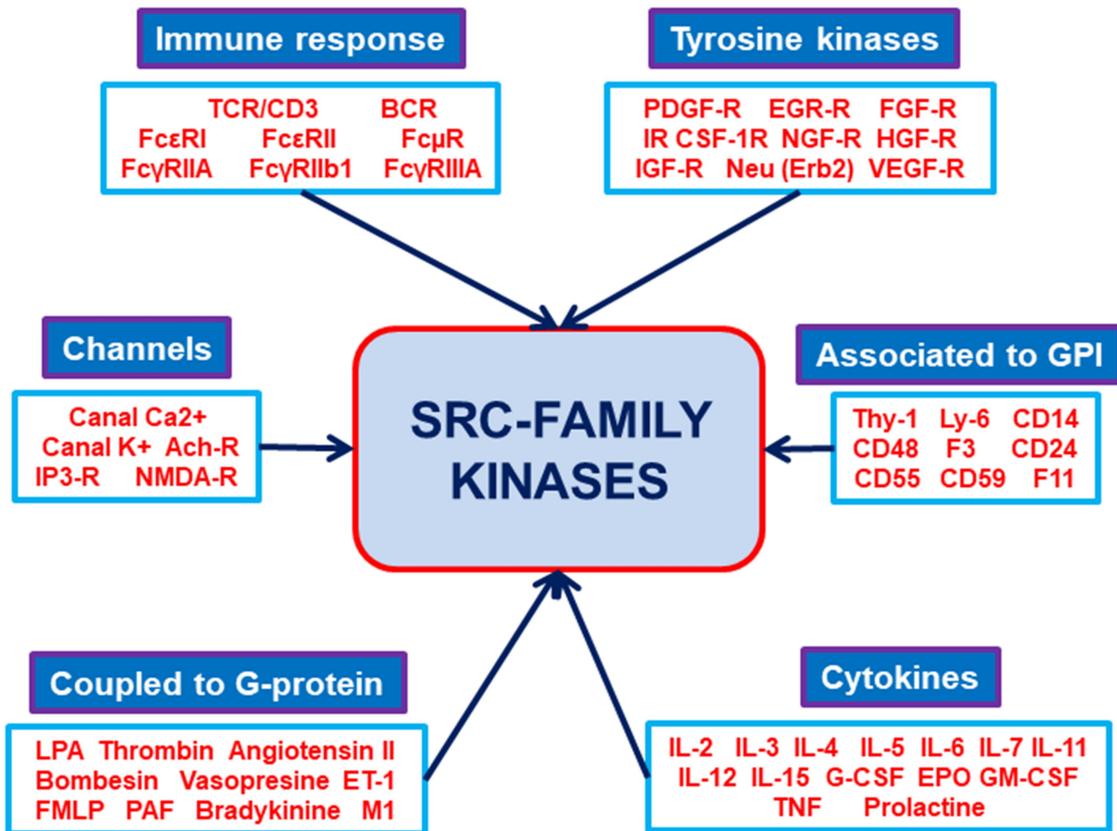
The Casitas B-cell lymphoma (Cbl) family of E3 ubiquitin ligases has been associated with the ubiquitination of SFK. Once activated, Src binds and phosphorylates Cbl, leading to the proteasomal degradation of itself and Cbl ([Yokouchi et al, 2001](#)). Moreover, Kyo et al ([Kyo et al, 2003](#)) demonstrated that Cbl is associated with the activated Lyn producing its polyubiquitination and its degradation, mechanism that could be extended to other proteins of the family.

Further, the protein Cbp/PAG1 that interact with Csk to produce the inactivation of the SFK can also interact with another protein, called SOCS1 (Suppressor Of Cytokine Signaling 1), in erythroid cells. In this case, Cbp/PAG1 interacts with Lyn firstly producing a down regulation in its activation, but then Cbp interacts with SOCS1 leading to an increased ubiquitination and degradation of Lyn ([Ingleby et al, 2006](#)).

Moreover, the relative expression of one SFK can induce some phenotypical and functional changes. In fact, depending of the mice genetic background, mast cells responses from the 129/Sv or C57BL/6 mice markedly differ, taking in consideration the different level expression of Lyn and Fyn in these cells according the mice genotype ([Yamashita et al, 2007](#)).

## I.VI. Physiological roles of SFK

It has been demonstrated that SFK play a crucial role in the triggering of several signaling pathways through a huge number of cellular receptors, structurally and functionally different, present in various kind of cells. The main receptors are those involved in the immune response, those coupled to G-proteins and those associated to cytokines as well as tyrosine kinase and cytokines receptors (**Figure 5**) ([Thomas and Brugge, 1997](#)).



**Figure 5:** Related-receptors to SFK activation.

Several cellular receptors have been implicated in the activation of SFK such as, receptors involved in the immune response, tyrosine kinase receptors, receptors associated to glycosyl-phosphatidylinositol, ions channels, coupled receptors to G-protein and cytokine receptors ([Thomas and Brugge, 1997](#)).

In the same way that SFK receive activation signals from several cellular receptors, once activated, this family can interact with a large number of substrates. However, it is difficult to establish whether SFK are responsible for phosphorylation of these substrates following receptor activation, due to not only SFK are activated, but multiple protein tyrosine kinases are phosphorylated by most receptor pathways, being able to phosphorylate the same substrates. Further, to determine which cellular

responses are regulated by each substrate phosphorylation event is also hard to elucidate. Nevertheless, the identification of potential substrates candidates has been possible by the analysis of constitutively activated variants of these kinases. Around 519 substrates have been postulated for SFK ([Takeda et al., 2010](#)), being many of them phosphorylated by several SFK members, reinforcing the idea of the overlapping functions in this family. Moreover, the phosphorylation cluster analysis grouped these substrates into 10 categories ([Takeda et al., 2010](#)). In **Table 5**, a synthetic list of identified favorite substrates of SFK is shown. However, although the most important role of SFK is the activation of substrates through phosphorylation by the kinase domain, it has been demonstrated that they can act as adaptor molecules through the use of their SH2 and SH3 domains. For example, in Lyn deficient mice expressing a kinase-dead allele of Lyn, an improvement in most of the principal abnormalities observed in Lyn deficient mice was found ([Verhagen et al., 2009](#)) and a regulation of the prolactin receptor-induced Jak2 signaling through the SH2/SH3 domains in a kinase-independent mechanism was shown for Src protein in human breast cancer cell lines ([Garcia-Martinez et al., 2010](#)).

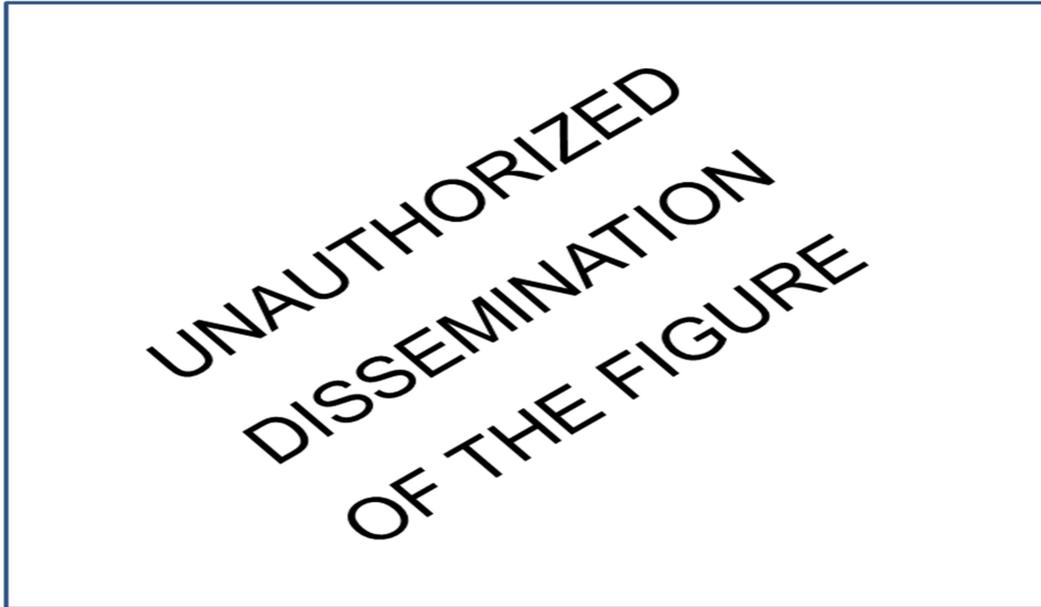
Therefore, the interaction of SFK with several protein tyrosine kinase receptors and the subsequent phosphorylation of a large amount of substrates lead to SFK play a crucial role in the regulation of a huge amount of cellular processes like: adhesion and spreading, migration, focal adhesion formation and disassembly, cell cycle progression, apoptosis, differentiation, survival as well as gene transcription and angiogenesis ([Espada and Martin-Perez, 2017](#); [Maa and Leu, 2016](#); [Zhang et al., 2014](#)) (**Figure 6**). However, to know about the normal function of one member of the SFK has been not possible yet taking in consideration that invalidation of only one member of this family in mice does not lead to a strong swift in mice (**Table 6**), reinforcing the idea of compensation effect by other SFK expressed in targeted tissues, although it is noteworthy that certain specific defects have been reported in those mice ([Lowell et al., 1996](#)). In fact, during viral infection Lck, Hck and Fgr are equally induced and all them contribute to a negative regulatory feedback mechanism ([Liu et al., 2017](#)). Moreover, it has been described that Fgr, Fyn, Lyn and Src play a key role in the initiation and propagation signals after platelet activation; however, the deletion of only one of these proteins does not hamper platelet activation, although it is worth to note that a delay is observed in the absence of Lyn, but no effect is appreciated in the absence of Src ([Severin et al., 2012](#)). Either the single deficiency in Hck, Fgr or Lyn or the double deficiency Hck/Fgr and Fgr/Lyn do not impair the autoantibody-induced arthritis in mice; only the triple knockout mice were completely protected ([Kovacs et al., 2014](#)),

confirming the redundant role of SFK in different cellular processes. Nevertheless, no redundant roles in ligand sensing threshold mediated by immunoreceptors have been demonstrated for Lyn and Fyn. Receptors can discriminate between negative and positive signals depending of the ligand avidity and the phosphorylation status. Thus, the phosphorylation of SH2-containing inositol phosphatase 1 (SHIP1) by Lyn results in a homeostatic response, but the phosphorylation of the same protein by Fyn leads to inflammation ([Mkaddem et al, 2017](#)), confirming that depending of the ligand interaction Fyn and Lyn present opposite roles.

SFK Substrates	Functions
FAK, CDCP1	Tyrosine kinase, binding and integrin signalization
Paxilin, p130Cas	Integrin signalization
Caveolin	Structure and functions of caveolae
Talin	Acting binding and integrin signalization
Tubulin	Cytoskeleton
Syk / ZAP70, Tec kinases, CTK, Pyk2, BTK, Axl, c-MET	Tyrosine kinase, signal transduction
PLC $\gamma$	Phospholipase C PI-specific
PKC $\delta$	Ser / Thr kinases
SHIP, SHP-1, SHP-2	Phosphatases
p190	Rho activator GTPase
RasGAP	Ras activator GTPase
Tom1L1	Negative regulator of mitogenesis
Shc, Vav2, Abl	Adaptor molecules, Mitogenesis
Cbl	Interact with Csk
STAT1, STAT3	Transcription factors
GluN2A, GluN2B, GluA2, SIRP $\alpha$ , GABAR $\gamma$ 2	Brain functions

**Table 5:** Favorite substrates of SFK

A huge amount of 519 substrates have been proposed for SFK phosphorylations which have been classified into 10 categories ([Takeda et al, 2010](#)). Here a synthetic list of favorite substrates in presented taking in consideration that most of them are phosphorylated by several SFK ([Ohnishi et al, 2011](#); [Wortmann et al, 2011](#); [Takeda et al, 2010](#); [Emaduddin et al, 2008](#); [Franco et al, 2006](#)).



**Figure 6:** Implication of SFK in different signaling pathways

SFK are controlled by several receptors and after their activation, SFK are able to phosphorylate several substrates triggering various signaling pathways ([Roskoski, 2015](#)).

#### **a) Physiological functions of SFK in tissues**

##### **(i) In bones**

The protein Src has a crucial role in the normal functioning of osteoclasts ([Horne et al, 2005](#)), which are cells implicated in bone degradation during development and repair processes of bones ([Huang et al, 2018](#)). Indeed, Src deficient mice develop osteoporosis due to the dysfunctional activity of osteoclasts ([Lowe et al, 1993](#)). Nevertheless, other SFK are expressed in osteoclasts such as Hck, Fgr, Lyn and Fyn; but only Hck is overexpressed in Src deficient osteoclasts ([Lowell et al, 1996](#)). This overexpression has been related with a compensation in the phenotype because Src and Hck double deficient mice develop a more severe phenotype of osteoporosis, which is not observed, for example, in Src and Fgr double deficient mice, where the phenotype is similar to those observed in Src deficient mice ([Miyazaki et al, 2006](#)). Moreover, the role of Hck in this compensation has been linked to the recruitment of osteoclast precursors ([Verollet et al, 2013](#)). Lyn has a negative role in osteoclastogenesis due to the interaction with phospholipase C gamma 1 (PLC $\gamma$ 1)-mediated calcium signaling leading to an inhibition in the osteoclast differentiation ([Yoon et al, 2009](#)), although no impact of Lyn is observed in the activity of mature osteoclasts ([Kim et al, 2009](#)) ([discussed in II.III. b\) \(iii\) Osteoclast](#)). Furthermore, the

implication of Fyn in osteoclast lineage cells is through the promotion of proliferation and survival processes and decreasing apoptosis in these cells ([Kim et al, 2010](#)).

SFK	KO deficient mice phenotype
Src	Osteoporosis, osteoclasts defected
Fyn	Abnormal hippocampus development, spatial representation problems, altered thymocyte signalization
Yes	No particular phenotype
Fgr	No particular phenotype
Lyn	Age-altered function of B cells, development of a lupus-related syndrome (glomerulonephritis)
Hck	No particular phenotype
Lck	Altered TCR signalization, altered T cell development
Blk	No particular phenotype

**Table 6:** Phenotype of mice deficient for SFK

No special phenotypes have been found in mice deficient for one member of SFK, indicating a redundant role of these proteins in several cellular processes.

(ii) In the nervous system

Src protein is essential in the maintenance of the blood brain barrier, which is crucial controlling the access of different molecules to the brain ([Alluri et al, 2015](#); [Daneman and Prat, 2015](#)). Moreover, this protein is well expressed in neurons, but although no brain phenotype has been found in Src deficient mice, a reduction in glioma cell infiltration of the brain parenchyma has been observed ([Lund et al, 2006](#)); showing an implication of this tyrosine kinase in the regulation of the extracellular matrix. Besides Src, neurons express Fyn, Lyn, Yes and Lck ([Lowell and Soriano, 1996](#)); but only Fyn deficient mice develop defects in the hippocampus presenting problems in the spatial representation ([Grant et al, 1992](#)). Fyn is also expressed in astrocytes and microglia ([Brozzi et al, 2009](#)) playing an important role in the regulation of neuroinflammatory responses ([Ko et al, 2018](#)), and an implication of Fyn in the disruptive action of amyloid- $\beta$  in the intracellular signaling cascade ([Kaufman et al, 2015](#)), important for the physiological synapsis, has highlighted Fyn as a potential target for Alzheimer's disease treatment ([Nygaard, 2018](#)). In fact, the Src inhibitor Saracatinib, which failed reaching the primary endpoint in CRC patients ([Reddy et al,](#)

2015), has been tested in a phase IIa clinical trial with Alzheimer's disease patients (<http://clinicaltrials.gov> Identifier: NCT02167256).

### (iii) In the hematopoietic system

SFK has a crucial role in lymphoid and myeloid cell signalization, having an essential function in hematopoietic homeostasis.

Regarding lymphocytic cells, the invalidation of Fyn, Blk, Hck or Fgr does not have serious implications in the development or function of B cells, where they are expressed. Nevertheless, Lyn deficient mice develop a lupus-like phenotype as a result of defects in BCR signaling ([Lowell and Soriano, 1996](#); [Hibbs et al, 1995](#); [Nishizumi et al, 1995](#)), indicating the crucial role of Lyn in B cell development and function ([discussed in II.III b\) \(i\) B cells](#)). Lck is the principal SFK expressed in T cells and it has been demonstrated that Lck deficient mice present a strong defect in the progression of T cells through thymus selection ([Molina et al, 1992](#)), producing thymus atrophy. Moreover, the germline invalidation of Lck and Fyn, the other SFK expressed in T cells, abrogates completely the double negative stage of thymocyte development ([Groves et al, 1996](#)), indicating the crucial role of these proteins in T cell development. Additionally, an increase in tyrosine phosphorylation is considered a hallmark in the process of spermatozoid capacitation in mice and, recently it has been demonstrated that the autophosphorylation process of Lck, leading to an upregulation in its kinase activity, is a fundamental event during capacitation ([Singh et al, 2017a](#)).

On the other hand, Hck, Fgr, Fyn and Lyn are the main SFK expressed in myeloid cells ([Mkaddem et al, 2017](#); [Parravicini et al, 2002](#)), which are involved in different signaling pathways in these cells. However, the invalidation of Hck or Fgr does not produce a remarkable phenotype in these mice ([Lowell et al, 1994](#)); which can be explained by Lyn compensation, taking into account that Lyn is highly implicated in the development of myeloid cells. Nonetheless, double invalidated mice for Hck and Fgr have serious defects in neutrophil function and can develop an immunodeficiency syndrome ([Lowell et al, 1996](#)).

## **b) Physiological functions of SFK in biological processes**

### (i) In cell differentiation

SFK are implicated in several cell differentiation processes such as keratinocyte and macrophage differentiation processes. Tyrosine phosphorylation is required in the differentiation process carried out by keratinocytes, which is a Fyn-dependent activity. Fyn deficient keratinocytes have an abnormal process of differentiation and present

morphological defects ([Calautti et al., 1995](#)), suggesting an alteration in the skin differentiation process from Fyn deficient mice. Src, Lyn, Fyn and Yes are normally expressed in these cells, but only Fyn has an increase in its activity during the differentiation of keratinocytes leading to the recruitment of junctional components to form adherent junctions ([Fenton and Denning, 2015](#)), through the regulation of calcium flux in cell-cell adhesion process ([Tu et al., 2011](#)). Osteoclastogenesis is the differentiation process of osteoclasts and a negative role of Lyn during this process it has been previously demonstrated ([discussed in II.III b \(iii\) Osteoclast](#)). However, although Fyn is not considered a key protein in this cellular process, a partial reduction in osteoclastogenesis has been observed in Fyn deficiency cells ([Kim et al., 2010](#)). SFK such as Src, Lyn and Hck are also involved in monocytes/macrophages differentiation ([Endele et al., 2017](#)) and a crucial function of Hck in this process has been reported through its interaction with the macrophage colony-stimulating factor (M-CSF) receptor ([Suzu et al., 2005](#)).

#### (ii) In mitochondrial functions

The SFK Src, Fyn, Fgr and Lyn can be permanently found within mitochondria ([Salvi et al., 2005](#)), although other studies have elucidated that these proteins might be translocated into the mitochondria after extracellular receptor activation ([Boerner et al., 2004](#)) through different adaptor proteins phosphorylating subunits of complex I, II and IV as well as pyruvate dehydrogenase enzyme ([Hebert-Chatelain, 2013](#)). Thus, the implication of SFK in mitochondria functions is through the regulation of the oxidative phosphorylation (OXPHOS) process in charge of the most part of the energy production used by cells. For example, a study showed that once Fyn is translocated to the mitochondria, it can regulate ROS production and cell death in cardiomyocytes ([Matsushima et al., 2016](#)), being involved in the phosphorylation of the translational machinery in this organelle ([Koc et al., 2017](#)). Moreover, intramitochondrial Src kinase inhibition drives in a diminished mitochondrial respiration process correlated with lower phosphorylation in subunits of the complex I in cancer cells ([Hebert-Chatelain et al., 2012](#)), suggesting that SFK might be target in cancer treatment.

As during my thesis, my principal project was to study the role of the Lyn protein tyrosine kinase in the context of cell death and inflammation, I decided to focus in more details on this family member.

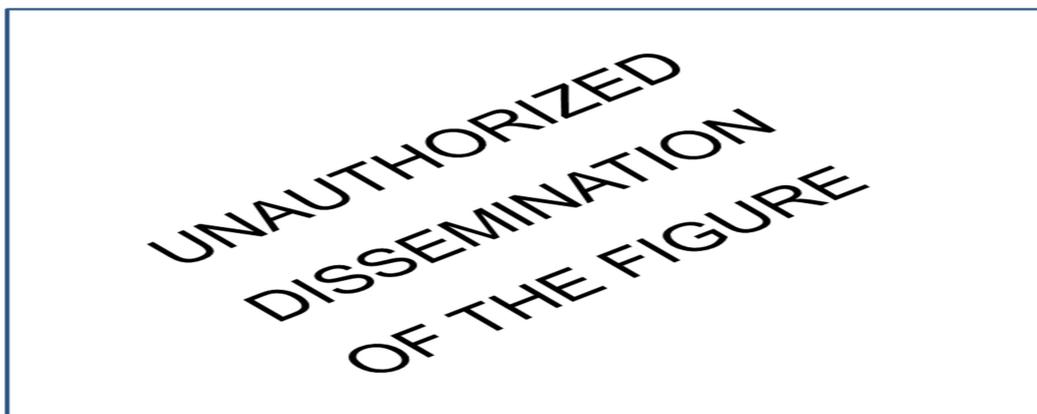
## II. Lyn tyrosine kinase

### II.I. General presentation of Lyn tyrosine kinase

The Lck/Yes-related Novel tyrosine kinase, Lyn, was identified by Yamanashi et al in 1987 ([Yamanashi et al, 1987](#)). In fact they found through the screening of a human cDNA library made from placental RNA, under conditions of reduced stringency and using a v-yes probe, DNA clones from a novel genetic locus; which encodes for a novel tyrosine kinase that they called Lyn. Moreover, a high homology between Lyn and Lck was demonstrated, and with regard to Src, Yes, Fgr, a high homology was also observed, but less than that seen between Lyn and Lck, confirming that this new protein shared a high degree of homology with the rest of members of the SFK. Furthermore, its coding gene is located on chromosome 8 q13-qter ([Yamanashi et al, 1987](#)). It is noteworthy that translocations in this chromosome have been evidenced associated to diverse pathologies. Indeed a fusion gene (ETV6-Lyn, previously known as TEL-Lyn) was identified in patients with myelofibrosis driving in chromosomal abnormalities (12;8)(p13;q11q21) ([Tanaka et al, 2010](#)), fusion gene that has been later associated with myeloproliferative neoplasm ([Takeda et al, 2011](#)) and recently with acute myeloid leukemia ([Ma et al, 2017](#)); enhancing its broaden disease association.

The structure of Lyn protein tyrosine kinase is similar to that observed in the other members of the SFK (**Figure 7**). Lyn has only one palmitoylation site in the unique domain. Furthermore, the autophosphorylation site is found in Tyr397, and the phosphorylation on Tyr508 renders the protein in the inactive form ([Ingleby, 2012](#)). Additionally, two isoforms of the protein, obtained by alternative splicing, can be found: p56 (LynA, 512 amino acids) and p53 (LynB, 491 amino acids) ([Stanley et al, 1991](#)). The difference between these two isoforms is the absence of 21 amino acids in the unique domain of p53, corresponding to those ranging from the position 24 to 44 in p56 ([Yi et al, 1991](#)); thus Tyr32, which modulates activity and interactions, is an isoform-specific motif only found in p56 or LynA. Related functions have been observed for these two isoforms of Lyn. However, few studies have described a significant isoform-specific function. For example, after BCR stimulation by anti-immunoglobulin (Ig)M, the down-regulation signaling via each isoform was slightly different ([Yamanashi et al, 1991](#)). Moreover, if mast cells derived from Lyn deficient mice are reconstituted with LynA, a better PLC $\gamma$  phosphorylation in response to Fc $\epsilon$ RI ligation is obtained; however, if these cells are reconstituted with LynB, a LynB-SHIP1 complex is formed and a decrease in calcium and degranulation processes is produced; although it is worth noting that reconstitution with both isoforms led to normalized responses

indicating a complementation of both isoforms for mast cell activation ([Alvarez-Errico et al, 2010](#)). Additionally, it has been reported that LynA is the primary mediator in the downstream signaling after 3-iodobenzyl analog of the kinase inhibitor PP1 (3-IB-PP1) stimulation in macrophages, and LynB produces negative feedback signaling, taking negative-regulatory effects ([Freedman et al, 2015](#)).



**Figure 7:** Schematic representation of Lyn tyrosine kinase

Functional domains and motifs of Lyn protein kinase: amino terminal lipid modification, Unique domain (UN), where the LynA isoform-specific motif Tyr32 is found; SH3 and SH2 domains, the proline motif in the hinge region (P) and the SH1 domain (kinase activity) are shown; myristoylation is indicated in blue and palmitoylation is shown in light blue. Moreover, phosphorylated motifs such as Tyr397 (active kinase) and Tyr508 (inactivated kinase) are described. Further, molecular interactions between SH3 domain and proline region as well as between SH2 domain and C-terminal motif are indicated ([Ingley, 2012](#)).

Lyn presents a similar structure to the other members of SFK being regulated as well by SH2/SH3 interactions and phosphorylation (Tyr397: activator, Tyr508: inhibitor) (**Figure 7**).

## II.II. Localization of Lyn tyrosine kinase

### a) Within the tissues

In the first years after the discovery of this protein, Lyn was considered an exclusive hematopoietic-specific SFK with a strong expression in B cells, platelets and macrophages and with a relative marked expression in granulocytes and erythrocytes ([Stanley et al, 1991](#); [Yi et al, 1991](#)). However, presently is known that Lyn can be equally expressed in physiological conditions in other tissues such as brain ([Chen et al, 1996](#)), kidneys ([Pontrelli et al, 2006](#)), prostate ([Goldenberg-Furmanov et al, 2004](#)), colon ([Bates et al, 2001](#)), lung ([Zhao et al, 2006](#)) and skin ([Marchetti et al, 2009](#)), indicating that Lyn can be an important tyrosine kinase regulating several signaling pathways in different tissues.

## **b) Within the cells**

Like all SFK, Lyn is attached to the plasma membrane by a myristoylation modification in Gly2 and also by a palmitoylation modification in Cys3 (**Figure 7**). Inside the plasma membrane Lyn is going to be principally located in the caveolin regions and in lipid rafts, where it can be more active after receptor signaling ([Young et al, 2003](#)), although it has been demonstrated in Csk deficient cells that Lyn localization in the lipid rafts plays a suppressive role with respect to the SFK-mediated transformation ([Oneyama et al, 2009](#)). However, Lyn is not only found attached to the plasma membrane. In fact, Lyn can be located in Golgi apparatus where it is synthesized, although is not only found in the cytoplasmic face of caveolin-positive-containing Golgi membranes to be transported to plasma membrane, but in the caveolin negative membranes, suggesting that Lyn moves apart from caveolin within the Golgi apparatus possibly to recycling endosomes that moves to the plasma membrane ([Okamoto et al, 2018](#)). Further, in stimulated macrophages Lyn is strongly associated with M-CSF receptor, but is also observed in endocytic vesicles such as macropinosomes ([Dwyer et al, 2016](#)) specialized in the internalization of small amount of M-CSF receptor. Lyn is constitutively present in the interne structure of the mitochondria and its activation in this organelle is associated with the proliferative status of the cell ([Tibaldi et al, 2008](#)) and cell fractionation experiments have shown a nuclear localization of Lyn ([Yoshida et al, 2000](#)), which has been associated with its kinase activity and lipid modifications ([Ikeda et al, 2008](#)). Additionally, Lyn can be present in a multi-client chaperone complex with the heat shock protein (HSP)-90 in chronic lymphocytic leukemia (CLL) cells ([Guo et al, 2017](#)) contributing to the over-activity of the BCR signaling.

It has been described that SFK are implicated in monolayer maintenance and tight junction formation ([Dorfel and Huber, 2012](#)), and although Lyn is located in plasma membrane in polarized epithelial cells ([Tsukita et al, 1991](#)); recently it has been shown that Lyn is translocated to the endomembranes upon depolarization by the loss of cell-cell interaction and calcium deprivation ([Morinaga et al, 2017](#)). Finally, it has been demonstrated that subcellular localization of Lyn is essential for its normal function. Specifically, in our lab it was demonstrated that Lyn can be a substrate from caspases producing a caspase-cleaved form of Lyn (Lyn $\Delta$ N or cLyn), which is immediately relocated from the plasma membrane to the cytosol ([Luciano et al, 2003](#); [Luciano et al, 2001](#)), functioning as a suppressor of the apoptosis ([discussed in II.III. c](#)) [\(i\) Implication of Lyn tyrosine kinase in cell death](#)).

## II.III. Role of Lyn tyrosine kinase

### a) In signaling

One of the principal functions of Lyn is the transduction of signals after the activation of different cellular receptors. In fact, Lyn has been associated to BCR ([Hibbs \*et al.\*, 2002](#)), FcεR1 ([Gilfillan and Rivera, 2009](#)), erythropoietin receptor ([Ingleby \*et al.\*, 2005](#)), c-kit ([Linnekin \*et al.\*, 1997](#)), granulocyte macrophage colony stimulator factor (GM-CSF) receptor ([Scapini \*et al.\*, 2009](#)), granulocyte colony-stimulator factor (G-CSF) receptor, interleukin (IL)-4 and other cytokine receptors ([Hibbs and Harder, 2006](#)), as well as integrins ([Nakata \*et al.\*, 2006](#)). Moreover, Lyn can have an essential role in the nuclear translocation of EGF receptor ([Iida \*et al.\*, 2013](#)).

In response to the activation of these receptors, Lyn can activate a large number of signaling molecules such as the ITAM and immunoreceptor tyrosine-based inhibitory motifs (ITIM) on FcγR ([Xu \*et al.\*, 2005](#)), as well as phosphatidylinositol 3 kinase (PI-3K), CD45, FAK (PTK2), PLCγ 1/2, ras-GTPase activating protein (ras-GAP), Hematopoietic lineage cell specific protein (HS)1 ([Ingleby \*et al.\*, 2000](#)), Cbp/PAG1 ([Ingleby, 2009](#)), the signal transducer and activator of transcription (STAT)5 ([Chin \*et al.\*, 1998](#)) and mitogen-activated protein kinases (MAPK) ([Ingleby, 2012](#)), among others; demonstrating that Lyn can participate in the regulation of different signaling pathways related with essential biologic processes (**Figure 8**). Moreover, it was recently demonstrated that interferon (IFN) regulatory factor 5 (IRF5), a crucial transcription factor in the pathogenesis of systemic lupus erythematosus (SLE), is a new substrate of Lyn. Indeed, Lyn inhibit the ubiquitination and phosphorylation of IRF5 in the Toll like receptor (TLR)-MyD88 pathway leading to the suppression of its transcriptional activity, which is mediated by a kinase-independent mechanism ([Ban \*et al.\*, 2016](#)), indicating that Lyn may have a role as specific suppressor of the TLR-MyD88-IRF5 pathway in SLE pathogenesis. Cortactin, a regulator of actin cytoskeleton, has been recently postulated as a new substrate of Lyn. In fact, after its Lyn phosphorylation in Y421, this factor is implicated in cell migration and cancer progression in CLL patients through the release of matrix metalloproteinase (MMP)-9 and motility of neoplastic cells. The treatment of cancer cells with the Src inhibitor PP2 produces lower levels of cortactin phosphorylation and the decrease of MMP-9 in culture medium ([Martini \*et al.\*, 2017](#)).

Therefore, Lyn has important functions in different hematopoietic cell types through the implication in different signaling pathways, and it has been demonstrated that Lyn can have a dual role by both activating and inhibiting these pathways; being a signaling modulator. In fact, Lyn exercises a positive role in TLR4-induced response.

Lipopolysaccharide (LPS)-TLR-4 engagement immediately activates Lyn and other SFK in macrophages/monocytes, mast cells and dendritic cells ([Avila et al, 2012](#); [Smolinska et al, 2008](#)), driving in the release of pro-inflammatory cytokines. However, the restoration of Lyn catalytic activity during LPS stimulation induces TLR4 phosphorylation, playing an inhibitory role in TLR4 signaling through disruption in the MyD88/TLR4 complex; impairing LPS-induced pro-inflammatory response ([Mitchell et al, 2018](#)). Moreover, Lyn plays a pivotal role in TLR-2 dependent internalization of *Pseudomonas aeruginosa* by alveolar epithelial cells ([Kannan et al, 2006](#)) and mediating bacteria phagocytosis and autophagosome maturation in alveolar macrophages ([Li et al, 2016](#)), being a crucial factor in the regulation of NF-κB activation after TLR-2-mediated activation of the innate immune response in human mononuclear cells ([Toubiana et al, 2015](#)). However, Lyn can also negatively regulate activation of innate cells after TLR engagement. In fact, activation of mouse bone marrow-derived macrophages (BMDM) deficient for Lyn produced more pro-inflammatory cytokines than WT BMDM after LPS-TLR4 engagement ([Keck et al, 2010](#)).



**Figure 8:** Lyn regulation of signaling pathways

Lyn is implicated in the regulation of different signaling pathways, through the transmission of signaling from different receptors and by the direct interaction or by phosphorylation processes in several target proteins, presenting a positive (enhancement) and a negative (inhibition) role in these cellular pathways ([Ingley, 2012](#)).

## b) In cells

### (i) B cells

Lyn has a crucial role in the BCR signaling. Indeed, in mature B cells, once BCR is activated by antigen-binding, Lyn becomes active and leads to the phosphorylation of ITAM motifs, beginning the signal amplification cascade (activation of the spleen (Syk) and brutus (Btk) tyrosine kinases, leading to the phosphorylation of several substrates (PLC $\gamma$ 2 and PI-3K), which propagate the BCR signaling (calcium flux as well as MAPK family proteins, such as ERK-1/2 and p38) and driving in the activation of transcription factors implicated in the regulation of proliferation, differentiation, antibody production or apoptosis ([Stevenson et al, 2011](#); [Irish et al, 2006](#); [Xu et al, 2005](#)) (**Figure 9**).

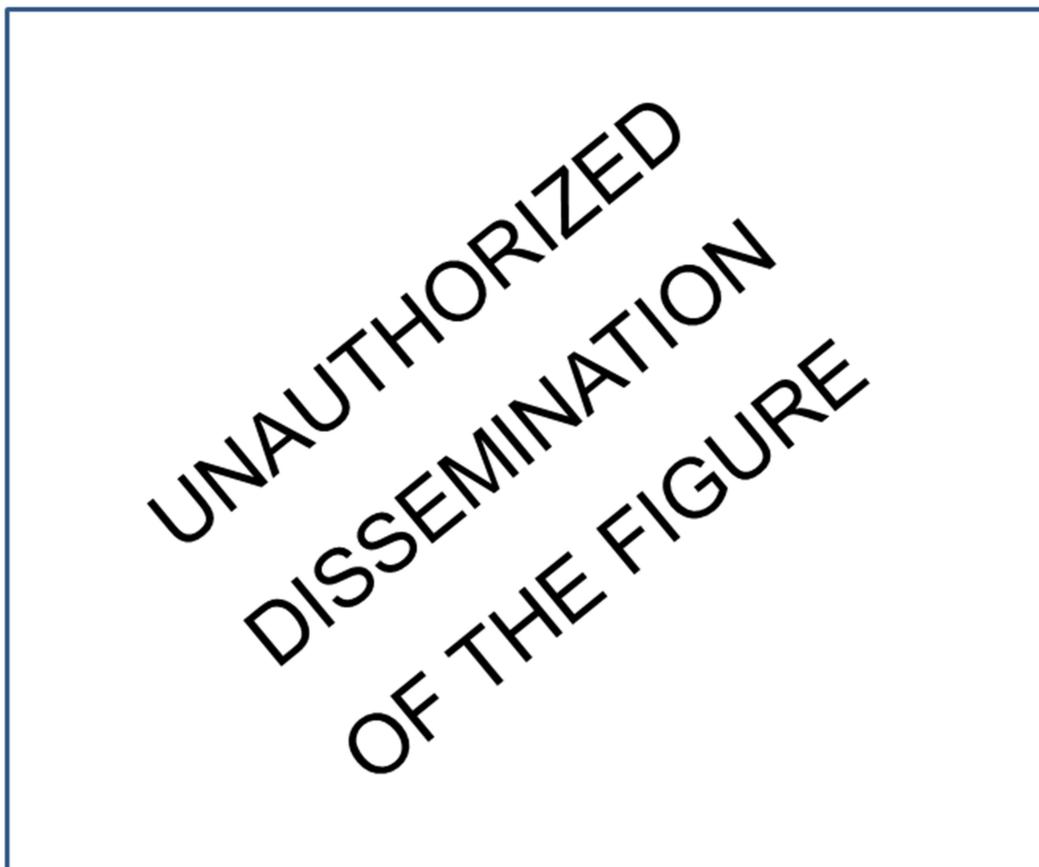


**Figure 9:** BCR signaling

After antigen binding Lyn, Btk and Syk are activated and the adaptor molecule BLNK connects these kinases to their principal effectors such as PI3K and PLC $\gamma$ 2 leading to Akt activation and Ca<sup>2+</sup> release, driving in the activation of several transcription factors related with several cellular processes ([Stevenson et al, 2011](#)).

In the activation of BCR, Lyn has a positive regulatory effect. For example, Lyn is a key mediator in various signaling pathways through the BCR such as CD19/CD21 and CD180 ([Xu et al, 2005](#)). In fact, Lyn was required for CD19 tyrosine phosphorylation in its ITAM motifs ([Hibbs et al, 2002](#)), leading to a signal amplification

loop in mouse B cells, indicating that Lyn is crucial for antigen-receptor induced signal transduction through CD19 signaling pathway ([Gauld and Cambier, 2004](#); [Fujimoto et al, 2000](#)). Additionally, Lyn has a positive role as substrate of CD148, a receptor-like protein tyrosine phosphatase, in B1 cell BCR signaling that was not evidenced for B2 BCR signaling ([Skrzypczynska et al, 2016](#)). However, a negative regulatory effect of Lyn has been also elucidated. For example, CD5 negatively regulates BCR stimulation through the association with SHP-1, which is Lyn dependent ([Ochi and Watanabe, 2000](#)) and CD22, which is quickly phosphorylated after BCR engagement in its ITIM motifs by Lyn leads to the recruitment of SHP-1 and SHP-2 to inhibit BCR signaling ([Ohashi and DeFranco, 2002](#)). Therefore, the positive regulatory role of Lyn is given by the phosphorylation of kinases and the negative regulator role of Lyn is through the activation of phosphatases (**Figure 10**).



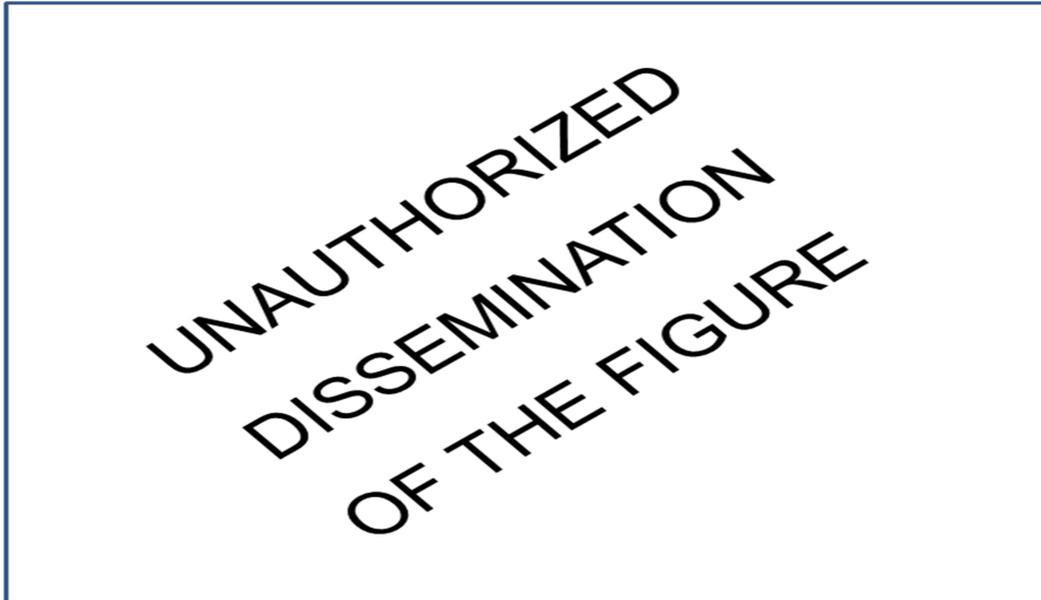
**Figure 10:** Positive and negative regulatory effect of Lyn

Lyn contributes to the positive regulation of signaling through the phosphorylation of ITAM motifs promoting B cell activation. The negative regulation of signaling is through the phosphorylation of ITIM motifs in inhibitory cell surface receptors leading to the recruitment of inhibitory phosphatases ([Xu et al, 2005](#)).

This functional duality of Lyn is well supported by the phenotype of Lyn deficient mice. These mice, which are viable, have a normal development of pro-B cells; however the quantity of peripheral mature B cells is decreased, indicating that Lyn is important in the survival and selection process of mature B cells ([Chan et al, 1997](#)). Moreover, Lyn deficient B cells have a highly rate of proliferation with an increase activation of the MAPK signaling pathway that is not observed in Wild Type (WT) B cells, indicating that other SFK can compensate the absence of Lyn in the initiation process, but the down-regulation signaling process seems to be non-redundant and Lyn deficient cells present a strong defective inhibitory mechanism ([Gross et al, 2011](#)). Thus, the inhibitory function of Lyn is dominant with regard to the activation role of this protein. Nevertheless, in B cells from Lyn<sup>up/up</sup> mice (mice with a gain-of-function mutation in Lyn gene, which express a constitutive active form of Lyn) (**Figure 11**) a decrease in the proliferation rate has been observed after BCR stimulation and constitutive tyrosine phosphorylation from both negative and positive regulators for BCR signaling have been observed ([Hibbs et al, 2002](#)).

Lyn deficient mice develop an autoimmune disease that resembles to human SLE with a large number of autoantibodies-secreting plasma cells, which scape to negative selection process, resulting in a glomerulonephritis ([Gutierrez et al, 2010](#)). Moreover, it is worth to note that in SLE patients a significant reduction number of B cells and less Lyn expression within these cells have been reported ([Liu et al, 2013](#)). Nevertheless, Lyn deficiency in B cells have not only been associated with the phenotype developed in Lyn deficient mice, but there is an important contribution of Lyn deficiency in macrophages and dendritic cells ([Lamagna et al, 2013](#); [Scapini et al, 2010](#)), although another study showed that Lyn deficiency in B cells is enough to induce the immune-complex producing glomerulonephritis in mice ([Lamagna et al, 2014](#)). Moreover, and similar to that found in Lyn deficient mice, Lyn<sup>up/up</sup> mice develop autoantibodies and glomerulonephritis ([Hibbs et al, 2002](#)), confirming the idea that Lyn-dependent signaling pathways are essential in the regulation of B cell homeostasis and activation and therefore an upregulation or a downregulation in Lyn expression/activation drives in the development of autoimmune processes. However, it is worth noting that mice expressing Lyn protein, but with a defective kinase activity (Lyn<sup>Mld4/Mld4</sup> mice) ([Verhagen et al, 2009](#)), (WeeB mice) ([Barouch-Bentov et al, 2009](#)) (**Figure 11**) develop an attenuated autoimmune phenotype compared to Lyn deficient mice. Both mutant mice have B cell hyperactivity explained by the loss of Lyn kinase activity, but autoantibodies production is only observed in WeeB mice. Indeed, in these mice, the suppression of the MyD88-mediated activation of IRF5 is totally lost ([Ban et](#)

[al, 2016](#)); indicating that Lyn functions independent of its kinase activity is implicated in the development of autoimmune-like phenotype.



**Figure 11:** Lyn mutations in genetically engineered mice

- A. Lyn<sup>up/up</sup> mice contain a point mutation of the C-terminal tyrosine, generating a phenylalanine (Y508F) that is unable to be phosphorylated.
- B. Lyn<sup>Mld4/Mld4</sup> mice contain a point mutation of a threonine at the end of the activation loop to a lysine (T410K), which inhibits the activity of the enzyme.
- C. WeeB mice contain a point mutation in the glycine loop, a glutamic acid is changed to a glycine (E260G), inhibiting binding of Mg-ATP, resulting in an inactive enzyme. ([Ingley, 2012](#)).

#### (ii) Myeloid cells

Deletion or overactivation of Lyn is not only related with defects in the lymphoid system. Indeed, abnormal development of the myeloid system has been also observed in Lyn deficient mice and Lyn<sup>up/up</sup> mice ([Harder et al, 2001](#)), showing the implication of Lyn, through a positive (ITAM phosphorylation) and a negative role (ITIM phosphorylation), in the regulation of myeloid cells such as macrophages, dendritic cells, basophils and neutrophils (**Table 7**).

Lyn has been reported to be a crucial regulator in the G-CSF signalization in neutrophils, showing a positive role in differentiated cells and a negative effect in the response of immature neutrophils to this growth factor ([Sampson et al, 2007](#)). In Lyn deficient mice the uncontrolled humoral self-response producing the lupus-like nephritis phenotype is partially mediated by hyper-proliferative basophils ([Charles et al, 2010](#)), which leads to a peripheral basophilia (accumulation of basophils in secondary lymphoid organs) in these mice.

Deficiencies in the erythroid compartment of Lyn deficiency mice have been also described ([Harder et al, 2004](#)). Indeed, being Lyn the most important SFK in mature erythrocytes ([De Franceschi et al, 1997](#)), it is involved in the erythropoietin receptor signaling and ion transporters in these cells ([Maccaglia et al, 2003](#)) as well as in the morphology regulation of erythrocytes ([Ingleby, 2009](#)). Moreover, Lyn regulates signaling through integrins and FcγR, which is essential in the processes of adhesion and aggregation carried out by mature platelets ([Brunati et al, 2005](#)). Finally, Lyn is implicated in the signaling by thrombopoietin receptor in megakaryocytes ([Santini et al, 2002](#)) as well as in the regulation of mast cell degranulation through its interaction with FcεRI ([Hernandez-Hansen et al, 2004](#); [Odom et al, 2004](#)).

Receptor	Ligand	Cell type	Function
<b>Negative role</b>			
Type I cytokine receptors	GM-CSF, G-CSF, IL-3, IL-6	MP, Mφ, DC, MC	Proliferation, survival
Immunoglobulin superfamily receptors	M-CSF	Mφ, MC	Proliferation
FcεRI	IgE	MC, basophils	Proliferation, degranulation, cytokine production
Integrin	Extracellular matrix proteins, counter receptors	Mφ, PMN, MP	Adhesion, activation, survival
<b>Positive role</b>			
Type I cytokine receptors	IL-5, G-CSF, GM-CSF	PMN, E	Survival, proliferation
Seven transmembrane α-helical receptors	Chemokines	PMN, Mφ, MP, MC	Activation, migration

**Table 7:** Dual role of Lyn modulating myeloid-cell signaling

Specific receptors, ligands, cell type and functions that are either positively or negatively regulated by Lyn activity in myeloid cells: MP, myeloid precursors; Mφ, macrophages; DC, dendritic cells; MC, mast cells; PMN, neutrophils; E, eosinophils; FcεRI, Fc receptor ε I; CSF, colony-stimulating factor; IL-3, interleukin-3; SCF, stem cell factor; IgE, immunoglobulin E ([Scapini et al, 2009](#)).

### (iii) Osteoclast

A negative role in osteoclastogenesis has been described for Lyn. In fact, Lyn impairs osteoclastogenesis through suppression of the receptor activator of nuclear factor (NF)-κB (RANK) ligand-mediated Grb2-associated binder 2 (Gab2) signaling, which is produced by the activation of the SHP-1-dependent inhibitory signaling

pathway ([Kim et al, 2009](#)). The nuclear factor of activated T cells c1 (NFATc1) is an osteoclastogenic gene induced by the binding of RANK ligand to RANK, which is calcium dependent through the activation of PLC $\gamma$  ([Koga et al, 2004](#)). Indeed, the negative role of Lyn in osteoclast differentiation has been also reported by the fact that a downregulation in Lyn expression by siRNA stimulates RANK ligand-induced NFATc1 activation by enhancing PLC $\gamma$ -mediated calcium flux ([Yoon et al, 2009](#)).

### **c) In biological processes**

#### **(i) Implication of Lyn tyrosine kinase in cell death**

The involvement of Lyn in the regulation of cell death has been well established in the study of the apoptosis ([discussed in Error! No se encuentra el origen de la referencia.](#)), a highly regulated process of programmed cell death. In fact, this protein can exhibit, dependent of the context, an anti-apoptotic or a pro-apoptotic role.

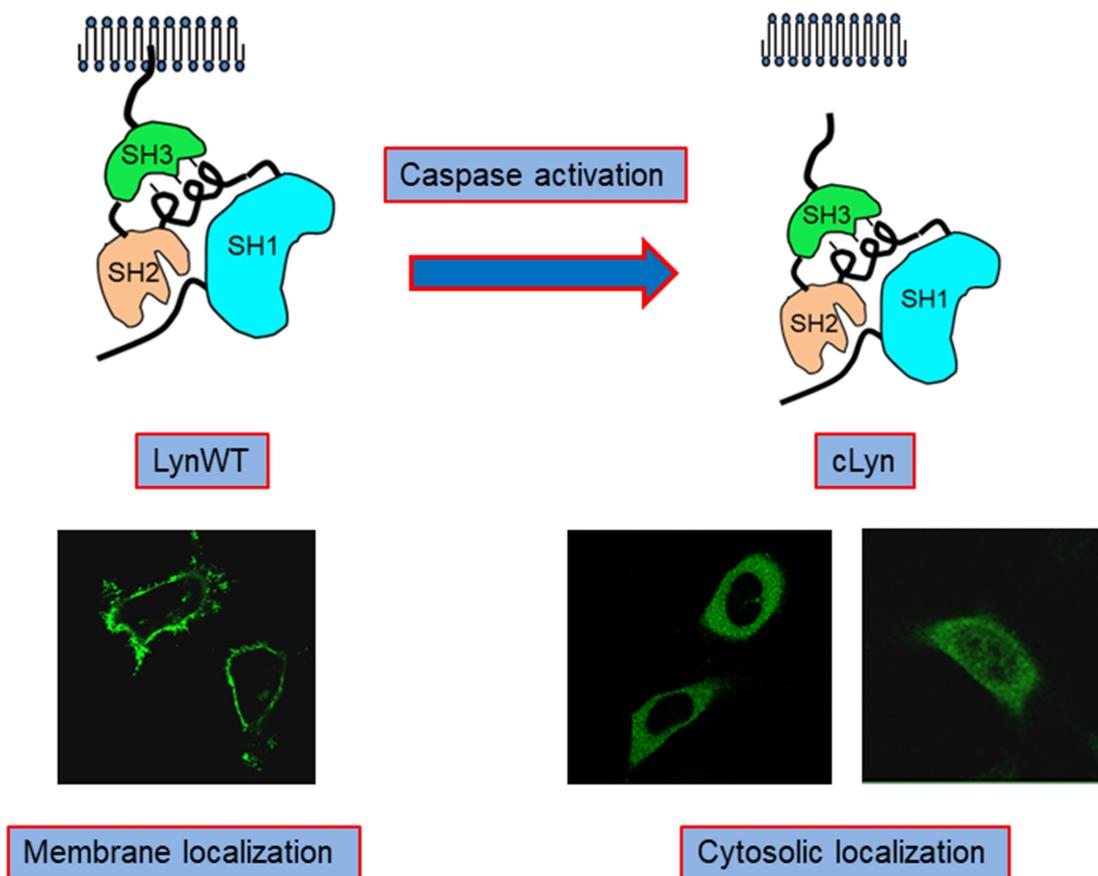
The pro-apoptotic role of Lyn has been shown in studies related with DNA damage ([Uckun et al, 1996](#)), being able to stabilize p53 in the nucleus, which assist its pro-apoptotic effects ([Ren et al, 2002](#)). Through the activation of stress signaling pathways in response to genotoxic agents, Lyn is activated and required for the activation of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) leading to the apoptosis of these cells ([Yoshida et al, 2000](#)). However, in response to these genotoxic agents, Lyn can be also translocated to sphingomyelin and cholesterol-enriched membrane domains, which in turn leads to neutral sphingomyelinase activation and the generation of the pro-apoptotic lipid second messenger, ceramide ([Grazide et al, 2002](#)). Dasatinib is a dual BCR-Abl (Abelson murine leukemia viral oncogene homolog 1)/SFK tyrosine kinase (Src, Lyn, Yes, Fyn) inhibitor, which has been used to treat chronic myeloid leukemia (CML) and Philadelphia chromosome positive acute lymphoblastic leukemia (Ph+ALL) patients ([Lindauer and Hochhaus, 2014](#)). Indeed, the combined treatment of dasatinib and genotoxic agents enhances p53-mediated elimination of human acute myeloid leukemia (AML) stem cells ([Dos Santos et al, 2013](#)). Therefore, Lyn may act as a negative regulator of genotoxic apoptosis. Nevertheless, the pro-apoptotic role of Lyn is not only appreciated in response to DNA damage, but also in response to activation of the cell death receptor Fas. Mature B cells from Lyn deficient mice are resistant to apoptosis induced by the Fas ligand ([Wang et al, 1996](#)) and in response to tumor necrosis factor (TNF)- $\alpha$  and Fas ligand, SHIP1 is activated by Lyn through nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and relocated to the plasma membrane, where its association with integrins enhances neutrophils apoptosis, which

is fully abrogated in Lyn deficient neutrophils ([Gardai et al, 2002](#)). Moreover, another study showed that Lyn is required by latent membrane protein 2A (MP2A) transcript, which function as a BCR mimic, to upregulated Fas expression during normal Epstein-Barr virus infection and produced Fas-mediated apoptosis ([Incrocci et al, 2015](#)).

On the other hand, the protective role of Lyn to avoid apoptosis has been evidenced in the association of activated Lyn with different integrins in mouse brain and in oligodendrocytes to prevent acid sphingomyelinase activity and ceramide-mediated apoptosis ([Chudakova et al, 2008](#)). In the same way Lyn has a protective apoptosis role in liver regeneration. During the early phase of liver generation after hepatectomy although an increase in oxidative stress and mitochondrial membrane potential is found, no cytochrome c release is observed, which has been associated with Lyn translocation to the mitochondria preserving its integrity ([Gringeri et al, 2009](#)). The overexpression and overactivation of Lyn in primary AML cells have been critically associated to the maintaining of proliferation and anti-apoptotic pathways in these cells ([Dos Santos et al, 2008](#)). BCR-Abl is a specific chromosomal abnormality, which is essential for the initiation, transformation and progression of CML disease due to its constitutive tyrosine kinase activity ([Ren, 2005](#)) and it has been demonstrated that Lyn activation is essential in the survival of BCR-Abl positive leukemia cells, because Lyn deletion in these cells leads immediately to apoptosis ([Rubbi et al, 2011](#)). Moreover, it has been demonstrated that in B-CLL cells, the resistance to apoptosis associated to an overactivation of Lyn is related to the Lyn-dependent phosphorylation of caspase 8 zymogen. Tyr380 phosphorylation of caspase 8 leads to the formation of a homodimer, which confers resistance to apoptosis in B-CLL cells and is abrogated by the use of SFK inhibitors ([Zonta et al, 2014](#)). Further, the decrease in the antigen-induced apoptosis related with phosphatase PTPN22 overexpression in CLL cells has been linked to a selective uncoupling of the AKT pathway related with an inhibition of the negative role of Lyn in the BCR signaling ([Negro et al, 2012](#)) and the resistance to apoptosis observed in these cells is partially related to the deregulated activity by Lyn-mediated mechanisms of the proteins PP2A and SHP-1 ([Tibaldi et al, 2017](#)).

Caspases are normally active in apoptotic process ([discussed in \*\*¡Error! No se encuentra el origen de la referencia.\*\*](#)) and in this context a caspase-dependent cleavage of Lyn has been previously shown in my PhD lab, demonstrating that this cleaved-form of Lyn might act as a suppressor of apoptosis. It has been previously described that SFK such as Lyn and Fyn can be cleaved by caspase 3 and 7 in their unique region (Asp 18 and Asp 19, respectively) in cells undergoing apoptosis ([Luciano et al, 2001](#); [Ricci et al, 2001](#)), resulting in removal of the myristoylation and

palmitoylation sites. Thus, this new form of Lyn called Lyn $\Delta$ N (cLyn) will be relocated from the plasma membrane to the cytosol compartment (**Figure 12**). Once in the cytosol, they may negatively regulate the apoptosis induced by the BCR engagement ([Luciano et al, 2001](#); [Ricci et al, 2001](#)). The overexpression of cLyn in immature B cells, such as Ramos cells, leads to an inhibition of the apoptosis induced by BCR-mediated activation ([Luciano et al, 2003](#)), although the mechanism by which Lyn exerts its anti-apoptotic effect remains to be elucidated. However, it is possible to speculate that the new subcellular localization confers to cLyn access to new substrates with which the WT form of Lyn could not interact.



**Figure 12:** Subcellular localization of cLyn

Schematic representation of the subcellular localization of the native form of Lyn (LynWT) and the cytosolic form (cLyn).

In B-CLL cells Lyn is found rather in the cytosol than in the plasma membrane, whose cytosol overexpression has been associated to cell death resistance ([Contri et al, 2005](#)), and the overexpression of Lyn associated to an increase in its tyrosine kinase activity has been found in the resistance of different CML cells lines to imatinib ([Donato et al, 2003](#)). To analyze the role of cLyn in the context of CML, cLyn and its

catalytic inactive form (cLynKD) were studied in K562 cell line (an imatinib sensitive cell line derived from CML). The overexpression of cLyn led to an inhibition of the apoptosis induced by imatinib treatment, which was not found when cLynKD was overexpressed in the same cells. Moreover, treatment with Src inhibitors produced apoptosis in these cells confirming the role of cLyn as a negative regulator of apoptosis depending on its tyrosine kinase activity ([Gamas et al., 2009](#)). The caspase-dependent cleavage of Lyn leads to an anti-apoptotic effect of Lyn, and this can be related with the onset of resistance associated to imatinib treatment. In fact, imatinib resistance has been associated to an increase in the expression and/or in the activity of Lyn with an abnormal localization in the cytosol ([Gamas et al., 2009](#)).

Thus, the caspase-cleaved form of Lyn has an important role in cancer, notably implicated in treatment resistance, suggesting that Lyn deregulation could be linked to hematopoietic abnormalities. In fact, an increase in Lyn activity has been found in different leukemias and lymphomas. However, studies with Lyn deficient (Lyn<sup>-/-</sup>), Lyn hyperactivated (Lyn<sup>up/up</sup>) and Lyn inactivated (Lyn<sup>Mld4/Mld4</sup>) mice have demonstrated that Lyn is not so important in the beginning of transformation process of malignant cells, but rather Lyn plays a role as an oncogene after transformation initiation ([Donato et al., 2003](#)), contributing to the development and maintaining of neoplastic cells. Indeed, positive correlations between Lyn activity, and SFK in general, with disease severity have been shown ([Russello and Shore, 2003](#)) and good clinical responses have been reached in the treatment of CML patients with dasatinib ([Chen and Chen, 2015](#)), confirming the role of Lyn in hematopoietic abnormalities.

Several studies have demonstrated that Lyn is overexpressed and overactive in various hematological malignancies ([Ingleby, 2012](#)), being strongly implicated in leukemias and lymphomas such as CML ([Donato et al., 2003](#)), AML ([Dos Santos et al., 2008](#)), CLL ([Contri et al., 2005](#)), B-cell ALL ([Hu et al., 2004](#)), B-cell-CLL ([Contri et al., 2005](#)), B-Non Hodgkin's lymphomas ([Tauzin et al., 2008](#)) and more recently in myeloproliferative disorders ([Takeda et al., 2011](#)).

Considerable studies put in relation the tyrosine kinase Lyn with the development of solid cancer. In one study that compared 130 tumor lines through proteomic analysis, Lyn was found activated in most of all cell lines tested ([Du et al., 2009](#)). Lyn has been associated with prostate cancer ([Goldenberg-Furmanov et al., 2004](#)), colorectal cancer ([Bates et al., 2001](#); [Chen et al., 1999](#)), head and neck cancer ([Xi et al., 2003](#)), renal cancer ([Roseweir et al., 2016](#)), oral cancer ([Bundela et al., 2014](#)), gastric cancer ([Mello et al., 2015](#)), cervical cancer ([Liu et al., 2016a](#)), bladder cancer ([Thaper et al., 2017](#)), glioblastoma ([Stettner et al., 2005](#)), Ewing's sarcoma ([Guan et al., 2008](#)), and

breast cancer ([Choi et al, 2010](#); [Bougeret et al, 2001](#)). In fact, Lyn has been identified as a factor of poor prognosis in triple negative breast cancer ([Hochgrafe et al, 2010](#)) and the treatment with the experimental drug and BCR-Abl and Lyn selective inhibitor, Bafetinib, significantly decreased the levels of Claudin-2, a protein that enhances breast cancer liver metastasis ([Tabaries et al, 2015](#)), suggesting that Lyn-specific inhibitors could be a potential therapeutic target for the treatment of breast cancer-derived metastasis. Furthermore, it has been demonstrated that Lyn is the principal SFK activated in glioblastomas presenting a key role in PDGF-mediated migration of these cells ([Ding et al, 2003](#)). The association of Lyn with cancer metastasis has been linked to a role of Lyn in promoting epithelial-mesenchymal transition (EMT) ([Guarino, 2010](#)). In fact, silencing Lyn in highly aggressive triple negative breast cancer cells decreases cell migration and invasion ([Choi et al, 2010](#)). Moreover, it was recently demonstrated that Lyn promotes EMT, invasion and metastasis through the activation of SNAIL family of transcription factors, which transcriptionally repress E-cadherin expression starting mesenchymal phenotype development ([Thaper et al, 2017](#)). Furthermore, an essential role of Lyn in tumor formation microenvironment has been lately evidenced. Indeed, Lyn deficient macrophages were less efficient in supporting both human and mouse CLL cell survival, indicating that Lyn protein is key in the microenvironment supporting leukemic growth ([Nguyen et al, 2016](#)).

Like it was elucidated in leukemia and lymphoma studies, the expression of Lyn in solid tumors is not related with the cancer initiation, but with the maintaining of the phenotype. Although, according to the kind of solid tumor Lyn seems to have different expression levels. For example the expression of Lyn in metastatic colorectal cancer, which has been associated with CD24-induced ERK-1/2 activation ([Su et al, 2012](#)), has been found in metastatic cancer, but no in the first stages of cancer development or even in normal tissue ([Chen et al, 1999](#)). However, in prostate cancer a high Lyn expression is observed in primary prostate tissue, which has been linked to the regulation of cell migration, once transformation has been initiated ([Sumitomo et al, 2000](#)).

Therefore, all the evidence detailed above confirm that Lyn has a significant impact in solid tumor development as well as leukemias and lymphomas through its effects in the deregulation of several signaling pathways or acting as a suppressor of the programmed cell death process, apoptosis; which validates Lyn as a therapeutic target in the treatment of all these malignant diseases.

## (ii) Implication of Lyn tyrosine kinase in inflammation

Lyn can have an anti and a pro-inflammatory role depending on the context (**Table 8**). The protective role in inflammation has been reported in the maintaining of the lung vascular barrier function ([Han et al, 2013](#)). In fact, Lyn deficient mice were susceptible to develop endotoxin-induced lung inflammation through the activation of NF- $\kappa$ B signaling pathway, increased caspase-1 and IL-1 $\beta$  cleavage and activation ([Gao et al, 2015](#)), evidencing that Lyn could be a negative mediator in inflammation. Moreover, it was evidenced that Lyn expression in plasma cells acts as a regulatory threshold preventing excessive signaling in response to IL-6 and IL-3 cytokines ([Infantino et al, 2014](#)), suggesting that the plasmacytosis observed in Lyn deficient mice could be related to an excessive signal transduction mediated by these cytokines.

Roles of Lyn in INFLAMMATION			
Anti-inflammatory role		Pro-inflammatory role	
Cells	Function	Cells	Function
Lung vascular barrier	Protective role suppressing NF- $\kappa$ B	Mast cells	phosphorylation of ITAMs motifs interaction with DJ-1 proteins
Plasma cells	preventing excessive signaling		
Disease	Function	Disease	Function
Asthma	Reduced airway eosinophil infiltration, IL-13, ER stress	Asthma	Induced airway eosinophil influx
DSS-induced colitis	Protective role No enteric bacterial colonization	Diabetes	Increase glucose-induced innate immune response

**Table 8:** Roles of Lyn in inflammation

Lyn has an anti-inflammatory and a pro-inflammatory role depending of the context.

On the other hand, the pro-inflammatory role of Lyn has been described in studies related with allergic diseases like asthma and other immune-mediated diseases.

- Role of Lyn in allergy

Mast cells are known to be the principal effector cells in allergy development ([Bischoff, 2007](#)). In fact the binding of antigens to IgE associated to Fc $\epsilon$ RI on plasma membranes from mast cells produces the release of allergy-induced substances like

histamines and prostaglandins, leading to the secretion of inflammatory cytokines ([Simons and Simons, 2011](#)). In this context, Lyn is implicated in initial signaling events through the phosphorylation of ITAMs motifs, leading to the recruitment/activation and binding of Syk to the phosphorylated ITAMs and producing the allergic response ([Nam et al, 2017](#); [Mocsai et al, 2010](#)). Furthermore, it has been demonstrated altered levels of DJ-1, a redox sensitive protein, in patients with mast cell-related disorders ([Kim et al, 2016a](#); [Kim et al, 2013](#)). In fact, it was recently established that reduced levels of DJ-1 in a human mast cell derived line, significantly impaired Lyn activation; and no interaction between DJ-1 and Lyn in the lipids rafts was evidenced, indicating that mast cells need the early interaction between Lyn and DJ-1 proteins to activate Lyn and then the FcεRI to trigger the allergy response in these patients ([Kim et al, 2017a](#)).

- Controversial roles of Lyn in asthma

Asthma is one of the most chronic airway inflammatory diseases, leading to goblet cell metaplasia, mucus hypersecretion and eosinophil infiltration ([Balenga et al, 2015](#)). While a study showed that blocking Lyn activation significantly reduced airway eosinophil infiltration in a mouse model of asthma ([Adachi et al, 1999](#)), validating Lyn as a therapeutical target for this disease; Lyn deficient mice are prone to develop severe and persistent asthma ([Beavitt et al, 2005](#)), suggesting contradictory roles of this protein in asthma development. Moreover, Lyn deficiency led to mucus hypersecretion when mice were challenged with House Dust Mite (HDM) allergen, a mouse model of asthma ([Li et al, 2013](#)), indicating that Lyn can be a negative regulator in asthma progression. Nevertheless, nowadays it is well known that asthma is a T helper (Th)-2 pattern-mediated disease ([Fahy, 2015](#)) and Lyn is not expressed in this kind of cell population, but it has been described that Lyn can influence T-cell function through signaling modulation in those cells that interact with T cells ([Beavitt et al, 2005](#)). In fact, Lyn can regulate the expression of the transcription factor GATA-3 in basophils controlling the onset and extent of basophil-mediated Th2 differentiation ([Charles et al, 2009](#)); but more studies are needed to clarify the role of Lyn in this disease.

Mucins are the principal components of mucus ([Pelaseyed et al, 2014](#)), being MUC5AC one of the primary mucins in human airways, which has been implicated in pulmonary diseases through mucus hypersecretion ([Bonser et al, 2016](#)). Moreover, as Th2 pattern-mediated disease, IL-13 levels are significantly increased during asthma pathogenesis, which induce mucus production in the airways ([Kano et al, 2011](#)), through the activation of transcription factor STAT6. In fact, STAT6-deficient mice were protected from airway inflammation in a mouse model of allergic airway inflammation

([Miyata et al., 1999](#)). A recent study showed that Lyn overexpression reduced ovalbumin (OVA)-induced mucus hypersecretion, MUC5a expression, airway inflammation and IL-13 levels through the decrease of STAT6 expression and activation as well as by decreased STAT6 binding to the muc5ac promoter ([Wang et al., 2017b](#)); evidencing that Lyn protein kinase plays a crucial role in mucus hypersecretion in antigen-challenged mice. Furthermore, endoplasmic reticulum (ER) stress has been correlated with mucus secretion and asthma development ([Li et al., 2012](#)) and HDM induces ER stress in airway epithelial cells ([Hoffman et al., 2013](#)). Recently, it has been demonstrated that Lyn overexpression improved mucus hypersecretion and ER stress through the inhibition of AKT and NF- $\kappa$ B signaling pathways. Moreover, Lyn overexpression reduced IL-13-induced ER stress and MUC5AC expression in airway epithelial cells ([Wang et al., 2017b](#)); confirming the negative role of this protein in asthma pathology.

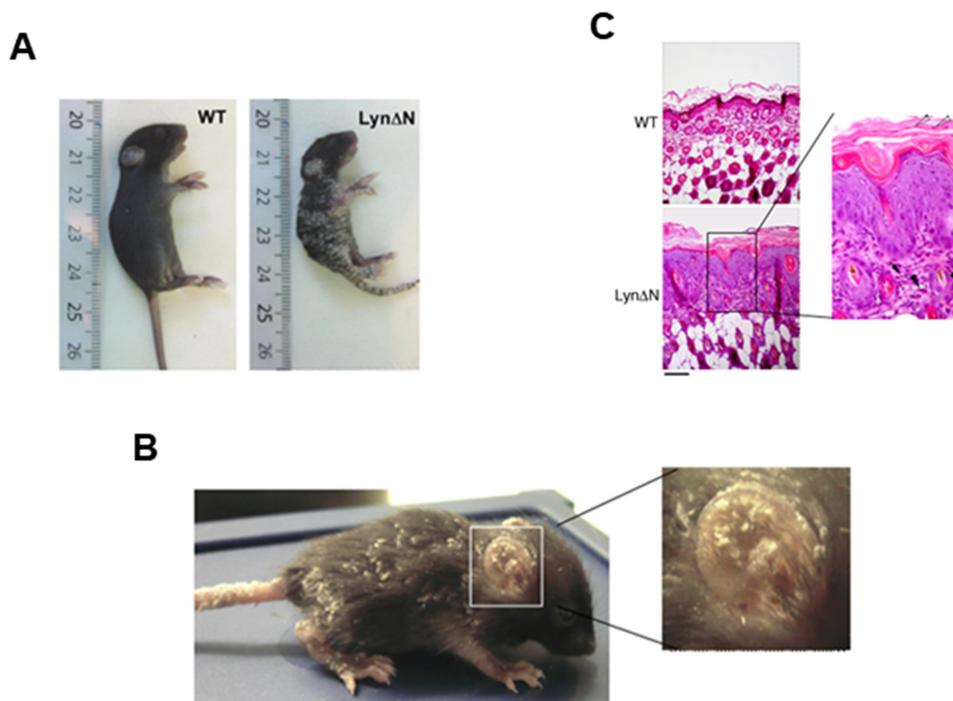
- Roles of Lyn in other inflammatory diseases

The role of Lyn in inflammation has also been evidenced in diabetes. Indeed the inhibition of the aldose reductase, an enzyme expressed in oxidative stress conditions and involved in ROS-mediated inflammation commonly observed in diabetes, prevents the high glucose-induced innate immune response by regulating the release of pro-inflammatory cytokines via a decrease in Lyn signaling ([Pal et al., 2017](#)).

In models of dextran sulfate sodium (DSS)-induced colitis a protective role of Lyn has been observed through elevated levels of IL-22 and IL-22-responsive factors in the colon through an enhanced TLR-dependent dendritic cell activation of group 3 innate lymphocytes ([Bishop et al., 2014](#)). However, it is noteworthy that this study was made with Lyn<sup>up/up</sup> mice, where Lyn is constitutively activated; and in the same study it was demonstrated that Lyn deficiency significantly increases susceptibility to DSS-induced colitis. Moreover, Lyn deficient mice have shown increased predisposition to enteric infection. In fact, these mice were highly susceptible to bacterial colonization and inflammation in the gut during gastroenteritis and typhoid models of salmonellosis ([Roberts et al., 2014](#)). Altogether, these results elucidate that Lyn have a pivotal role in controlling intestinal inflammatory responses, indicating that Lyn can be a possible target in the treatment of inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis.

A significant increase of SFK activation has been found in lesional skin biopsies from psoriasis patients ([Ayli et al., 2008](#)), indicating SFK activation is related with pathogenesis of hyperproliferative epidermal disorders. Furthermore the ubiquitous

expression of cLyn in mice led to the development of a chronic inflammatory syndrome resembling human psoriasis (**Figure 13**) ([Marchetti et al, 2009](#)). Indeed, six days after birth, mice develop skin lesions associated to an epidermal hyperplasia and to an altered keratinocyte differentiation as well as a significant increase in the immune cell infiltration and in the expression of proinflammatory cytokines. Further, albeit all cLyn mice do not reach adulthood, in those mice who arrive to the fifth or sixth week the skin phenotype disappears, indicating that skin phenotype can be reversible like it has been shown in mice with an overexpression of active STAT3 in the skin ([Sano et al, 2005](#)) or in psoriasis patients, where disease is through flare ups ([Conrad and Gilliet, 2018](#)), thus showing that cLyn mice develop an inflammatory phenotype resembling human psoriasis.



**Figure 13:** Principal phenotypical characteristic of cLyn mice

- A. cLyn ( $Lyn\Delta N$ ) transgenic mice developed skin defects characterized by scaling patches, being smaller than control mice.
- B. The 4-week-old  $Lyn\Delta N$  mice with skin defects resembling scalp psoriasis with a macroscopic view of scaly patches affecting ear.
- C. Histological comparison of skin sections from 2-week-old cLyn ( $Lyn\Delta N$ ) and control mice reflecting the hallmarks of psoriasis. Haematoxylin/eosin staining revealed a marked epidermis hyperplasia, a large number of nuclei in the cornified layer (arrowheads), hyperkeratosis and inflammatory infiltrates in the epidermis and dermis of cLyn skin (arrows). Bar scale: 100  $\mu\text{m}$  ([Marchetti et al, 2009](#)).

### III. Apoptosis

Apoptosis is a highly regulated process of programmed cell death, which normally occurs in the development of living organisms and as an homeostatic process in the maintenance of tissues, whose principal characteristics are the mitochondrial outer membrane permeabilization (MOMP), leading to Smac/Diablo and cytochrome c release to the cytosol as well as chromatin condensation, nuclear and chromosomal DNA fragmentation. Two pathways have been described in the induction of apoptosis: the intrinsic pathway (also known as mitochondrial pathway) and the extrinsic pathway (**Figure 14**) ([Nagata and Tanaka, 2017](#)), where the activation of initiator caspases results in the cleavage and activation of executioner caspases, which cleave several target proteins, leading to cell death ([Cullen and Martin, 2009](#)).



**Figure 14:** Two major apoptosis pathways

The extrinsic pathway is activated through the binding of death inducer factor to its receptor. The intrinsic pathway activates apoptotic members from Bcl-2 family leading to the apoptosome formation. In both pathways caspase 3 is activated, which cleaves more than 500 cytoplasmic proteins to induce apoptotic cell death ([Nagata and Tanaka, 2017](#)).

The first pathway is the most induced in mammals and is initiated through apoptotic intracellular signals (DNA damage, ER stress, growth factor deprivation) leading to the activation of pro-apoptotic members of B-cell lymphoma-2 (Bcl-2) family producing MOMP and the activation of caspase 9 through the apoptosome formation ([Pop et al, 2006](#)). Briefly, intracellular stress signals are sensed at the mitochondria level by Bcl-2 family members producing Bax/Bak oligomerization resulting in a pore formation and the subsequent MOMP and the release of Smac/Diablo and cytochrome c release into the cytosol. In the cytosol, the cytochrome c is enabled to associate to

Apaf-1 and pro-caspase 9 forming a complex called apoptosome. Once apoptosome is built, caspase 9 is activated leading to the subsequent activation of caspases 3 and 7, which can cleave specific substrates related to cell death by apoptosis ([Galluzzi et al., 2016a](#)).

In the second pathway, apoptosis is induced by TNF family death ligands such as FAS and TRAIL, which induce the multi-molecular death-inducing signaling complex (DISC) formation resulting in the homodimerization and the subsequent activation of effector caspases 8 and 10 ([Dickens et al., 2012](#)).

### III.I. Bcl-2 family members

The intrinsic pathway of apoptosis or the mitochondrial apoptosis ([Kapoor et al., 2013](#)) is tightly regulated by the members of the Bcl-2 family, which control the integrity of the mitochondria by preventing, in proliferating cells, MOMP and release of cytochrome c into the cytosol ([Birkinshaw and Czabotar, 2017](#); [Estaquier et al., 2012](#)). This family is composed by several members, which share BH (Bcl-2 homology) domains, classifying its member in those which have four BH domains and in those which have only one BH domain. Moreover, the different members of this family, according to its function, can be classified in two groups: the anti-apoptotic group (prevent MOMP) and the pro-apoptotic group (produce MOMP). The anti-apoptotic group is composed by Bcl-2, Bcl-XL, Bcl-W, Mcl-1, A1 and Bcl-B and all these members have four BH domains. Meanwhile the pro-apoptotic group is divided in two subgroups: one with four BH domains composed by Bak, Bax and Bok, and the other one with only one BH domain (BH3-only members) composed by Bim, Bid, Puma, Noxa, Bad, Hrk, Bmf and Bik (**Figure 15**) ([Cory et al., 2016](#)).

To promote apoptosis the domain from a BH3-only protein has to be inserted in the groove from one of the multi-BH domain proteins (Bak and Bax), which is mediated by conserved hydrophobic residues ([Liu et al., 2003](#)); and these protein-protein interactions between the Bcl-2 family members are going to decide whether cells live or die ([Edlich, 2017](#)). Therefore, the expression of the Bcl-2 family members is well-regulated. Indeed, all members of the family are regulated by several transcriptional, translational and post-translational mechanisms ([Kutuk and Letai, 2008](#)). Among all, post-translational modifications, is worth mentioning ubiquitination and cleavage as well as phosphorylation/dephosphorylation mechanisms.



**Figure 15:** Bcl-2 family members

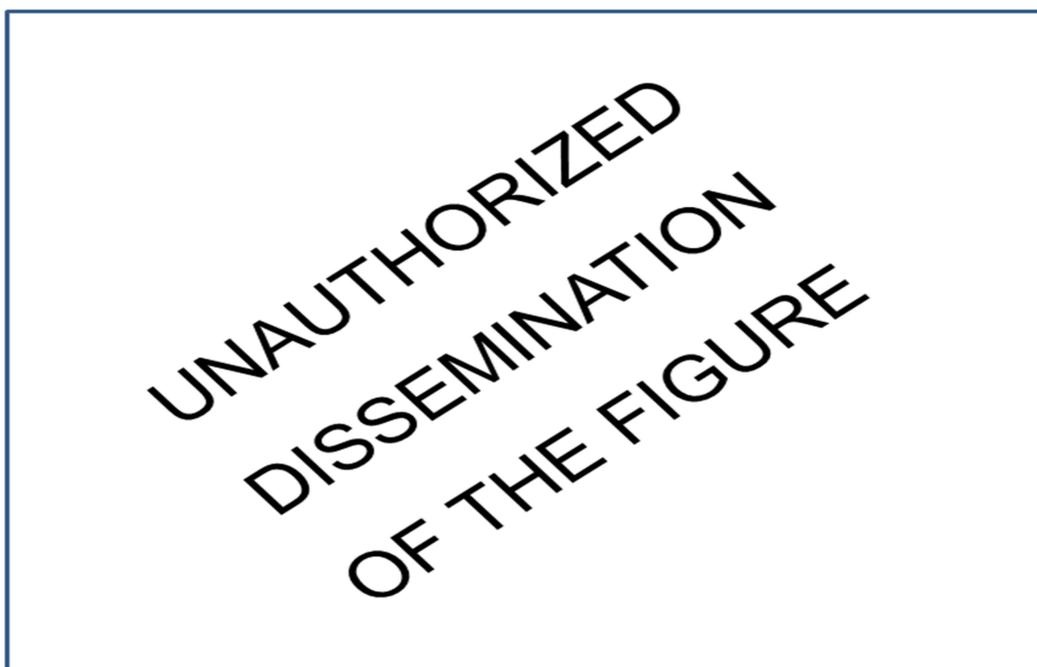
Pro-survival and pro-apoptotic members of Bcl-2 family: All proteins from this family share BH domains and a C-terminal trans-membrane domain (TM) for anchoring to organelles ([Shukla et al, 2017](#)).

### III.II. Bim

The **Bcl-2** interacting mediator of cell death (Bim) is a potent inducer of apoptosis and promotes apoptosis either by direct activation of pro-apoptotic members Bax/Bak or by inhibiting anti-apoptotic Bcl-2 members via its BH3 domain, driving the MOMP, and the subsequent events leading to cell death (**Figure 16**) ([Cory et al, 2016](#); [Brunelle and Letai, 2009](#)). Through alternative splicing three major isoforms of this protein are obtained: BimEL (extra-large, 22 kDa), BimL (large, 15.8 kDa) and BimS (small, 12.3 kDa) ([Shukla et al, 2017](#)) (**Figure 17**), being BimEL the predominant isoform in all body tissues.

Bim plays a key role in the induction of apoptosis after chemotherapeutic agent treatments such as imatinib, dasatinib, nilotinib and bortezomib ([Akiyama et al, 2009](#)). In fact, in CML patients, Bim has a crucial role in apoptosis induction after imatinib treatment via the mitochondrial pathway ([Kuroda et al, 2006](#)). Moreover, the successful treatment with dasatinib and nilotinib in imatinib resistant CML patients is the induction of cell death in a Bim-dependent manner ([Bhamidipati et al, 2013](#)). Bortezomib, an inhibitor of the 20S proteasome ([Chen et al, 2014](#)), increases Bim half-life and

decreases the binding with Mcl-1, ([Gomez-Bougie et al, 2007](#)); which promotes Bak/Bax dependent mitochondrial permeabilization leading to apoptosis. Therefore, Bim is a key regulator of immune homeostasis and a major obstacle for the development of cancer ([Bouillet et al, 1999](#)), and a negative regulation of its expression correlates with a decrease in the cellular apoptosis and an increase in the tumor growth ([Mestre-Escorihuela et al, 2007](#)).

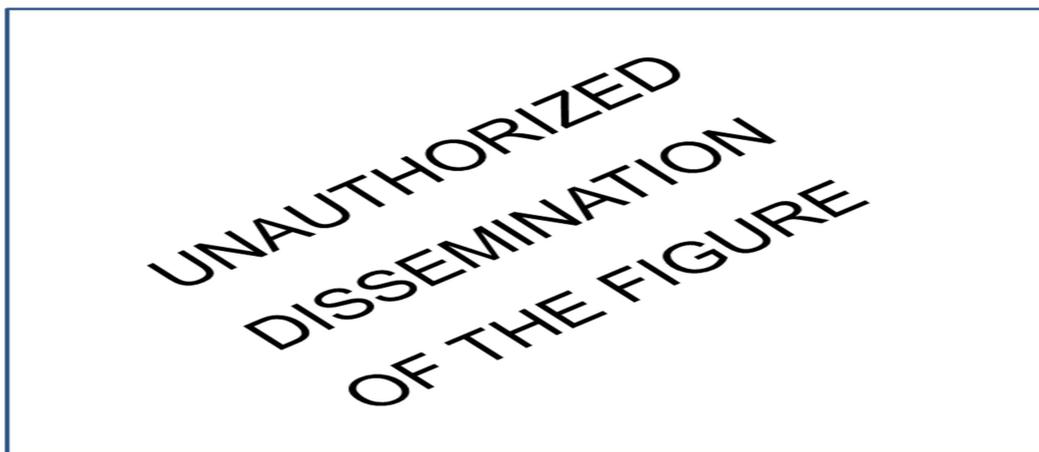


**Figure 16:** Bcl-2 family members control cell life and death

In healthy cells pro-survival factors prevent activation of Bax and Bak. Stress signal activate BH3-only proteins like Bim, which interact with pro-apoptotic members, resulting in the activation of Bax and Bak leading to the MOMP and subsequently cell death by apoptosis ([Cory et al, 2016](#)).

Bim is regulated at transcriptional level by epigenetic mechanisms and several transcription factors ([Ridinger-Saison et al, 2013](#)). In fact, Bim promoter acetylation has been associated with an increase in apoptosis in multiple myeloma cancer ([De Bruyne et al, 2010](#)) and transcription factors such as Forkhead-box class O (FoxO) 3a ([Liu et al, 2014](#)), Hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) ([Whelan et al, 2013](#)) and the chromatin regulator Brd4 ([Patel et al, 2014](#)) lead to a transcriptional repression of Bim. However, Run-related transcription factor 3 (Runx3) promotes Bim expression in breast cancer cells ([Merino et al, 2015](#)). At post-transcriptional level, Bim is regulated by various microRNA where nine potential sites have been identified in Bim RNA to be target from microRNA ([Koralov et al, 2008](#)). For example, miR-192 interacts with the region 3'-UTR from Bim gene and the expression of Bim is inhibited in esophageal squamous cell

carcinoma ([Li et al., 2015](#)), and downregulation of miR-101-3p in serum deprivation conditions increases Bim expression inducing apoptosis in endothelial cells ([Kim et al., 2017b](#)). Further, Bim is also regulated at post-translational level via phosphorylation and ubiquitination processes, principally. Indeed, it has been demonstrated that ERK1/2-dependent phosphorylation of human Bim at Ser residues (59, 69 and 77) reduces interaction with pro-survival proteins (Mcl-1 and Bcl-XL) ([Ewings et al., 2007](#); [Hinds et al., 2007](#)) and allowed its degradation by proteasome ([Wiggins et al., 2011](#)). Moreover, it has been described that JNK-mediated phosphorylation of Bim in Ser100, Thr112 and Ser114 enhances its stabilization and drives to a more effective activation of Bax/Bak in presence of apoptotic stimuli ([Geissler et al., 2013](#)). Furthermore, phosphorylation of Bim in Ser87 by protein kinase A increases its half-life and apoptotic potential ([Moujalled et al., 2011](#)). Additionally, phosphorylation on Ser93, Ser94 and Ser98 by Aurora A kinase leads to Bim ubiquitination and its subsequent degradation ([Moustafa-Kamal et al., 2013](#)). However, although ERK1/2-dependent phosphorylation of Bim is well-known in the literature, in knock-in mice expressing BimEL, which cannot be directly controlled by ERK1/2, there is no altered hematopoietic system homeostasis, indicating that the physiological regulation of Bim relies in other ERK independent mechanisms ([Clybouw et al., 2012](#)), such as the phosphorylation by other proteins, like SFK, on the tyrosine residues. In fact, it has been demonstrated in a model of fibrosarcoma cells that the particularly interesting cysteine-histidine-rich protein-1 suppresses Bim both transcriptionally and post-transcriptionally by promoting the activation of Src family kinases ([Chen et al., 2008](#)).



**Figure 17:** Bim isoforms

Predominant Bim isoforms, which are target of post-transcriptional modifications, are showed. The domain structure arising from alternative splicing, the number of amino acids as well as ERK 1/2 and JNK phosphorylation sites are presented ([Shukla et al., 2017](#)).

### III.III. Caspases

Caspases are CysteinyI ASPartate- specific proteASES encoded by 12 genes in humans and 10 in mice ([Songane et al., 2018](#)), which are going to cleave specific substrates after an aspartate residue. These proteases are firstly synthesized as inactive zymogens, which can be activated by other caspases or by auto-activation mechanisms. Moreover, according to the structure and functional similarities caspases have been classified in apoptotic and inflammatory caspases (**Figure 18**). The subfamily of apoptotic caspases is divided in two groups: initiator (Caspases 2, 8, 9 and 10) and executioner (Caspases 3, 6 and 7) caspases ([Fuentes-Prior and Salvesen, 2004](#)). On the other hand, the subfamily of proinflammatory caspases is composed by caspases 1, 4, 5 and 12 in humans and caspases 1, 11 and 12 in mice ([Fuentes-Prior and Salvesen, 2004](#)). It is worth noting that caspase 11 is the mouse orthologous of human caspases 4 and 5 ([Wang et al., 1998](#)). Moreover, caspase 12 has limited enzymatic activity in mice ([Roy et al., 2008](#)) and is completely inactive in humans ([Xue et al., 2006](#)). Furthermore, caspase 13 is another pro-inflammatory caspase and is the human orthologous of caspase 4 only found in bovine animals ([Evans et al., 2004](#)).



**Figure 18:** Domain organization and classification of human caspases

Caspases have been grouped according to their sequences similarities, being classified in inflammatory, initiator and effector caspases ([Fuentes-Prior and Salvesen, 2004](#)).

Initiator caspases are activated by proximity through signaling platforms such as the apoptosome in the case of pro-caspase 9 or the death-inducing signaling complex (DISC) for pro-caspases 8 and 10 ([Ramirez and Salvesen, 2018](#)), driving in the induction of intrinsic and extrinsic pathways of apoptosis, respectively. On the other hand inflammatory caspases, except caspase 12, are activated by proximity within large macromolecular complexes called inflammasomes ([Broz and Dixit, 2016](#)).

Although caspases have been classified in apoptotic and pro-inflammatory caspases, it is noteworthy these proteases have been implicated in other cellular processes. For example, caspase 2 has been found playing a crucial role in the cell cycle regulation ([Fava et al., 2012](#)), and caspase 3 as well as caspase 8 have been associated with differentiation processes in myeloid cells ([Solier et al., 2017](#)). In fact, caspase 3 and 8 inhibition impairs differentiation of erythrocytes ([Zermati et al., 2001](#)). Moreover, caspase 8 deletion in mouse spoils monocytes differentiation to macrophages ([Kang et al., 2004](#)) and caspase 3 deficient mice have a reduced number of osteoclasts because osteoclastic precursors are not able to differentiate to mature osteoclasts ([Szymczyk et al., 2006](#)).

Caspase are widely expressed proteins, although caspase 14 is only present in keratinocytes ([Denecker et al., 2007](#)). Indeed, caspase 14 has been considered a highly specialized protease with an essential function in the cornification process that take place in the terminal differentiation of keratinocytes leading to the formation of the stratum corneum in the epidermis ([Denecker et al., 2008](#)). Thus, this specific caspase has not been classified as apoptotic or pro-inflammatory. Additionally, other four caspases have been reported in mammals; although three of them (caspases 15, 17 and 18) are not expressed in placental mammals. The other one, caspase 16, exists as a pseudogene in humans and mice and it has not been detailed so far ([Eckhart et al., 2008](#)).

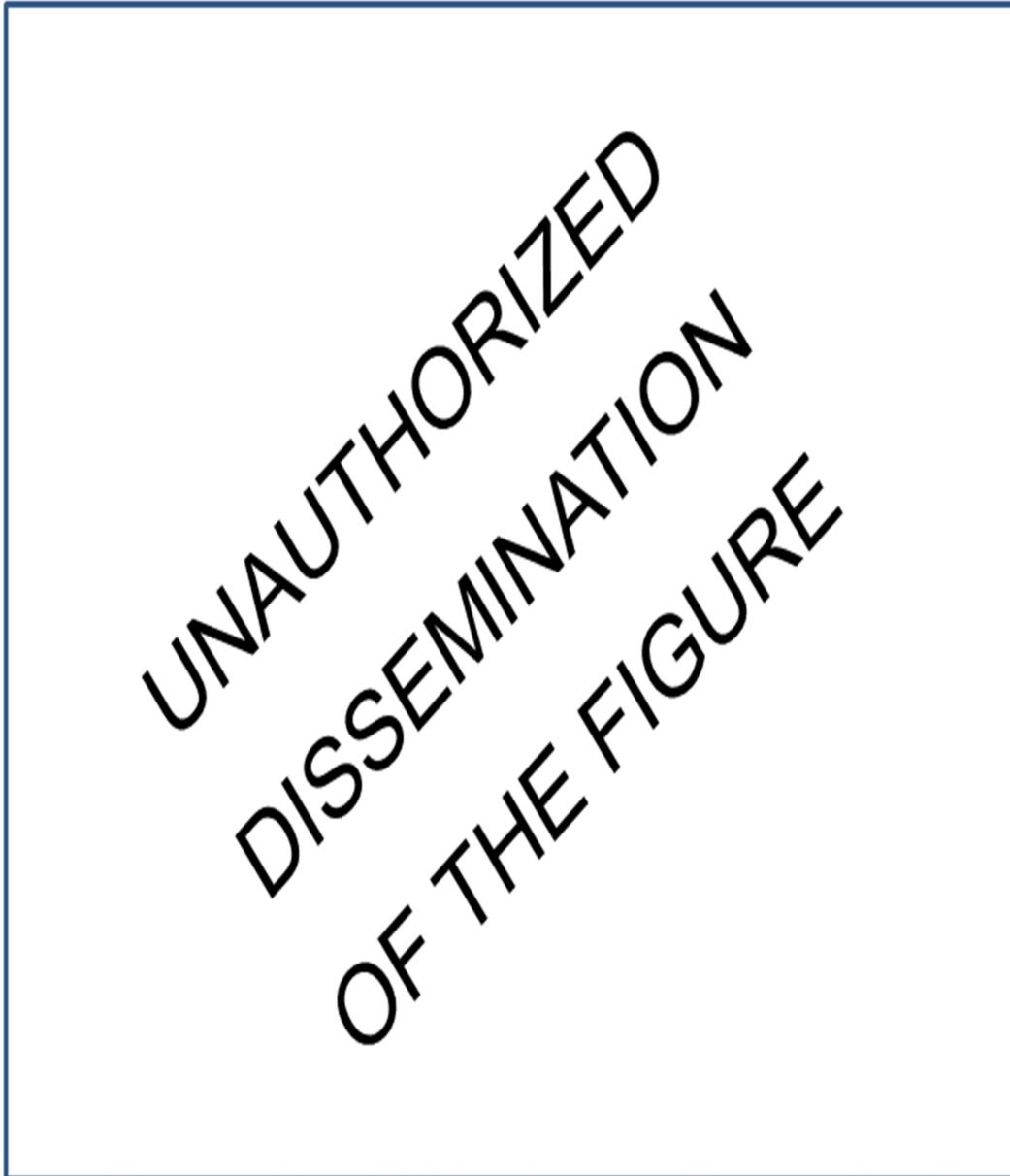
### **a) Caspases and inflammation**

Inflammasomes are macromolecular structures, which serve as platforms for the activation, through oligomerization, of pro-inflammatory caspases after the recognition of pathogen (PAMP) or damage (DAMP)-associated molecular patterns by the pattern recognition receptors (PRR) such as TLR, c-type lectin receptors, absent in melanoma 2 (AIM2)-like receptors (ALR) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) ([Ramirez and Salvesen, 2018](#); [Yi, 2017](#)). Once activated, pro-inflammatory caspases cleave precursors of pro-inflammatory cytokines IL-1 $\beta$  and IL-18, rendering these two cytokines in their active form. Moreover, these caspases also cleave Gasdermin D ([Liu et al., 2016b](#)), which induce pore formation in plasma

membrane producing the release of pro-inflammatory cytokines and cell death by pyroptosis ([Fink and Cookson, 2005](#)) (**Figure 19**).

Pro-inflammatory caspases have a CARD (**Figure 18**), which allows the interaction with CARD-containing partners such as the apoptosis-associated speck-like protein containing C-terminal CARD (ASC) ([Srinivasula \*et al.\*, 2002](#)). ASC links caspase-1 to NLR and ALR producing the formation of inflammasome structures and the subsequent activation of pro-inflammatory caspases. At the present time, 10 inflammasomes, leading to the activation of caspase 1, have been described such as NLRP1, NLRP3 and AIM2 ([Songane \*et al.\*, 2018](#)). The activation of caspase 1 depending of the adapter protein ASC is known as the canonical pathway of caspase activation ([Martinon \*et al.\*, 2002](#)). Nevertheless, the activation of caspase 11 and their human orthologous caspase 4 and 5 do not require ASC adaptor molecule, thus their activation is produced through the non-canonical inflammasome pathway ([Kayagaki \*et al.\*, 2011](#)). In fact, caspase 11 is an essential mediator of endotoxic shock acting as a sensor of cytosolic LPS during macrophage-mediated inflammatory response ([Hagar \*et al.\*, 2013](#)).

It has been demonstrated that caspase 8 can cleave the precursor of IL-1 $\beta$  ([Maelfait \*et al.\*, 2008](#)) and caspase 8 inflammasome is formed in dendritic cells in response to several pathogens ([Chen \*et al.\*, 2015](#); [Ganesan \*et al.\*, 2014](#); [Gringhuis \*et al.\*, 2012](#)). It is noteworthy that in some case caspase 8 inflammasome do not require pathogen internalization ([Gringhuis \*et al.\*, 2012](#)), which is essential for caspase 1 and 11-mediated inflammasomes. Moreover, caspase 2 has been implicated in inflammation signaling. Indeed, under ER stress, NLRP-3 inflammasome is recruited towards the mitochondria driving in caspase 2 activation and producing mitochondrial damage ([Bronner \*et al.\*, 2015](#)). Furthermore, caspase 3, 6 and 7 have been also involved modulating inflammation. For example, the release of caspase 6 by sensor neurons drives in microglial activation and TNF production leading to the induction of chronic pain ([Berta \*et al.\*, 2014](#)).



**Figure 19:** Canonical and non-canonical inflammasomes

The NLR and ALR members assemble canonical inflammasomes, where caspase-1 is the central mediator, while caspase-11 acts as cytosolic danger sensor in non-canonical inflammasome ([Lamkanfi and Dixit, 2014](#)).

Therefore, the simple classification of caspases in apoptotic and pro-inflammatory cannot be restrictive and completely absolute taking into account that all mammalian caspases are totally involved in inflammation and immunity ([Songane et al, 2018](#)).

It is noteworthy that caspases are implicated in the control of organism homeostasis by favoring the establishment and preservation of normal tissue architecture ([Galluzzi et al, 2016b](#)), through the regulation of inflammatory responses to

both microbial and neoplastic challenges ([Maltez et al, 2015](#)). However, in pathophysiological scenarios with a deregulation in the inflammatory response, an important role of pro-inflammatory caspases has been demonstrated. For example, a crucial role of pro-inflammatory caspases has been described in the pathogenesis of IBD ([Dupaul-Chicoine et al, 2010](#)). IBD is mediated by recurrent and chronic inflammation of the gastrointestinal tract manifested in two principal forms: Crohn's disease and ulcerative colitis ([Galgut et al, 2017](#)). In this context, increased levels of IL-1 $\beta$  and IL-18 pro-inflammatory cytokines, which are processed upon inflammasome-mediated caspase-1 activation, have been observed ([Becker et al, 2013](#)), indicating a role of caspase 1 in driving intestinal inflammation. Moreover, a positive correlation between the expression of caspase 4 and 5 with disease severity was found in ulcerative colitis patients ([Flood et al, 2015](#)), demonstrating that the enhanced expression of pro-inflammatory caspases is associated to IBD pathogenesis. Another example in the implication of pro-inflammatory caspases in inflammatory processes is the link between inflammasome-mediated caspase activation and the pathogenesis of several metabolic disorders such as type 2 diabetes, gout and obesity ([Wen et al, 2012](#)). On the other hand, pro-inflammatory caspases have been also related with epithelial cancers such as CRC. CRC is one of the most common solid cancers in humans and an inflammatory microenvironment has been postulated as essential for tumor progression ([Terzic et al, 2010](#)). Recently, a high expression of caspase 4 and 5 was observed in neoplastic epithelial intestinal cells ([Flood et al, 2015](#)), validating both caspases as possible therapeutic target for the treatment of CRC.

Although the pathological role of pro-inflammatory caspases is well documented in neoplastic diseases, IBD and metabolic disorders, few things are known about their role in psoriasis. Interestingly, an increased activity of caspase 1 has been described in lesional skin from psoriasis patients ([Marchetti et al, 2009](#); [Johansen et al, 2007](#)), which has postulated caspase 1 as a potential target in the treatment of psoriasis disease. Indeed, it was recently demonstrated that the suppressive effects of methotrexate in the immunopathogenesis of psoriasis is partially justified by the decrease levels in protein and mRNA expression of caspase 1 and IL-18 in treated patients ([Thirupathi et al, 2016](#)). The NLRP-3 inflammasome is activated in skin inflammatory diseases, and recently, it has been demonstrated that NLRP-3 deficient mice present reduced histological score after IMQ treatment through a decrease in IL-1 $\beta$  production ([Irrera et al, 2017](#)), confirming the role of this inflammasome in psoriasis. Moreover, the genetic ablation of caspase 1 and 11 in a mouse model of chronic proliferative dermatitis leads to a significant reduction in the skin inflammatory syndrome as well as a delayed onset in the phenotype ([Douglas et al, 2015](#)). The

mRNA expression of caspase 1, 4 and 5 has been demonstrated in psoriasis skin, but only caspase 5 was found up-regulated ([Salskov-Iversen et al, 2011](#)). Moreover, protein expression of caspase 5 has been detected in lesional psoriasis skin ([Zwicker et al, 2017](#)), although it is worth noting that it has been shown in only one patient; and polymorphisms in this caspase have been related with psoriasis susceptibility in the Han Chinese population ([He et al, 2015](#)). Furthermore, in response to inflammatory stress, keratinocytes are able to activate caspase 5 ([Zwicker et al, 2017](#)) and secrete IL-1 $\beta$  and IL-18 ([Goblos et al, 2016](#); [Feldmeyer et al, 2007](#)), suggesting that caspase activation inside these cells leading to secretion of pro-inflammatory cytokines IL-1 $\beta$  and IL-18 contributes to psoriasis development.

## IV. Psoriasis disease

### IV.I. Is it psoriasis an autoimmune disease or an autoinflammatory syndrome?

Autoimmune diseases are pathological conditions caused by an increase in the immunological response against the own cells and tissues in the body ([Lu, 2013](#)), characterized by the manifestation of a humoral and cellular response against the target tissue ([Leo et al, 2010](#)). Currently, although the specific etiology of the majority of autoimmune diseases remains to elucidate, a general consensus exists regarding the onset of these diseases, defined by the combination of genetic predisposition and environmental triggers ([Ngalamika et al, 2012](#); [Selmi et al, 2012a](#); [Selmi et al, 2012b](#)). Recently, it has been postulated that epigenetic regulation plays a key role in the development and pathogenesis of most of them ([Lu, 2013](#)).

Autoinflammatory diseases are a relatively new and expanding group of self-directed inflammatory disorders, clinically described as periodic fever syndromes but also with episodes of acute inexplicable inflammation involving the innate immune system ([Doria et al, 2012a](#); [Doria et al, 2012b](#); [Kastner et al, 2010](#)). They are characterized by inflammatory episodes at disease-prone sites, in the absence of autoreactive T cells and high autoantibody titers ([Goldbach-Mansky and Kastner, 2009](#)).

Autoimmune diseases and autoinflammatory diseases share common characteristics, such as self-tissue directed inflammation in the absence of an obvious infectious trigger or injury, while differences in primary players are observed. In fact in autoinflammatory diseases the innate immune system directly causes tissue inflammation, while in autoimmune diseases the innate immune system activates the adaptive system and this later activates the inflammatory process ([Arakelyan et al, 2017](#)).

Psoriasis has been considered as a mixed disease between autoimmune and autoinflammatory diseases, sharing the acquired component through major histocompatibility complex (MHC) class I associations and autoinflammatory components ([McGonagle and McDermott, 2006](#)). However, it is worth to note that psoriasis has been never classified as an autoinflammatory disease ([Murthy and Leslie, 2016](#)), but as an autoimmune disease ([Carter and Zhao, 2010](#)), although not all the scientific community has considered this skin pathology as an autoimmune disease because no auto-antigen and no self-reactive T cells have been identified ([Fry et al, 2015](#)). Nevertheless, for those who reinforce the idea to present psoriasis as an

autoimmune disease, two theories have been evidenced. Firstly, the postulate of molecular mimicry ([Besgen et al, 2010](#)), justified by the homology found between streptococcal and keratin peptides, which are equally recognized by CD8 T cells in psoriasis patients ([Valdimarsson et al, 2009](#)), and second, the hypothesis that psoriasis is triggered by bacterial microbiota in the skin ([Fry et al, 2013](#)), which has been denoted by the fact that a high expression of TLR2 in psoriasis keratinocytes has been observed ([Baker et al, 2003](#)). This TLR is involved in the recognition of peptidoglycans from Gram-positive bacteria ([Lien et al, 1999](#)) and moreover, streptococcal and staphylococcal peptidoglycans have been found in antigen-presenting cells from psoriasis patients ([Baker et al, 2006](#)).

Therefore, actually, psoriasis is considered as a chronic immune-mediated skin disease that shares altered signaling pathways with other autoimmune diseases such as type I diabetes, SLE and rheumatoid arthritis ([Ayala-Fontanez et al, 2016](#)).

## IV.II. General characteristics of psoriasis disease

Its name itself comes from the Greek “psora” meaning itch or prickle and although it is a known disease since antiquity, it was not until 19<sup>th</sup> century that the establishment of psoriasis as a disease entity occurred; producing its definitive separation from leprosy and finishing ending hundreds of years of discussion about this topic ([Nestle et al, 2009](#)).

Psoriasis is a skin disease characterized by an aberrant proliferation of keratinocytes and an abnormal immune cell infiltration into the skin, whose etiology is unknown and its course is completely unpredictable ([Ogawa et al, 2018](#); [Sabat et al, 2007](#)). It is a very common skin pathology whose prevalence worldwide is 2-3 % ([Danielsen et al, 2013](#)). It is worth noting that in continental American native populations and in Australian aborigines psoriasis has never been reported, while in northern regions of Europe can reach a 7% of prevalence ([Raychaudhuri and Farber, 2001](#)), being in this way, the most widespread immune-mediated skin pathology in adults. Men and women are equally affected ([Gelfand et al, 2005](#)), albeit women manifest a more severe pattern of the disease ([Lebwohl, 2003](#)), like it was also demonstrated in females cLyn mice ([Marchetti et al, 2009](#)).

Psoriasis disease has been associated with the development of several comorbidities. Indeed, psoriasis patients can present cardiovascular diseases, hypertension, and obesity as well as type 2 diabetes, metabolic syndrome, Crohn's disease (**Table 9**) ([Brooks, 2018](#); [Furue et al, 2018](#); [Vena et al, 2010b](#); [Vena et al, 2010a](#)). Moreover, psoriasis patients can develop a disorder causing joint pain and

inflammation called psoriasis arthritis, whose prevalence is around 10% from all cases of psoriasis ([Boutet et al, 2018](#); [Schon and Boehncke, 2005](#)). This skin pathology imposes physical and social burdens that extend beyond of dermatological symptoms and interferes with daily performance, which negatively impact family and social relationship ([Patel et al, 2018](#); [Eghlileb et al, 2007](#)), leading to a high incidence of depression ([Poot, 2017](#); [Zieciak et al, 2017](#)) and increased tendencies to suicide ([Prabhakar et al, 2018](#); [Singh et al, 2017b](#)).

A small increased risk of solid cancers has been linked to psoriasis patients, mainly in those patients with high incidence in smoking and alcohol dinking. However a high risk to develop squamous cell carcinoma has been found in those patients previously treated with 8-methoxypsoralen plus ultraviolet light A (PUVA) ([Pouplard et al, 2013](#)). Some recent studies are indicating an association between the exposure to TNF inhibitors and the risk to develop non melanoma skin cancers ([Peleva et al, 2018](#)), suggesting that long-term pharmacovigilance is still required to establish a correlation between biological therapies and cancer development in psoriasis patients.

Classification of psoriasis comorbidities	
Clinical criteria	Comorbidities
Common diseases	Psoriasis arthritis    Crohn disease
Activation of cutaneous T cells	Cutaneous T cell lymphoma
Chronic/Systemic skin inflammation	Metabolic syndrome, Atherogenic dyslipidemia, Hypertension, Abdominal obesity, Diabetes and insulin resistance, predisposition to thrombosis, nonalcoholic hepatic steatosis
Impaired in quality of life	Anxiety, Depression, Smoking, Alcoholism
Related to treatment	Nephrotoxicity, Hepatotoxicity, Dyslipidemia, Skin cancer

**Table 9:** Classification of psoriasis comorbidities

A summarized list of the main associated comorbidities observed in psoriasis patients ([Grozdev et al, 2014](#)).

#### IV.III. Genetic susceptibility of psoriasis disease

Although the inheritance pattern in psoriasis disease has not been proven for all psoriasis cases ([Hebert et al, 2012](#)), a strong genetic background has been demonstrated. In fact, in monozygotic twins the rate of concordance is around 70% and in dizygotic twins is approximatively 20% ([Capon, 2017](#); [Bowcock, 2005](#)), showing an

involvement of genetic factors in psoriasis development. Indeed, by genome-wide association studies (GWAS), about 30 single nucleotide polymorphisms (SNPs) have been associated with a risk to develop psoriasis and at least 12 major psoriasis susceptibility (PSORS) loci have been identified, although only two gene mutations have been observed to induce psoriasis (IL36RN and CARD14) ([Lowes et al, 2014](#)).

PSORS1 was the first associated psoriasis susceptibility loci observed and it is located on chromosome 6p21, in the MHC class-1, encoding for human leukocytes antigens (HLA). HLACw6 is the haplotype more related with psoriasis susceptibility ([Stuart et al, 2002](#)) and it has been demonstrated that more than 60% of psoriasis patients bring along the HLACw0602 haplotype, conferring them a 20-fold increased risk to develop psoriasis ([Chen and Tsai, 2017](#); [Mallon et al, 1999](#)). Moreover, a gain-of-function mutation in PSORS2 or Caspase recruitment domain-containing protein (CARD)14 was found in this locus ([Van Nuffel et al, 2017](#); [Jordan et al, 2012a](#)), which should cause psoriasis by the increased induction of NF-κB signaling pathway leading to an increased expression of different chemokines involved in psoriasis development ([Lowes et al, 2014](#)). Nevertheless, although this mutation is present, an environmental trigger is needed to initiate the psoriasis disease process ([Jordan et al, 2012b](#)) and in CARD14 deficient mice an abrogation of induced psoriasis-like phenotype is obtained ([Tanaka et al, 2018](#)), indicating the key role of this psoriasis-susceptibly gene in the skin pathology. IL36RN is a gene encoding for the anti-inflammatory protein IL-36 receptor antagonist (IL36Ra) and mutations in this gene has been associated with a loss of protein activity and increased neutrophil infiltration in psoriasis patients ([Lowes et al, 2013](#)). SNPs identified in psoriasis patients have been associated with the IL-23/IL-17 axis ([Duffin and Krueger, 2009](#)), which have been involved in several immunological processes related with psoriasis pathogenesis such as keratinocytes differentiation, T cell proliferation, cytokines responses, among others ([Tsoi et al, 2012](#)). In **table 10** a summarized list of genetic loci found to be associated with psoriasis development is presented.

Furthermore, epigenetic alterations have been linked to psoriasis, in particular DNA methylation and histone modifications. In fact, a higher expression of DNA methyltransferases was observed in peripheral mononuclear cells (PBMC) from psoriasis patients compared to healthy donors ([Zhang et al, 2010](#)) as well as a general histone H4 hypoacetylation, which was negatively correlated with the disease activity ([Trowbridge and Pittelkow, 2014](#)). Moreover, several microRNA have been found modulated compared to healthy donors, showing the implication of these non-coding RNA in psoriasis pathogenesis. Indeed, an over-expression of miR-203 was found in

patient skin, leading to a negative regulation of STAT3 pathway ([Sunkoly et al, 2007](#)). In the same way, miR-146, which targets TNF- $\alpha$  regulators like IRAK1 and TRAF6, was described upregulated in psoriasis skin ([Shams et al, 2018](#); [Sunkoly et al, 2007](#)), but miR-125, a suppressor of keratinocyte proliferation, was seem downregulated ([Xu et al, 2011](#)); validating the role of microRNA modulating keratinocyte proliferation in psoriasis skin.

Genetic associations reported in Psoriasis		
Genetic locus identified	Predicted candidates Genes associated	Variant ID
6p21.33 (PSORS1)	HLA-Bw7, DR4, DR7, HLA-A1, HLA-B13, B17, B27, B39, B57, HLA-Cw6, Cw7, PSORS1C3, CCHCR1, CDSN, POU5F1, CDKAL1, IRF4	rs1265181, rs720465, rs130065, rs3130455, rs3130457, rs1265159, rs10484554, rs10484554-rs130065, rs6908425
17q25.3 (PSORS2)	NAT9, SLC9A3R1, RAPTOR, CMRF35A1, CMRF35A2-6, CMRF35H, CARD14, STAT3	rs734232, rs11652075, rs2019154
4q (PSORS3)	IL2, IL21	rs2282276
1q21 (PSORS4)	LOR, S100A8, S100A9, PGLYRP3, PGLYRP4, LCE3A, LCE3B/3C deletion, LCE3D, LCE3E, IVL, FLG	rs4112788, rs4112788, rs4085613, rs4845454, rs1886734, rs512208
3q21 (PSORS5)	CSTA, SLC12A8	rs9813946, rs9831295, rs1554241, rs651630
19p13 (PSORS6)	Jun-B, ILF3, BSG	rs8259
1p (PSORS7)	EPS15, IL23R, PTPN22, RUNX3, TNFRSF9	rs11209026, rs1217414, rs7536201
16q (PSORS8)	NOD2, CARD15, CYLD	rs2076752, rs2066844, rs12925755
4q31-34 (PSORS9)	IL15	rs10519613, rs2322301
18p11.23 (PSORS10)		
5q31.1-q33.1 (PSORS11)	IL12B, IL13, TNIP1	rs3213094, rs20541, rs1024995
20q13 (PSORS12)	RNF114, SPATA2, ZNF313	rs2235617, rs495337
6q21 (PSORS13)	TRAF3IP2, TAGAP	rs240993, rs33980500

**Table 10:** Genetic-associated loci related with psoriasis development

A summarized list of the principal genetics associations reported in psoriasis patients ([Chandra et al, 2015](#)).

#### IV.IV. Environmental triggers of psoriasis disease

Various are the environmental factors that have been associated with a predisposition toward psoriasis disease development such as physical trauma, drug reactivity, psychological stress and smoking ([Ayala-Fontanez et al, 2016](#)). Physical trauma was the first reported environmental factor to trigger psoriasis. Studies from Heinrich Koebner demonstrated that after skin injury, psoriasis lesions emerged in previously normal-appearing skin ([Sagi and Trau, 2011](#)). Currently, it has been accepted that Koebner response in psoriasis patients has prevalence about 30% ([Ladizinski et al, 2013](#)), although psoriasis development after skin injury may be dependent on the season of the year, being more common in winter. This aspect was also dependent from pre-existing disease because in psoriasis patients, Koebner's trauma is an exacerbating factor of psoriasis ([Ganguly et al, 2018](#); [Kalayciyan et al, 2007](#)). Several drugs have been associated with psoriasis development and its exacerbation ([Gupta et al, 2014](#); [O'Brien and Koo, 2006](#)). The most usually described drugs to induced psoriasis disease are lithium, beta-blockers, tetracycline, anti-malarials and non-steroidal anti-inflammatory medications; although today, TNF- $\alpha$  blockers ([Denadai et al, 2013](#)), IL-6R blockers ([Grasland et al, 2013](#)) and medications against INF- $\alpha$ , - $\beta$ , - $\gamma$  ([Basavaraj et al, 2010](#)) have been also shown to trigger psoriasis onset. Moreover, imiquimod, a ligand for TLR 7/8, which is used in clinical for the topical treatment of genital and perineal warts, has been reported to induced or exacerbate psoriasis disease ([Fanti et al, 2006](#); [Gilliet et al, 2004](#); [Geisse et al, 2002](#)). Infections by microorganisms have been also reported as psoriasis triggers. In fact, *Streptococcus pyogenes* infection has been linked to psoriasis onset and exacerbation of chronic disease ([Naldi et al, 2001](#)) and *Staphylococcus aureus*, *Helicobacter pylori* and *Candida albicans* gave the same potentiation ([Campanati et al, 2015](#); [Sigurdardottir et al, 2013](#); [Onsun et al, 2012](#)). The relationship between pathogen colonization and psoriasis development is given by the release of exotoxins and peptidoglycan from these microorganisms, which can activate T cells producing an abnormal immunological response ([Baker et al, 2006](#)). Possibly, stress is the most common trigger known by the worldwide population to aggravate psoriasis disease ([Snast et al, 2017](#)), which is explained by increased level expression of hormone stress called Corticotrophin-release hormone (CRH) that have been reported in psoriasis skin ([Weigl, 2000](#)). Although the mechanism through which this hormone can modulate inflammatory response is not yet clear, CRH can act on keratinocytes to stimulate IL-6 production and exacerbate psoriasis in this way ([Zbytek et al, 2002](#)). Finally, another environmental trigger is smoking ([Nguyen et al, 2018](#)). Indeed, one study showed that

the risk to develop psoriasis was higher in current smokers than in individuals who have never smoked ([Naldi et al, 2005](#)). Furthermore, combination of genetic susceptibility and smoking increase the risk of psoriasis onset. Presence of HLACw6 haplotype in smoker individuals produces 11-fold increased risk of psoriasis susceptibility compared to non-smokers without this haplotype ([Jin et al, 2009](#)).

In general, although several studies evidenced the present understanding of genetic predisposition and environmental factors with psoriasis disease development, more studies are needed to fully demonstrate the direct relationship between these factors and psoriasis onset.

#### IV.V. Clinical classification of psoriasis

Because no diagnostic criteria have been described to diagnose psoriasis disease, only a detailed morphologic assessment of a skin lesion is the clinical pattern to diagnose a psoriasis patient. Thus, taking into account this morphological classification, psoriasis has been divided into several clinical phenotypes, which are exemplified in **table 11** ([Raychaudhuri et al, 2014](#)).

Clinical classification of psoriasis disease	
Clinical criteria	Clinical phenotypes
Age of onset	Type I: before 40 years, type II: after 40 years
Degree of severity (%BSA)	Mild (<5%), moderate (5-10%), severe (>10%)
Pattern of distribution	Localized psoriasis / widespread psoriasis Inverse, flexor, seborrheic
Morphology	Plaque, guttate, erythrodermic, pustular, rupioid, elephantine
Anatomical site	Scalp, palmoplantar, genital, nail, anal
Stage of development	Stable plaque, unstable eruptive plaque

**Table 11:** Clinical classification of psoriasis disease

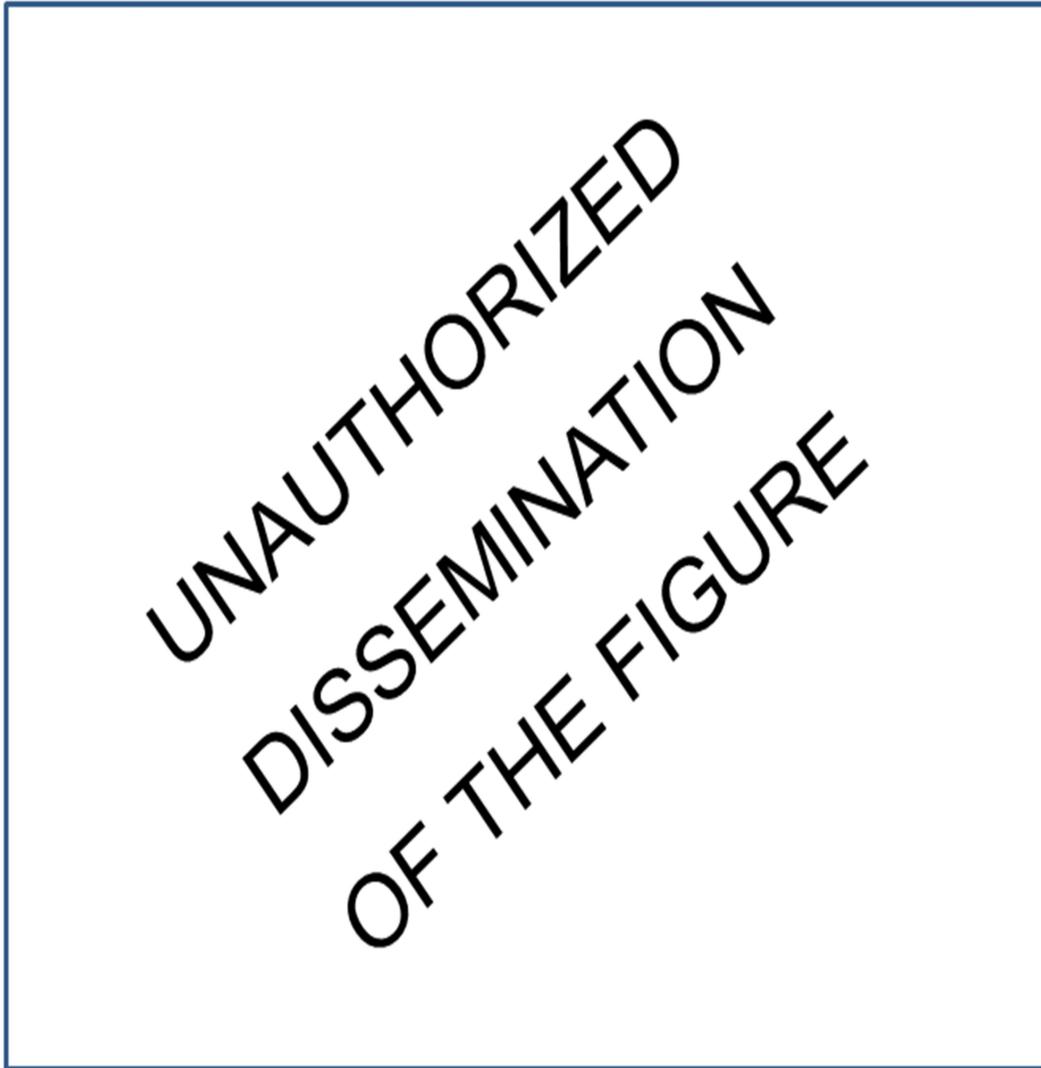
According to several clinical criteria, psoriasis can be classified in different subtypes. BSA: body surface area ([Raychaudhuri et al, 2014](#)).

Plaque psoriasis is the most common manifestation of psoriasis disease, affecting almost 90% of all psoriasis cases described worldwide ([Raychaudhuri et al, 2014](#); [Levine and Gottlieb, 2009](#)) and is commonly known as psoriasis vulgaris. Lesions, which are generally symmetric, are characterized by chronic erythematous and scaly plaque, which are well demarcated and covered with silvery scales ([Lebwohl,](#)

[2003](#)). These lesions can appear in any part of the body, but generally arise over elbows, knees, umbilicus, lumbosacral region and scalp, being the last one the most common site of involvement ([Griffiths and Barker, 2007](#); [Raychaudhuri and Gross, 2000](#)).

#### IV.VI. Histological features of psoriasis

Three are the principal characteristics of lesional skin in psoriasis disease observed by hematoxylin and eosin staining: i) epidermal hyperplasia, ii) dilated and prominent blood vessels in the dermis and iii) epidermal and dermal infiltration of immune cells. In non-lesional skin, histology is like in normal skin ([Ogawa \*et al.\*, 2018](#); [Griffiths and Barker, 2007](#)) (**Figure 20**). The significant thickness of epidermis is called acanthosis and is histologically characterized by elongated rete ridges, which is a product of the marked movement of keratinocytes through the epidermis, which is reduced to 4-5 days producing a tenfold acceleration compared to normal skin ([Lowes \*et al.\*, 2014](#)). In this process, an underexpression of keratinocytes differentiation markers (keratins K1 and K10) is found, leading to the loss of the normal granular layer. Moreover, a thickened stratum corneum called hyperkeratosis and a retention of nuclei in the upper layers and stratum corneum, which causes squamous cell layer thickening denominated parakeratosis, are seen. All these factors (acanthosis, hyperkeratosis and parakeratosis) lead to the production of desquamation (scaling) in the psoriasis plaque. Further, an increases K16 staining, marker of basal and non-differentiated keratinocytes is observed throughout the epidermis. The increased vascularity in the dermis is because of the production of angiogenic factors by keratinocytes, like the vascular endothelial growth factor (VEGF), leading to an abnormal dermal vascular proliferation and angiogenesis, which is the basis for the redness (erythema) of psoriasis lesions. The increased inflammatory cell infiltrate is composed of neutrophils (Kogoj pustules and Munro's microabscesses) in the stratum corneum and epidermis, mononuclear cells in the epidermis and mostly T, B and myeloid cells in the dermis ([Ayala-Fontanez \*et al.\*, 2016](#); [Boehncke and Schon, 2015](#); [Lowes \*et al.\*, 2014](#); [Griffiths and Barker, 2007](#); [Lowes \*et al.\*, 2007](#)).



**Figure 20:** Histological features of psoriasis disease

- A. Keratin 16 stains basal epidermis in non-lesional skin but stains almost all full epidermis in lesional skin.
- B. Hematoxylin and eosin of non-lesional and lesional skin at the magnification. Lesional psoriasis exhibits a greatly thickened epidermis (acanthosis) with elongation into the dermis (rete ridges). Retention of the nuclei (parakeratosis) can be seen in the thickened stratum corneum.
- C. Increased numbers of CD3+ T cells in lesional psoriasis skin.
- D. Accumulation of neutrophils in the epidermis (Munro microabscesses) (white arrow). Infiltration of mononuclear cells in the epidermis and dermis (black arrowhead) ([Greb et al, 2016](#); [Lowes et al, 2014](#)).

#### **IV.VII. Clinical disease assessment**

In a clinical research point of view, psoriasis severity is focused in clinical signs such as area of involvement, degree of scaling (desquamation), erythema (redness) and induration (thickness) of the skin ([Finlay, 2005](#)). To analyze the area of

involvement or the percentage of the body surface area (BSA) affected by psoriasis, the area of the palm of the hand (handprint) is taken as 1% of the body ([Lowes et al., 2014](#)). To assess the parameters of severity (scaling, erythema and induration) a scale from 0 (no symptoms) to 4 or 5 (very marked symptoms), depending on the score system, has been established. Although several scoring systems have been proved ([Weisman et al., 2003](#)), two are the major scoring systems to classify the severity of psoriasis in patients: the psoriasis area and severity index (PASI) and the physician's global assessment (PGA) ([Ayala-Fontanez et al., 2016](#)). The PASI scale quantifies the extent of psoriasis disease as a degree of area (BSA) and intensity, from 0 to 4, of scaling, erythema and induration ([Finlay, 2005](#)), oscillating between 0 (no psoriasis) and 72 (the highest score reached). Taking into account the complicated nature of PASI estimation, PGA score was introduced as an alternative measure for clinical evaluation of patients. In PGA score system, the degree of BSA and intensity of scaling, erythema and induration is measured through a score from 0 (no psoriasis) to 5 (more severe disease) ([Langley and Ellis, 2004](#)). However, the PASI score is the most used score to assess the response to a therapy and albeit the absolute PASI score is frequently used to define severity in evaluated patients, PASI score is often used to define response to treatment. For example, for a patient to be included in a clinical trial a PASI score of 10 or greater is needed and this value is assigned as a baseline PASI score. However, after treatment evaluations of the PASI score are made at different time points and then improvement is calculated. Thus, PASI75 or PASI50 mean an improvement of 75% or 50%, respectively, compared to the baseline assigned PASI score. In general, to consider a therapy as clinically relevant a PASI75 is needed in almost all treated patients ([Feldman and Krueger, 2005](#)).

#### **IV.VIII. Psoriasis pathogenesis**

Until the 1990s, psoriasis was considered a disease produced by the high rate of proliferation and disorganized differentiation of keratinocytes, because the hyperplasia of the epidermis displayed the most obvious clinical and histological characteristics of the disease ([Bowcock and Krueger, 2005](#)). More recent evidences indicated that changes in the epidermis occur in response to immune cell infiltration, thus at present, psoriasis is recognized as an immune-mediated inflammatory disease caused by the inappropriate activation of immune cells and the aberrant proliferation of keratinocytes ([Ogawa et al., 2018](#)).

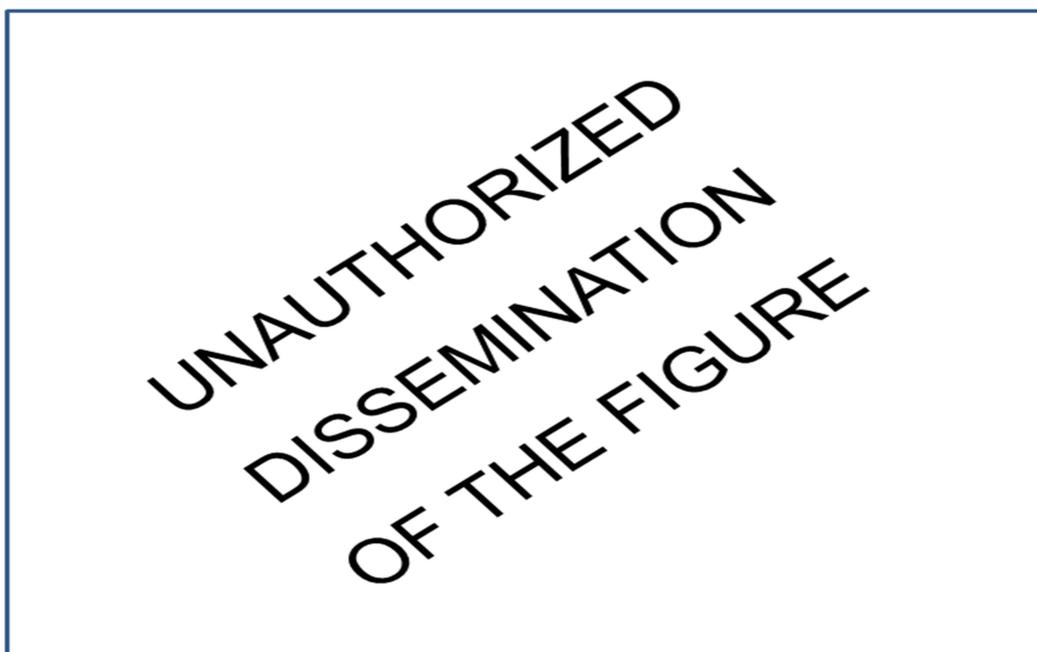
The administration to psoriasis patients of the fusion protein known as IL-2R $\alpha$ + (IL-2 plus diphtheria toxin), which resulted in decreased lymphocyte infiltration in skin biopsies and clinical remission ([Gottlieb et al., 1995](#)), was the first evidence that T cells

induced hyperplasia of epidermal keratinocytes and vascular alterations in skin lesions. The administration of another fusion protein, the antigen 4 of the cytotoxic T lymphocytes (CTLA-4) linked to the Fc portion of a human IgG, demonstrated that a continuous co-stimulation of T-cells was necessary to sustain the activity of the disease, which included excessive infiltration of dendritic cells and T cells into the skin ([Abrams et al, 1999](#)). Generally, the physiopathology of psoriasis disease is given by an abnormal activation and infiltration of T cells, which release cytokines that attract other immune cells and thus perpetuate the inflammatory cascade, which leads to inflammation and excessive production of keratinocytes, developing the typical psoriasis plaques ([Prinz, 2003](#)). Going further in details, dermal dendritic cells are activated, which produce release of pro-inflammatory cytokines such as TNF- $\alpha$ , IL-12, IL-6, TGF- $\beta$  and IL-23, leading to the activation of CD4+ and CD8+ cells ([Di Meglio et al, 2016](#); [Hijnen et al, 2013](#)). Upon T cell activation, they proliferate and migrate into the epidermis, where they interact with keratinocytes through the secretion of IL-17 and IL-22 ([Cheuk et al, 2014](#)). Moreover, T cells can recognize epidermal autoantigens such as keratin 7 or the antimicrobial peptide LL37 expressed by keratinocytes ([Lande et al, 2014](#)) driving to the epidermal hyperplasia and keratinocyte activation to secrete cytokines, chemokines and antimicrobial peptides, which produces the disease development and the maintaining of the inflammatory phenotype ([Conrad and Gilliet, 2018](#)). Therefore, psoriasis disease can be divided in two phases, the first one is the initiation phase, which a close interaction between external factors and genetic alterations that lead to the development of the phenotype. The second one is the chronic inflammatory phase characterized by recruitment and activation of inflammatory cells, which contribute to the amplification of skin phenotype ([Mahil et al, 2016](#)) (**Figure 21**).

#### **a) Principal cell types involved in psoriasis development**

The role of T cells in psoriasis has been well demonstrated, being the crucial immune cell population in psoriasis pathogenesis. In human lesional skin as well as in the bloodstream CD4+ and CD8+ T cell population are highly increased compared to healthy donors ([Hijnen et al, 2013](#)). They express chemokines receptors such as CCR6, CCR4 as well as CXCCR3, penetrating in the skin through interaction with endothelial cells expressing adhesion molecules such as P- and E-selectins ([Chiricozzi et al, 2018](#)). Different Th patterns and their counterparts T cytotoxic have been identified in psoriasis skin. In fact, three are the most relevant Th patterns described in psoriasis: Th1, Th17 and Th22, which secrete IFN- $\gamma$ /TNF- $\alpha$ , IL-22/IL-17 and IL-22, respectively ([Amatya et al, 2017](#); [Kagami et al, 2010](#)) and the continuous exposure to

IL-23 led to the proliferation of mature T cells ([Gaffen et al, 2014](#)). However, other patterns like Th2, Th9 and T regulatory cells have been also described in psoriasis pathogenicity ([Harden et al, 2015](#)). Furthermore, a significant increase of  $\gamma\delta$  T cells is found in lesional skin compared to healthy donors, and most of the IL-17 found in the skin is produced by these cells ([Mabuchi et al, 2011](#)), describing the essential function of this cell population in psoriasis pathogenesis ([O'Brien and Born, 2015](#)).



**Figure 21:** Inflammatory pathways in both phases of psoriasis

Psoriasis is mediated by the aberrant activation of dermal dendritic cells (light blue) producing TNF and IL-23 cytokines. These dendritic cells stimulate autoimmune CD4 (Th17) and CD8 (Tc17) cells to migrate into the epidermis, where they recognize epidermal autoantigens and produce Th17 cytokines IL-17 and IL-22. Th17 cytokines trigger the epidermal phenotype of plaque psoriasis, characterized by an abnormal keratinocyte hyperproliferation and the activation of keratinocytes to produce antimicrobial peptides and chemokines. **The TNF-IL-23-Th17 pathway** appears to be central in the pathogenesis of chronic plaque psoriasis (central portion of the figure). Another inflammatory pathway, mediated by plasmacytoid dendritic cells producing large amounts of type I IFNs, has emerged by studying early events in the pathogenesis of psoriasis. **The plasmacytoid dendritic cells-IFN pathway** is dominant in acute forms of psoriasis such as erythrodermic psoriasis (left part of the figure). Finally, analysis of pustular psoriasis has revealed that neutrophil infiltration triggered by IL17 is central for the cleavage and activity of IL-36, which in turn induces IL-1 production by dendritic cells and further stimulates Th17 polarization. **The IL-36-IL-1 pathway** appears to be dominant in pustular psoriasis (right part of the figure). ([Conrad and Gilliet, 2018](#)).

B cells have been also involved in psoriasis development although their role in this pathology is not completely understood. Indeed, regulatory B cell population is numerically decreased in psoriasis patients and no production of the anti-inflammatory

cytokine IL-10 by this cell population has been observed ([Mavropoulos et al, 2016](#)). Moreover, the number of splenic regulatory B cells have been found reduced in mouse models of psoriasis-like phenotype ([Yanaba et al, 2013](#)), and the treatment of psoriasis patients with recombinant IL-10 protein has showed an improvement in the skin pathology ([McInnes et al, 2001](#)) with a significant decrease in macrophages and T cells infiltration, indicating that the diminution of B regulatory cells is fundamental in psoriasis.

Dendritic cells are crucial cells in the secretion of pro-inflammatory cytokines and chemokines in the early stages of psoriasis and two main populations have been reported in the pathology: plasmacytoid and myeloid dendritic cells; principally localized in the dermis ([Chiricozzi et al, 2018](#)). In fact, the inhibition of plasmacytoid dendritic cells signalization blocks T cell activation and prevents psoriasis development ([Capon et al, 2002](#)). Langerhans cells are another kind of dendritic cells closely related with keratinocytes, but although these cells are able to present antigen to T cells, their role in psoriasis pathogenesis is still unclear.

Neutrophils are very important in the early stages of psoriasis and have been identified as a key factor in the recruitment of T cells and keratinocyte proliferation ([Reich et al, 2015](#)). Antimicrobial peptides are secreted by activated neutrophils as neutrophil extracellular traps, which have been found in lesional psoriasis skin biopsies ([Lin et al, 2011](#)) and they are able to release elastase, which induce keratinocyte proliferation and cytokine activation ([Meyer-Hoffert et al, 2004](#)).

Other important populations in psoriasis pathogenesis are macrophages, mast cells as well as NK and NKT cells. The role of the first one has not been well characterized yet, although CD163+ macrophages have been described in lesional skin and activated macrophages secrete TNF- $\alpha$  and regulate angiogenesis through the release of VEGF ([Fuentes-Duculan et al, 2010](#)). Mast cells are known to infiltrate lesional skin in the early phases of psoriasis contributing to the T cell-mediated skin inflammation by the production of IL-1 $\beta$  ([Bonnekoh et al, 2018](#)). In the other hand, NK cells play a role in psoriasis because they secrete IFN- $\gamma$ , TNF- $\alpha$  and IL-22 ([Dunphy and Gardiner, 2011](#)). NKT cells have been associated with the release of IFN- $\gamma$  and CD1d, an invariant stimulator of NKT cells, is highly expressed in the epidermis of psoriasis patients ([Bonish et al, 2000](#)).

## **b) Principal cytokines implicated in psoriasis pathogenesis**

Pro-inflammatory cytokines are pivotal mediators in psoriasis development and several studies have been made to analyze the effect of each cytokine in this disease.

In fact, this skin pathology has been considered as an inflammatory triangle conceived by the action of TNF, type I IFN and IL-17 ([Grine et al, 2015](#)) (**Figure 22**).



**Figure 22:** Inflammatory triangle in psoriasis pathogenesis

Overactivation of TNF can lead to inflammation and synergizing with IL-17, they enhance neutrophil recruitment. IL-17 mediates psoriatic inflammation acting, directly or indirectly, on keratinocytes. Plasmacytoid dendritic cell-derived type I IFNs initiate psoriasis and is normally silenced by TNF. Type I IFN can act on keratinocytes indirectly together with IL-17 through increase of IL-22 receptor ([Grine et al, 2015](#)).

TNF- $\alpha$  was the first cytokine to be targeted in psoriasis treatment. Increased levels of this cytokine have been described in lesional skin and serum from psoriasis patients ([Arican et al, 2005](#)) and the production of this pro-inflammatory mediator is mediated by keratinocytes, T cells and dendritic cells ([Kim et al, 2014](#); [Mabuchi et al, 2012](#); [Chu et al, 2011](#)). Nowadays it is considered an upstream cytokine in the IL-23/IL-17 pathways acting as inducer of IL-23 production by dendritic cells ([Zaba et al, 2009a](#); [Zaba et al, 2009b](#)).

IFN- $\alpha$  is produced by plasmacytoid dendritic cells activating myeloid dendritic cells to produce IL-12, IL-15, IL-18 and IL-23 ([Lande and Gilliet, 2010](#)), being considered one of the principal initiators of skin inflammation acting as an upstream cytokine along the IL-23/IL-17 axis. IFN- $\alpha$ -induced genes are upregulated in psoriasis

skin compared to non-lesional skin and healthy donors, and its neutralizations prevents the spontaneous development of psoriasis lesions in mice xenotransplanted with non-lesional skin obtained from psoriasis patients ([Nestle et al, 2005](#)). High IFN- $\gamma$  levels have been detected in lesional skin correlating with disease severity ([Johnson-Huang et al, 2010](#)). In vitro stimulation of keratinocytes with this cytokine alters the expression of around 1200 genes, although the transcriptome modulated by this cytokine is weak in comparison with those regulated by IL-17 ([Chiricozzi et al, 2014](#)).

IL-17A is the most known member of the IL-17 family, whose concentration has been observed strongly increased in psoriasis patients correlating with the high number of circulating IL-17-producing cells in this disease ([Chiricozzi et al, 2016a](#); [Chiricozzi et al, 2016b](#)). Pro-inflammatory effects from this cytokine are mainly exerted on keratinocytes leading to the activation and proliferation of these cells, but also IL-17 induces the production of IL-19, a cytokine that once secreted by keratinocytes drives in positive feedback for these cells ([Witte et al, 2014](#)). The most relevant proof of the crucial role of IL-17 in psoriasis pathogenesis is given by the strong improvement achieved with IL-17 blockers in psoriasis patients ([Conrad and Gilliet, 2018](#)) reverting clinical, histological and molecular features of this disease.

IL-22 receptor and IL-22 cytokine levels are increased in psoriasis epidermis and effects of this cytokine are mainly directed to keratinocytes leading to migration, inhibition of differentiation and inducing chemokines production, resulting in epidermal thickness ([Mashiko et al, 2015](#); [Sa et al, 2007](#)). Indeed, blocking IL-22 has resulted in a significant decrease in the induction of psoriasis-like phenotype ([Van Belle et al, 2012](#)) and its pro-inflammatory activity is enhanced by the synergism with IL-17 and TNF- $\alpha$  ([Tohyama et al, 2009](#)). However, the development of an IL-22-neutralizing antibody was discontinued ([Gottlieb, 2005](#)), demonstrating that pathological effects of this cytokine are more relevant in mouse model than in psoriasis patients.

IL-23 is the key effector cytokine in the IL-17 signaling pathway ([Chiricozzi et al, 2018](#)). A large amount of cells in psoriasis disease are able to secrete IL-23 pro-inflammatory cytokine such as keratinocytes, dendritic cells and macrophages ([McGeachy et al, 2007](#)), being myeloid cells the principal source of this cytokine. IL-23 contributes to the differentiation, survival and expansion of IL-17-producing T cells ([Chiricozzi et al, 2014](#)) and lesional psoriasis samples have shown increased levels of IL-23p19 compared to non-lesional skin ([Sofen et al, 2014](#)), which has been associated with a marked infiltration of myeloid dendritic cells ([Zaba et al, 2009a](#)). In fact, in IL-23p19 deficient mice a fully protection to develop psoriasis-like phenotype has been shown ([van der Fits et al, 2009](#)) and the clinical improvement obtained in psoriasis

patients treated with IL-23 neutralizing antibodies ([Fotiadou et al, 2018](#)) have demonstrated the importance of this cytokine in psoriasis pathogenesis.

Other cytokines such as IL-1 $\beta$  and IL-18 are taken in the last years a prominent role in psoriasis development ([discussed in ¡Error! No se encuentra el origen de la referencia.](#)), indicating that the development of psoriasis phenotype is the outcome of multiple cytokine-mediated signaling pathways.

#### IV.IX. Keratinocytes and psoriasis

Keratinocytes, the principal cells in the epidermis, are crucial in both early stages and chronic phase of psoriasis disease. Indeed psoriasis pathogenesis is mediated by a keratinocyte-activation induced epidermal hyperplasia and cytokine-mediated inflammation, which in part, are released by these epidermis cells ([Sun et al, 2017](#)).

Antimicrobial peptides are released by keratinocytes and have a crucial role in psoriasis pathogenesis because they are induced by the IL-17 signaling pathway in psoriasis pathogenesis ([Morizane and Gallo, 2012](#)) and led to the secretion of pro-inflammatory cytokines and chemokines such as IL-6, IL-8 and CXCL10, mediating the recruitment of macrophages and neutrophils ([Gambichler et al, 2011](#)). In fact, LL37 complexed to self-DNA or RNA can be also recognized by myeloid dendritic cells via TLR7 and TLR8, leading to the dendritic cell maturation and to the secretion of TNF- $\alpha$  and IL-6 ([Lande et al, 2014](#); [Ganguly et al, 2009](#)); which supports the effect of the TLR7/8 agonist imiquimod inducing a psoriasis-like phenotype in mice. Furthermore S100 calcium binding proteins such as S100A12 (calgranulin C), S100A7 (psoriasin), S100A8, S100A9 and S100A15 play a major inflammatory role in psoriasis because they can activate TLR ([Son et al, 2016](#); [Wilsmann-Theis et al, 2016](#); [Vogl et al, 2007](#)) and together with the  $\beta$ -defensins act as chemotactic proteins for the immune cell infiltration leading to the amplification of the immune response ([Kolbinger et al, 2017](#); [Wolf et al, 2008](#)).

Keratinocytes secrete IL-1 $\beta$  and IL-18, two cytokines activated through the activation of inflammatory caspase 1 in multi-protein complexes called inflammasomes ([Lamkanfi and Dixit, 2014](#)), being keratinocytes immunocompetent cells. IL-1 $\beta$  and IL-18 are implicated in the differentiation of Th17 and Th1 cells, and once differentiated these cells form a positive feedback loop, leading to the secretion of more cytokines (IL-17, IL-22) and to the proliferation and activation of other keratinocytes ([Chung et al, 2009](#)). In fact, keratinocytes are the main cell type expressing IL-17R in the skin ([Nogales et al, 2008](#)), and the combined stimulation by IL-17, TNF- $\alpha$ , IFN- $\gamma$  and IL-22 lead to the regulation of a huge amount of genes in human keratinocytes and to the

self-amplifying loop ([Morizane et al, 2018](#); [Chiricozzi et al, 2011](#)), showing synergistic effects. In fact, the combined cytokine stimulation drives in the activation of NF- $\kappa$ B pathway and the transcriptional factor I $\kappa$ B $\zeta$  ([Morizane et al, 2018](#)), being this latter a key driver in psoriasis pathogenesis through the regulation of IL-17 signaling pathway ([Johansen et al, 2016](#); [Johansen et al, 2015](#)). During inflammatory states keratinocytes are able to secrete VEGF, which induces angiogenesis and the proliferation of endothelial cells, driving in the formation of the psoriasis plaques ([Patel et al, 2018](#)).

MicroRNAs are also involved in keratinocyte proliferation in psoriasis. In fact, levels miR-6731-5p have been found diminished in IL-22-stimulated keratinocyte leading to an activation of the antimicrobial peptide S100A7 ([Qiao et al, 2018](#)), and levels of miR-122-5p and miR-548a-3p were observed increased in the same conditions of stimulation leading to an increase in keratinocyte ERK/MAPK signaling pathways ([Jiang et al, 2017](#)) and keratinocyte proliferation ([Zhao et al, 2017](#)), respectively.

Cyr61 (Cysteine-rich angiogenic inducer 61, now named Cyr61/CCN1), a secreted matricellular protein, has been found elevated in psoriasis patients; modulating key function in keratinocytes ([Quan et al, 2015](#)) enhancing epidermal hyperplasia and inflammation through keratinocyte activation ([Sun et al, 2017](#)). In fact, Cyr61/CCN1 stimulates chemokine ligand (CCL)-20 production leading to an hyperproliferation on keratinocytes ([Li et al, 2017](#)), being implicated in IL-8 synthesis and release by these cells in a JNK/NF- $\kappa$ B-dependent pathway ([Wu et al, 2017](#)). Moreover, keratinocyte stimulation with Cyr61/CCN1 drives in IL-1 $\beta$  production by p38 signaling pathways ([Sun et al, 2017](#)), indicating that Cyr61/CCN1 is a new and key driver in psoriasis pathogenesis through the activation of keratinocytes and could be a new trigger for psoriasis treatment development drugs.

## IV.X. Psoriasis treatment

Patients with psoriasis often require lifelong treatments ([Kim et al, 2012](#)). Currently, the dermatological medical community has several therapeutic alternatives. In general, therapy begins with the safest option and advances to the most aggressive to achieve the control of symptoms in psoriasis patients. Among the safest option for patients there are two examples. Firstly, phototherapy, which includes treatment with ultraviolet light B (UVB) and PUVA ([Morita, 2018](#); [Shirsath et al, 2018](#); [Menter et al, 2008](#)) and second, the use of anti-inflammatory creams for the skin (topical therapy) ([Traub and Marshall, 2007](#)). The action of phototherapy is given by the induction of apoptosis of inflammatory cells, the increase in the production of IL-10 and the switch to Th2 and T regulatory populations ([Tartar et al, 2014](#)). Topical therapy is principally

based in topical corticosteroids ([Greb et al, 2016](#)), which induce keratinocyte apoptosis and topical retinoids ([Lebwohl et al, 1998](#)), which lead to a normal keratinocyte differentiation and suppression of immune response.

In addition, oral systemic therapies are also prescribed for patients in the first moments of the disease development. In fact, the small inhibitory molecules of T cells that act as immunosuppressive agents, such as methotrexate, acitretin and cyclosporine are widely used in psoriasis patients owing to their low cost ([Heydendael et al, 2003](#)). However, most patients treated with these oral systemic therapies are bad responders. Most of them present some contraindications and they are not able to maintain the clinical response after discontinuing the treatment and the majority of them become intolerant because of their adverse effects. Therefore, the search of biological drugs (injectable systemic therapies) against specific targets of the disease has been a very active research field in recent years ([Garcia-Valladares et al, 2011](#); [Kurzeja et al, 2011](#); [Alwawi et al, 2009](#)).

Biological agents (**Table 12**) such as monoclonal antibodies and fusion proteins that bind to specific antigens and cytokines as well as small inhibitor molecules (**Table 12**) implicated in the inhibition of different signaling pathways may offer safer systemic treatment options for patients with moderate to severe psoriasis ([Kim et al, 2012](#)). Due to its specificity, biological treatments for psoriasis prevent collateral damage to organs that can occur with the application of methotrexate, acitretin and cyclosporine.

Although most of the biological agents available are effective in achieving a 75% improvement of PASI score over a 12-week period of treatment, more data on long-term treatments are required. Therefore, when choosing the most effective biological agent for psoriasis treatment, multiple factors must be considered, such as the patient's preference, cost, tolerance, adverse events, the dose scheme and the mode of administration ([Kim et al, 2012](#)).

Albeit the first approved biological drugs for psoriasis treatment targeted T cell activation (alefacept) and T cell transmigration (efalizumab), today both of them have been discontinued ([Menter et al, 2008](#)). However, a novel antibody against the pan T cell marker (CD6) involved in co-stimulation, adhesion and maturation of T cells has been approved in Cuba ([Hernandez et al, 2016](#)) and India ([Parthasaradhi et al, 2017](#)) for the treatment of psoriasis. Itolizumab (Alzumab) treatment leads to a significant decrease in T cell activation, pro-inflammatory cytokine secretion and T cell infiltration to the inflammation site ([Aira et al, 2016](#); [Aira et al, 2014](#)).

Actually and due to the deeper knowledge of psoriasis pathogenesis, several topical, systemic and biologic agents have been approved for psoriasis treatment (**Table 12**) ([Greb et al, 2016](#)), although taking in consideration that the physiopathology

of psoriasis remains unknown, it is worth noting that additional therapies in the future should be developed in a more personalized way with the patients ([van de Kerkhof, 2018](#)).

<b>Biological therapies in psoriasis treatment</b>		
<b>Molecule</b>	<b>Mechanism</b>	<b>Status January 2018 (concerning psoriasis)</b>
<b>Etanercept</b>	Anti-TNF	Approved
<b>Adalimumab</b>	Anti-TNF	Approved
<b>Infliximab</b>	Anti-TNF	Approved
<b>Certolizumab</b>	Anti-TNF	Awaiting approval in psoriasis
<b>Ustekinumab</b>	Anti-IL12/23 p40	Approved
<b>Secukinumab</b>	Anti-IL17A	Approved
<b>Ixekizumab</b>	Anti-IL17A	Approved
<b>Brodalumab</b>	Anti-IL17 receptor	Approved
<b>Guselkumab</b>	Anti-IL/23 p19	Approved
<b>Tildrakizumab</b>	Anti-IL/23 p19	Awaiting approval in psoriasis
<b>Risankizumab</b>	Anti-IL/23 p19	Phase III trial
<b>Apremilast</b>	PDE4 inhibitor	Approved
<b>Tofacitinib</b>	JAK3 inhibitor	Development in psoriasis stopped
<b>Itolizumab</b>	Anti-CD6	Approved (Cuba, India)

**Table 12:** Targeted therapies in psoriasis treatment

A summarized list of the principal therapies to treat psoriasis ([Conrad and Gilliet, 2018](#)).

#### **IV.XI. Mouse models of psoriasis disease**

Several mouse models have been developed to better understand the pathogenesis of psoriasis, which have been classified as xenografts, allografts, transgenics, targeted mutations and spontaneous models ([Hawkes et al, 2018](#); [Nakajima and Sano, 2018](#); [Gudjonsson et al, 2007](#)). In total, 27 models have been proposed to study psoriasis disease ([Wagner et al, 2010](#)) and although none of them represents all cellular and molecular features of psoriasis disease, sub-elements of this disease are appreciated across all of them. In fact, similarities in gene expression patterns between humans and the different mouse models of psoriasis were found,

particularly related with epidermal hyperplasia and keratinization. However, several differences were observed in immune-associated gene expression across the models ([Swindell et al, 2011](#)). Six are the major mouse models that have been studied in the understanding of psoriasis pathogenesis: 1) overexpression of human amphiregulin in the basal epidermal layer (K14-AREG) ([Hawkes et al, 2018](#); [Cook et al, 1997](#)), 2) basal keratinocyte-specific overexpression of a constitutively active mutant of STAT3 (K5-Stat3c) ([Hawkes et al, 2018](#); [Nakajima and Sano, 2018](#); [Sano et al, 2005](#)), 3) overexpression of the endothelial specific receptor tyrosine kinase in basal keratinocytes (K5-Tie2) ([Hawkes et al, 2018](#); [Wolfram et al, 2009](#)), 4) overexpression of the latent form of transforming growth factor beta 1 in basal keratinocytes (K5-TGF- $\beta$ 1) ([Hawkes et al, 2018](#); [Li et al, 2004](#)), 5) Imiquimod cream application in shaved and epilated mouse backs (IMQ) ([Hawkes et al, 2018](#); [van der Fits et al, 2009](#)) and 6) ear intradermal injections of mouse recombinant IL-23 protein (IL-23) ([Hawkes et al, 2018](#); [Hedrick et al, 2009](#)). However, in our lab, another mouse model has been developed and also recapitulates the main characteristics of psoriasis pathogenesis. This murine model is named cLyn ([Marchetti et al, 2009](#)) and it was previously described ([discussed in Role of Lyn in other inflammatory diseases](#)). Recently, another mouse model has been characterized ([Nakajima and Sano, 2018](#)). The serine palmitoyltransferase (SPT)-targeted mice under the keratin 5 promotor is a new model in which mice keratinocytes are not able to produce ceramide leading to a phenotype characterized by skin barrier disruption and developing most of the principal features of psoriasis disease.

Three of these principal models were used in this work for the development of *all in vivo* experiments (IMQ, IL-23 and cLyn mice). In the three models a significant increase in the erythema, scaling and thickness of the treated-skin is observed compared to control mice. Through H&E-stained sections an increased epidermal hyperplasia is found and moreover, parakeratosis and hyperkeratosis as well as no terminal keratinocyte differentiation, analyzed by the loss of involucrin marker, are observed in treated skin. Immunohistochemical staining showed a significant increase of antigen-presenting cells composed, mainly, by dermal (dDC) and plasmacytoid (pDC) dendritic cells. Further, an increase in T cells and neutrophils were described, although no differences in macrophages were appreciated to compare with control-treated skin. Moreover, in treated skin, an increased vascularization has been demonstrated. In addition, the development of the psoriasis-like phenotype in these models is fully dependent of the IL-23/IL-17 axis and TNF- $\alpha$  signaling, which have been described as crucial signaling pathways in psoriasis pathogenesis. Thus, these models evidence several similarities with human psoriasis skin regarding the principal features

of this disease, the composition of the immune cell infiltrate and the process of neoangiogenesis ([Hawkes et al, 2018](#); [Hedrick et al, 2009](#); [Marchetti et al, 2009](#); [van der Fits et al, 2009](#)).

IMQ is a potent immune activator and a TLR 7/8 agonist, which it is used in clinic to treat genital and perianal warts produced by papilloma virus infection ([Beutner and Tyring, 1997](#)), although its prescription has been extended to the treatment of actin keratosis and superficial basal cell carcinoma ([Geisse et al, 2002](#)). However, an exacerbation of psoriasis was observed in well-controlled psoriasis patients who were treated with IMQ ([Fanti et al, 2006](#)); leading to the development of IMQ topical treatment in mice as a new model for the study of psoriasis disease. A daily topical dose of 62.5 mg (3.125 mg of active compound) of IMQ cream (Aldara 5%, Meda) is applied for 6 days in shaved and epilated mouse backs to produce the main features of psoriasis disease ([van der Fits et al, 2009](#)). As a TLR 7/8 agonist, the first cells activated by IMQ treatment are monocytes, macrophages and pDC ([Schon and Schon, 2007b](#)). In fact, one of the most important hallmarks of IMQ treatment is the increased infiltration of pDC and type I IFN activity ([Gilliet et al, 2004](#)), producing the migration of Langerhans cells from the skin to the draining lymph nodes ([Suzuki et al, 2000](#)). However, keratinocytes do not express TLR 7/8 ([Lebre et al, 2007](#)), but an increased production of pro-inflammatory cytokines have been observed after IMQ treatment of keratinocytes, which have been explained by the fact that IMQ can activate keratinocytes through the adenosine receptor signaling pathway ([Schon and Schon, 2007a](#)).

In the IL-23 model, 500 ng of mouse recombinant IL-23 protein are diluted in PBS and intradermally injected into the ears of mice daily for eight days or each other day for 12 days. Although acanthosis, parakeratosis and hyperkeratosis as well as dermal immune cell infiltration are appreciated through the injections, the IL-23 model fails in the infiltration of CD8+ T cells into the epidermis ([Chan et al, 2006](#); [Kopp et al, 2003](#)).



## **RESULTS**



## **RESUME ARTICLE 1:**

### ***The oncogenic tyrosine kinase Lyn impairs the pro-apoptotic function of Bim***

The Src family kinase, composed of 8 members, has a key role in the control of many biological processes such as differentiation, migration and survival. Lyn, one of these proteins, has a well-established function in hematopoietic cells, participating in the regulation of different hematological abnormalities. Indeed, it has been demonstrated that Lyn play an important role in the maintaining of several leukemia phenotypes and a dysfunctional regulation of the protein has been found in various solid tumors. Recently it was shown that Lyn can have an anti-apoptotic role. In fact, the caspase-cleaved form of Lyn (cLyn), which is going to be relocated from the plasma membrane into the cytosol, can negatively control apoptosis after the binding of IgM to the BCR, indicating that cLyn can have access to new substrates with which the native form of the protein cannot interact. Moreover, it was evidenced that apoptosis inhibition by Lyn has been associated to the intrinsic pathway or the mitochondrial pathway and the chemoresistance of cancer cells to some treatments such as imatinib has been associated to an increase in the activation and expression of Lyn as well as its cytosolic localization. Therefore, we focused our study to determine the molecular mechanism of this anti-apoptotic function of Lyn.

Taking into account that Bcl-2 family members are crucial proteins in the regulation of mitochondrial apoptosis and specially that Bax and Bak, the essential effectors controlling apoptosis intrinsic pathway, need to be activated by Bim, we drove our work to determine whether the interaction of Lyn with Bim could explain the anti-apoptotic effects of Lyn. Bim is a highly regulated protein by post-translational modifications. In fact, it has been demonstrated that the phosphorylation of Ser/Thr residues of Bim is key in the modulation of the pro-apoptotic functions of the BH3-only protein Bim. However, nothing is known about the putative tyrosine phosphorylation of this Bcl-2 family member and its potential impact on Bim function and subsequent Bax/Bak-mediated cytochrome c release and apoptosis. Bim has three major isoforms (EL, L and S). We found that Lyn was able to interact with BimEL and BimL. Moreover, BimEL was phosphorylated onto tyrosine residues 92 and 161 by Lyn, leading to an inhibition of its pro-apoptotic function. Going more in details within the mechanism we showed that Lyn-dependent tyrosine phosphorylation of Bim increases its interaction with anti-apoptotic members such as Bcl-xL, therefore limiting mitochondrial outer membrane permeabilization and subsequent apoptosis.

Altogether, these results showed a direct link between Lyn and Bim, confirming the implication of Lyn in the resistance of cancer cells to chemotherapeutic treatments through the Bim regulation by its tyrosine phosphorylation. Therefore, we demonstrated a molecular mechanism through which the oncogenic tyrosine kinase Lyn negatively regulates the mitochondrial apoptotic pathway, demonstrating its suppressor role in apoptosis.



# The oncogenic tyrosine kinase Lyn impairs the pro-apoptotic function of Bim

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## Abstract

Phosphorylation of Ser/Thr residues is a well-established modulating mechanism of the pro-apoptotic function of the BH3-only protein Bim. However, nothing is known about the putative tyrosine phosphorylation of this Bcl-2 family member and its potential impact on Bim function and subsequent Bax/Bak-mediated cytochrome *c* release and apoptosis. As we have previously shown that the tyrosine kinase Lyn could behave as an anti-apoptotic molecule, we investigated whether this Src family member could directly regulate the pro-apoptotic function of Bim. In the present study, we show that Bim is phosphorylated onto tyrosine residues 92 and 161 by Lyn, which results in an inhibition of its pro-apoptotic function. Mechanistically, we show that Lyn-dependent tyrosine phosphorylation of Bim increases its interaction with anti-apoptotic members such as Bcl-xL, therefore limiting mitochondrial outer membrane permeabilization and subsequent apoptosis. Collectively, our data uncover one molecular mechanism through which the oncogenic tyrosine kinase Lyn negatively regulates the mitochondrial apoptotic pathway, which may contribute to the transformation and/or the chemotherapeutic resistance of cancer cells.

## Introduction

The Src family of non-receptor tyrosine kinases (SFKs) plays an important role in the control of many biological processes, such as proliferation, differentiation, survival, and cell motility [1]. There are eight SFKs that all share a common structure consisting of six distinct regions (for

detailed see ref. [2]). SFKs are activated by a wide variety of cell-surface receptors to orchestrate intracellular signaling networks in many cell types [3]. In addition to their relevant role in physiological conditions, SFKs also participate in oncogenesis [4]. Overexpression or excessive activation of SFKs is frequently observed in tumors, playing a central role in the regulation of multiple signaling pathways important for tumor progression, such as apoptosis [4]. Moreover, their activation level often correlates with disease severity and metastatic potential.

In physiological conditions, the SFK Lyn plays a key role in the regulation of B lymphocyte and myeloid cell homeostasis [5–7]. Furthermore, several studies have shown that Lyn is overactive in hematological malignancies, including chronic myelogenous leukemia (CML) [8], B-cell chronic lymphocytic leukemia B [9], and Burkitt lymphoma [10], and in solid cancers [11, 12]. During tumorigenesis, Lyn is involved in tumor progression [2] and resistance to cancer treatments [9, 13] rather than in neoplasia initiation. Resistance to chemotherapeutic agents can be linked to an increase in Lyn expression and activity as well as an aberrant localization within the cytosol [9, 14]. Interestingly, we showed that the cytosolic form of Lyn (hereafter

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Patrick Auberger and Sandrine Marchetti contributed equally to this work.

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cLyn) behaves as an inhibitor of the mitochondrial apoptosis pathway, and this effect relies on its kinase activity [9, 14, 15]. Therefore, Lyn inhibition by pharmacological inhibitors or siRNA in resistant cells resensitizes them to apoptotic death induced by chemotherapeutic treatment [13]. Although Lyn-mediated regulation of apoptosis has previously been linked to the regulation of caspase-8 activation [16], the direct regulation of the apoptotic mitochondrial pathway has not yet been addressed.

The intrinsic apoptotic pathway is tightly controlled by the Bcl-2 family proteins, which are primary regulators of mitochondrial outer membrane permeabilization (MOMP), an essential event in mitochondrial apoptosis, resulting in the release of cytochrome *c* into the cytosol to activate executioner caspases [17]. The Bcl-2 family is composed of anti-apoptotic members, (Bcl-2, Mcl-1, and Bcl-xL), that are required for cell survival to counteract the pro-apoptotic effector (Bax and Bak) and BH3-only (such as Bim and Bid) protein functions [18]. Among the pro-apoptotic, the BH3-only proteins act as stress sentinels that relay apoptotic signals to the mitochondria and induce MOMP via Bax/Bak activation [17]. BH3-only proteins are regulated both transcriptionally and by post-translational modifications, such as phosphorylation [19, 20]. For example, phosphorylation of Bim on serine/threonine residues by kinases such as ERK and JNK [21, 22] represents an important regulatory mechanism of its pro-apoptotic function that primarily modulates its degradation. Although Bim is a key regulator of immune homeostasis and a major obstacle to the development of autoimmune diseases and cancer [23], the importance of ERK regulation of Bim function *in vivo* seems to be limited, as knock-in mice expressing BimEL, one of the molecular forms of Bim obtained by alternative splicing [24], that cannot be directly controlled by ERK do not exhibit altered hematopoietic system homeostasis [25]. These observations suggest that other regulations of Bim could be implicated in such aspects.

In the present study, we report that BimEL can be directly phosphorylated by Lyn on tyrosine residues 92 and 161 and that the Lyn-dependent tyrosine phosphorylation of BimEL inhibits MOMP and subsequent apoptosis.

## Results

### Lyn interacts with BimEL and BimL but not BimS

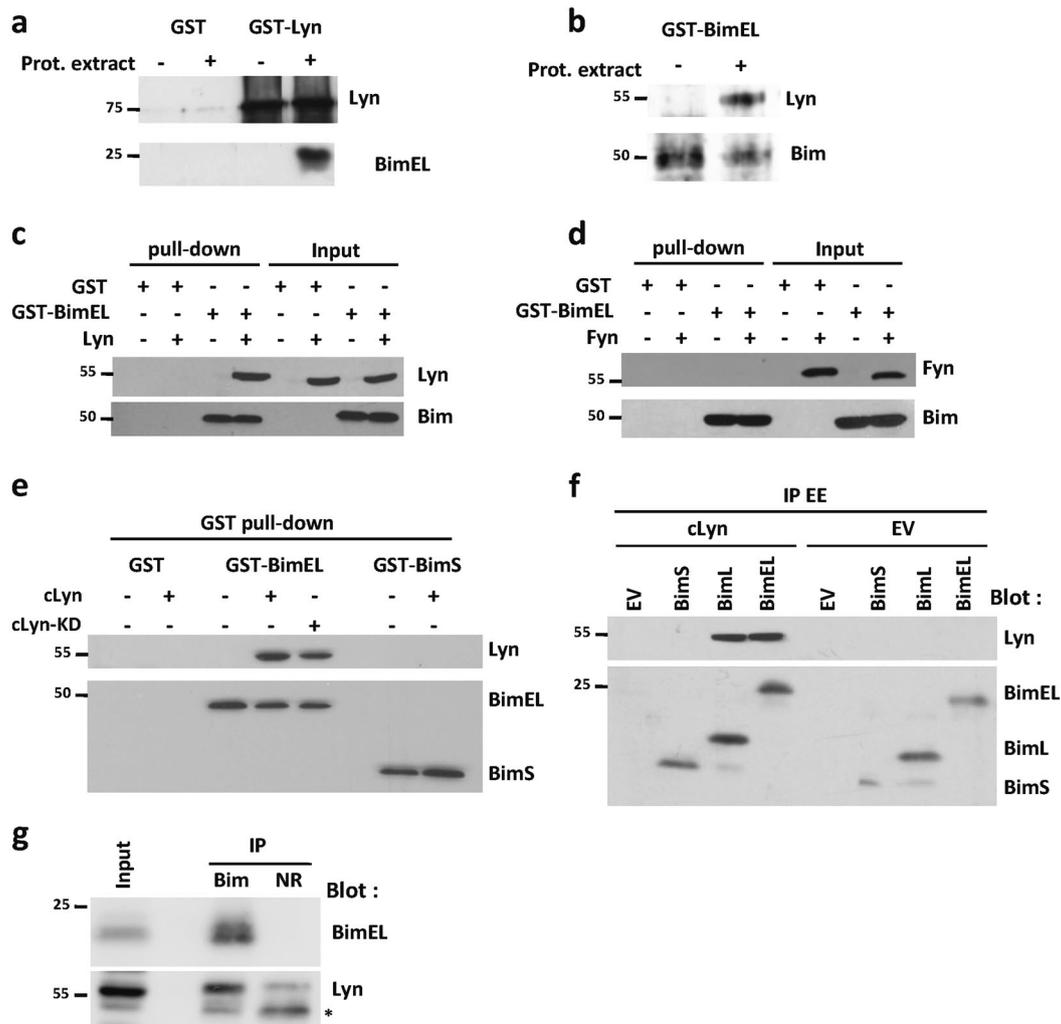
Based on our previous results showing that the cytosolic form of Lyn (cLyn) can inhibit the mitochondrial pathway upstream of caspase activation [14, 15], we determined whether Lyn can regulate one of the Bcl-2 family members. We focused our attention on Bim because of its major role in the context of B-cell receptor-mediated and imatinib-

induced apoptosis [26, 27]. To identify whether Lyn is involved in Bim regulation, we first analyzed their putative interaction. Using a GST pull-down approach with cell protein extract (Fig. 1a, b) or recombinant protein (Fig. 1c), we identified an interaction between BimEL and Lyn. Interestingly, this interaction seemed to be specific to Lyn, as no interaction was observed with Fyn (Fig. 1d), another SFK known to negatively regulate apoptosis [28]. Moreover, to determine whether Lyn activity is involved in its interaction with Bim, an inactive form of cLyn (cLyn Kinase Dead, cLynKD) was used. We found that both forms of cLyn, WT and KD, interact with Bim, indicating that Lyn activity is not required for its interaction with Bim (Fig. 1e, Supplementary Fig. 1a).

Bim exists as three major isoforms, obtained by alternative splicing (BimEL, BimL, and BimS) [24]. We next wanted to know which one of these isoforms Lyn interacts with. We confirmed that BimEL interacts with cLyn (Fig. 1f, Supplementary Fig. 1b) and showed that BimL, but not BimS (Fig. 1e, f), could bind to Lyn. Finally, the interaction between endogenous BimEL and Lyn was also observed (Fig. 1g). Thus, Lyn binds to BimEL and BimL in a kinase-independent manner.

### Bim is phosphorylated on tyrosine residues by Lyn

To assess whether Bim could be a direct substrate of Lyn, recombinant GST-Bim was subjected to an *in vitro* kinase assay with an active Lyn recombinant protein. As shown in Fig. 2a, b, the mouse BimEL and BimL isoforms, but not BimS, were phosphorylated in the presence of Lyn. We also confirmed that a commercially available recombinant human BimL protein was phosphorylated *in vitro* by Lyn (Supplementary Fig. 2a), suggesting that upon binding, Lyn could phosphorylate BimEL and BimL. We then investigated whether Bim tyrosine phosphorylation could occur within cells by transfecting BimEL with the WT or inactive (KD) form of cLyn into HEK293 cells. To increase the pool of tyrosine-phosphorylated proteins, transfected cells were treated with pervanadate, a tyrosine phosphatase inhibitor. As shown in Fig. 2c (Supplementary Fig. 2b), a tyrosine-phosphorylated form of Bim was observed in the presence of cLyn and pervanadate (lane 1), but not when cLyn was inactive (lane 3). Conversely, the ERK-dependent phosphorylation of BimEL on serine 65 was increased in cells treated with pervanadate but in a Lyn-independent manner. Similar results were obtained when cells were treated with the Src kinase inhibitor PP2 to inhibit Lyn activity (Fig. 2d, Supplementary Fig. 2c). Moreover, inhibition of either the MEK/ERK pathway (U0126) or the JNK pathway (SP600125) had no impact on Bim tyrosine phosphorylation (Fig. 2d). Altogether, these results suggest that tyrosine phosphorylation of Bim can occur independently of Ser/Thr



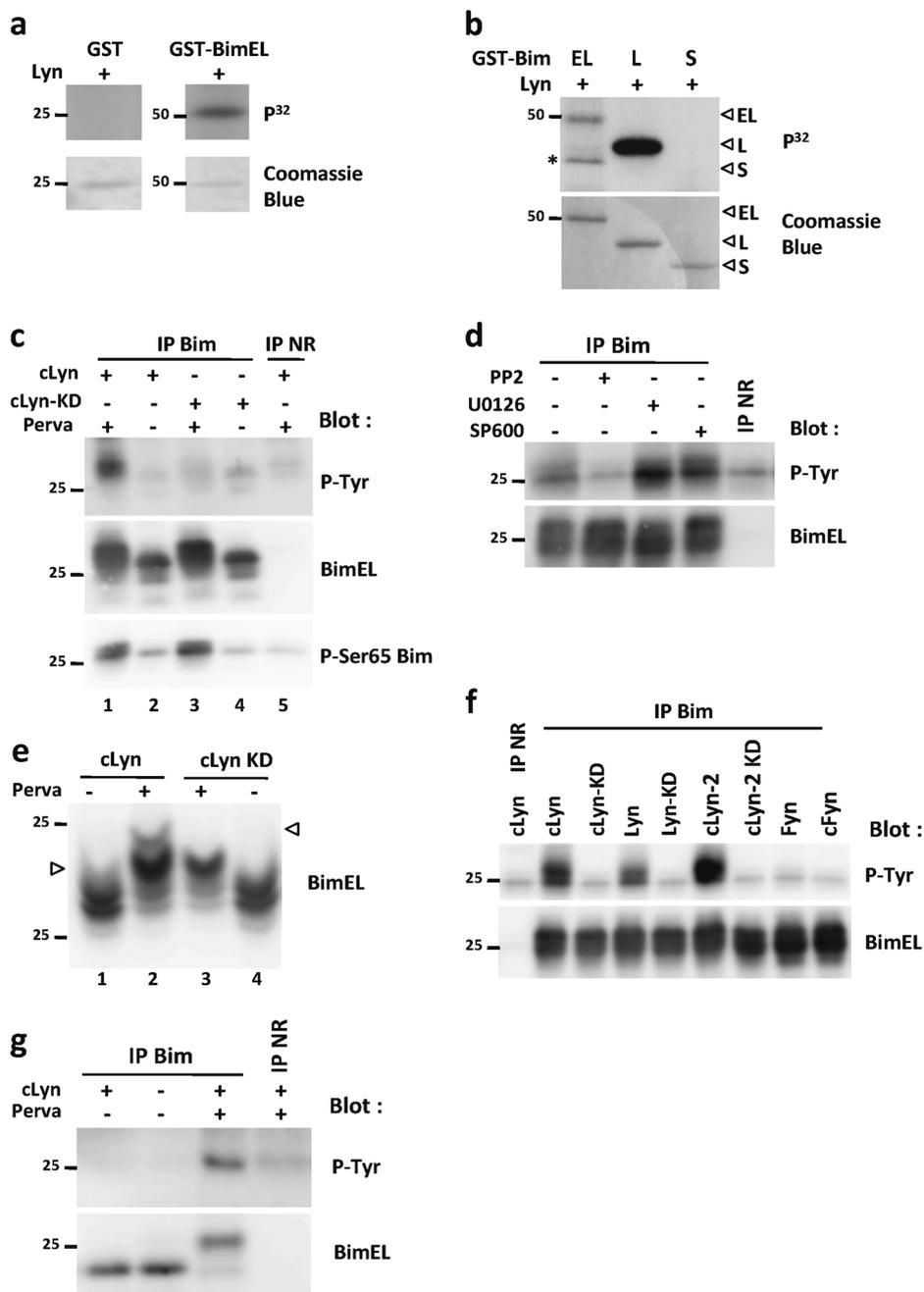
**Fig. 1** Lyn interacts with BimEL and BimL. **a, b** Recombinant GST and GST-Lyn proteins (**a**) or recombinant GST-BimEL (**b**) were incubated with protein extracts from Ramos cells. After GST pull-down, the interaction between Lyn and Bim was analyzed by WB. **c** Recombinant GST and GST-BimEL were incubated with a recombinant Lyn protein produced by TNT. After GST pull-down, protein interactions were analyzed by WB. **d** Same experiment as depicted in **c** but with a recombinant Fyn protein. **e** Recombinant GST, GST-BimEL, and GST-BimS were incubated with recombinant Lyn WT or its inactive form (KD). After GST pull-down, protein interactions were analyzed by WB. The input is presented in Supplementary Fig. 1a. **f** HEK293 cells were transfected with vectors encoding EE-tagged BimEL, BimL, or BimS proteins in the presence or absence of cLyn.

Twenty-four hours after transfection, cell extracts (input) were immunoprecipitated with an anti-EE antibody, and protein interactions were analyzed by WB. Cells were treated with the pan-caspase inhibitor Q-VD-OPh (20 μM) during transfection to prevent Bim-dependent cell death. The input is presented in Supplementary Fig. 1b. **g** WEHI-231 cell extracts were immunoprecipitated with an anti-Bim antibody or a nonrelevant Ig (NR), and protein interaction was analyzed by WB. The asterisk indicates cross-reactivity with the antibody light chain used in the IP. All GST proteins were purified from bacteria. The recombinant Lyn and Fyn proteins were obtained by *in vitro* transcription and translation in reticulocyte lysates. EV, empty vector

kinase-dependent phosphorylation, already known to regulate Bim functions [17, 20]. Furthermore, the tyrosine phosphorylation of BimL was detected in cells (Supplementary Fig. 2d), and the human BimEL was also phosphorylated on tyrosine residues in a Lyn-dependent manner (Supplementary Fig. 2e), indicating that this phosphorylation event is not restricted to the murine BimEL.

We next analyzed the phosphorylation of Bim using Phos-tag SDS gels, which are made with a particular

acrylamide that binds to phosphorylated tyrosine, serine, and threonine residues, producing stepwise gel shifts for each phosphorylated residue [29]. As shown in Fig. 2e, BimEL is a highly phosphorylated protein, with several shifted bands in all conditions tested. However, only the WT form of cLyn induced an additional shifted band of transfected BimEL (lane 2 versus lane 3, right arrowhead), which occurred independently of pervanadate treatment (lane 1 versus lane 4, left arrowhead).



Then, we compared the ability of different forms of Lyn, active or inactive (KD), to phosphorylate Bim on tyrosine residues. To do so, we analyzed (i) the native anchored form (Lyn) and (ii) two different cytosolic forms of Lyn, the caspase-cleaved form of Lyn (cLyn) [14, 30] and one in which acylated residues were mutated (cLyn-2), preventing anchoring to the plasma membrane (Fig. 2f, Supplementary Fig. 2f). Although all active Lyn constructs could induce tyrosine phosphorylation of Bim, the two cytosolic forms appeared to be more effective than the native anchored form

of Lyn. However, neither the WT nor the cytosolic form of Fyn could generate the tyrosine phosphorylated form of Bim. These results support the idea that Lyn, and more efficiently its cytosolic forms, phosphorylates Bim on tyrosine residues.

Finally, we detected endogenous tyrosine-phosphorylated BimEL both in HEK293 cells transfected with cLyn and treated with pervanadate (Fig. 2g, Supplementary Fig. 3a), and in activated WEHI-231, a murine B-cell line activated upon BCR activation through IgM

◀ **Fig. 2** Bim is phosphorylated on tyrosine residues by Lyn. **a, b** In vitro Lyn phosphorylation assay of GST-BimEL, GST-BimL, and GST-BimS. GST was used as a negative control. Proteins were separated by SDS-PAGE, and gels were stained with Coomassie blue and exposed for autoradiography. **c** HEK293 cells were co-transfected with vectors encoding BimEL and the WT (cLyn) or inactive form of Lyn (cLyn-KD). Twenty-four hours after transfection, cells were treated with or without sodium pervanadate ( $\text{H}_6\text{Na}_3\text{VO}_{10}$ ), an inhibitor of tyrosine phosphatases, for 15 min. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine, anti-Bim and anti-P-Ser65 Bim antibodies. Non-relevant Ig (IP NR) served as an immunoprecipitation control. The input is presented in Supplementary Fig. 2b. **d** HEK293 cells were co-transfected with vectors encoding BimEL and cLyn. Twenty-four hours after transfection, cells were pre-treated for 30 min either with a Src inhibitor (PP2, 10  $\mu\text{M}$ ), a MEK1/2 inhibitor (U0126, 10  $\mu\text{M}$ ) or a JNK inhibitor (SP600125, 20  $\mu\text{M}$ ). Then, cells were treated for 15 min with sodium pervanadate. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine, anti-Bim, and anti-P-Ser69 Bim antibodies. The input is presented in Supplementary Fig. 2c. **e** The same extracts used in **c** were run on Phos-tag SDS gels, which are made with a special acrylamide that binds to each serine/threonine and tyrosine phosphorylated residues and produces gel shifts in a given protein. The membrane was immunoblotted with an anti-Bim antibody. The arrowhead indicates Bim-phosphorylated forms dependent on Lyn kinase activity, observed in the presence (right arrowhead) and in the absence (left arrowhead) of sodium pervanadate stimulation. **f** HEK293 cells were co-transfected with vectors encoding BimEL and the WT or inactive forms (KD) of cytosolic or membrane-anchored Lyn and Fyn. For Lyn, we compared two different cytosolic forms of Lyn, the caspase-cleaved form of Lyn (cLyn) and another one in which acylated residues were mutated (cLyn-2). Twenty-four hours after transfection, cells were treated for 15 min with sodium pervanadate to inhibit tyrosine phosphatases. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-Bim antibodies. The input is presented in Supplementary Fig. 2f. In all transfection experiments, the cells were treated with the pan-caspase inhibitor Q-VD-OPh (20  $\mu\text{M}$ ) to prevent Bim-dependent cell death. **g** HEK293 cells were transfected with a vector encoding cLyn or an empty vector. Twenty-four hours after transfection, cells were treated with or without sodium pervanadate, an inhibitor of tyrosine phosphatases, for 15 min. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-Bim antibodies. The input is presented in Supplementary Fig. 3a

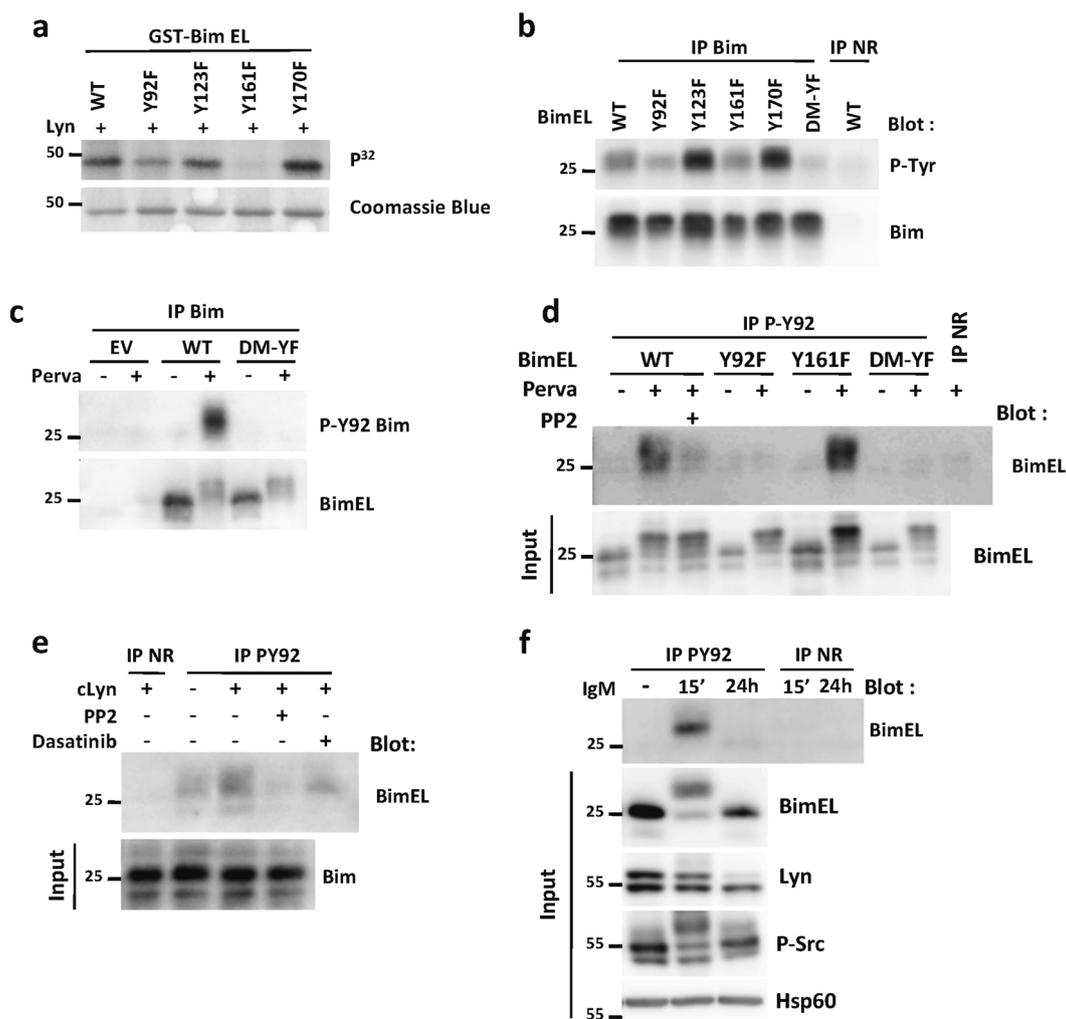
(Supplementary Fig. 3b) where Lyn and Bim are known to control B-cell response [2, 23]. Altogether, these data suggest that Lyn-mediated phosphorylation of Bim can occur at the endogenous level in physiological conditions.

### BimEL is phosphorylated by Lyn on tyrosine residues 92 and 161

An analysis of the mouse BimEL sequence revealed four tyrosine residues, Y92, Y123, Y161, and Y170, with the last three being common to BimL (Supplementary Fig. 4a). To identify the tyrosine residue(s) targeted by Lyn, we performed an in vitro kinase assay with recombinant active Lyn and GST-Bim mutants, whereby each tyrosine was

individually mutated to phenylalanine (Y to F) by site-directed mutagenesis. In contrast to the Y123F and Y170F mutants, which were phosphorylated to the same extent as WT BimEL, the mutation of tyrosine 92 or 161 significantly reduced Bim phosphorylation, indicating that both residues are phosphorylated by Lyn (Fig. 3a). The same experiments conducted with a single tyrosine mutant of BimL confirmed that tyrosine 105 (Y161 in BimEL) was targeted by Lyn (Supplementary Fig. 4b). To confirm that both tyrosine residues 92 and 161 are also phosphorylated by Lyn within cells, we co-transfected cells with each single mutant or the double mutant Y92/161F (DM-YF) and cLyn (Fig. 3b, Supplementary Fig. 4c). A reduction in the tyrosine phosphorylation of both Y92F and Y161F single mutants compared to WT BimEL was observed, whereas mutation of the other two tyrosine residues had no impact. Notably, the Y92F mutant was the most affected. However, phosphorylation was completely abolished when both tyrosine residues (92 and 161) were concomitantly mutated (double mutant-YF: DM-YF) (Fig. 3b, Supplementary Fig. 5a). Furthermore, mutating Y105 of BimL abolished its tyrosine phosphorylation, suggesting that this residue was also targeted by Lyn within cells (Supplementary Fig. 4d). We generated Bim mutants that could not be phosphorylated on Y92 and Y161 and Ser65 (S65G/DM-YF), one of the serine residues targeted by ERK [21]. As we observed with pharmacological inhibitors of Lyn and ERK pathway (Fig. 2d), the tyrosine phosphorylation of Bim occurred independently of the Ser65 phosphorylation (Supplementary Fig. 4e). However, there are many Ser and Thr residues in Bim that can be phosphorylated, and we cannot completely exclude that one or more other phosphorylated sites could affect the tyrosine phosphorylation mediated by Lyn. Therefore, BimEL is phosphorylated on tyrosine residues 92 and 161 in a Lyn-dependent manner, and this event is independent of Bim phosphorylation by ERK or JNK.

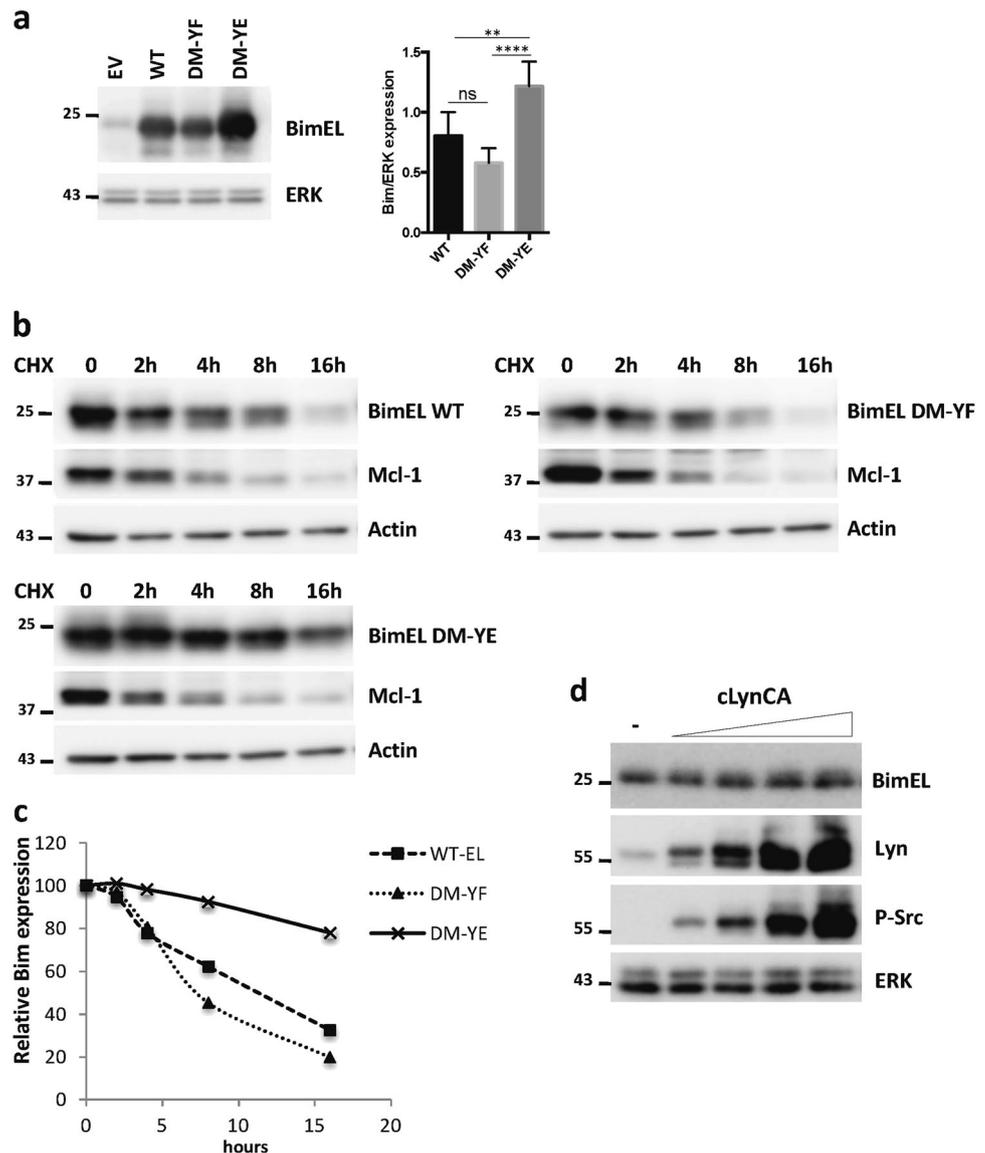
To better examine Bim tyrosine phosphorylation, we generated phospho-specific polyclonal antibodies against synthetic peptides containing either the Y92 or Y161 phosphorylated residues. After affinity purification against the nonphosphopeptide to remove any cross-reactivity, we were only able to obtain an antibody specific for phosphorylated tyrosine 92, which specifically detected ectopically expressed WT BimEL but not a nonphosphorylatable double mutant (Fig. 3c, Supplementary Fig. 5b). Immunoprecipitation experiments confirmed that this antibody recognizes phosphorylated Bim when Lyn is active and tyrosine 92 is not mutated (Fig. 3d, Supplementary Fig. 5c), and allowed the detection of tyrosine-phosphorylated Bim in the absence of sodium pervanadate stimulation, when a constitutively active form of cLyn was expressed (Supplementary Fig. 5d). Interestingly, proteasome inhibition by velcade did not modify the level of tyrosine phosphorylated



**Fig. 3** BimEL is phosphorylated by Lyn on tyrosine residues 92 and 161. **a** In vitro Lyn phosphorylation assay with recombinant WT GST-BimEL and nonphosphorylatable mutants (Y to F) of GST-BimEL. The proteins were separated by SDS-PAGE, and gels were stained with Coomassie blue and exposed for autoradiography. **b** HEK293 cells were co-transfected with vectors encoding cLyn and WT BimEL or nonphosphorylatable single mutants (Y to F) of BimEL. The non-phosphorylatable Y92F/Y161F double mutant was also tested (DM-YF). Twenty-four hours after transfection, sodium pervanadate, an inhibitor of tyrosine phosphatases, was added for 15 min. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-Bim antibodies. Cells were treated with the pan-caspase inhibitor Q-VD-Oph (20  $\mu$ M) during transfection to prevent Bim-dependent apoptosis. The input is presented in Supplementary Fig. 4c. **c** HEK293 cells were co-transfected with vectors encoding cLyn and WT BimEL or the non-phosphorylatable Y92F/Y161F (DM-YF) double mutant of BimEL. Twenty-four hours after transfection, cells were treated with or without sodium pervanadate for 15 min to inhibit tyrosine phosphatases. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phospho-Bim (Y92) and anti-Bim antibodies. The input is presented in Supplementary Fig. 5b. **d**

HEK293 cells were co-transfected with vectors encoding cLyn and WT or mutant forms of BimEL (single mutants Y92F and Y161F and the double mutant Y92/Y161F). Twenty-four hours after transfection, cells were treated with or without sodium pervanadate for 15 min to inhibit tyrosine phosphatases. Cell extracts (input) were immunoprecipitated for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody. When indicated, a Src kinase inhibitor (PP2, 10  $\mu$ M) was added 1 h before pervanadate stimulation. The input is presented in Supplementary Fig. 5c. **e** HEK293 cells were transfected with a vector encoding cLyn or an empty vector. Twenty-four hours after transfection, cells, which were either pre-treated or not for 30 min with Src inhibitors (PP2, 10  $\mu$ M or Dasatinib, 100 nM), were then treated with or without sodium pervanadate for 15 min. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-Bim antibodies. The input is presented in Supplementary Fig. 5e. **f** Ramos cells were stimulated for the indicated time points with 10  $\mu$ g/ml goat anti-human IgM, and then treated with sodium pervanadate for 10 min. The cell extracts (input) were immunoprecipitated for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody. Nonrelevant Ig (IP NR) served as an immunoprecipitation control

**Fig. 4** Phosphomimetic (DM-YE) double mutant is more stable than WT BimEL and nonphosphorylatable (DM-YF) double mutants of BimEL. **a** HEK293 cells were co-transfected with vectors encoding for WT or mutants forms of BimEL. Twenty-four hours after transfection, Bim expression was evaluated by WB. Histograms represent the mean  $\pm$  SD of the Bim/ERK quantification corresponding to five independent experiments. Student's *t*-test was applied, \**p* < 0.05; \*\**p* < 0.01; \*\*\*\**p* < 0.0001; n.s. non significant. **b** Bax/Bak DKO MEFs were transfected with pMIG vector encoding WT or mutants forms of BimEL, and GFP through an IRES sequence. After GFP FACS sorting, the cells were treated with cycloheximide (10  $\mu$ g/ml) and then harvested at the indicated times. BimEL and Mcl-1 expression was analyzed by WB. Actin was used as a loading control. **c** Quantification of the WB presented in **b** (Bim/actin ratio in % of control). **d** HeLa cells were transfected with increasing amount of cLynCA, and 24 h after transfection, endogenous BimEL expression was analyzed by WB



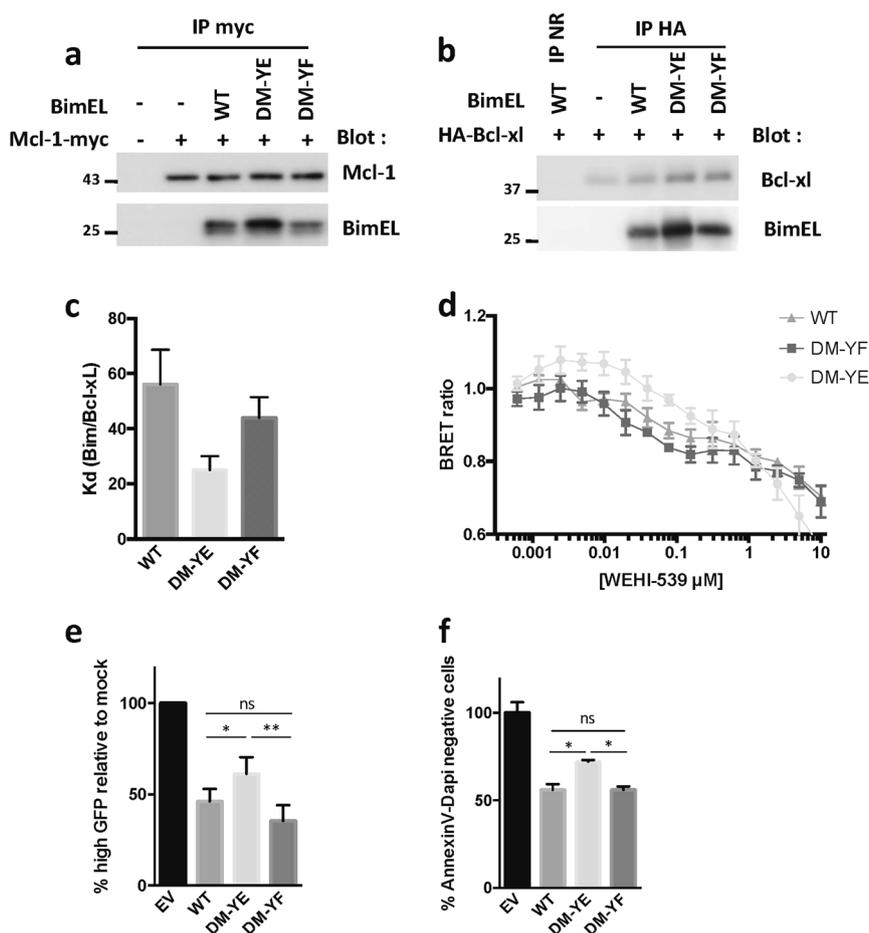
Bim. Finally, as the S65G Bim mutant was immunoprecipitated with the P-Y92 Bim antibody, we confirmed that both phosphorylation events could occur independently of each other (Supplementary Fig. 5d). Altogether, these data validated this antibody as a tool to analyze Lyn-dependent Bim tyrosine phosphorylation in both physiological and pathological conditions.

Therefore, to observe the endogenous tyrosine 92 phosphorylation of Bim, we first transfected HEK293 cells with cLyn and treated them with sodium pervanadate. Interestingly, Bim phosphorylation on tyrosine 92 was observed preferentially when cLyn was expressed and active, as PP2 or Dasatinib treatment, two Src kinase inhibitors, fully abolish the detection of phosphorylated Bim (Fig. 3e, Supplementary Fig. 5e). Next, we investigated whether this phosphorylation could also occur in the mouse (WEHI-231) and human (Ramos) lymphoma B-cell lines. As expected,

B-cell receptor engagement induced activation of Lyn as early as 15 min after stimulation (Fig. 3f and Supplementary Fig. 5f). Concomitantly, in this condition, phosphorylation of Bim on tyrosine 92 was observed (Fig. 3f, Supplementary Fig. 5f) both in mouse and human cells, indicating that Bim phosphorylation on tyrosine 92 can occur when Lyn is activated through B-cell receptor stimulation.

### Bim phosphorylation on tyrosine residues 92 and 161 increases its stability

Serine/threonine phosphorylation of Bim by several survival kinases regulates its pro-apoptotic function, primarily by modulating its stability [19, 21]. Therefore, we investigated the effect of Bim tyrosine phosphorylation on its pro-apoptotic function and/or stability by comparing nonphosphorylatable (DM-YF) Bim to phosphomimetic (DM-YE)



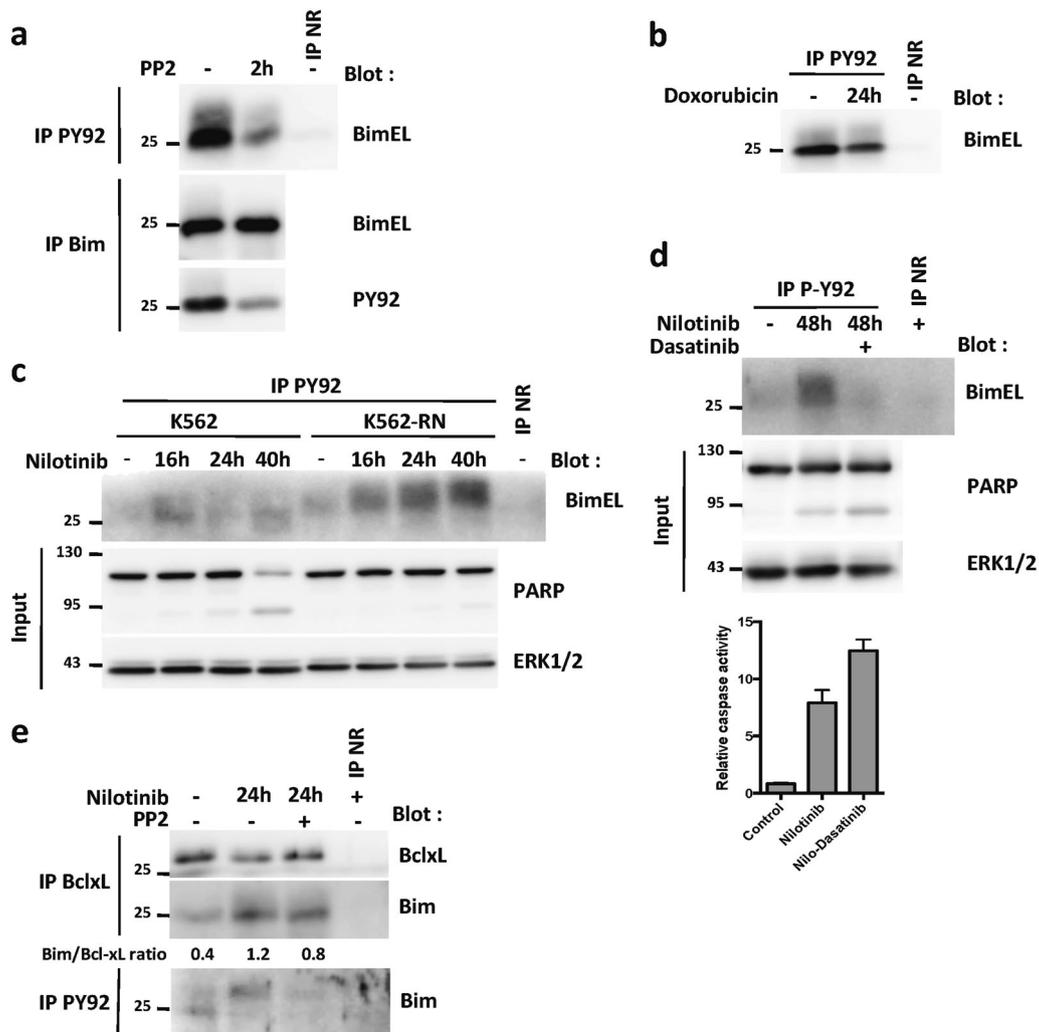
**Fig. 5** Lyn-dependent tyrosine phosphorylation of BimEL inhibits its pro-apoptotic function. **a**, **b** HEK293 cells were co-transfected with vectors encoding WT or double mutant forms of BimEL (nonphosphorylatable DM-YF or phosphomimetic DM-YE) and either Myc-tagged Mcl-1 (**a**) or HA-tagged Bcl-xL (**b**). Twenty-four hours after transfection, cell extracts (input) were immunoprecipitated with the respective anti-tag antibody, separated by SDS-PAGE and immunoblotted with anti-Bim and anti Mcl-1 or Bcl-xL antibodies. The inputs are presented in Supplementary Fig. 8a, b. **c** The Kd was determined by measuring the RLuc-BimEL variant/YFP-Bcl-xL BRET ratio that corresponded to a relative affinity determined at 50% of the BRET saturation plateau. **d** The BH3-mimetic (WEHI-539) response of the RLuc-BimEL variant/YFP-Bcl-xL BRET signals was assessed by concentration curve experiments. The data are presented as the mean  $\pm$  SD, and are representative of three independent experiments. **e** HeLa cells stably expressing Smac-GFP were co-transfected with vectors encoding RFP and WT or double mutant forms of BimEL

(nonphosphorylatable DM-YF or phosphomimetic DM-YE). Cells were treated with the pan-caspase inhibitor Q-VD-OPh (20  $\mu$ M) during transfection to prevent Bim-dependent cell death. Fifteen hours after transfection, the RFP+ cells (transfected cells) were analyzed for GFP expression by flow cytometry. The percentage of high-GFP-expressing cells (corresponding to cells with Smac-GFP within the mitochondria) was plotted relative to the control cells (EV, empty vector). Data are presented as the mean  $\pm$  SD ( $n = 4$ ). Student's *t*-test was applied, \* $p < 0.05$ ; \*\* $p < 0.01$ ; n.s. non significant. **f** HeLa cells were co-transfected with vectors encoding membrane-GFP (pBB14) and WT or double mutant forms of BimEL (nonphosphorylatable DM-YF or phosphomimetic DM-YE). Twenty-four hours after transfection, cell death of the transfected population (GFP+ cells) was monitored by flow cytometry using Annexin V-PE and DAPI. The percentage of AnV-DAPI-negative cells (live cells) was plotted by normalizing to the control cells (EV, empty vector). Data are presented as the mean  $\pm$  SD ( $n = 2$ ). Student's *t*-test was applied, \* $p < 0.05$ ; n.s. non significant

double mutants of Bim, to get rid of the Lyn kinase. Each variant was expressed in DKO Bax/Bak MEFs, because they are defective in the mitochondrial apoptotic pathway, and immunofluorescence analyses revealed that both mutants were localized at the mitochondria, similar to the BimEL WT form (Supplementary Fig. 6). These results confirmed the mitochondrial localization of BimEL but also suggested that tyrosine phosphorylation of BimEL, based

on the phosphomimetic mutant localization, did not influence its subcellular distribution.

We next compared the basal expression of BimEL WT and double mutants (DM-YF and DM-YE) in HEK-293 cells. Transfection of BimEL variants indicated that DM-YE mutant protein expression was higher than that of the WT form and the DM-YF mutant (Fig. 4a), despite an equivalent transfection efficiency, indicating that the



**Fig. 6** Tyrosine 92 phosphorylation of BimEL in human cancer cell lines is associated with cellular survival. **a** Sudh14 were pre-treated or not with 10  $\mu$ M PP2 for 2 h or **b** with 2  $\mu$ M of doxorubicin to induce apoptosis for 48 h, before sodium pervanadate treatment for 10 min. Cell extracts (input) were immunoprecipitated either for phospho-Y92 Bim or Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim or PY92 Bim antibody. The respective inputs are presented in Supplementary Fig. 10a, c. Nonrelevant Ig (IP NR) served as an immunoprecipitation control. **c** K562 cells sensitive and resistant to nilotinib (K562-RN) were stimulated with 20 nM of nilotinib for the indicated time points and then cells were treated with sodium pervanadate for 10 min. Cell extracts (input) were immunoprecipitated for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody. PARP cleavage was analyzed to monitor

apoptosis induction. The input is presented in Supplementary Fig. 10e. Nonrelevant Ig (IP NR) served as an immunoprecipitation control. **d** K562-RN cells were treated with nilotinib for 48 h. 100 nM Dasatinib, a dual Src-Abl kinase inhibitor, was added 2 h before 10 min of sodium pervanadate treatment. Cell extracts (input) were used as in **c**. The input is presented in Supplementary Fig. 10f. Histogram represents caspase activity monitored on the same protein extracts. **e** K562-RN cells were treated with nilotinib for 24 h. 10  $\mu$ M PP2 was added 2 h before 10 min of sodium pervanadate treatment. Cell extracts (input) were immunoprecipitated for phospho-Y92 Bim or Bcl-xL, separated by SDS-PAGE and immunoblotted with indicated antibodies. The input is presented in Supplementary Fig. 10g. Nonrelevant Ig (IP NR) served as an immunoprecipitation control

phosphomimetic mutant (DM-YE) should be post-translationally stabilized. To confirm this finding, we next performed cycloheximide experiments on sorted DKO Bax/Bak MEFs infected with WT and mutant BimEL-expressing retroviruses. As expected, the phosphomimetic mutant (DM-YE) had an increased half-life (>16 h) compared to WT BimEL (10 h) and the nonphosphorylatable (DM-YF) mutant (8 h) (Fig. 4b, c), whereas the clearance of Mcl-1, a

highly labile protein, was equivalent between each condition. Notably, the half-life of endogenous BimEL was not significantly different from the exogenous WT BimEL, although their basal expression was different (Supplementary fig. 7a). Thus, the differences in basal expression level do not affect the half-life of Bim, indicating that the increased half-life of the phosphomimetic mutant cannot be attributed to its increased basal expression. Interestingly,

transfection of cLynCA, a cytosolic form of Lyn with constitutive tyrosine kinase activity in HEK293 cells, leads to an increase in BimEL protein expression (Fig. 4d). Hence, this result suggests that Lyn-dependent tyrosine phosphorylation of BimEL increases its stability.

### Tyrosine phosphorylation of Bim inhibits its pro-apoptotic function

The key role of Bim in apoptosis regulation has been well established [31, 32]. However, non-apoptotic Bim functions, such as negative regulation of autophagy [33] or prosurvival signaling [34], have recently been described. To examine whether tyrosine phosphorylation could impact this, we used GFP FACS-sorted DKO Bax/Bak MEFs expressing Bim WT and double mutants (DM-YF and DM-YE) and confirmed that the phosphomimetic mutant (DM-YE) exhibited higher expression than both the WT form and the nonphosphorylatable mutant (DM-YF) (Supplementary Fig. 7a). Furthermore, none of the BimEL variants expressed affected either the basal and EBSS-induced autophagy, as determined by LC3 I-II conversion (Supplementary Fig. 7b, c), or cell proliferation (Supplementary Fig. 7d, e). Based on these results, we speculate that tyrosine phosphorylation could regulate the canonical pro-apoptotic function of BimEL.

Interactions between anti-apoptotic Bcl-2 proteins and pro-apoptotic BH3-only proteins are pivotal in regulating apoptosis [17]. In proliferating cells, the pro-apoptotic function of Bim is silenced by its interaction with anti-apoptotic Bcl-2 members, such as Mcl-1 and Bcl-xL, thus preventing unwanted cell death. Therefore, we transiently co-transfected HEK293 cells with each BimEL variants and either Mcl-1-myc or Bcl-xL-HA, and immunoprecipitations were performed (Fig. 5a, b, Supplementary Fig. 8a, b). Although the amount of IP-transfected Mcl-1 or Bcl-xL was equivalent in all conditions, more DM-YE BimEL mutant was co-immunoprecipitated compared to its WT or the DM-YF counterpart, suggesting that the phosphomimetic mutant interacted more with the anti-apoptotic proteins.

To confirm that the tyrosine phosphorylation of Bim changes its ability to interact with anti-apoptotic members, the spatial proximity between transiently transfected luciferase-fused Bim variants and YFP-fused Bcl-xL was measured by bioluminescence resonance energy transfer (BRET) in live cells [35]. Consistently, the relative affinity (Kd) of the Bim phosphomimetic mutant (DM-YE) for Bcl-xL was lower than those of the other BimEL variants (Fig. 5c), indicating that Bim tyrosine phosphorylation improves its interaction with anti-apoptotic members. It is worth noting that all Bim mutant fusion proteins had the same luciferase activity indicating that they were expressed at the same level (Supplementary Fig. 8c). Moreover, Bim DM-

YE/Bcl-xL binding was more resistant to WEHI-539 BH3-mimetic treatment (Fig. 5d), confirming the higher affinity of the Bim phosphomimetic mutant for Bcl-xL.

We further explored whether modulation of Bim binding capability of those mutants could also influence its pro-apoptotic function and consequently cell death. To answer that we first monitored MOMP induced by Bim transfection into HeLa Smac-GFP cells [36]. Flow cytometry analysis showed a decrease in Smac-GFP fluorescence intensity induced by actinomycin D treatment in the presence of Q-VD-OPh, as previously described [36], and upon Bim transfection (Supplementary Fig. 9a). Based on this, we compared the potential of each BimEL variant in the absence of Lyn overexpression, but expressed at the same protein level, to induce MOMP. Interestingly, the BimEL phosphomimetic mutant (DM-YE) induced MOMP with a lower efficiency than the WT form and the nonphosphorylatable mutant (DM-YF) (Fig. 5e), the latter having the strongest effect. In addition, in an *in vitro* MOMP assay using isolated mitochondria, Lyn could inhibit the mitochondrial release of Smac induced by BimEL and BimL, but not BimS (Supplementary Fig. 9b).

Analysis of phosphatidylserine exposition confirmed that the Bim phosphomimetic mutant (DM-YE) displayed a decreased pro-apoptotic potential compared to the WT form and the nonphosphorylatable mutant (DM-YF) (Fig. 5f). These results suggest that despite an increased protein stability, the phosphomimetic BimEL mutant exhibits reduced pro-apoptotic potential.

Altogether, these results suggest that the tyrosine phosphorylation of BimEL by Lyn inhibits its pro-apoptotic function via its increased affinity for the anti-apoptotic members of the Bcl-2 family.

### Tyrosine 92 phosphorylation of BimEL in human cancer cell lines is associated with cellular survival

To address the impact of Bim tyrosine phosphorylation in pathological conditions, we first checked the presence of PY92-Bim in several hematological cancer cell lines. By immunoprecipitation, we identified that Bim was phosphorylated on tyrosine 92 in Sudhl4, a B-cell lymphoma cell line (Fig. 6a, Supplementary Fig. 10a), and in LAMA-84 and K562, two CML (Supplementary Fig. 10b, Fig. 6c) in a Lyn-dependent manner, as confirmed by PP2 treatment (Fig. 6a, Supplementary Fig. 10a) and silencing of Lyn by shRNA expression in K562 cells (data not shown). Interestingly, this phosphorylation event was reduced when cells undergo apoptosis, visualized by PARP cleavage (Fig. 6b–d, Supplementary Fig. 10c), suggesting that tyrosine 92 phosphorylation of Bim inhibits its pro-apoptotic function. To confirm this, we compared the tyrosine phosphorylation

status of K562 cells sensitive and resistant (K562-RN) to nilotinib treatment, whose resistance has been attributable to overexpression of Lyn, with a large proportion into the cytosol [13, 14]. Upon nilotinib treatment, Bim tyrosine 92 phosphorylation was decreased in K562-sensitive cells along with induction of apoptosis, while in K562-RN nilotinib-resistant cells, Y92 phosphorylation was maintained (Fig. 6c). Dasatinib treatment, a dual Src-Abl kinase inhibitor, inhibited PY92-Bim in K562-RN cells. Interestingly, inhibition of Bim tyrosine phosphorylation increased apoptosis induced by nilotinib treatment, confirming that Bim tyrosine phosphorylation is Lyn dependent (Fig. 6d, Supplementary Fig. 10f) and suggesting that this event is implicated in cell death resistance of K562-RN cells. Finally, we further demonstrated in these cells an increased binding between Bim and Bcl-xL when Bim is phosphorylated on tyrosine 92 (Fig. 6e), as PP2 treatment reduced the level of Bim co-immunoprecipitated with Bcl-xL. Altogether, these data demonstrate that Bim tyrosine phosphorylation by the Src tyrosine kinase Lyn is a novel mechanism of Bim pro-apoptotic function inhibition implicated in chemo-resistance of cancer cells.

## Discussion

The Bcl-2 proteins have been implicated in the control of the mitochondrial apoptotic pathway [17], and, importantly, the cellular decision to live or die relies on complex interactions between the anti-apoptotic and pro-apoptotic members of this family. Regulation of BH3-only protein expression represents a key event in controlling the commitment to mitochondrial apoptotic cell death. In a large majority of tumors, the levels of pro- and anti-apoptotic Bcl-2 proteins are altered, leading to evasion of apoptosis, a hallmark of cancer development [37]. Negative regulation of Bim expression has been associated with decreased apoptosis and accelerated tumor growth [38, 39] as well as resistance to various chemotherapeutic agents, such as imatinib in CML cells [27, 40]. Bim expression is tightly regulated at several levels, one of which is the post-translational regulation by phosphorylation that controls its pro-apoptotic activity and stability [41]. For example, ERK-mediated phosphorylation of BimEL negatively regulates the BimEL pro-apoptotic function by targeting it for proteasomal degradation [21, 42]. However, deciphering the effect of a single phosphorylation event on the pro-apoptotic function of Bim remains difficult, as it is regulated by several phosphorylation events [41] that can occur at the same time within the same molecule. In accordance to that, our results also demonstrate that tyrosine phosphorylation of Bim could occur at the same time as ERK1/2-dependent phosphorylation.

This complex code of Bim phosphorylation is supported by a study by Clybourn and colleagues, who showed that knock-in mice expressing a mutated Bim that cannot be regulated by ERK display a normal hematopoietic system homeostasis [25], suggesting that other processes or phosphorylation events are critical for regulating BimEL function. In our study, we identified that BimEL is directly phosphorylated on two tyrosine residues by the non-receptor tyrosine kinase Lyn and that this leads to an inhibition of Bim pro-apoptotic function. Surprisingly, we found that a Bim phosphomimetic mutant is more stable but less able to induce cell death, an outcome that is the opposite of what is known about Bim regulation by Ser/Thr kinases such as ERK and JNK [21, 22]. Indeed, stabilizing Bim expression has been associated with an increase in Bim pro-apoptotic function, whereas proteasomal degradation is able to inhibit its function. This observation suggests that the Bim phosphorylation status, rather than its expression level, may be a good marker of its pro-apoptotic function. Therefore, further experiments are required to better understand how tyrosine phosphorylation of Bim can increase its expression. However, as Bcl-xL is a highly stable protein [43], one hypothesis would be that the increase in protein stability of the BimEL phosphomimetic mutant is related to its stronger interaction with Bcl-xL, possibly preventing its subsequent degradation. Hence, as Bim is a direct activator BH3-only protein [17], its stronger interaction with anti-apoptotic molecules when phosphorylated on tyrosine residues should be considered as a cell survival mechanism, which is consistent with the reduced pro-apoptotic potential of BimEL phosphomimetic mutant.

Here, we demonstrated for the first time the direct regulation of one of the Bcl-2 family proteins by a member of the SFKs. Src kinases are divided into two subgroups: the Src-related kinases (Src, Yes, Fyn, and Fgr) and the Lyn-related kinases (Lyn, Hck, Lck, and Blk) [44]. As we established that Lyn but not Fyn induces Bim tyrosine phosphorylation, it would be interesting to determine whether other Lyn-related kinases could also fulfill this function. Inhibition of BH3-only protein function by tyrosine phosphorylation has already been described for Bak and Puma [45, 46] and has been linked to the negative regulation of protein stability, at least for Puma. Whereas HER2 is responsible for Puma tyrosine phosphorylation [45], the kinase implicated in Bak phosphorylation remains to be identified. Hence, the role of Src family kinases, and especially Lyn, in the direct control of other Bcl-2 proteins needs to be more carefully examined. However, as not all Src kinases are expressed in all cell types, it may be challenging to determine which cellular contexts are optimal for deciphering the potential tyrosine phosphorylation of Bcl-2 proteins regulated by one specific member of this family. Our data also showed that the cytosolic form of Lyn

regulates Bim function. In addition, we observed that the native anchored form of Lyn can phosphorylate BimEL (Fig. 2f). Thus, this observation suggests that Lyn-dependent BimEL tyrosine phosphorylation could be a more general mechanism and could be important in other contexts than chemotherapeutic resistance associated with the cytosolic localization of Lyn.

Mice lacking Bim exhibit lymphocyte hyperplasia and antibody-mediated autoimmune pathology [23], demonstrating that Bim is an important regulator of B-cell homeostasis. Notably, the tyrosine kinase Lyn also plays important roles in regulating myeloid and B-cell functions [6, 47], and although several studies have noted a link between Lyn and Bim, none of them have addressed the direct regulation of Bim by tyrosine phosphorylation. B cells isolated from Lyn-deficient mice display an increased expression of this BH3-only protein [48], and treatment with dasatinib, a dual ABL/Lyn inhibitor, increased the expression of Bim but not the expression of other BH3-only proteins in acute lymphoblastic leukemia [49]. These observations would indicate that Lyn negatively regulates Bim expression, which disagrees with our results showing that once phosphorylated by Lyn, Bim is more stable. However, we must consider that Lyn likely regulates Bim expression at different levels through both direct and indirect mechanisms. Indeed, Src kinases are implicated in various signaling cascades; in particular, Lyn has been shown to phosphorylate several signaling molecules such as PI3K, STAT-5 or MAP kinase [2], which could be involved in an indirect Lyn-dependent regulation of Bim. Importantly, Src has previously been shown to regulate apoptosis through the degradation of the BH3-only protein Bik upon activation of the ERK pathway [50], and we cannot rule out the possibility of such indirect regulation by Lyn. Regarding this hypothesis, we have observed that Lyn inhibition, following dasatinib or PP2 treatment, increased Bim expression both at the protein and mRNA level (data not shown), indicating that Lyn could regulate Bim at both the transcriptional and post-translational levels.

In our study, we identified that BimEL is phosphorylated on tyrosine residues 92 and 161. Notably, these two residues among all the tyrosine residues present in the BimEL sequence were the most evolutionarily conserved (Supplementary Fig. 11). An *in silico* analysis with NetPhos software predicted Y92 (Y96 in the human protein) to be a potential targeted site for phosphorylation in both human and mouse BimEL, whereas Y161 was predicted to be phosphorylated only in mouse BimEL. Moreover, recently, two different tyrosine phosphoproteomic studies identified Bim as a phosphotyrosine protein [51, 52] but only confirmed the Y92 residue. Unlike our results, these studies also identified Y172 (Y170 in the mouse protein) as a potential phosphorylated residue, but there is no indication

about which kinase would be responsible for Tyr172 phosphorylation. Thus, we cannot exclude that this phosphorylation could be mediated by another member of the SFKs or another tyrosine kinase. Interestingly, in HEK293 cells, the observation of a slight tyrosine phosphorylation of Bim in the presence of the tyrosine phosphatase inhibition by pervanadate but when Lyn was not overexpressed (Fig. 3e, Supplementary Fig. 5a) support the notion that other SFKs or other tyrosine kinases could be implicated in such post-translational modification of Bim. Indeed, the NetPhos software analysis predicted that BimEL could be phosphorylated on Y172 in an EGFR-dependent manner, and the phosphorylation of Y172 has been observed in breast cancer cells [51, 52]. Thus, supplementary experiments are needed to determine whether Y172 could be a target of EGFR signaling.

In summary, our study identifies a new regulatory mechanism of Bim pro-apoptotic function, which depends on its tyrosine phosphorylation by the oncogenic tyrosine kinase Lyn. The integration of this event in the complex regulation of Bim should facilitate a better understanding of its role in the physiological control of the hematopoietic system homeostasis. Moreover, Bim tyrosine phosphorylation may also be relevant in cancer resistance to chemotherapeutic agents and more particularly in contexts where both Lyn and Bim are key mediators of this resistance.

## Materials and methods

### Reagents and antibodies

Reagents and antibodies are detailed in *Supplementary Information*.

### Cell culture

HEK293 and HeLa cells were purchased from ATCC. WEHI-231 was kindly provided by Dr. Andreas Villunger (Medical University of Innsbruck, Austria). Immortalized MEFs (WT, Bax/Bak dKO), Sudhl4, and HeLa Smac-GFP [37] cells were kindly provided by Dr. Jean-Ehrland Ricci (C3M, Nice, France). LAMA84 and K562 sensitive and resistant to nilotinib [13] were kindly provided by Dr. Jean-Max Pasquet (Université de Bordeaux, France).

HEK293, HeLa, HeLa Smac-GFP, and MEFs cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin, 100 µg/ml streptomycin and 1 mM pyruvate. For WEHI-231 cells, 50 µM 2-mercaptoethanol was added to the previously described medium. Ramos (Burkitt lymphoma), Sudhl4 (DLBCL), LAMA84 (CML), K562 (CML) cells were maintained in

RPMI supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-mercaptoethanol. K562 expressing a doxycycline-inducible Sh-RNAi specific for Lyn [14] were cultured with zeocin (200 µg/ml) and blasticidin (10 µg/ml). All cell lines were maintained in 5% CO<sub>2</sub>/95% air in a humidified incubator. All cells were tested for mycoplasma contamination.

To inhibit tyrosine phosphatases, cell-permeable sodium pervanadate (H<sub>6</sub>Na<sub>3</sub>VO<sub>10</sub>) was generated by combining 250 µl of sodium orthovanadate Na<sub>3</sub>VO<sub>4</sub> (100 mM) with 1.97 ml of PBS and 280 µl of hydrogen peroxide (30%). After an incubation at room temperature for 10 min, catalase (Sigma) was added to inactivate the H<sub>2</sub>O<sub>2</sub>. The resulting sodium pervanadate solution (10 mM) was immediately added to cells at a concentration of 100 µM.

### Transfection and infection

The experimental procedures are detailed in *Supplementary Information*.

### Immunoprecipitation

To analyze Bim phosphorylation, cells were lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% NaDOC). To analyze the interaction of Bim with anti-apoptotic Bcl-2 members and Lyn, cells were lysed in CHAPS buffer (20 mM Tris pH 7.5, 137 mM NaCl, 2 mM EDTA, 2% CHAPS, 10% glycerol). In both cases, protein extracts were prepared in ice-cold lysis buffer supplemented with protease inhibitors (10 µg/ml leupeptin, 5 µg/ml aprotinin, 1 mM PMSF) and phosphatase inhibitors (1 mM Na<sub>3</sub>VO<sub>4</sub>, 100 µM NaF). Pre-cleared lysates (0.4–3.5 mg) were incubated with the appropriate antibody (1–3 µg) with protein A/G plus sepharose beads. For the analysis of endogenous BimEL tyrosine phosphorylation, antibody for the IP was crosslinked to protein A/G plus beads according to the manufacturer's protocol (ThermoScientific). After 16 h at 4 °C under end-over-end agitation, the beads were washed six times by centrifugation in ice-cold corresponding lysis buffer. The immunoprecipitated protein complexes were then resuspended in Laemmli buffer and boiled for 5 min at 95 °C before immunoblot analysis.

### Western blotting

The experimental procedures are detailed in *Supplementary Information*.

### Plasmids and site-directed mutagenesis

The experimental procedures are detailed in *Supplementary Information*.

### In vitro kinase assay

The experimental procedures are detailed in *Supplementary Information*.

### In vitro transcription/translation and GST pull-down

The experimental procedures are detailed in *Supplementary Information*.

### Analysis of Smac release from isolated mitochondria

The experimental procedures are detailed in *Supplementary Information*.

### Annexin V-DAPI staining

Twenty-four hours after transfection, HeLa cells were resuspended in 200 µl of buffer (150 mM NaCl, 10 mM HEPES pH, 5 mM KCl, 1 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>) and incubated with recombinant Annexin V-PE (BD Biosciences) for 10 min at room temperature. DAPI (0.5 µg/ml) (Molecular Probes) was then added, and the samples were analyzed immediately by flow cytometry using a MACS-Quant Analyzer (Miltenyi Biotec).

### Immunofluorescence

The experimental procedures are detailed in *Supplementary Information*.

### BRET experiment

The experimental procedures are detailed in *Supplementary Information*.

### Caspase activity measurement

The experimental procedures are detailed in *Supplementary Information*.

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**Author contributions** SM and PA conceived the project. LEA, EV, PG, and SM conducted the experiments and were assisted by PC, LS, SO, EP. FG and PPJ contributed to the BRET experiments. SM wrote the paper. PA, J-ER, LEA, BB-M, AJ, GR, FL, FG, and PPJ reviewed and edited the paper. SM and PA supervised the study and obtained funding.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interests.

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## **Supplementary Materials and Methods**

### **Reagents**

RPMI, DMEM and fetal calf serum (FCS) were purchased from ThermoFischer Scientific. Sodium fluoride, phenylmethylsulfonyl fluoride (PMSF), aprotinin, and leupeptin were purchased from Sigma-Aldrich. Sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ) was purchased from New England Biolabs. PP2 and U0126 were purchased from Tocris. Dasatinib was purchased from Selleckchem. SP600125 was purchased from Calbiochem. Coomassie Blue was purchased from ThermoFisher Scientific. The active Lyn recombinant protein was purchased from Millipore. The TNT-coupled reticulocyte lysate system was purchased from Promega. Recombinant BimL was obtained from R&D systems. Q-VD-OPh was obtained from SM Biochemicals LLC. The unconjugated goat F(ab')<sub>2</sub> anti-mouse and anti-human IgM antibodies were purchased from Jackson Immunoresearch. The Phos-Tag M series was obtained from WAKO. The JetPEI transfection reagent was purchased from Ozyme.

### **Antibodies**

Antibodies for western blotting, immunostaining and immunoprecipitation were obtained from the following suppliers: anti-Bim (#2933) for immunostaining and western blotting, anti Bim (#2819) for immunoprecipitation, anti-Phospho-Bim (Ser69) (#4585), anti-HA (#3724), anti-Lyn (#2732), anti-Phospho-Src (Y416) (#2101), anti-Fyn (#4023), anti-PARP (#9542), anti-ERK1/2 (#4695), anti-Phospho-ERK1/2 (#4370), anti-cleaved caspase-3 (#9661), anti-Bcl-xL (#2762), anti-LC3 B (#2775), anti-Phospho-S6 ribosomal (#4858), anti-Phospho-Jun (#2361), and anti-Phospho-Tyrosine (#9411) were purchased from Cell Signaling Technology. Anti-Lyn (sc-15 and sc7274), anti-mcl-1 (sc-819), anti-hsp90 (sc-69703), anti-myc 9E10 (sc-40), anti-TOM20 (sc-11415), and anti-Hsp60 (sc-1722) were purchased from Santa Cruz Biotechnology. Anti-Smac was purchased from R&D systems. Anti-Phospho-Bim (Y92) was custom made by ThermoFisher Scientific. Mouse anti-rabbit IgG (Conformation Specific) mAbs were purchased from Cell Signaling Technology. Secondary antibodies coupled to Alexa Fluor 488, Alexa Fluor 594 and Alexa Fluor 647 for immunofluorescence were obtained from Molecular Probes (ThermoFisher Scientific).

### **Transfection and infection**

For plasmid transfections, cells were seeded twenty-four hours prior transfection and were transfected with the JetPei method according to the manufacturer's instructions.

For the cell cycle experiments, cells were permeabilized in 70% ethanol overnight at -20°C, washed with PBS, incubated with PBS, RNase (20  $\mu\text{g/ml}$ ) and propidium iodide (50  $\mu\text{g/ml}$ ,

Sigma Aldrich) for 30 minutes at 4°C, and then analyzed by flow cytometry (MacsQuant Cytometers, Miltenyi Biotec).

DKO Bax/Bak MEFs were seeded in a 6-well plate ( $1.25 \times 10^5$  cells/well) and incubated for 24 h with 500  $\mu$ l of retroviral particles. The cells were washed with PBS, cultured for 2 days and then analyzed by FACS for GFP.

### **Western blotting**

Proteins were separated on polyacrylamide gels and transferred onto PVDF membranes. After nonspecific binding sites saturation, the membranes were incubated overnight at 4°C with a primary antibody in blocking buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% Tween-20, 3% BSA, 0.5% gelatin). The membranes were washed three times in TNA-1% NP40 (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP40) and incubated with the HRP-conjugated secondary antibody for 1 h at room temperature. The immunoblots were visualized using an enhanced chemiluminescence detection kit (Pierce).

Bim phosphorylation was also analyzed by immunoblotting using acrylamide conjugated with Phos-tag, a phosphate-binding chemical. SDS-PAGE was performed using 50  $\mu$ M Phos-tag Acrylamide (Wako) and 100  $\mu$ M MnCl<sub>2</sub> per the manufacturer's instructions.

### **Plasmids and site-directed mutagenesis.**

Mouse BimEL, BimL and BimS cDNA cloned into pEF PGKhygro vector, were previously described<sup>21</sup>. Mouse BimEL and BimS cloned into pGEX-6P1 and mouse Bim L into pGEX-4T1 were previously described<sup>21,54</sup>. Tyrosine mutations to phenylalanine in both the pEF PGK hygro- Bim and pGEX-Bim constructs were generated by QuikChange site-directed mutagenesis (Stratagene) using the following pairs of oligonucleotides: 5'-CCGGTCCTCCAGTGGGTTTTTCTCTTTGACAC-3' and 5'-GTGTCAAAGAGAAAAACCCACTGGAGGACCGG-3' (Tyr92 to Phe mutation); 5'-CCAGGCCTTCAACCACTTTCTCAGTGCAATGGC-3' and 5'-GCCAT TGCACGGAGAAAGTGGTTGAAGGCCTGG-3' (Tyr123 to Phe mutation); 5'-GTTCAACGAACTTTTACAAGGAGGGTG-3' and 5'-CACCTCCTTGTGAAAGTTT CGTTGAAC-3' (Tyr161 or Tyr105 to Phe mutation); 5'-GTGTTTGCAAATGATTTCC GCGAGGCTGAAGAC-3' (Tyr170 to Phe mutation); 5'-CCGGTCCTCCAGTGGGGA GTTCTCTTTTGTGACAC-3' and 5'-GTGTCAAAGAGAACTCCCACTGGAGGACCG G-3' (Tyr92 to Glu mutation); and 5'-GTTCAACGAACTGAGACAAGGAGGGTG-3' and 5'-CACCTCCTTGTCTCAGTTTCGTTGAAC-3' (Tyr161 to Glu mutation). The pEF PGKhygro mBimEL S65G was previously described<sup>21</sup>. The plasmid encoding human BimEL was a generous gift from Dr David Rubinsztein<sup>34</sup>.

The mouse BimEL WT form and mutants were PCR amplified from the pEF PGKhygro plasmid and subcloned into the pMIG-GFP viral vector containing an IRES-controlled GFP cassette for co-expression. For BRET experiments, the mouse BimEL variants were PCR amplified and subcloned into the Renilla luciferase fusion protein expression vector pRLuc provided by Dr Philippe Juin. All constructs were confirmed by sequencing.

The pcDNA3-Lyn WT, LynKD and cLyn (cytosolic and caspase-cleaved form of Lyn, Lyn $\Delta$ N) constructs were also previously described<sup>30</sup>. To obtain the kinase-dead form of cLyn (cLyn-KD), the lysine residue at position 275 in the putative ATP-binding site was replaced with an arginine using QuikChange site-directed mutagenesis (Stratagene). The primers used were previously described<sup>30</sup>.

To obtain the constitutive active form of cLyn (cLynCA), an Xba-1 fragment containing the mutation (Y508K) obtained from the pcDNA3-LynCA construct was subcloned into an Xba-1-digested pcDNA3-cLyn construct.

The cytosolic form of Lyn (cLyn-2) obtained by mutation of the two acylation motifs of Lyn (Glycine at position 2 and Cysteine at position 3 to Alanine) was generated from pcDNA-Lyn WT constructs using QuikChange site-directed mutagenesis (Stratagene). The primers used were as follows: sense 5'-CGCGTAGCGAGAAATAT

GGCAGCTATTAAATCAAAAAGG-3' and antisense 5'- CCTTTTTGATTTAATAGCTG  
CCATATTTCTCGCTACGCG-3'. Lyn was PCR amplified from pcDNA3-LynWT and then subcloned into pGEX-5X3.

The myc-tagged Mcl-1 construct cloned into the pcDNA3-myc-his vector was previously described<sup>55</sup>. The HA-tagged Bcl-xL cloned into the pcDNA3 vector was a generous gift from Dr Jean-Ehrland Ricci. The pHcRed1 vector was obtained from Clontech. pBB14, expressing a membrane-anchored GFP, pBB14 was a gift from Lynn Enquist (Addgene plasmid # 18657).

### **In vitro kinase assay**

GST fusion proteins were produced from BL21 bacteria transformed with either the pGEX-6P vector for BimEL and BimS, as previously described<sup>21</sup>, the pGEX-4T1 vector for BimL, as previously described<sup>54</sup>, or pGEX-5X3-Lyn.

The assay was performed in kinase buffer (30 mM HEPES pH 6.8, 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>) containing 20 ng of recombinant active Lyn (Millipore), 1  $\mu$ g of GST protein, and 10  $\mu$ Ci ATP- $\gamma$ -<sup>32</sup>P (Amersham Biosciences) for 30 min at 30°C. The reaction was stopped by the addition of Laemmli sample buffer and resolved by SDS-PAGE. <sup>32</sup>P-labeled proteins were electrophoresed on polyacrylamide gels. The gels were autoradiographed using Amersham hyperfilm.

### **In vitro transcription and translation**

Wild-type Lyn and Fyn constructs were transcribed and translated using the Promega TNT-coupled reticulocyte lysate system in the presence of cold methionine as previously described<sup>55</sup>. The recombinant proteins contained in the cell lysates were used for GST pull-down assays.

### **GST pull-down**

Purified GST fusion proteins (20  $\mu$ g) were incubated for 16 h at 4°C with either a protein extract from Ramos B cells (2 mg) or a recombinant protein produced in vitro in reticulocyte lysates. The protein complexes were then pulled down with glutathione sepharose beads for 2 h at 4°C. After 6 washes, the GST pull-down protein complexes were analyzed by WB.

### **Analysis of Smac release from isolated mitochondria**

Mitochondria were isolated from HeLa cells using a differential centrifugation method as previously described<sup>56</sup>. Briefly, the cells were centrifuged at 1,000  $\times$  g for 5 min at 4°C, washed, and resuspended in mitochondrial isolation buffer (MIB) buffer (10 mM HEPES-KOH pH 7.4, 250 mM d-mannitol, 0.5 mM EGTA) supplemented with protease inhibitors (20  $\mu$ g/ml leupeptin, 20  $\mu$ g/ml aprotinin, and 1 mM PMSF). The cells were homogenized in a Dounce homogenizer. The homogenates were centrifuged twice at 1,000  $\times$  g for 5 min at 4°C to pellet nuclei and unbroken cells. The supernatants were centrifuged at 10,000 g for 10 min at 4°C, and the mitochondrial pellet was washed twice with MIB buffer. The mitochondria were resuspended in MIB buffer and used within 1 h.

To assess mitochondrial protein release, 250 ng of purified GST-BimEL, L and S were pre-incubated with or without 500 ng of recombinant Lyn (Millipore) for 15 min at 30°C. The GST-Bim +/- Lyn protein samples were then incubated with 25  $\mu$ g of purified mitochondria in a total volume of 30  $\mu$ l of MIB buffer for 1 h at 30°C. The samples were then centrifuged to separate the mitochondrial and supernatant fractions, and denatured protein samples in Laemmli Buffer were analyzed by western blotting for Smac and Hsp60 expression.

### **Immunofluorescence**

Cells were grown on coverslips for 24 h and then fixed in 3.3% paraformaldehyde, permeabilized in PBS containing 0.1% Triton X-100, blocked in 2% fetal bovine serum (FBS), and stained with anti-Bim (C34) and anti-cytochrome-c antibodies. The cells were next labeled with the respective Alexa Fluor-coupled secondary antibody (Molecular Probes, Thermo). The cells were observed by confocal microscopy (Nikon).

### **BRET experiment**

The BRET experiment was performed as previously described<sup>36</sup>. Briefly, BimEL and mutated coding sequences were subcloned into the pRLuc-C2 luciferase expression vector (BioSignal Packard) to produce the donor moiety in the BRET assay. The acceptor moiety was obtained by cloning the sequence of Bcl-xL A221R into the pEYFP-C1 vector (BD Biosciences). Dose response curves were generated according to the following protocol: cells were plated in 12-well plates and transfected 24 h later using Lipofectamine 2000 (Life Technologies) with 200 ng of donor plasmid (pRLuc-BIM-EL, pRLuc-BIM-EL-YE, or pRLuc-BIM-EL-YF) and 500 ng of acceptor plasmid (pEYFP-BCL-xL-A221R) per well (day 0). On day 1, the cells were trypsinized and seeded in 96-well white plates before being treated on day 2 with increasing concentrations of WEHI-539. Sixteen hours later, the cells were washed once with PBS, and BRET was then monitored using a Mithras LB 940 reader (Berthold Technologies) after the injection of the luciferase substrate Coelenterazine-h (5  $\mu$ M final) (Interchim). The data are presented as the mean  $\pm$ SD of three independent experiments.

### **Caspase activity measurement**

Assay (in triplicate) was performed with 15  $\mu$ g of protein prepared from control or stimulated cells. Briefly, cellular extracts were incubated in a 96-well plate, with 0.2 mM of Ac-DEVD-AMC (7-amino-4methylcoumarin) as substrates for various times at 37°C as previously described<sup>14</sup>. Caspase activity was measured following emission at 460 nm (excitation at 390 nm) in the presence or not of 10  $\mu$ M of Ac-DEVD-CHO. Enzyme activities were determined as initial velocities expressed as relative intensity per min and per mg of protein.

## **Legends of supplementary figures**

### **Supplementary Figure 1:**

- (a) Input related to figure 1e.
- (b) Input related to figure 1f.

### **Supplementary Figure 2:**

- (a) In vitro phosphorylation of recombinant human BimL by Lyn. Proteins were separated by SDS-PAGE, and the gels were stained with Coomassie blue and exposed for autoradiography.
- (b) Input related to figure 2c.
- (c) Input related to figure 2d.
- (d-e) HEK293 cells were transfected with vectors encoding cLyn and BimL (d) or human BimEL (e). Twenty-four hours after transfection, cells were treated with or without sodium pervanadate for 15 min to inhibit tyrosine phosphatases. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-Bim antibodies. Nonrelevant Ig (IP NR) served as an immunoprecipitation control.
- (f) Input related to figure 2f.

### **Supplementary Figure 3:**

- (a) Input related to figure 2g.
- (b) WEHI-231 cells, a mouse B lymphoma cell line, were stimulated for 16 h with 5  $\mu\text{g/ml}$  goat anti-mouse IgM in the presence or absence of velcade (10 nM), a proteasome inhibitor. Twenty-four hours after transfection, sodium pervanadate, an

inhibitor of tyrosine phosphatases, was added for the last 15 min of stimulation. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with the appropriate antibodies.

Nonrelevant Ig (IP NR) served as an immunoprecipitation control. The black arrowhead indicates the tyrosine phosphorylated form of Bim. The asterisk indicates cross-reactivity with the antibody light chain used in the IP.

#### **Supplementary Figure 4:**

(a) Schematic representation of the tyrosine residues present in the mouse BimEL and BimL proteins. The black box indicates the BH3 domain.

(b) In vitro Lyn phosphorylation assay with recombinant WT and nonphosphorylatable mutants (Y to F) of GST-BimL.

(c) Input related to figure 3b.

(d-e) HEK293 cells were co-transfected with vectors encoding (d) cLyn and WT or a nonphosphorylatable Y105F mutant of BimL, or (e) cLyn and WT or mutant forms of BimEL (double mutant Y92/Y161F, single mutant S65G, triple mutant DM-YF/S65G). Twenty-four hours after transfection, cells were stimulated with sodium pervanadate for 15 min. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with the indicated antibodies.

Nonrelevant Ig (IP NR) served as an immunoprecipitation control.

#### **Supplementary Figure 5:**

(a) HEK293 cells were co-transfected with vectors encoding cLyn and WT or the nonphosphorylatable Y92F/Y161F (DM-YF) double mutant of BimEL. Twenty-four hours after transfection, cells were treated with or without sodium pervanadate for 15

min to inhibit tyrosine phosphatases. Cell extracts (input) were immunoprecipitated for Bim, separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine and anti-Bim antibodies. Nonrelevant Ig (IP NR) served as an immunoprecipitation control.

(b) Input related to figure 3c.

(c) Input related to figure 3d.

(d) HEK293 cells were co-transfected with vectors encoding a constitutively active cLyn (cLynCA) and WT or mutant forms of BimEL (Y92F, Y161F, DM-Y92/Y161F or S65G). Twenty-four hours after transfection, cells were pre-treated for 5h with the proteasome inhibitor velcade (50nM) and then stimulated or not with sodium pervanadate for 15 min to inhibit tyrosine phosphatases. Cell extracts (input) were immunoprecipitated for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody.

(e) Input related to figure 3e.

(f) WEHI-231 cells were stimulated for the indicated time points with 5  $\mu$ g/ml goat anti-mouse IgM in the presence or absence of velcade® (10 nM), a proteasome inhibitor. The cell extracts (input) were immunoprecipitated for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody.

Nonrelevant Ig (IP NR) served as an immunoprecipitation control.

### **Supplementary Figure 6:**

DKO Bax/Bak MEFs were transfected with a vector encoding EE-tagged WT or double mutant forms of BimEL. Then, cells were fixed, permeabilized and immunostained for Bim (anti-EE, red) and Tom20 (green). Cells were observed by

confocal microscopy (Nikon). The merge panel shows that both mutants localized to mitochondria, similar to WT BimEL.

### **Supplementary Figure 7:**

Bax/Bak DKO MEFs transduced with a pMIG vector encoding for WT or mutant forms of BimEL and GFP, were sorted for GFP and used to analyze the impact of the expression of BimEL variants on autophagy and cell proliferation in an apoptosis-deficient background.

**(a)** Expression of Bim and basal autophagy (monitored by LC3-I/II conversion) were analyzed by WB.

**(b)** Cells were cultured in DMEM or EBSS for indicated time points in the presence or absence of bafilomycin (40 nM), an inhibitor of late stage of autophagy, which induces LC3-II accumulation. P-S6R was used as a control of autophagy induction. Note that the autophagy induction is the same in all cell lines tested, indicating that neither WT Bim nor the nonphosphorylatable or phosphomimetic mutants modulate this pathway, at least in MEFs.

**(c)** Basal autophagy in the absence and presence of bafilomycin (40 nM) was evaluated by analyzing the conversion of LC3-I/II by WB. Note that neither the expression of WT Bim nor that of the Bim mutants was modulated by bafilomycin, indicating that none of the variants was degraded in an autophagy-dependent manner.

**(d)** Cell cycle was evaluated by flow cytometry 24 h and 48 h after cell plating. Histogram graphs represent the percentage of cells in each phase of the cell cycle (n=2). Error bars represent the standard error of duplicate samples from the same experiment.

(e) Cells were seeded at a density of 1000 cells per well in 6-well plate, and proliferation was evaluated by staining cell clones with crystal violet after 5 days in culture.

**Supplementary Figure 8:**

(a) Input related to figure 5a.

(b) Input related to figure 5b.

(c) Luciferase activity of each fusion Bim-Luciferase variants used for BRET assay reported in fig. 5d.

**Supplementary Figure 9:**

(a) Representative GFP fluorescence profile of surviving (blue) or apoptotic (red) HeLa Smac-GFP cells. Note that when the cells undergo apoptosis, upon actinomycin D treatment or BimEL transfection, the GFP fluorescence shifts from high to low intensity.

(b) WB analysis of Smac release from isolated mitochondria incubated for 30 min at 37°C with GST-BimEL, GST-BimL and GST-BimS in the presence or absence of recombinant active Lyn. Lyn and GST-Bim proteins were mixed together 30 min before the incubation with mitochondria.

**Supplementary Figure 10:**

(a) Input related to figure 6a.

(b) LAMA84 were pre-treated or not with 10 $\mu$ M PP2 for 2h before sodium pervanadate treatment for 10min. The cell extracts (input) were immunoprecipitated

for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody.

(c) Input related to figure 6b.

(d) K562 cells were treated with 20nM of nilotinib for 24h and 48h time and then treated with sodium pervanadate for 10min. The cell extracts (input) were immunoprecipitated for phospho-Y92 Bim, separated by SDS-PAGE and immunoblotted with an anti-Bim antibody. PARP cleavage was analyzed to monitor apoptosis induction. Nonrelevant Ig (IP NR) served as an immunoprecipitation control.

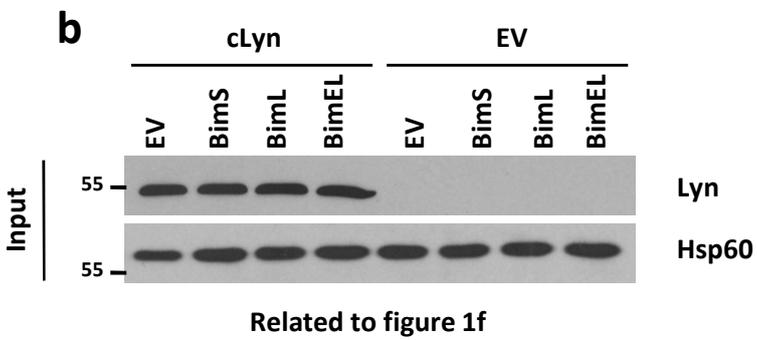
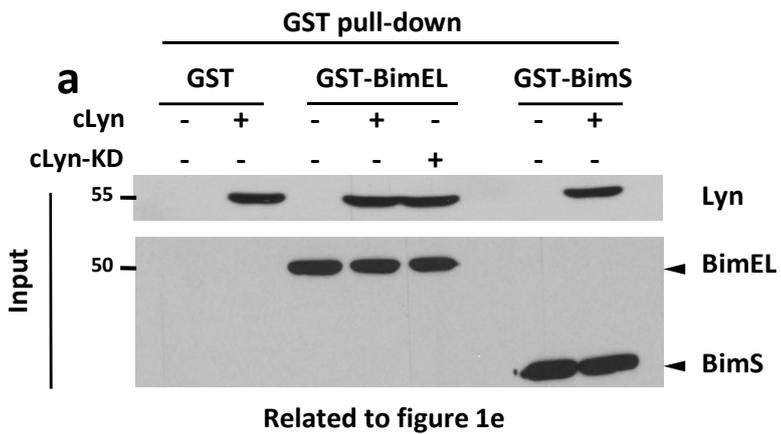
(e) Input related to figure 6c.

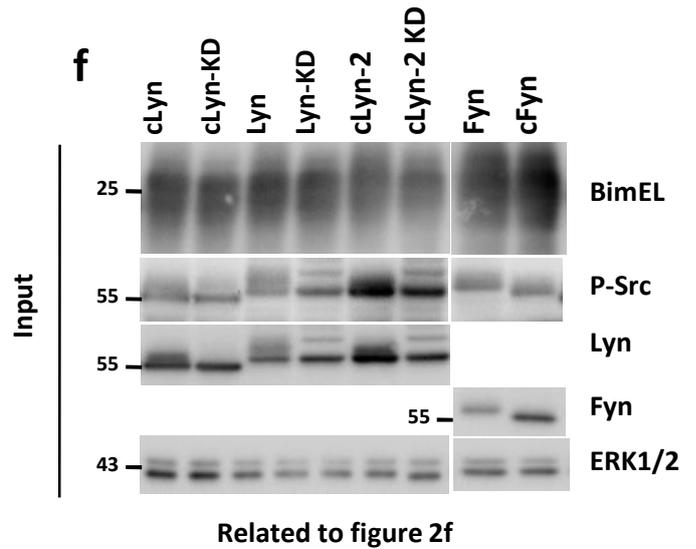
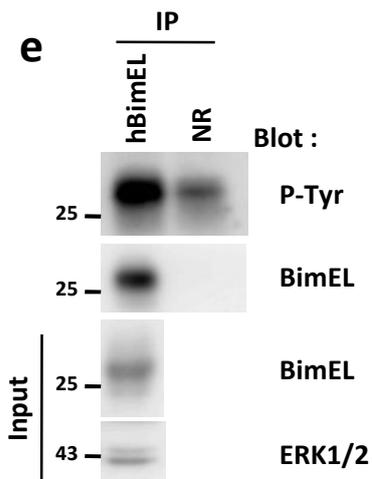
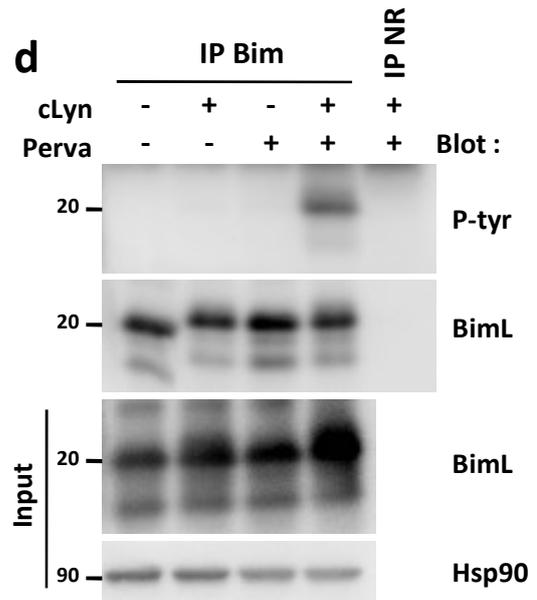
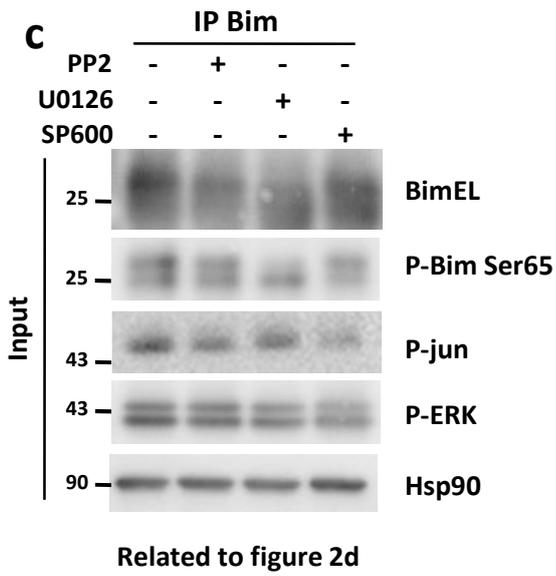
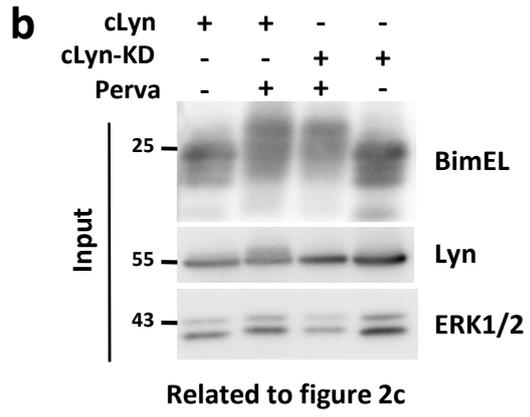
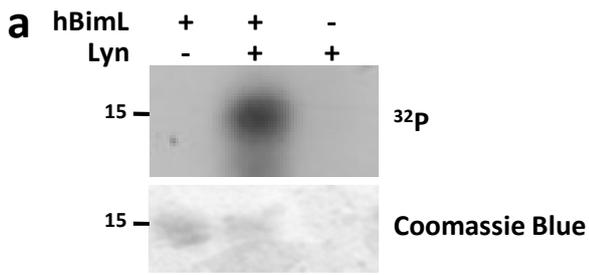
(f) Input related to figure 6d.

(g) Input related to figure 6e.

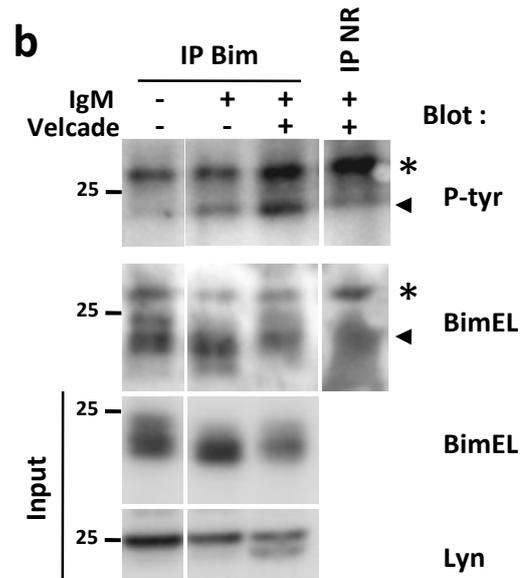
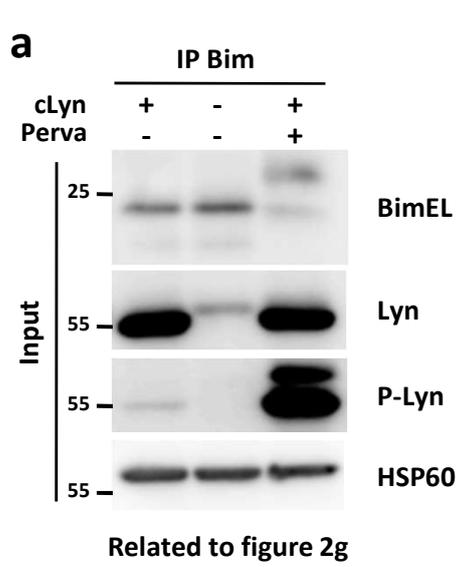
**Supplementary Figure 11:**

Sequence alignment of the Bim protein and its homologs between human, mouse, zebrafish, xenopus, drosophila and C. elegans. Note that Y92 and Y161 are the 2 most evolutionarily conserved tyrosine residues.

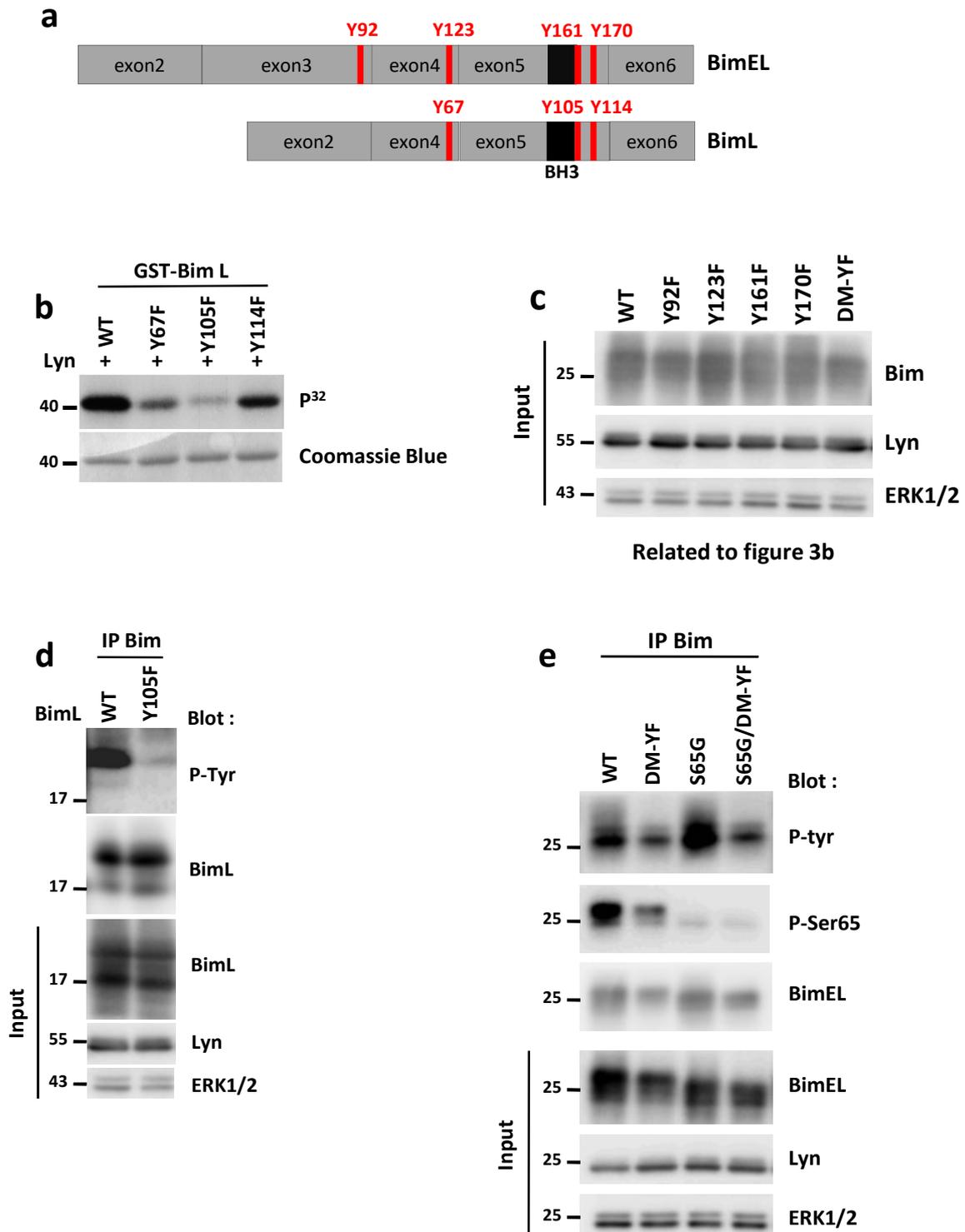




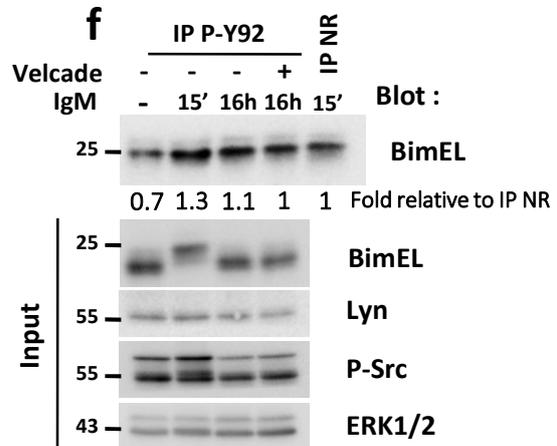
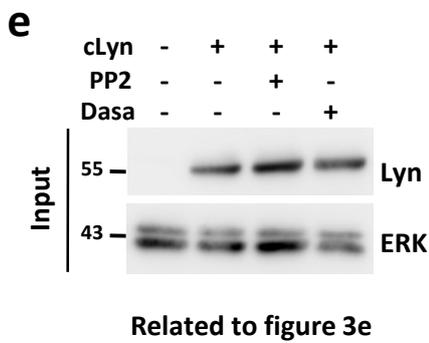
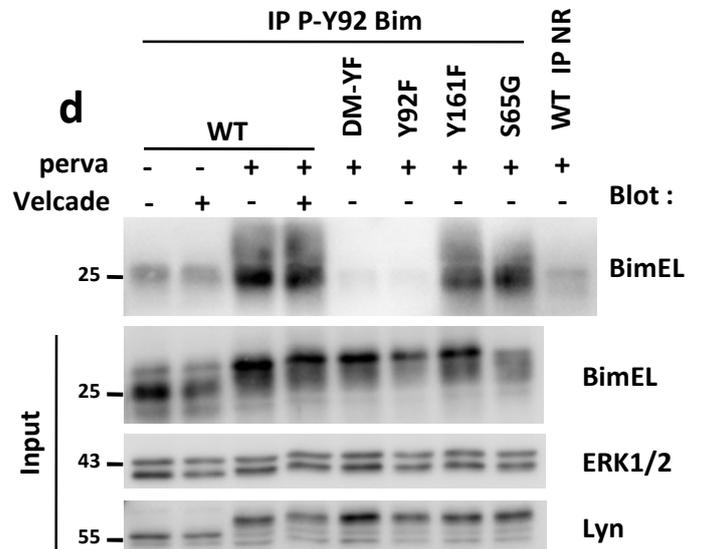
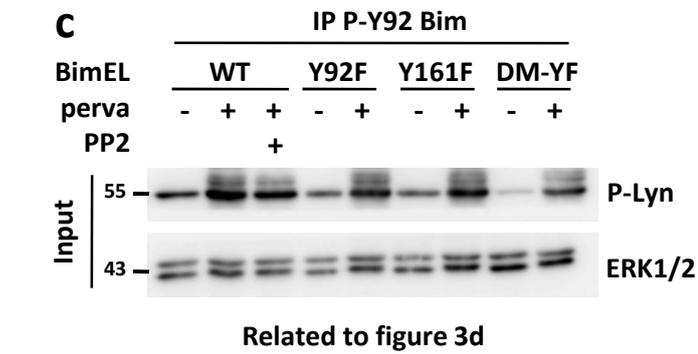
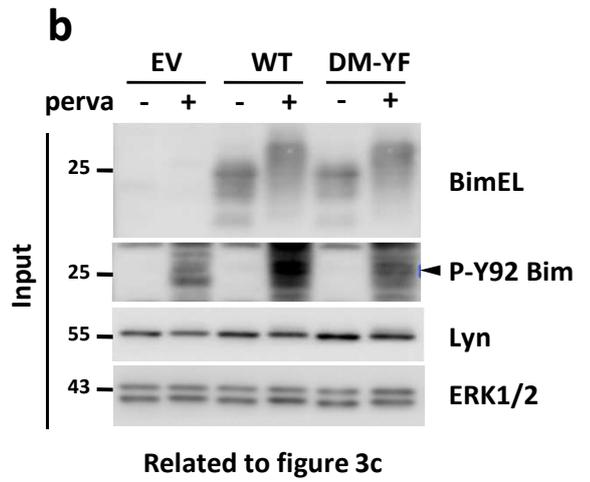
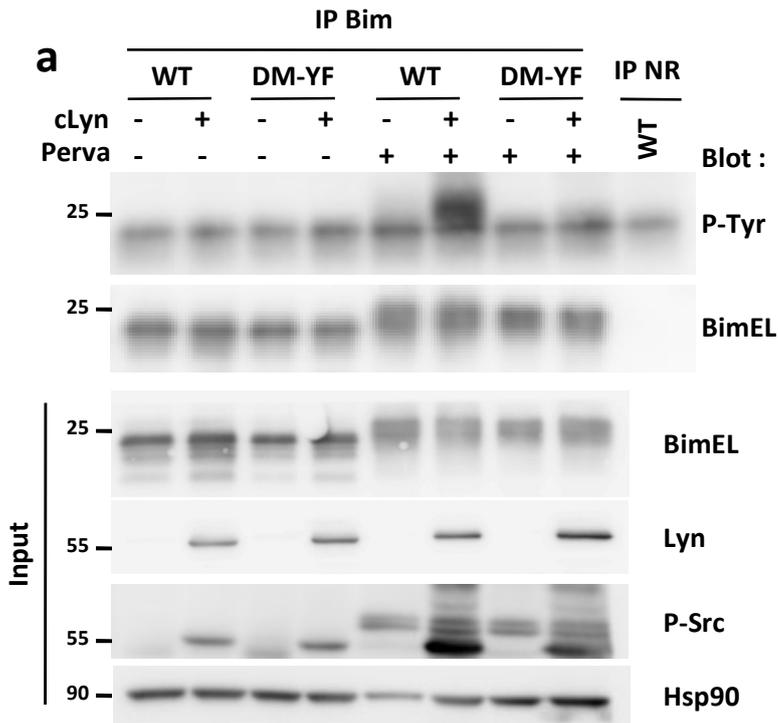
Supplementary Figure 2



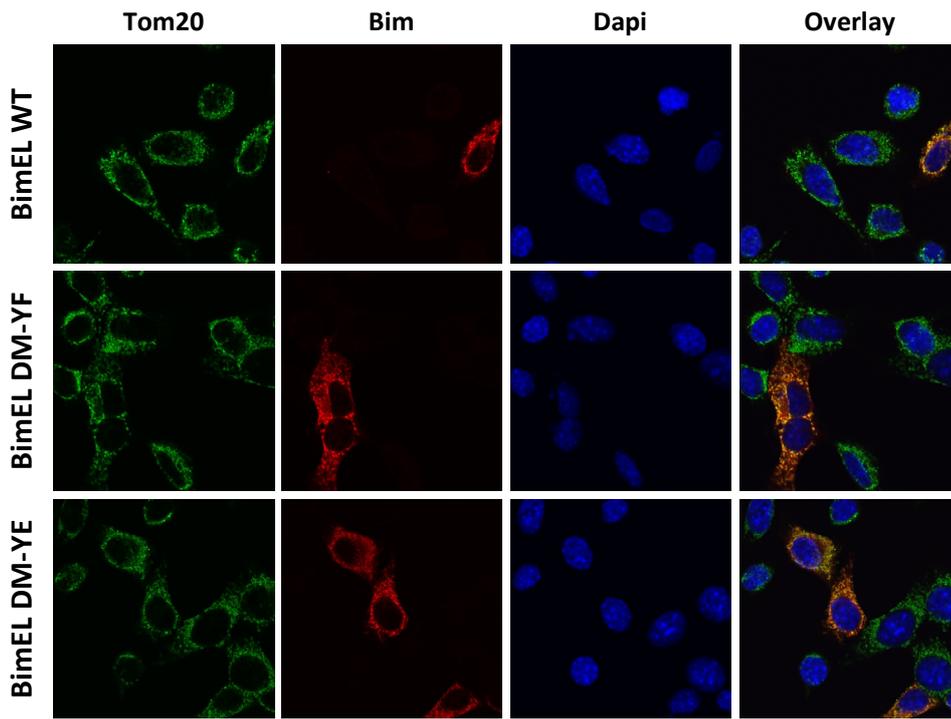
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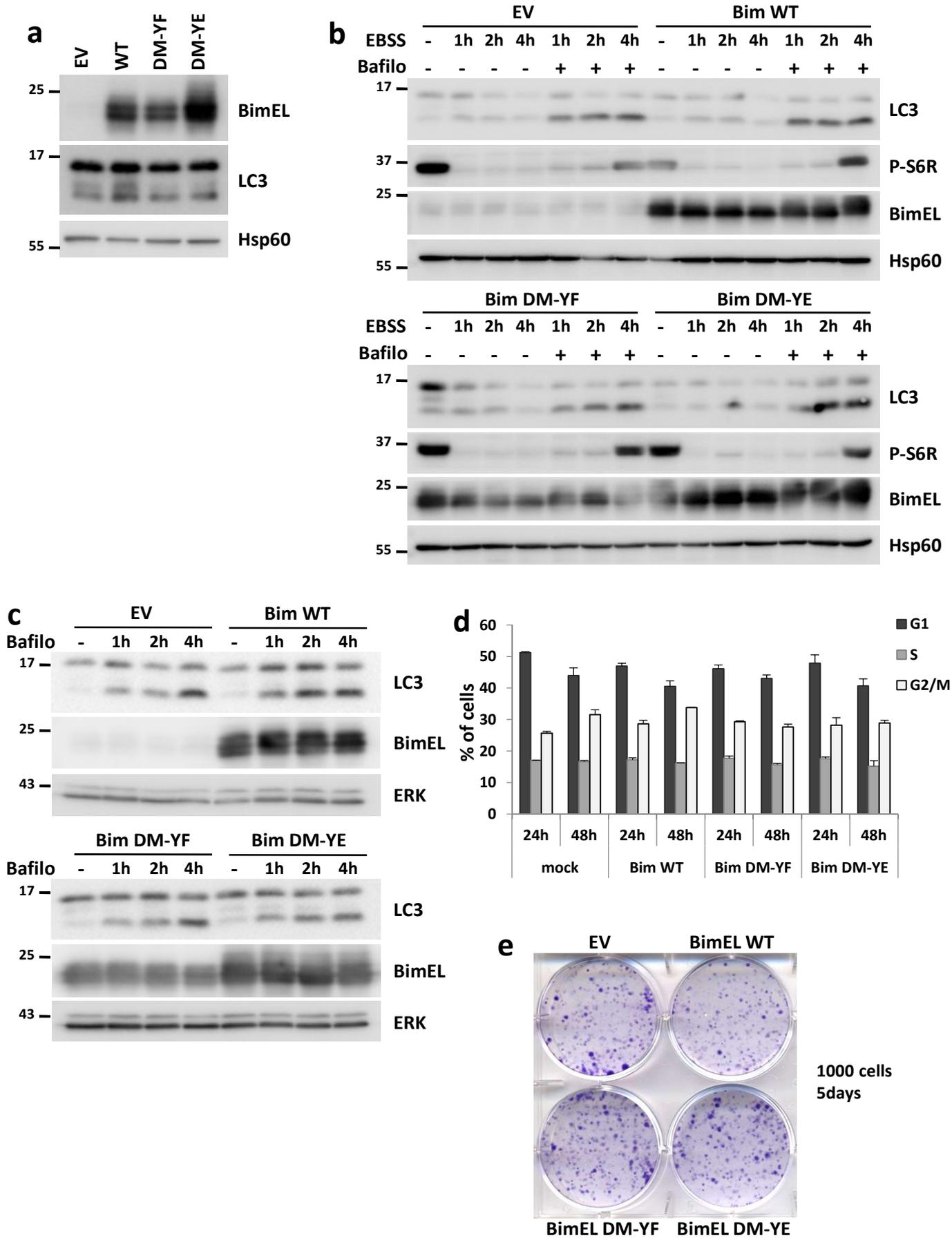
Supplementary Figure 4



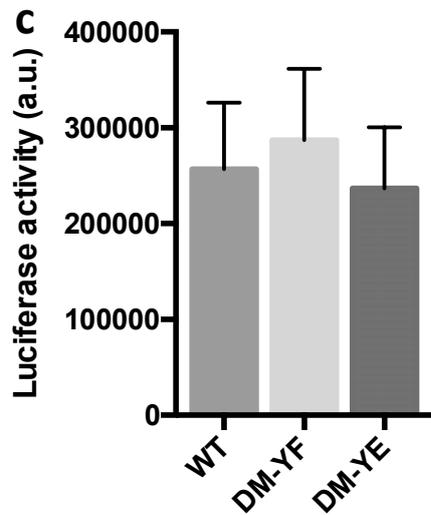
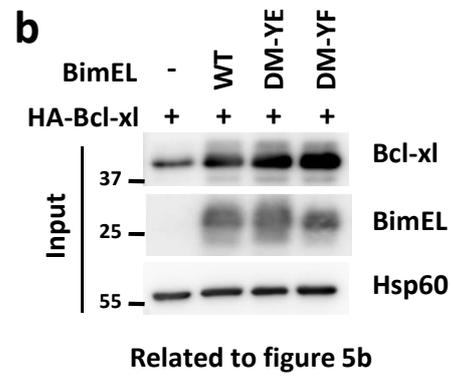
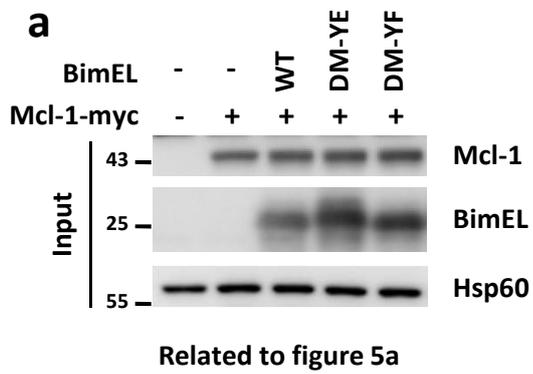
Supplementary Figure 5

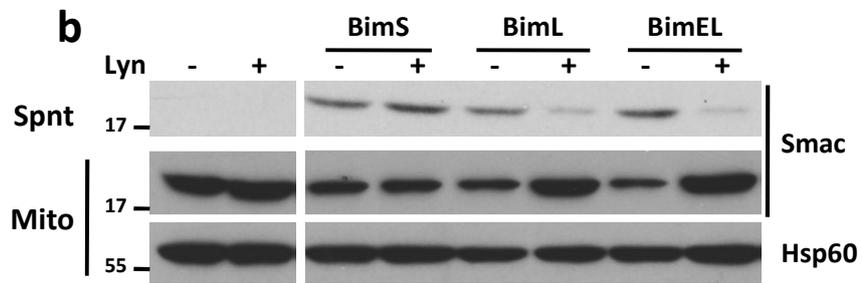
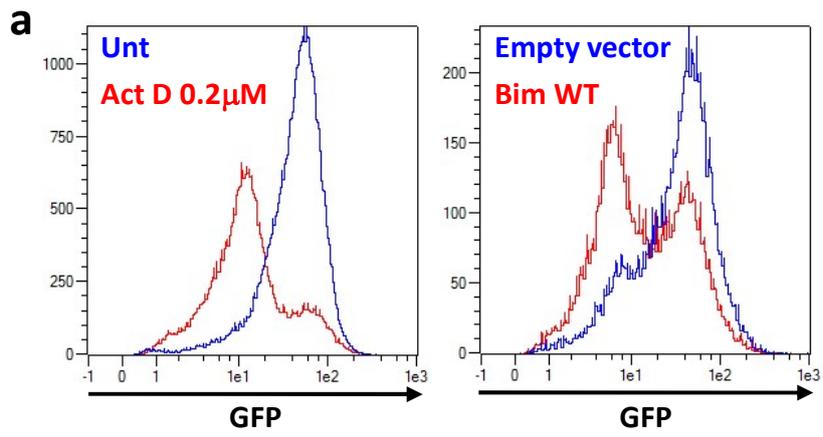


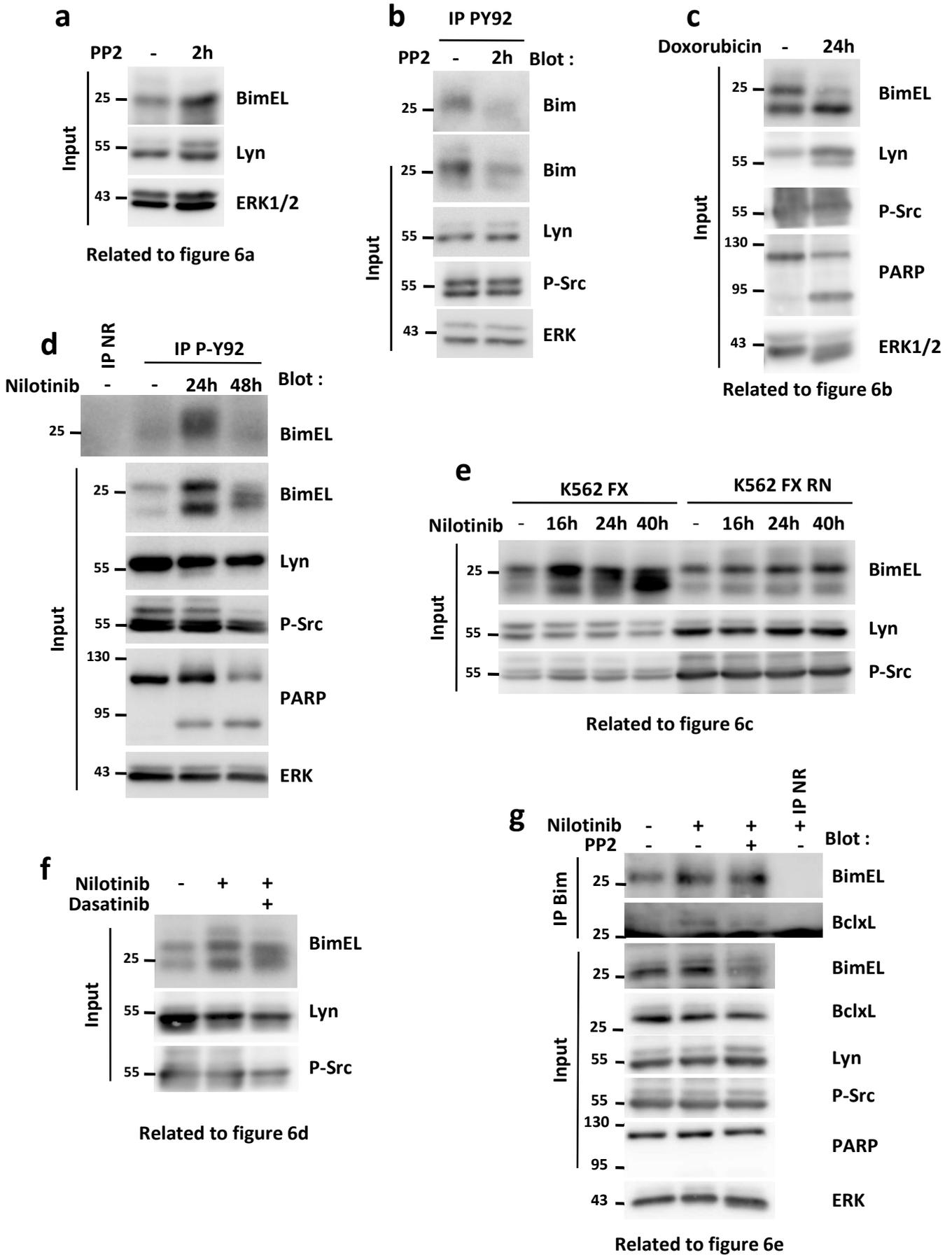
Supplementary Figure 6



Supplementary Figure 7







Supplementary Figure 10

Human  
 Mouse  
 Danio rerio  
 Xenopus  
 Drosophilla  
 C. Elegans

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  ---MAKQPSDVSSECDR-EGRQLQPAERPPQLRP-----G
  ---MAKQPSDVSSECDR-EGGQLQPAERPPQLRP-----G
  -----MSDTSREQTLANGPASQSGES-----TG
  ---MAKQPSVLSPECNSGEGGQLQSTSRRHSHRPRR-----RG
  MAPTTSPPPKLAKFKSSSLDHEIYTANRRGTIATASSDWKALRG
  -----
  Human
  Mouse
  Danio rerio
  Xenopus
  Drosophilla
  C. Elegans
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  TEPQGNPD----GEGDRCPHGSPQGPLAPPASPGPFATRSPLFI
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  SPFQGNOS---DEGGSSSASTPWGPTLSPYSPSSFVNRSPhCM
  GPGGSVFPN92---SNGRSLHAGGPMTRAASTSSIASSTRMTNYQ123
  -----MIMLTFASTSS--D
  Human
  Mouse
  Danio rerio
  Xenopus
  Drosophilla
  C. Elegans
  LLSRSSSGYFSFDTDRSPAPMSCDKSTQTPSPPCQ--AFNHLYS.
  LLSRSSSGYFSFDTDRSPAPMSCDKSTQTPSPPCQ--AFNHLYS.
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  LVSKTSSGYFTFEG--SPGPVSCDKSTQTPSPPCQ--AFNHLLS.
  INQKCLCGQYIRARLRRAGVLNRKVTQRI161RNILD170EGSSHVVYE'
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  . . . . . : :
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  ----PVDQMTPELW--IAQELRRIG-----DEFNASFTP----
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  : .: :* .::
  
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Supplementary Figure 11



## RESUME ARTICLE 2:

### *Caspase 1/11 deficiency or pharmacological inhibition mitigates the psoriasis-like phenotype in mice*

Inflammatory caspases, clustering caspases 1, 4 and 5 in human as well as caspases 1 and 11 in mice, are activated through multiprotein complex known as inflammasomes; enacting important functions in inflammation regulation by directly cleaving their target proteins, notably the pro-inflammatory cytokines IL-1 $\beta$  and IL-18. Albeit they are principally activated in presence of microbial products, important functions for these caspases have been described in sterile inflammation, prominently in intestinal inflammation and metabolic disorders. Psoriasis is a chronic immune-mediated skin pathology, where the expression and activation of inflammatory caspases has been reported, although the function of these caspases has not been well described. Thus, in this study, we analyzed the role of inflammatory caspases in psoriasis disease.

First, lesional and non-lesional skin biopsies from psoriasis patients were compared and a significant increase in the activation and expression of inflammatory caspases were found in lesional skin, being this study the first one evidencing that caspase 4 and 5 are activated in lesional skin from psoriasis patients.

Then, we wanted to know if these caspases were involved in psoriasis pathogenesis, and to answer that, inflammatory caspase deficient mice (dKO mice) were challenged with three mouse models of psoriasis-like disease. The crossing of cLyn mice with dKO mice or the treatment of dKO mice with the psoriasis-like mouse models, IMQ or IL-23, shown that the absence of inflammatory caspases led to a delay in the psoriasis-like phenotype compared to WT mice, corroborated by a significant decrease in the epidermal hyperplasia, immune cell infiltration and pro-inflammatory cytokine production. To confirm these results, WT mice were concomitantly treated with IMQ and the inflammatory caspase inhibitor AC-YVAD-CMK and similar results were obtained, indicating that inflammatory caspases are implicated in psoriasis pathogenesis, but either their invalidation or their pharmacological inhibition partially impact in the induced psoriasis-like phenotype.

Keratinocytes are able to secrete IL-1 $\beta$  and IL-18 in response to stress conditions. Here, we demonstrated that keratinocytes are primed to activate caspase 5 and to release the pro-inflammatory cytokine IL-1 $\beta$  under mimicking psoriasis conditions of stimulation with TNF- $\alpha$  and IL-17A, two of the principal pro-inflammatory cytokines involved in psoriasis pathogenesis, indicating that although immune cells are

a key source of caspase activation and IL-1 $\beta$  production, keratinocytes could be also an important wellspring. Therefore, we wanted next to know the respective contribution of keratinocytes or immune cells through inflammatory caspase activation in psoriasis disease. Bone marrow transfer experiments demonstrated that the expression/activation of inflammatory caspases in immune cells was sufficient to develop a complete cutaneous inflammatory response.

Altogether, these results show that inflammatory caspases are involved in psoriasis pathogenesis and although keratinocytes are able to activate inflammasome leading to caspase activation and pro-inflammatory cytokine production, the activation of inflammatory caspases in the recruited immune cells is enough to fully develop the induced psoriasis-like phenotype.

## **Caspase 1/11 deficiency or pharmacological inhibition mitigates psoriasis-like phenotype in mice**

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### **Keywords**

Inflammatory response, pro-inflammatory caspases, pro-inflammatory cytokines, keratinocytes, human skin biopsies, psoriasis

**Running title:** Inflammatory caspases are involved in psoriasis

## **Abstract**

Inflammatory caspases, activated within inflammasome, are responsible for the maturation and secretion of IL-1 $\beta$ /IL-18. While their expression in psoriasis was demonstrated several years ago, little is known about the role of inflammatory caspases in the context of psoriasis. Here, we confirmed that caspase-1, -4 and -5 are activated in lesional skin from psoriasis patients. We showed in three psoriasis-like models that inflammatory caspases are activated, and accordingly, caspase-1/11 invalidation or pharmacological inhibition by AC-YVAD-CMK injection induced a decrease in ear thickness, erythema, scaling, inflammatory cytokine expression and immune cell infiltration in mice. Interestingly, we observed that keratinocytes were primed to secrete IL-1 $\beta$  when cultured in condition mimicking psoriasis. Generation of chimeric mice by bone marrow transplantation was carried out in order to decipher the respective contribution of keratinocytes and/or immune cells in the activation of inflammatory caspases during psoriasis-like inflammatory response. Our data showed that the presence of caspase-1/11 in the immune system is sufficient for a fully inflammatory response whereas the absence of caspase-1/11 in keratinocytes/fibroblasts had no impact. In summary, our study indicates that inflammatory caspases activated in immune cells are implicated in psoriasis pathogenesis.

## Introduction

Caspases are a family of proteases (Cysteine-ASPartic proteASES), highly conserved evolutionarily, with a key role in apoptosis and inflammatory signaling pathways (Lamkanfi et al., 2007). In relation to their biological function, caspases have been classified in apoptotic (2, 3, 6, 7, 8, 9 and 10) and pro-inflammatory (1, 4, 5, 11 and 12) (Li and Yuan, 2008), mainly involved in cell death signaling pathways and regulation of cytokine maturation during inflammation, respectively. Nevertheless, pro-inflammatory caspases have been also implicated in pyroptosis, another programmed cell death (Lamkanfi and Dixit, 2014, Man and Kanneganti, 2015). Although proinflammatory caspases 1 and 12 are functional orthologous between human and mice; caspases 4 and 5, only present in human, are the homologous of mouse caspase 11 (Liu and Lieberman, 2017).

Pro-inflammatory caspases, firstly produced as inactive zymogens, are activated through multiprotein complexes called inflammasomes after cellular stimulation via engagement of pattern recognition receptors (Martinon et al., 2002). Once activated, inflammatory caspases mediated immune response against infectious stress through the maturation and secretion of pro-inflammatory cytokines such as interleukin (IL)-1 $\beta$  and IL-18. Caspase 1 activation and subsequent IL-1 $\beta$  production have been associated with a large variety of inflammatory and autoimmune diseases (reviewed in (Gabay et al., 2010, McIlwain et al., 2013, Patel et al., 2017)) such as rheumatoid arthritis, type 2 diabetes (Ruscitti et al., 2015) and inflammatory bowel diseases (IBD) (Perera et al., 2017).

Psoriasis is a chronic auto-inflammatory/autoimmune skin disease (Lowe et al., 2014) characterized by an intense dialogue between keratinocytes and immune cells, affecting 2% of the worldwide population (Parisi et al., 2013). Psoriasis has been classified according to several clinical manifestations, psoriasis vulgaris being the most common type, whose principal feature is the development of red patches with silver scales called psoriasis plaques (Deng et al., 2016). A large and complex network of several proinflammatory cytokines such as IL-1- $\alpha$ , IL-1 $\beta$ , IL-17, IL-18, IL-22, IL-23, interferon (IFN)- $\gamma$  and tumor necrosis factor (TNF)- $\alpha$  have been associated with the development and establishment of psoriasis plaques (Nickoloff et al., 2007), highlighting a key role of these cytokines in the pathogenesis of psoriasis.

Albeit the role of IL-1 and IL-18 cytokines has been well described in psoriasis pathogenesis (Companjen et al., 2004, Rabeony et al., 2015, Shimoura et al., 2017, Singh et al., 2016, Uribe-Herranz et al., 2013), little is known about the contribution of pro-inflammatory caspases in this context. It has been showed that activated caspase 1 is increased in lesional skin samples from psoriasis patients compared to non-lesional samples (Johansen et al., 2007, Marchetti et al., 2009). Moreover, an increase in the mRNA expression of caspase 5 was found in lesional biopsies (Salskov-Iversen et al., 2011) and a constitutive

expression of this caspase was observed in the epidermis of normal skin (Zwicker et al., 2017). However, the contribution of inflammatory caspases in psoriasis pathogenesis is controversial as Cho et al reported a decrease in the epidermal thickness associated with a significant reduction in IL-1 $\beta$  levels in caspase 1 -/- mice treated with IMQ (Cho et al., 2012) whereas another recent study concluded that the invalidation of caspase 1 had no impact in reducing the psoriasis-like phenotype after IMQ treatment (Rabeony et al., 2015).

Therefore, we decided to address the implication of pro-inflammatory caspases in psoriasis pathogenesis. We first find in a large cohort of psoriasis patient biopsies that caspase 1 and 5 are activated in all lesional samples and that caspase 4 is activated in most lesional biopsies. Further, caspase 1/11 deficiency and pharmacological inhibition of pro-inflammatory caspases significantly reduce skin inflammatory disease in three mouse models of psoriasis like-phenotype. Moreover, stimulation of normal human keratinocytes (NHK) with TNF- $\alpha$  and IL-17A demonstrates that keratinocytes are primed to activate caspase 5 and secrete IL-1 $\beta$ . Finally, by adoptive transfer experiments we show that pro-inflammatory caspases activation in immune cells is sufficient to induce a complete inflammatory response in mice.

## Results

### **Pro-inflammatory caspases are activated in lesional skin biopsies from psoriasis patients**

Although increased mRNA expression of pro-inflammatory caspases 1, 4 and 5 in lesional biopsies from psoriasis patients has been already observed (Salskov-Iversen et al., 2011, Zwicker et al., 2017) and expression of cleaved caspase 5 could be detected in one psoriasis patient (Zwicker et al., 2017), we decided to assess in 25 psoriasis patients, the expression of caspases to confirm their activation status. To do that, western blot analysis of skin extracts was performed from non lesional and lesional biopsies. Pro-caspase 1, 4 and 5 were all expressed in non-lesional biopsies; however only a strong increase of pro-caspase 5 was observed in lesional skin as compared to non-lesional paired and healthy donor skin (Figure 1a, b, d; Figure S1a). Interestingly, high levels of cleaved caspase 1 and 5 were observed in lesional skin from all analyzed patients (Figure 1a-d; Figure S1a) and cleaved caspase 4 was seen in most analyzed lesional biopsies (Figure 1a, b, d; Figure S1a). Moreover, an increase in inflammasome component expression along with the mature forms of IL-1 $\beta$  and IL-18 was found (Figure S1b), as previously reported (Dombrowski et al., 2011, Johansen et al., 2007). Altogether, these data suggest that pro-inflammatory caspase activity is regulated in injured skin from psoriasis patients.

### **Deficiency in pro-inflammatory caspases 1/11 impaired the psoriasis-like disease development in different mouse models**

While the role of inflammasome-processed cytokines IL-1 $\beta$  (Feldmeyer et al., 2010) and IL-18 (Companjen et al., 2004) is well-known in psoriasis, the role of proinflammatory caspases in this scenario is controversial. Therefore, to clearly define the role of pro-inflammatory caspases in psoriasis-like disease, we decided to use three different mouse models: i) the Lyn $\Delta$ N transgenic mice (Marchetti et al., 2009), which develop spontaneously psoriasis after birth; ii) the IMQ-induced skin dermatitis (van der Fits et al., 2009), and iii) the IL-23 intradermal ear injections (Hedrick et al., 2009).

First, we used the Lyn $\Delta$ N mice (Marchetti et al., 2009) which recapitulate the main features of human psoriasis, as illustrated by the increased skin expression of the principal cytokines implicated in psoriasis pathogenesis (Figure S2a). Interestingly, an increased mRNA expression of some inflammasome components, such as caspase-11, Absent In Melanoma (AIM)-2 and NLRP-3 (Figure S2b), associated to an increase in IL-1 $\beta$  expression both at the mRNA and protein level was observed (Figure S2b-c). Next, we crossed Lyn $\Delta$ N mice with C1/C11 deficient mice (Kuida et al., 1995) to obtain Lyn $\Delta$ N C1/C11  $-/-$  mice, named hereafter Lyn $\Delta$ N dKO mice. Strikingly, the absence of pro-inflammatory caspases hampered the skin

inflammatory phenotype developed by Lyn $\Delta$ N mice over time, based on the adapted PASI score that we established (Figure 2a and Figure S3a), producing a significant delay in the initiation of the psoriasis-like phenotype in Lyn $\Delta$ N dKO mice. Moreover, both the no weight gain linked to the onset of the disease and the reported death of Lyn $\Delta$ N mice (Marchetti et al., 2009) were significantly reduced in Lyn $\Delta$ N dKO mice (Figure S3b, Figure 2b). The improved phenotype observed in Lyn $\Delta$ N dKO mice was associated to a decrease in epidermal hyperplasia (Figure 2c, d), along with a reduction in the mRNA expression of inflammasome components such as AIM-2 and NLRP-3 and several pro-inflammatory cytokines (Figure 2e), with an exception for IL-23 and TNF- $\alpha$ , for which similar levels were obtained. Concomitantly, the absence of pro-inflammatory caspases significantly reduced the levels of both IL-1 $\beta$  and IL-18 protein expression observed in Lyn $\Delta$ N mice skin (Figure 2f). Therefore, activation of caspase 1/11 is necessary for the development of a complete inflammatory skin disease in the Lyn $\Delta$ N mice model.

To confirm the contribution of pro-inflammatory caspases in psoriasis pathogenesis, we induced a psoriasis-like phenotype either by topical IMQ cream application onto the back skin (Figure 3) or by IL-23 intradermal injections into ears (Figure S4) of WT and C1/C11 deficient mice, hereafter named dKO mice. In both models (IMQ and IL-23), the absence of pro-inflammatory caspases delayed the onset of the clinical score measured. Indeed, erythema, scaling and cumulative scores quantified in the IMQ model (Figure 3a) and ear thickness measured in the IL-23 model (Figure S4a, b) were significantly reduced in dKO mice compared to WT mice. Further, H&E staining of skin sections revealed a decrease in epidermal hyperplasia (Figure 3b, c; Figure S4c, d), along with a reduction in the inflammatory cell infiltration (Figure 3d; Figure S4e), with a reduced number of CD45.2<sup>+</sup> immune cells into the dKO mice skin, evidenced more precisely by a significant decrease number of inflammatory monocytes and neutrophils. Additionally, inflammasome components, pro-inflammatory cytokine mRNA expression (Figure 3e, Figure S4f and Figure S5) and IL1b protein level were significantly reduced in dKO mice in both models (Figure 3f, Figure S4g). Surprisingly, IL-18 protein level was only differentially modulated in the IMQ model. Altogether, these data indicate that regulation of pro-inflammatory caspase activity is necessary to induce a maximal inflammatory response in psoriasis.

### **Pharmacological inhibition of caspases 1/11 by AC-YVAD-CMK compound reduces IMQ-induced psoriasis like phenotype in mice**

It has been previously demonstrated in mice suffering from acute gastric injury that treatment with the selective caspase 1 inhibitor AC-YVAD-CMK led to a protection through the attenuation of NLRP-3 inflammasome activity (Zhang et al., 2016). Therefore, we next decided to analyze the effect of AC-YVAD-CMK treatment in the IMQ model. To do that, WT

mice were treated with IMQ cream together with either DMSO or AC-YVAD-CMK compound injected intra-peritoneally.

While the co-treatment had no additive effect on both weight loss (Figure S6a) and splenomegaly induced by IMQ treatment (Figure S6b) as reported (van der Fits et al., 2009), a significant reduction in erythema, scaling and cumulative score was achieved in mice treated with the selective caspase 1 inhibitor (Figure 4a). It is worth noting that the delay observed upon pharmacological inhibition of caspase 1 started later compared to deficient mice for C1/11 (dKO mice) (Figure 3a). However, the skin phenotype attenuation was associated with a significant decrease in epidermal hyperplasia (Figure 4b, c) and mRNA expression of different inflammasome components and pro-inflammatory cytokines (Figure 4d; Figure S6c). Additionally, the pharmacological inhibition of caspase 1 was linked to a significant decrease in IL-1 $\beta$  and IL-18 protein levels (Figure 4e and f). Finally, no induction in caspase 11 protein expression was observed in AC-YVAD-CMK treated mice (Figure 4f). Altogether these data confirm that pro-inflammatory caspase activation is required for the complete achievement of the inflammatory process implicated in psoriasis pathogenesis.

### **Primary human keratinocytes are primed to secrete IL-1 $\beta$ in mimicking psoriasis condition in an inflammasome dependent manner**

Psoriasis is characterized by a dialogue between keratinocytes in the epidermis and the immune cell infiltration, mainly in the dermis, through the release of cytokines. Thus, to decipher in which skin compartments inflammatory caspases and IL-1 $\beta$  were induced upon IMQ treatment in mice, epidermis/dermis dissociation experiment was performed (Figure S7). A significant increase in mRNA expression of caspase 1 and IL-1 $\beta$  was found both in epidermis and dermis compartment, while caspase 11 expression was only increased in the dermis. We next wanted to know whether pro-inflammatory caspases could be activated in normal human keratinocytes (NHK) in vitro upon stimulation with two cytokines implicated in psoriasis pathogenesis: IL-17A and TNF- $\alpha$  (Johansen et al., 2016). As expected, a significant increase in the mRNA expression of IL-6 and IL-8 (Bertelsen et al., 2017, Fujishima et al., 2010) was observed, validating our in vitro model (Figure S8a). While no difference in caspase 1 and 4 (Figure 5a and e) expression levels was observed, a significant increase of caspase 5, IL-1 $\beta$ , NLRP-1 and NLRP-3 expression (Figure 5a-d and f), both at the mRNA and protein level, was observed. Moreover, it is interesting to note that there was not only an increase of pro-caspase 5 but also of its activated form at long-term stimulation time points (Figure 5d). Moreover, to know if those primed keratinocytes could be responsible for IL-1 $\beta$  release upon inflammasome activation (Stout-Delgado et al., 2012), nigericin was added to NHK pre-treated for 24h with TNF- $\alpha$  + IL-17A. Interestingly, a significant increase in the release of mature IL-1 $\beta$  and activated caspase 1 was observed (Figure 5g and h), confirming

that keratinocytes in a psoriasis environment can be implicated in IL-1b secretion in an inflammatory caspase dependent manner.

### **Caspase 1/11 activation in immune cells is sufficient for the development of psoriasis-like disease in mice**

As we showed that 1) the activation of inflammatory caspases is necessary to obtain a complete psoriasis-like disease, and 2) inflammatory caspase activation can take place both in immune cells and keratinocytes, we decided to perform bone marrow transplantation experiments to elucidate in which cell type their activation is responsible for the psoriasis pathogenesis. As expected, dKO mice receiving dKO bone marrow showed a significant decrease in the adapted PASI score (Figure 6a), epidermal hyperplasia (Figure 6b, c), mRNA expression of inflammasome components and pro-inflammatory cytokines (Figure 6d) and IL-1 $\beta$  protein levels (Figure 6e) compared to WT mice transplanted with WT bone marrow. After IMQ treatment, WT mice transplanted with dKO bone marrow showed a significant reduction in erythema and scaling scores (Figure 6a), epidermal hyperplasia (Figure 6b, c), mRNA expression of different inflammasome components and pro-inflammatory cytokines (Figure 6d) and IL-1 $\beta$  protein levels (Figure 6e) compared to WT mice, which received WT bone marrow. The reduction in the phenotype observed in this chimeric mouse is comparable to what was observed in dKO mice reconstituted with dKO bone marrow. On the opposite, dKO mice transplanted with WT bone marrow developed a phenotype comparable to WT mice reconstituted with WT bone marrow. These results provided evidence of a major role of inflammatory caspase activation into immune cells rather than keratinocytes to trigger a complete psoriasis-like disease.

Furthermore, similar results were obtained with the IL-23 mice model (figure S9), reinforcing the notion that activation of pro-inflammatory caspases in immune cells is sufficient to induce a complete pro-inflammatory response leading to a psoriasis-like phenotype in treated mice.

## Discussion

Psoriasis is a chronic inflammatory skin disease with an unpredictable course (Sabat et al., 2007). While many efforts have been done to identify molecular factors implicated in the initiation and maintenance of the disease, which allowed the identification of new biological drugs targeting TNF- $\alpha$ , IL17 and IL-23 (Lowe et al., 2014), the etiology of psoriasis is still a matter of concern. In this context, controversial results are found in the literature regarding the impact of inflammasome pathway leading to inflammatory caspase activation and mature IL-1b/IL-18 secretion in psoriasis. Indeed, besides strong demonstration of the implication of IL-1 family members in psoriasis (Rabeony et al., 2015), the role of inflammatory caspases in this respect is still a matter of debate as Cho et al (Cho et al., 2012) and Rabeony et al (Rabeony et al., 2015) reported opposite results using IMQ psoriasis-like model. However, our results argue in favor of a major role of inflammatory caspases in cytokine maturation in psoriasis as the invalidation or the pharmacological inhibition of pro-inflammatory caspases leads to a significant reduction in the induced inflammatory phenotype in several psoriasis mouse models. Moreover, even if other proteases such as caspase-8 and neutrophil derived proteases have been reported to participate in the maturation of IL-1b and IL-18 (Clancy et al., 2017, Pierini et al., 2012), our study pointed out inflammatory caspases as major contributors of IL1b/IL-18 maturation in skin disease. This finding is supported by the study of Douglas et al, showing that atopic dermatitis/psoriasis-like disease developed by Sharpin (cpdm) mice, is significantly reduced when caspase 1/11 are deficient (Douglas et al., 2015). Indeed, neither lesions nor epidermal hyperplasia were observed, demonstrating the main role of pro-inflammatory caspases in the induction of dermatitis and the function of inflammasome activation as an initiating signal in Sharpin mice. Interestingly, the delay in the dermatitis onset in Sharpin-C1/C11  $-/-$  mice is consistent with the delay observed in Lyn $\Delta$ N dKO mice used in the present study, showing the improvement in psoriasis-like phenotype through the invalidation of pro-inflammatory caspases. It is worth noting that while genetic ablation of TNFR1 in Sharpin (Rickard et al., 2014) or LynDN (Marchetti et al., 2009) mice produced a complete rescue of the phenotype, the invalidation of inflammatory caspases was associated with a delay in the inflammation symptoms, demonstrating that TNF signaling is one of the principal pathway to induce skin inflammation, while inflammatory caspases pathway is more implicated in the maintenance and exacerbation of the disease. Additionally, the level of psoriasis-like phenotype inhibition in the absence of pro-inflammatory caspases is consistent with other studies where other important components for psoriasis disease development, like T cells, are deleted (van der Fits et al., 2009).

Although it was previously demonstrated that cleaved-form of caspase-5 is present in lesional skin biopsies from psoriasis patients (Zwicker et al., 2017), we confirmed in a large

cohort of lesional and non-lesional skin biopsies that cleaved forms of caspase-1, 4 and 5 are found in all lesional samples of tested psoriasis patients whereas no cleaved forms are found in healthy donor samples. Moreover, cleaved forms of proinflammatory caspases were associated with an increase in the NLRP-3 and AIM-2 inflammasomes. Caspase inhibitors have been evaluated in clinical trials, specifically the small molecule inhibitors VX-740 and VX-765, also known as Pralnacasan and Belnacasan, respectively; which are potent and selective inhibitors of caspase 1 and caspase 4 (Vertex Pharmaceuticals). In our study, we co-treated mice with IMQ and the selective caspase-1 inhibitor AC-YVAD-CMK and a significant decrease in the erythema, scaling and pro-inflammatory cytokine production was found, reinforcing the notion that targeting pro-inflammatory caspases could be of interest to treat psoriasis disease. Indeed, in a model of acute gastric injury, mice were pre-treated with AC-YVAD-CMK and an inhibition in the production of IL-1 $\beta$  was observed leading to the protection of these mice, an effect correlated with the impairment of NLRP-3 inflammasome activity (Zhang et al., 2016). Moreover, like in AC-YVAD-CMK treated mice, Zhang et al found a strong diminution in other pro-inflammatory cytokines including IL-6, IL-8 and TNF- $\alpha$ . Actually, not only caspase inhibitors are evaluated as potential drugs to treat psoriasis disease, but IL-1, IL-18 and inflammasome inhibition could be possible targets to treat inflammatory skin diseases (Fenini et al., 2017). In this line, psoriasis patients were treated in an open-label clinical trial I/II with the human monoclonal antibody against IL-1 $\alpha$ , MABp1, and an encouraging clinical response was achieved that must be improved with an increase in dose/frequency (Coleman et al., 2015).

In our study, we showed that keratinocytes cultured in conditions mimicking psoriasis (TNF- $\alpha$  + IL-17A) are primed to secrete IL-1 $\beta$  in an inflammasome-dependent manner. Recently, it has been demonstrated that the stimulation of keratinocytes with TNF- $\alpha$  or with IL-17A only did not trigger caspase activation (Zwicker et al., 2017), but that the combination of at least two pro-inflammatory cytokines is necessary (Cho et al., 2012), suggesting that in the presence of several pro-inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-17 and IL-22) a strong increase in pro-inflammatory caspases could be obtained. This hypothesis is in agreement with what happens in a psoriatic skin, where keratinocytes are in direct contact with a huge amount of pro-inflammatory cytokines secreted from the recruited immune cells and from themselves. Our data showing that keratinocytes are primed to secrete IL-1 $\beta$  through the activation of inflammasome reinforces the idea that keratinocytes are immunological active cells. However, bone marrow transplantation experiment indicated that the activation of pro-inflammatory caspases in immune cells is sufficient to induce a fully inflammatory response in mice.

In conclusion, the findings presented herein confirm that pro-inflammatory caspases are involved in psoriasis pathogenesis, highlighting the notion that control of pro-inflammatory

caspace activation and IL- $\beta$  maturation/secretion is essential for the improvement of chronic inflammatory disease.

## **Materials and methods**

### **Study approval.**

This study was conducted on biopsies from 25 patients enrolled in a study registered in clinicaltrials.gov under the identifier NCT01538342: Role of Tyrosine Kinase Lyn and Cleaved Form by Caspases in Psoriasis. This study was approved by the local ethics committee (Comité de Protection des Personnes Sud-Méditerranée V, n° 2011-A00786-35.065), and was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practices guidelines. All patients and healthy donors included signed informed consent before their inclusion in the study. The ethics review board from the French Minister for the High Education and Research approved all animal studies together with the Institutional Animal Care and Use Committee of the Centre Méditerranéen de Médecine Moléculaire (C3M), Nice, France (INSERM U1065).

**Mice.** Mice were housed under specific pathogen-free conditions and fed with standard laboratory food. All mice (WT, C1/C11 deficient and Lyn $\Delta$ N) were on a C57BL/6J genetic background and were used at 8-10 weeks of age, except for adoptive transfer, where mice were irradiated and reconstituted at six weeks. Both males and females were used in this study. WT mice (C57Bl6/J) were purchased from Envigo and C1/C11 deficient mice were a gift from Dr Richard A Flavell (Yale University, New Haven, CT, USA).

### **Experimental protocols**

To evaluate the skin phenotype of LynDN mice, an adapted version of the clinical PASI score was used by analyzing the scaling. To do that, we divided the body in six-regions and we assigned a percentage as followed: Head: 16%, front legs: 6%, abdomen: 27%, hind legs: 12%, tail: 12% and back: 27%. Moreover, according to the percentage of desquamation, a number (#) was assigned to each region (0%: 0, <10%: 1, 10-29%: 2, 30-49%: 3, 50-69%: 4, 70-89%: 5 and 90-100%: 6). Then, the adapted version of PASI score was calculated as following (%head\*# + %front legs\*# + %abdomen\*# + %hind legs\*# + %tail\*# + %back\*#). Therefore, our adapted PASI score took values between 0 (no psoriasis) and 6 (maximal score).

To induce psoriasis-like phenotype, mice were treated either with Aldara cream corresponding to 3,125 mg of IMQ (daily for six days) in the backs after shaving and depilation or with mouse recombinant IL-23 (500 ng diluted in PBS) by intradermal injection in the ears (each other day for 12 days). The severity of inflammation in the back skin through erythema (redness) and scaling (desquamation) was assessed by an adapted version of the clinical PASI score, 0: none; 1: slight; 2: moderated; 3: strong; 4: very strong

phenotype. Moreover, a cumulative score, resulting from the sum of erythema and scaling, was calculated. The severity of inflammation in the ears was measured through the thickness of the ears using a caliper Mitutoyo. The change from baseline was determined.

To evaluate the effect of AC-YVAD-CMK compound in our model of IMQ-induced psoriasis-like phenotype, mice were divided in four groups. 1) Vaseline plus Dimethyl sulfoxide (DMSO) diluted in PBS (1:66, v/v, 10 mL/kg), 2) Vaseline plus AC-YVAD-CMK, 3) IMQ plus DMSO diluted in PBS (1:66, v/v, 10 mL/kg) and 4) IMQ plus AC-YVAD-CMK. The compound AC-YVAD-CMK was prepared in DMSO and PBS (1:66, v/v) and administered by intraperitoneal injections (8 mg/kg) one hour before starting IMQ or Vaseline treatment.

For the adoptive transfer experiment, six weeks WT and C1/C11 deficient mice were lethally irradiated at 7 Gy and then reconstituted with  $3 \times 10^6$  bone marrow cells from donor mice. Donor bone marrow cells were harvested from the hind limbs (femur and tibia) and a single cell suspension of bone marrow cells was made. All recipient mice were left for 8 weeks to ensure recovery and then protocols to induce psoriasis-like phenotype (IMQ and IL-23) were performed.

**Statistics.** The number of animals used in each experiment is described in the figure legends. All statistical analyses were performed in GraphPad Prism software (GraphPad, version 6) and results are represented as mean  $\pm$  SD. To compare means between two groups, the unpaired two-tailed Student t test was used. To compare means between three or more groups, the one-way ANOVA with the uncorrected Fisher's Least Significance Difference (LSD) multiple comparison test was applied. Kaplan-Meier survival analyses were performed and survival curves were compared using long-rank tests (Mantel-Cox test), (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant).

### **Conflict of interest**

There is no conflict of interest declared by the authors.

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### **Author contributions**

LEA and DG obtained the majority of data presented herein and were assisted by CRB, JC, LM, PLO and PC. JPO, JPL and TP included patients and performed clinical examination. SM and LEA wrote and edit the manuscript. JER and PA provided intellectual input and edit the paper. SM conceived the project, supervised the study and obtained funding. All authors reviewed and approved the final version of the manuscript prior to its submission for publication.

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## Figure legends

### **Figure 1: Pro-inflammatory caspases are activated in lesional skin biopsies from psoriasis patients.**

Western Blot analysis of healthy donors (HD) and non-lesional (NL) and lesional (L) skin biopsies from psoriasis patients: **a**, **b** and **c**: Caspase 1, 4 and 5 were blotted in HD and in NL and L biopsies from different psoriasis patients showing caspase activation in lesional samples. **d**: Total of analyzed patients showing in how many patients activated caspases are present.

\* Unspecific band.

### **Figure 2: Backcrossing of Lyn $\Delta$ N mice with C1/C11 deficient mice leads to a reduction in psoriasis-like phenotype.**

Lyn $\Delta$ N mice were crossed with C1/C11 deficient mice (dKO mice). Newborn mice, Lyn $\Delta$ N C1/C11 deficient mice (Lyn $\Delta$ N dKO mice), were sacrificed, together with WT and Lyn $\Delta$ N mice, 11 days after birth and abdomen skin was harvested. **a**: Adapted PASI score was assessed at three time points (see Materials and Methods), number of analyzed mice is showed below the graph. **b**: Survival of WT, Lyn $\Delta$ N and Lyn $\Delta$ N dKO through the eleven days of the study. **c**: Representative histological sections stained with haematoxylin and eosin (10X and 20X), scale bars 50  $\mu$ m. **d**: Epidermal hyperplasia quantification through haematoxylin and eosin staining, (WT, n = 5; Lyn $\Delta$ N, n = 7; Lyn $\Delta$ N dKO, n = 10). **e**: Skin was processed for total RNA isolation and gene expression was determined by qPCR (WT, n = 4; Lyn $\Delta$ N, n = 4; Lyn $\Delta$ N dKO, n = 8). **f**: Total protein was prepared and IL-1 $\beta$  (WT, n = 5; Lyn $\Delta$ N, n = 8; Lyn $\Delta$ N dKO, n = 14) and IL-18 (WT, n = 3; Lyn $\Delta$ N, n = 5; Lyn $\Delta$ N dKO, n = 7) levels were quantified by ELISA.

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. One-way ANOVA with the uncorrected Fisher's LSD multiple comparison test was applied to determine statistical significance in the PASI score. Long-rank test (Mantel-Cox test) was applied for survival experiment and Student t test was applied for the rest of panels, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant.

### **Figure 3: Deficiency in C1/C11 genes in mice decreases IMQ-induced psoriasis-like phenotype.**

WT and C1/C11 deficient mice (dKO mice) were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream and after sacrificed them back skin was harvested. **a:** Severity of IMQ-induced psoriasis like phenotype represented by Erythema, Scaling and Cumulative score measurement, (Vasel, n = 2; IMQ-WT, n = 4; IMQ-dKO, n = 8). **b:** Representative histological sections stained with haematoxylin and eosin (10X and 20X), scale bars 50  $\mu$ m. **c:** Epidermal hyperplasia quantification through haematoxylin and eosin staining, (Vasel, n = 3; IMQ-WT, n = 4; IMQ-dKO, n = 8). **d:** The number of immune cell populations was quantified by FACS, (Vasel, n = 3; IMQ-WT, n = 4; IMQ-dKO, n = 8). **e:** Skin was processed for total RNA isolation and gene expression was determined by qPCR (Vasel, n = 3; IMQ-WT, n = 4; IMQ-dKO, n = 8). **f:** Skin was processed for total protein extraction and IL-1 $\beta$  and IL-18 levels were quantified by ELISA, (Vasel, n = 3; IMQ-WT, n = 4; IMQ-dKO, n = 8).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant.

**Figure 4: Inhibition of Caspase-1 by AC-YVAD-CMK compound reduces IMQ-induced psoriasis-like phenotype in mice.**

WT mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream in combination with dimethylsulfoxide (DMSO) or the caspase-1 inhibitor AC-YVAD-CMK (8 mg/kg). **a:** Severity of IMQ-induced psoriasis like phenotype represented by Erythema, Scaling and Cumulative score measurement, (Vasel, n = 2; IMQ, n = 4). **b:** Representative histological sections stained with haematoxylin and eosin (10X), scale bars 50  $\mu$ m. **c:** Epidermal hyperplasia quantification through haematoxylin and eosin staining, (Vasel, n = 2; IMQ, n = 4). **d:** Skin was processed for total RNA isolation and gene expression was determined by qPCR (Vasel, n = 2; IMQ, n = 4). **e:** Skin was processed for total protein extraction and IL-1 $\beta$  and IL-18 levels were quantified by ELISA, (Vasel, n = 2; IMQ, n = 4). **f:** Total protein was prepared and Western Blot analyses were performed and densitometry quantification was made, (Vasel, n = 2; IMQ, n = 4).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant.

**Figure 5: Primary human keratinocytes are primed to secrete IL-1 $\beta$  and activate caspase 5 in mimicking psoriasis conditions.**

Primary human keratinocytes were stimulated with TNF- $\alpha$  (10 ng/ml) and IL-17A (200 ng/ml) at different time points and after total RNA isolation, caspase 1, caspase 4, caspase 5 (**a**); IL-

1 $\beta$  (**b**); and NLRP-1 and NLRP-3 (**c**) gene expression was determined by qPCR, (n = 3). **d**, **e**, and **f**: Cells were lysed and Western Blot analyses were performed, (n = 3). **g**: Primary human keratinocytes were stimulated with TNF- $\alpha$  (10 ng/ml) and IL-17A (200 ng/ml) in the presence or not of nigericin to activate inflammasome, then IL-1 $\beta$  and caspase 1 expression was determined intracellular and extracellularly, (n = 3). **h**: After keratinocytes stimulation with TNF- $\alpha$  (10 ng/ml) and IL-17A (200 ng/ml) in the presence of nigericin at two time points, supernatant was harvested and IL-1 $\beta$  secretion was quantified by ELISA, (n = 3).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\*, p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, ns: non-significant. T: TNF- $\alpha$  (10 ng/ml), I: IL-17A (200 ng/ml).

**Figure 6: Caspase 1/11 deficiency in immune cells is enough to reduce IMQ-induced psoriasis like phenotype.**

WT and C1/C11 deficient (dKO) mice were irradiated and then reconstituted with bone marrow from WT (WT $\rightarrow$ WT and WT $\rightarrow$ dKO) and dKO (dKO $\rightarrow$ dKO and dKO $\rightarrow$ WT) mice, which were left to recuperate for 8 weeks. Then, the four groups of mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream. After treatment back skin was harvested. **a**: Severity of IMQ-induced psoriasis like phenotype represented by Erythema, Scaling and Cumulative score measurement, (n = 6). **b**: Representative histological sections stained with haematoxylin and eosin (10X), scale bars 50  $\mu$ m. **c**: Epidermal hyperplasia quantification through haematoxylin and eosin staining, (for each group of mice, Vasel, n = 3; IMQ, n = 6). **d**: Skin was processed for total RNA isolation and gene expression was determined by qPCR (for each group of mice, Vasel, n = 2; IMQ, n = 6). **e**: Skin was processed for total protein extraction and IL-1 $\beta$  levels were quantified by ELISA, (for each group of mice, Vasel, n = 3; IMQ, n = 6).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. One-way ANOVA with the uncorrected Fisher's LSD multiple comparison test, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\* p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, ns: non-significant, ND: non-detected.

## **Supplementary Materials and Methods**

**Biopsies.** Four-millimeter punch biopsies were collected from healthy donors and from psoriasis patients. In healthy donor, biopsies were taken in the upper thigh. In psoriasis patients, biopsies were taken from the center of a chronic plaque (lesional biopsy) and from non lesional skin. All biopsies were frozen immediately in liquid nitrogen and then stored at -80 °C.

**Reagents.** Aldara (5% IMQ) cream was purchased from MEDA Pharma. Mouse recombinant protein IL-23 was purchased from eBioscience Systems. AC-YVAD-CMK was obtained from Sigma-Aldrich. ELISA kits for mL-1 $\beta$  and mL-18 cytokines were provided by R&D and MBL international, respectively. ELISA kits for hIL-1 $\beta$  was purchased from e-bioscience. TRIzol reagent was acquired from Life Technologies. All TaqMan reagents were bought from Thermo Fisher Scientific. Dispase enzyme was obtained from Sigma-Aldrich. Human recombinant proteins IL-17A and TNF- $\alpha$ , for keratinocyte stimulation, were purchased from PeproTech.

**Antibodies.** Rabbit polyclonal antibodies against caspase-1 (sc-622, sc-515) and caspase-5 (4429) were purchased from Santa Cruz Biotechnology and from Cell Signaling Technology, respectively. Goat polyclonal antibodies against caspase-4 (sc-1229) and IL-1 $\beta$  (AF-401-NA) were obtained from Santa Cruz Biotechnology and from R&D Systems, respectively. Mouse monoclonal antibodies against HSP-70 (sc-66048) and HSP-90 (sc-69703) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against Actin (A2228) was purchased from Sigma-Aldrich. Rat monoclonal antibody against caspase-11 (17D9) was obtained from Novus Biologicals.

**H&E staining.** skin biopsies were fixed in 10% neutral-buffered formalin and then include in paraffin. Five-micrometer thick sections were cut and stained with H&E (Sigma-Aldrich). To quantify epidermal hyperplasia, 14 assessments were made in both portions of the tissue. Histopathological examinations were performed on a Nikon light microscope.

**RNA extractions and qPCR assessments.** Total RNA was extracted from skin tissues using TRIzol reagent and reverse transcribed using random primer from Promega and SuperScript II reverse transcriptase from ThermoScientific. For qPCR analysis, different TaqMan sondes were used (Thermo Fisher Scientific), (Mouse Taqman, RPLP0: Mm00725448, IL-17: Mm00439618\_m1, IL-6: Mm00446190\_m1, IL-22: Mm01226722\_g1, IL-23: Mm00518984\_m1, TNF- $\alpha$ : Mm00443258\_m1, IL-1 $\beta$ : Mm00434228\_m1, NLRP-3:

Mm00840904\_m1, AIM-2: Mm01295719\_m1 and Caspase 11: Mm00432304\_m1; Human Taqman, RPLP0: Hs99999902\_m1, IL-6: Hs00174131\_m1 and IL-8: Hs00174103\_m1.

**FACS.** Two 8-millimeter punches were taken and single-cell suspensions of ear and back skin were obtained as described previously (Riol-Blanco et al., 2014). Briefly, for the ear skin digestion, dorsal and ventral parts of the ear were mechanically separated and for the back skin digestion, all hypodermis was mechanically eliminated. Then, a digestion for 90 minutes at 37°C in gentleMACS tubes (Miltenyi) with gentle agitation in freshly prepared digestion mix consisting of DMEM (Gibco) supplemented with HEPES (Invitrogen), 2% FCS, 100 µg/mL Liberase TM (Roche), 100 µg/mL DNase I (Roche) and 0.5 mg/mL Hyaluronidase (Sigma) was produced. After enzymatic digestion, the mixture was processed using a gentleMACS homogenizer (Miltenyi) in order to obtain a cell suspension, which was then filtered through a 70 µm cell strainer (BD). Cells were then resuspended in FACS buffer for analysis (PBS with 2mM EDTA and 2% FCS (GIBCO)).

For FACS staining, the following antibodies were used: FITC-conjugated anti-Ly-6G (clone 1A8; BD Pharmingen), PE-conjugated anti-Ly-6C (clone HK1.4; Biolegend), PerCP/Cy5.5-conjugated anti-CD45.2 (clone 104; Biolegend), VB conjugated anti-CD11c (clone HL3; BD Pharmingen), Alexa647-conjugated anti-CD11b (clone M1/70; Biolegend) and APC-Cy7-conjugated anti I-A/I-E (clone M5/114.15.2; Biolegend). For analysis, cells were acquired on a MACSQuant FACS (Miltenyi) and analyzed using the MACSQuantify Software (Miltenyi).

**Tissue dissociation.** Mouse tissues and psoriasis patient skin biopsies were homogenized with a CK14 Soft Tissue Homogenizing Kit (VWR) in a Precellys 24 (Bertin Corporation) and cells were lysed in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 10 mg/ml leupeptin, 5 mg/ml aprotinin and 1% Triton X-100. Supernatants were harvested, dosed and stored immediately at -80 °C.

**Pro-inflammatory cytokines assays.** IL-1β and IL-18 protein levels were evaluated using ELISA kits according to the manufacturer's instructions. Results were determined at 450 nm and corrected at 560 nm using a microplate reader (Corning).

**Dermis-Epidermis dissociation.** Two 8-millimeter punches were obtained from mice backs and after removing hypodermis, punches were put, dermis face down, in a dish containing 1% dispase for 1h30 at 37 °C. Then, epidermis and dermis were mechanically separated and immediately frozen for total RNA isolation.

**Western blotting.** Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). After blocking nonspecific binding sites, the membranes were incubated with specific antibodies. Then, membranes were washed and incubated

further with horseradish peroxidase conjugated antibody. The immunoblots were visualized using an enhanced chemiluminescence detection kit (Pierce).

**Keratinocyte culture**. Normal Human Keratinocytes were a gift from Dr Karine Bille and Dr Corine Bertolotto (C3M, Nice, France). NHK were culture in presence of irradiated fibroblasts and a special medium consisting of 60% DMEM, 30% F-12 NutMix and 10% FCS supplemented with Cholera toxin 0,1 nM, Adenine 0,18 mmol/L, Insulin 5 ug/mL, Hydrocortisone 0,4 ug/ml, Triiodothyronine 2nmol/L and EGF 10 ng/ml.

These cells were stimulated at different time points with both IL-17A (200 ng/ml) and TNF- $\alpha$  (10 ng/ml) cytokines and in presence of basal KGM medium plus supplemented solutions (PromoCell).

## Supplementary Figure Legends

### Supplemental Figure 1:

Western Blot analysis of non-lesional (NL) and lesional (L) skin biopsies from psoriasis patients: **a**: Caspase 1, 4 and 5 were blotted in NL and L biopsies from different psoriasis patients showing caspase activation in lesional samples. **b**: Inflammasome and pro-inflammatory cytokines were blotted in NL and L biopsies from different psoriasis patients.

### Supplemental Figure 2:

Lyn $\Delta$ N and WT mice were sacrificed 11 days after birth and skin was harvested. **a-b**: Skin was processed for total RNA isolation and gene expression was determined by qPCR, (WT, n = 5; Lyn $\Delta$ N, n = 7). **c**: Total protein was prepared and IL-1 $\beta$  was quantified by ELISA, (WT, n = 5; Lyn $\Delta$ N, n = 10).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\* p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, ns: non-significant.

### Supplemental Figure 3:

Lyn $\Delta$ N mice were backcrossing with C1/C11 deficient mice (dKO mice). Newborn mice, Lyn $\Delta$ N C1/C11 deficient mice (Lyn $\Delta$ N dKO mice), were sacrificed, together with WT and Lyn $\Delta$ N mice, 11 days after birth. **a**: Mice pictures. **b**: Mice weight taken at three time points, number of analyzed mice is show below the graph.

Errors bars represent mean  $\pm$  SD. One-way ANOVA with the uncorrected Fisher's LSD multiple comparison test, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\* p  $\leq$  0.001.

### Supplemental Figure 4:

WT and C1/C11 deficient (dKO) mice were treated, each other day, for 12 days with vehicle (phosphate buffered saline, PBS) and mouse recombinant protein IL-23 (500 ng). After treatment, ears were harvested. **a**: Measurement of ear thickness throughout the study. **b**: Increase in ear thickness at the end of the study, (PBS, n = 2; IL-23, n = 5). **c**: Representative histological sections stained with haematoxylin and eosin (40X), scale bars 50  $\mu$ m. **d**: Quantification of the epidermal hyperplasia through haematoxylin and eosin staining, (PBS, n = 2; IL-23, n = 5). **e**: The number of immune cell populations per ear was quantified by FACS, (PBS, n = 2; IL-23, n = 5). **f**: After total RNA isolation, gene expression was determined by qPCR, (PBS, n = 4; IL-23-WT, n = 7; IL-23-dKO, n = 10). **g**: Total protein was prepared, and then IL-1 $\beta$  and IL-18 levels were quantified by ELISA, (PBS, n = 2; IL-23, n = 5).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\* p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, ns: non-significant.

### **Supplemental Figure 5:**

WT and C1/C11 deficient mice (dKO mice) were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream. **a:** Skin was processed for total RNA isolation and gene expression was determined by qPCR (Vasel, n = 3; IMQ-WT, n = 4; IMQ-dKO, n = 8).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ .

### **Supplemental Figure 6:**

WT mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream in combination with dimethylsulfoxide (DMSO) or the caspase-1 inhibitor AC-YVAD-CMK (8 mg/kg). **a:** Mice were weighted each day of treatment, (Vasel, n = 2; IMQ, n = 4). **b:** Splenomegaly after six daily DMSO or AC-YVAD-CMK treatment of mice, (Vasel, n = 2; IMQ, n = 4). **c:** Skin was processed for total RNA isolation and gene expression was determined by qPCR (Vasel, n = 2; IMQ, n = 4).

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WT mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream and after sacrificed them back skin was harvested. Then dermis and epidermis were analyzed separately. **a:** Total RNA isolation was performed and gene expression was determined by qPCR, (Vasel, n = 4; IMQ, n = 7).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , ns: non-significant.

### **Supplemental Figure 8:**

Primary human keratinocytes were stimulated with TNF- $\alpha$  (10 ng/ml) and IL-17A (200 ng/ml) at different time points and after total RNA isolation, IL-6 (**a**) and IL-8 (**b**) gene expression was determined by qPCR, (n = 3).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ . T: TNF- $\alpha$  (10 ng/ml), I: IL-17A (200 ng/ml).

### **Supplemental Figure 9:**

WT and dKO mice were irradiated and then reconstituted with bone marrow from WT (WT $\rightarrow$ WT and dKO $\rightarrow$ WT) and dKO (dKO $\rightarrow$ dKO and WT $\rightarrow$ dKO) mice, which were left to recuperate for 8 weeks. Then, the four groups of mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream. **a:** Splenomegaly after six daily Vasel or IMQ treatment of mice, (for each group of mice, Vasel, n = 3; IMQ, n = 6).

Errors bars represent mean  $\pm$  SD. One-way ANOVA with the uncorrected Fisher's LSD multiple comparison test, ns: non-significant.

**Supplemental Figure 10:**

WT and C1/C11 deficient (dKO) mice were irradiated and then reconstituted with bone marrow from WT (WT $\rightarrow$ WT and dKO $\rightarrow$ WT) and dKO (dKO $\rightarrow$ dKO and WT $\rightarrow$ dKO) mice, which were left to recuperate for 8 weeks. Then, the four groups of mice were treated, each other day, for 12 days with vehicle (phosphate buffered saline, PBS) and mouse recombinant protein IL-23 (500 ng). After treatment ears were harvested. **a:** Increase in ear thickness at the end of the study, D12-D0 (for groups WT $\rightarrow$ WT and dKO $\rightarrow$ WT, PBS, n = 4; IL-23, n = 5; for groups dKO $\rightarrow$ dKO and WT $\rightarrow$ dKO, PBS, n = 6; IL-23, n = 8). **b:** Quantification of the epidermal hyperplasia through haematoxylin and eosin staining, (PBS, n = 2; IL-23, n = 3).

Errors bars represent mean  $\pm$  SD. One-way ANOVA with the uncorrected Fisher's LSD multiple comparison test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant.

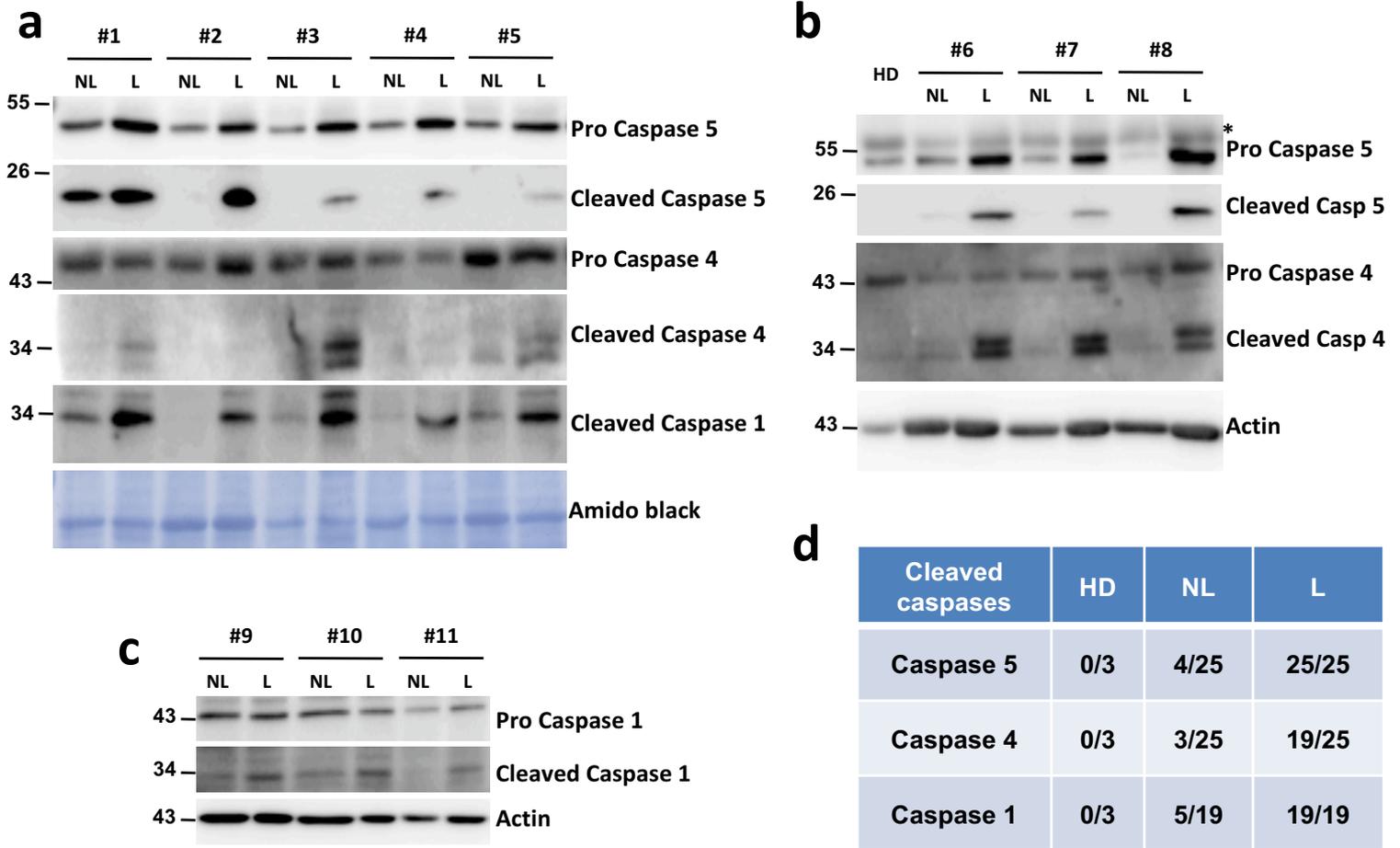


Figure 1, Aira-Caspases

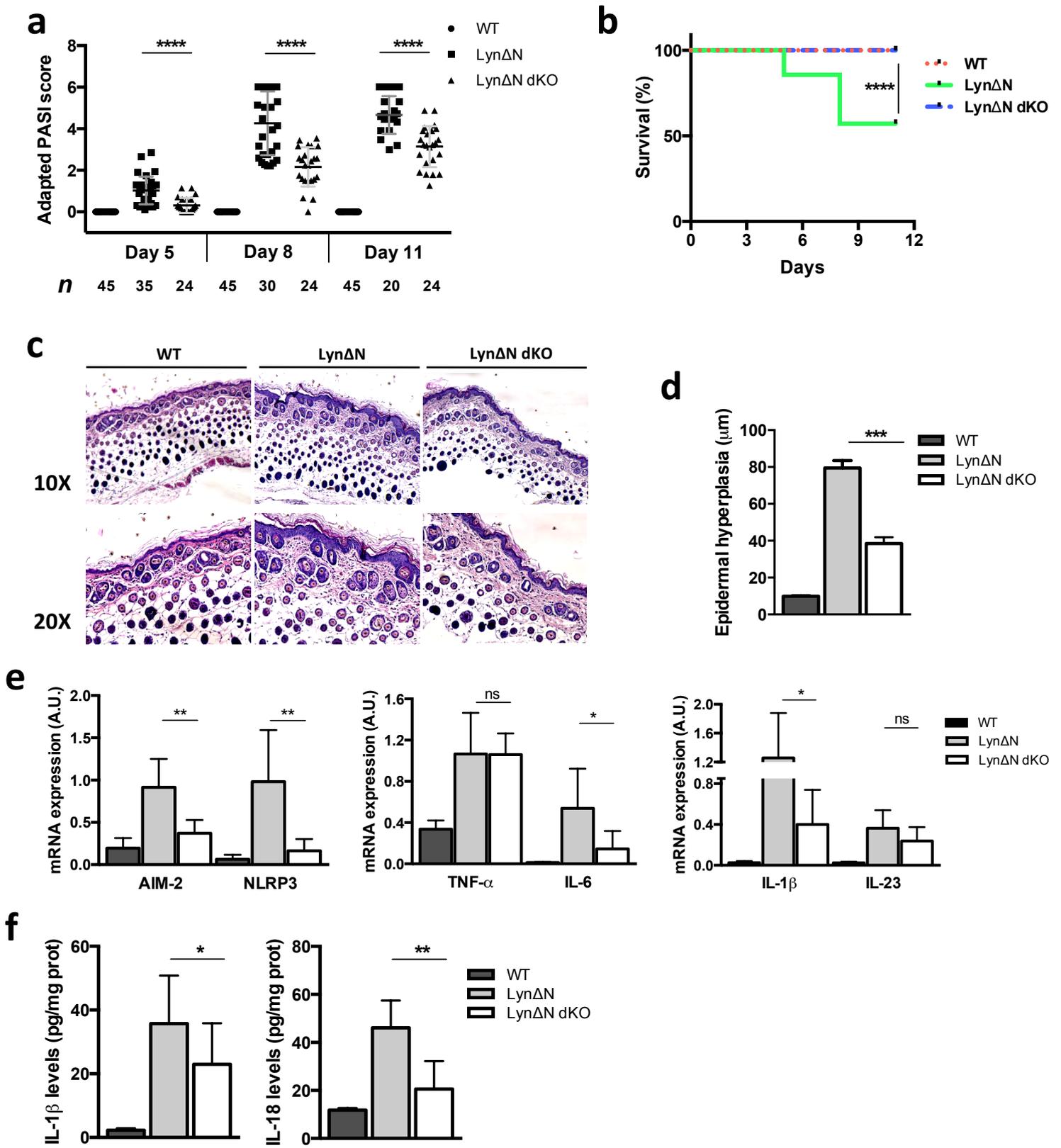


Figure 2, Aira-Caspases

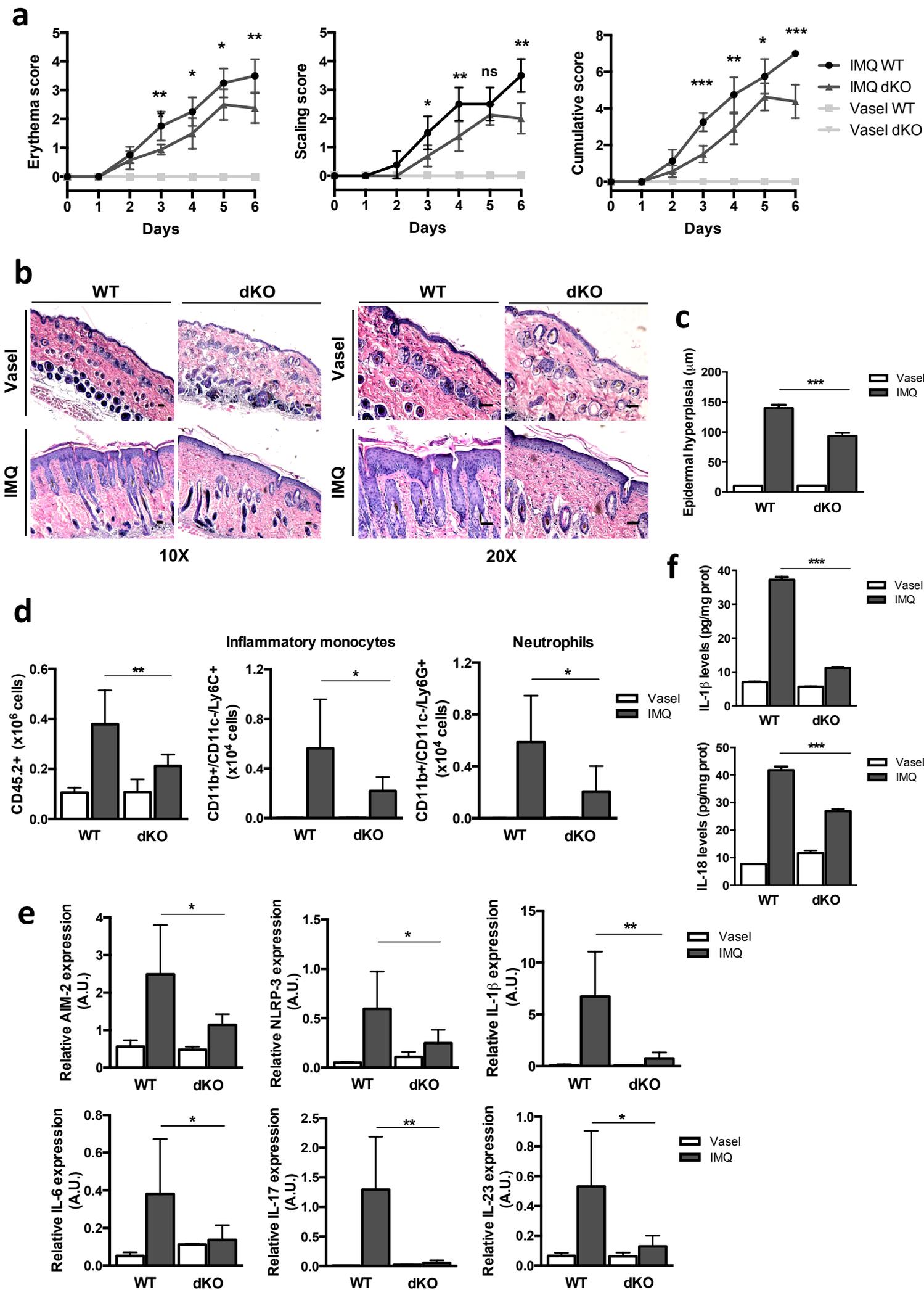


Figure 3, Aira-Caspases

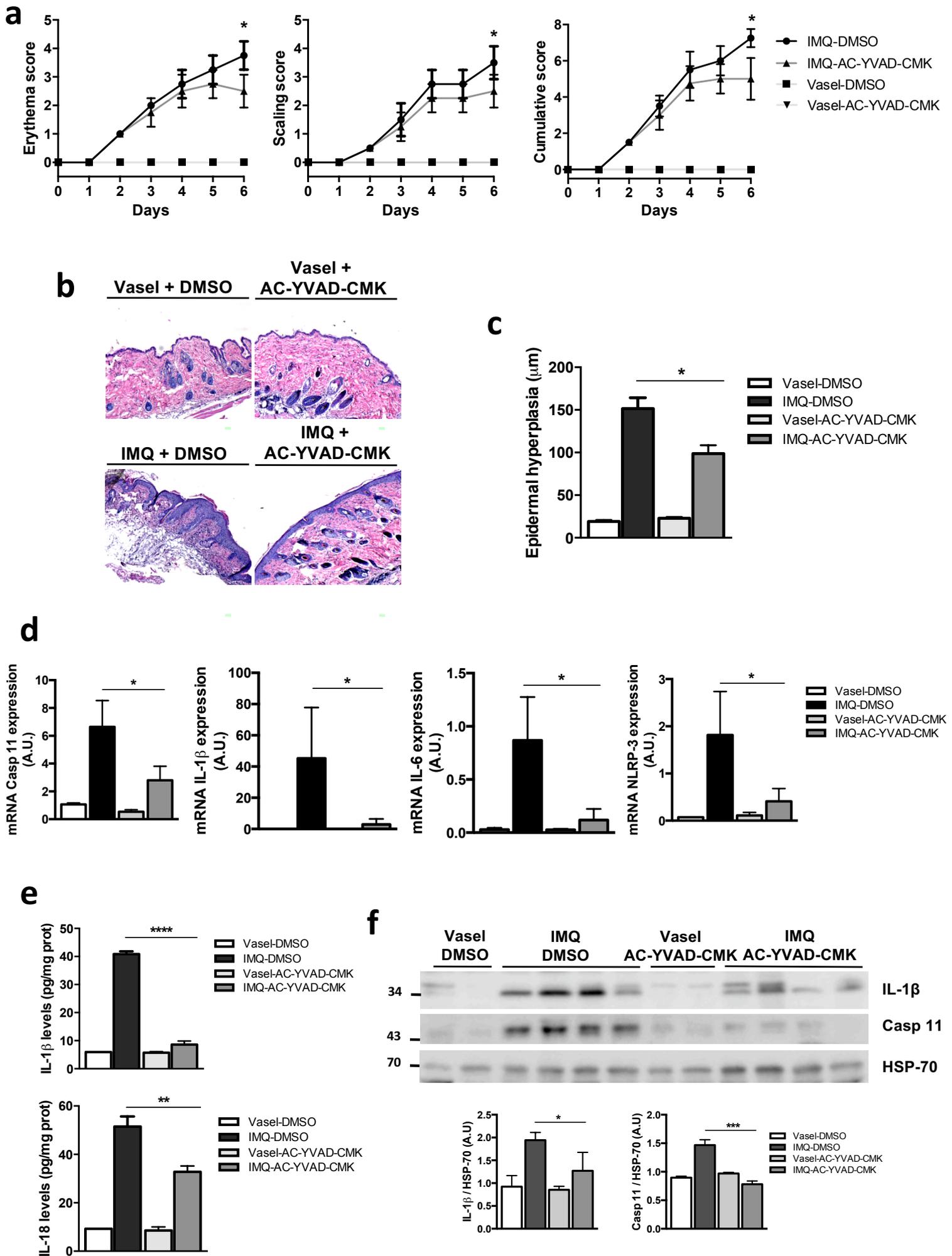


Figure 4, Aira-Caspases

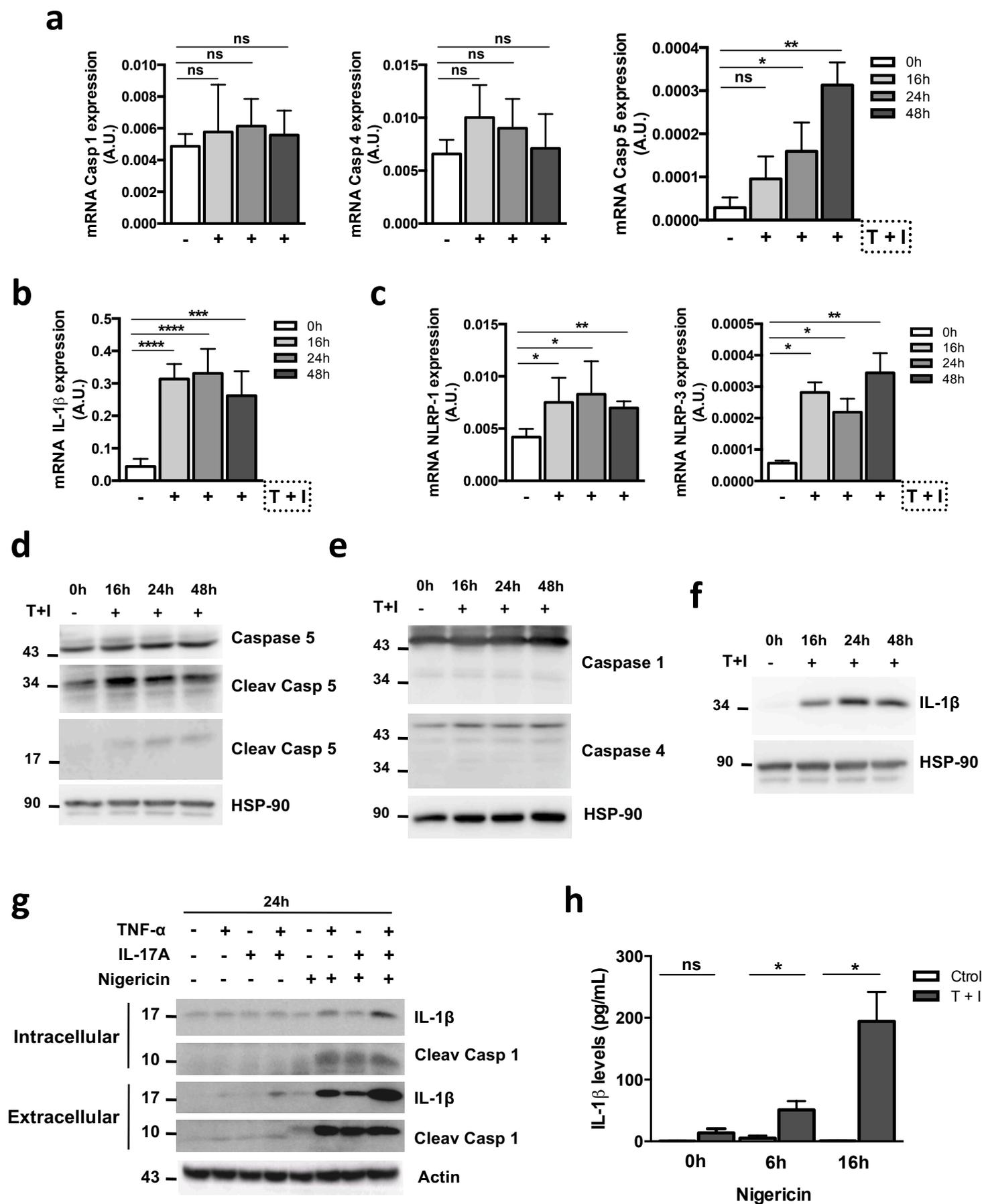


Figure 5, Aira-Caspases

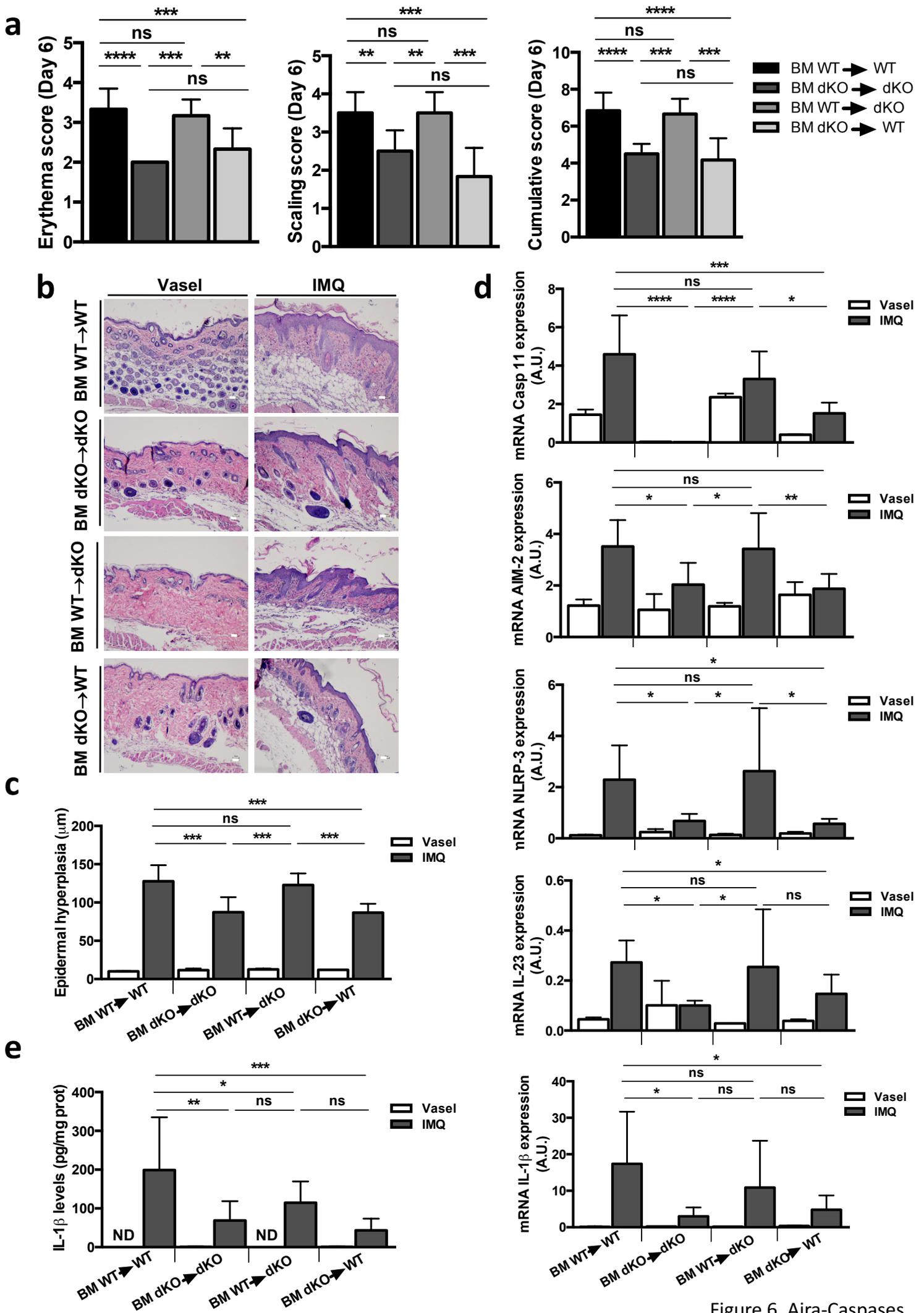


Figure 6, Aira-Caspases

## **Supplementary Materials and Methods**

**Biopsies.** Four-millimeter punch biopsies were collected from healthy donors and from psoriasis patients. In healthy donor, biopsies were taken in the upper thigh. In psoriasis patients, biopsies were taken from the center of a chronic plaque (lesional biopsy) and from non lesional skin. All biopsies were frozen immediately in liquid nitrogen and then stored at -80 °C.

**Reagents.** Aldara (5% IMQ) cream was purchased from MEDA Pharma. Mouse recombinant protein IL-23 was purchased from eBioscience Systems. AC-YVAD-CMK was obtained from Sigma-Aldrich. ELISA kits for mL-1 $\beta$  and mL-18 cytokines were provided by R&D and MBL international, respectively. ELISA kits for hIL-1 $\beta$  was purchased from e-bioscience. TRIzol reagent was acquired from Life Technologies. All TaqMan reagents were bought from Thermo Fisher Scientific. Dispase enzyme was obtained from Sigma-Aldrich. Human recombinant proteins IL-17A and TNF- $\alpha$ , for keratinocyte stimulation, were purchased from PeproTech.

**Antibodies.** Rabbit polyclonal antibodies against caspase-1 (sc-622, sc-515) and caspase-5 (4429) were purchased from Santa Cruz Biotechnology and from Cell Signaling Technology, respectively. Goat polyclonal antibodies against caspase-4 (sc-1229) and IL-1 $\beta$  (AF-401-NA) were obtained from Santa Cruz Biotechnology and from R&D Systems, respectively. Mouse monoclonal antibodies against HSP-70 (sc-66048) and HSP-90 (sc-69703) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against Actin (A2228) was purchased from Sigma-Aldrich. Rat monoclonal antibody against caspase-11 (17D9) was obtained from Novus Biologicals.

**H&E staining.** skin biopsies were fixed in 10% neutral-buffered formalin and then include in paraffin. Five-micrometer thick sections were cut and stained with H&E (Sigma-Aldrich). To quantify epidermal hyperplasia, 14 assessments were made in both portions of the tissue. Histopathological examinations were performed on a Nikon light microscope.

**RNA extractions and qPCR assessments.** Total RNA was extracted from skin tissues using TRIzol reagent and reverse transcribed using random primer from Promega and SuperScript II reverse transcriptase from ThermoScientific. For qPCR analysis, different TaqMan sondes were used (Thermo Fisher Scientific), (Mouse Taqman, RPLP0: Mm00725448, IL-17: Mm00439618\_m1, IL-6: Mm00446190\_m1, IL-22: Mm01226722\_g1, IL-23: Mm00518984\_m1, TNF- $\alpha$ : Mm00443258\_m1, IL-1 $\beta$ : Mm00434228\_m1, NLRP-3:

Mm00840904\_m1, AIM-2: Mm01295719\_m1 and Caspase 11: Mm00432304\_m1; Human Taqman, RPLP0: Hs99999902\_m1, IL-6: Hs00174131\_m1 and IL-8: Hs00174103\_m1.

**FACS.** Two 8-millimeter punches were taken and single-cell suspensions of ear and back skin were obtained as described previously (Riol-Blanco et al., 2014). Briefly, for the ear skin digestion, dorsal and ventral parts of the ear were mechanically separated and for the back skin digestion, all hypodermis was mechanically eliminated. Then, a digestion for 90 minutes at 37°C in gentleMACS tubes (Miltenyi) with gentle agitation in freshly prepared digestion mix consisting of DMEM (Gibco) supplemented with HEPES (Invitrogen), 2% FCS, 100 µg/mL Liberase TM (Roche), 100 µg/mL DNase I (Roche) and 0.5 mg/mL Hyaluronidase (Sigma) was produced. After enzymatic digestion, the mixture was processed using a gentleMACS homogenizer (Miltenyi) in order to obtain a cell suspension, which was then filtered through a 70 µm cell strainer (BD). Cells were then resuspended in FACS buffer for analysis (PBS with 2mM EDTA and 2% FCS (GIBCO)).

For FACS staining, the following antibodies were used: FITC-conjugated anti-Ly-6G (clone 1A8; BD Pharmingen), PE-conjugated anti-Ly-6C (clone HK1.4; Biolegend), PerCP/Cy5.5-conjugated anti-CD45.2 (clone 104; Biolegend), VB conjugated anti-CD11c (clone HL3; BD Pharmingen), Alexa647-conjugated anti-CD11b (clone M1/70; Biolegend) and APC-Cy7-conjugated anti I-A/I-E (clone M5/114.15.2; Biolegend). For analysis, cells were acquired on a MACSQuant FACS (Miltenyi) and analyzed using the MACSQuantify Software (Miltenyi).

**Tissue dissociation.** Mouse tissues and psoriasis patient skin biopsies were homogenized with a CK14 Soft Tissue Homogenizing Kit (VWR) in a Precellys 24 (Bertin Corporation) and cells were lysed in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 10 mg/ml leupeptin, 5 mg/ml aprotinin and 1% Triton X-100. Supernatants were harvested, dosed and stored immediately at -80 °C.

**Pro-inflammatory cytokines assays.** IL-1β and IL-18 protein levels were evaluated using ELISA kits according to the manufacturer's instructions. Results were determined at 450 nm and corrected at 560 nm using a microplate reader (Corning).

**Dermis-Epidermis dissociation.** Two 8-millimeter punches were obtained from mice backs and after removing hypodermis, punches were put, dermis face down, in a dish containing 1% dispase for 1h30 at 37 °C. Then, epidermis and dermis were mechanically separated and immediately frozen for total RNA isolation.

**Western blotting.** Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). After blocking nonspecific binding sites, the membranes were incubated with specific antibodies. Then, membranes were washed and incubated

further with horseradish peroxidase conjugated antibody. The immunoblots were visualized using an enhanced chemiluminescence detection kit (Pierce).

**Keratinocyte culture**. Normal Human Keratinocytes were a gift from Dr Karine Bille and Dr Corine Bertolotto (C3M, Nice, France). NHK were culture in presence of irradiated fibroblasts and a special medium consisting of 60% DMEM, 30% F-12 NutMix and 10% FCS supplemented with Cholera toxin 0,1 nM, Adenine 0,18 mmol/L, Insulin 5 ug/mL, Hydrocortisone 0,4 ug/ml, Triiodothyronine 2nmol/L and EGF 10 ng/ml.

These cells were stimulated at different time points with both IL-17A (200 ng/ml) and TNF- $\alpha$  (10 ng/ml) cytokines and in presence of basal KGM medium plus supplemented solutions (PromoCell).

## **Supplementary Figure Legends**

### **Supplemental Figure 1:**

Western Blot analysis of non-lesional (NL) and lesional (L) skin biopsies from psoriasis patients: **a**: Caspase 1, 4 and 5 were blotted in NL and L biopsies from different psoriasis patients showing caspase activation in lesional samples. **b**: Inflammasome and pro-inflammatory cytokines were blotted in NL and L biopsies from different psoriasis patients.

### **Supplemental Figure 2:**

Lyn $\Delta$ N and WT mice were sacrificed 11 days after birth and skin was harvested. **a-b**: Skin was processed for total RNA isolation and gene expression was determined by qPCR, (WT, n = 5; Lyn $\Delta$ N, n = 7). **c**: Total protein was prepared and IL-1 $\beta$  was quantified by ELISA, (WT, n = 5; Lyn $\Delta$ N, n = 10).

A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \* p  $\leq$  0.05, \*\* p  $\leq$  0.01, \*\*\* p  $\leq$  0.001, \*\*\*\* p  $\leq$  0.0001, ns: non-significant.

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A.U: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ .

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WT mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream in combination with dimethylsulfoxide (DMSO) or the caspase-1 inhibitor AC-YVAD-CMK (8 mg/kg). **a:** Mice were weighted each day of treatment, (Vasel,  $n = 2$ ; IMQ,  $n = 4$ ). **b:** Splenomegaly after six daily DMSO or AC-YVAD-CMK treatment of mice, (Vasel,  $n = 2$ ; IMQ,  $n = 4$ ). **c:** Skin was processed for total RNA isolation and gene expression was determined by qPCR (Vasel,  $n = 2$ ; IMQ,  $n = 4$ ).

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#### **Supplemental Figure 8:**

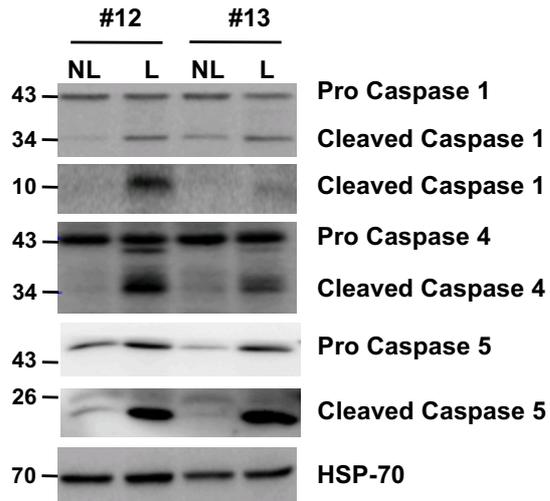
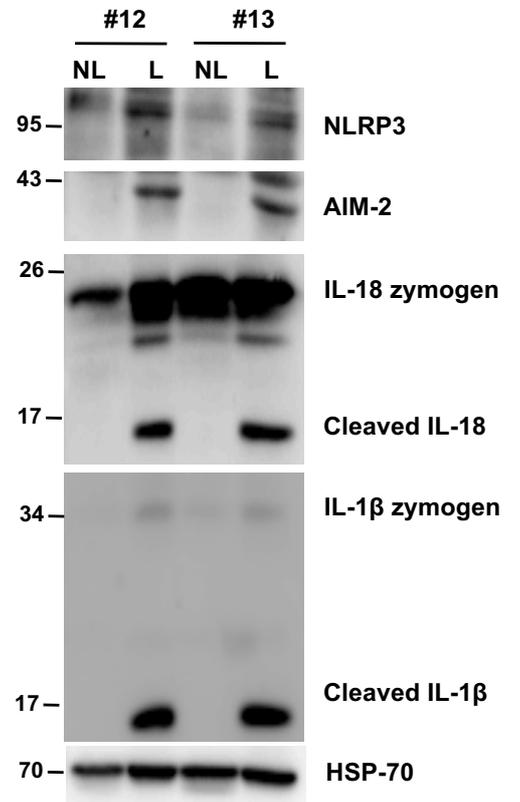
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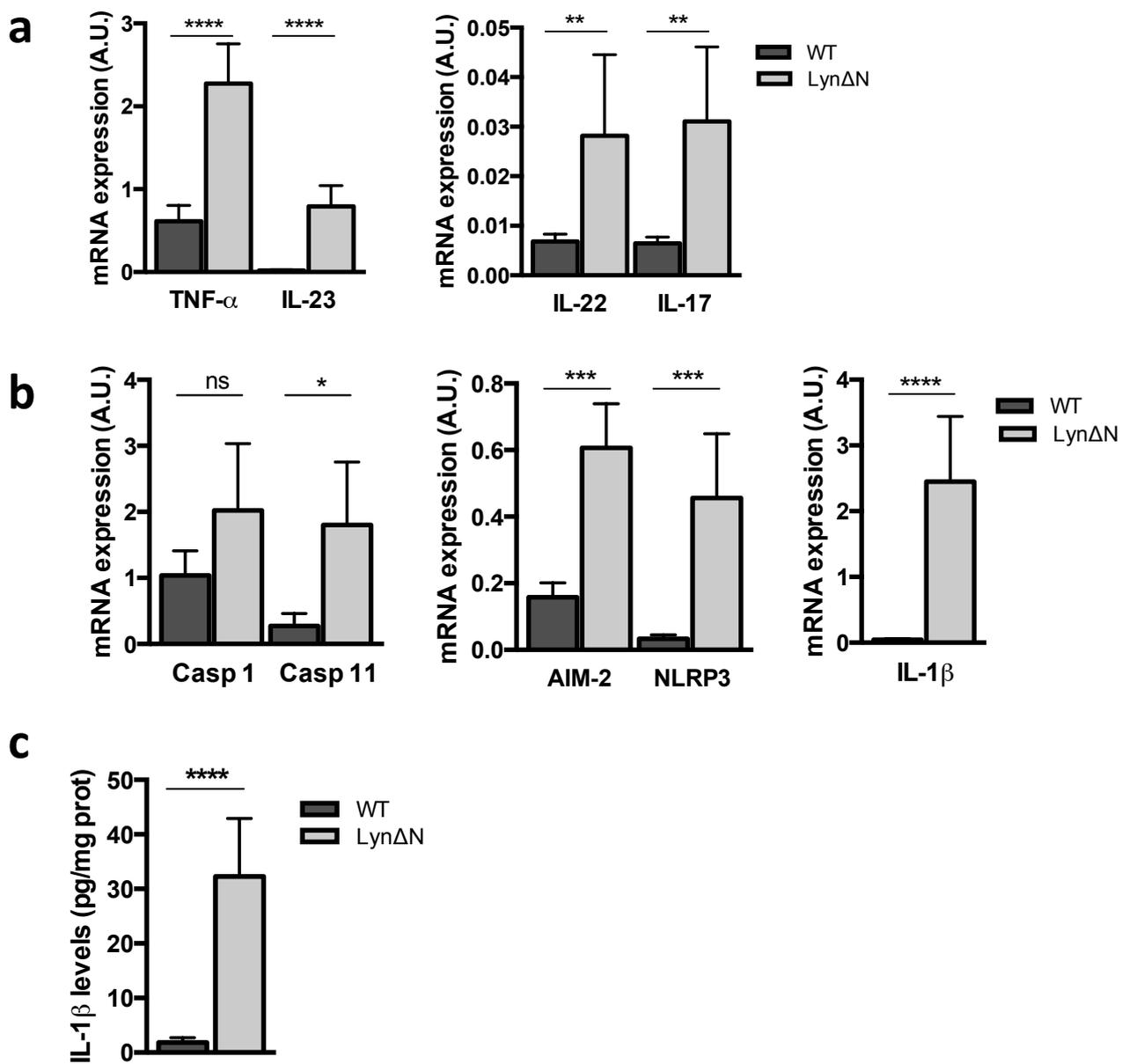
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T: TNF- $\alpha$  (10 ng/ml), I: IL-17A (200 ng/ml).

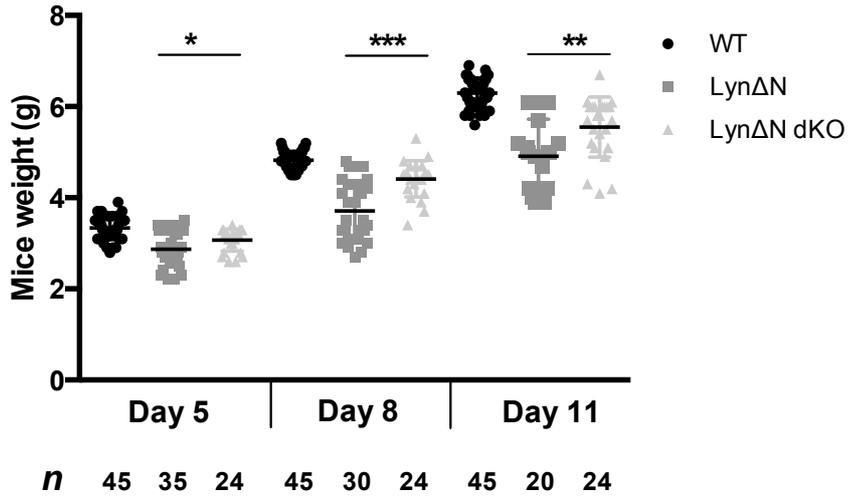
#### **Supplemental Figure 9:**

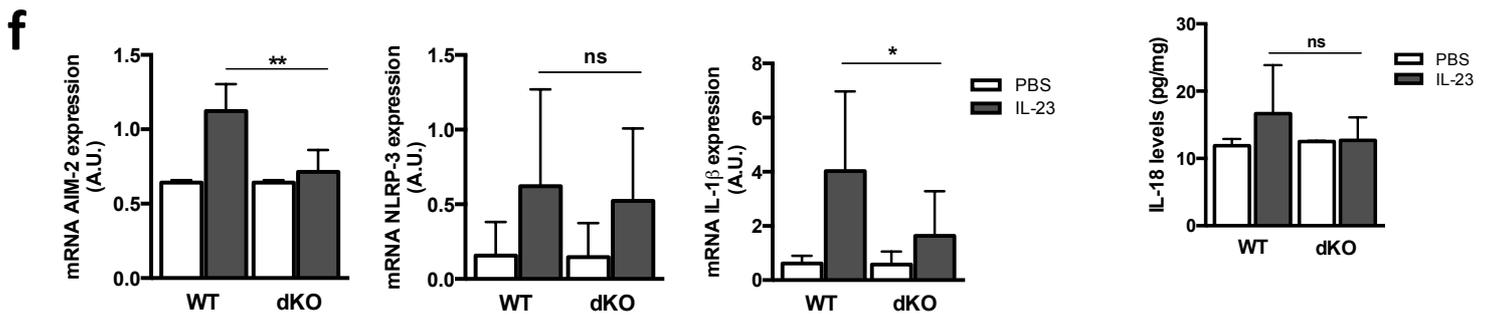
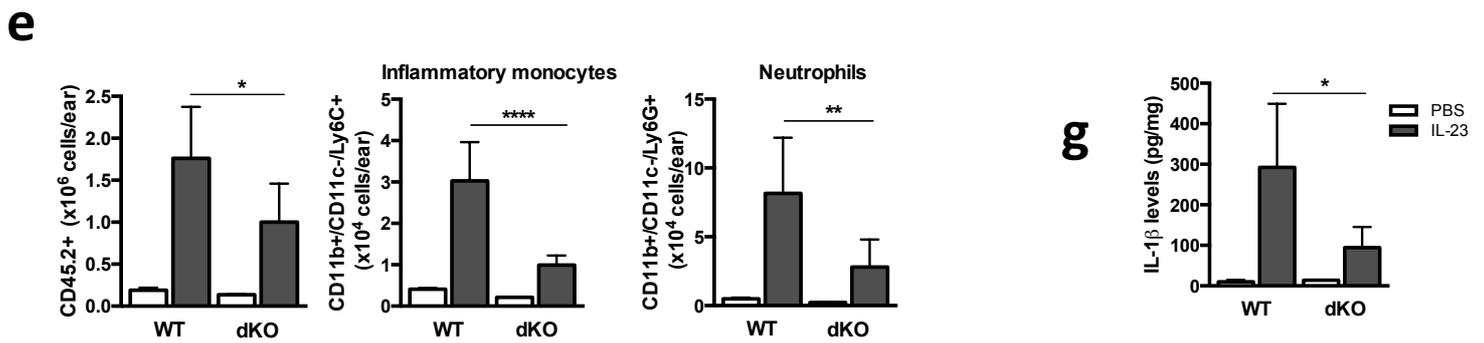
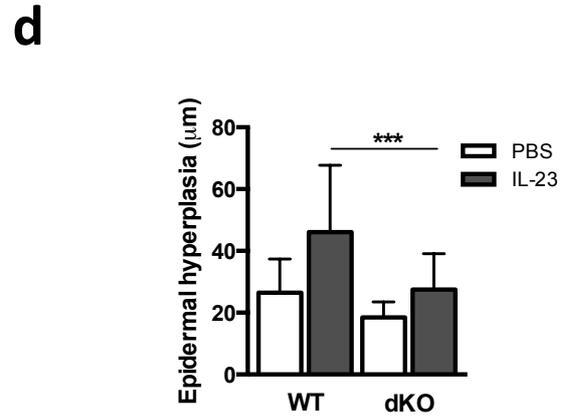
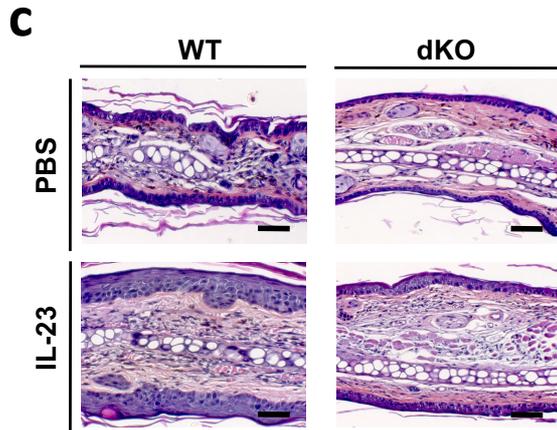
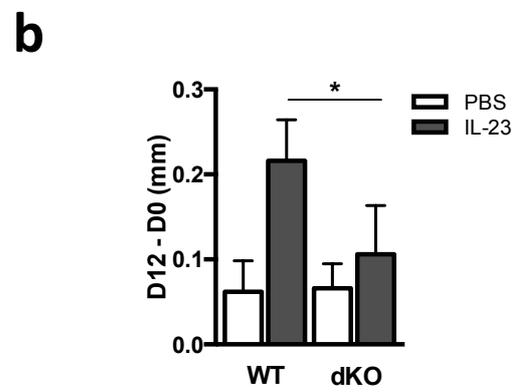
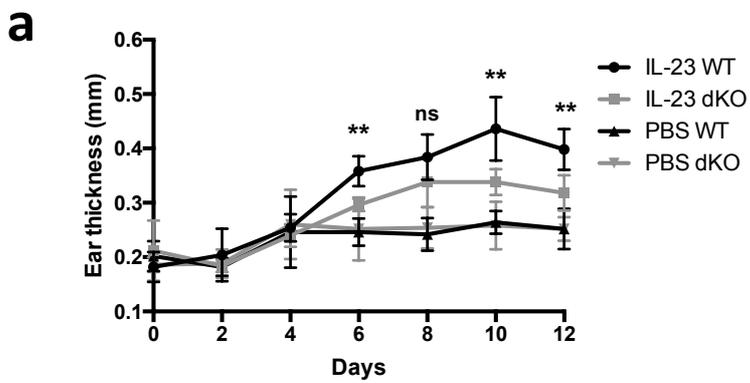
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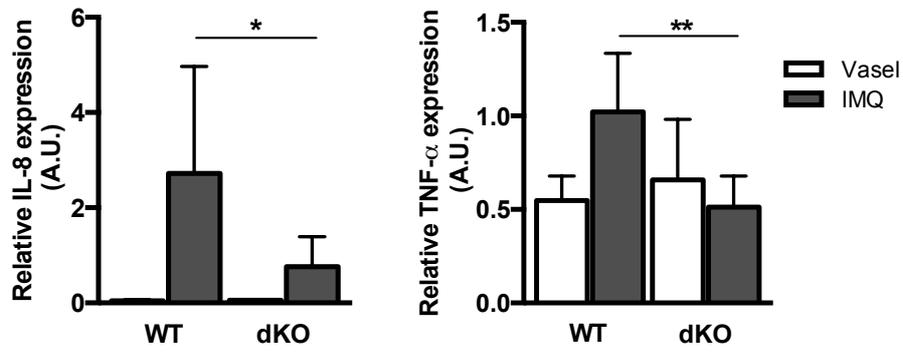
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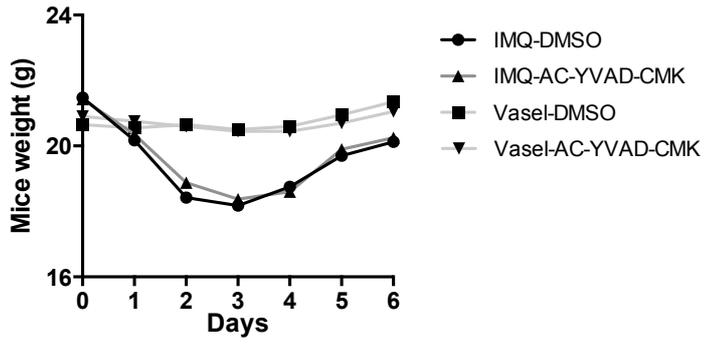
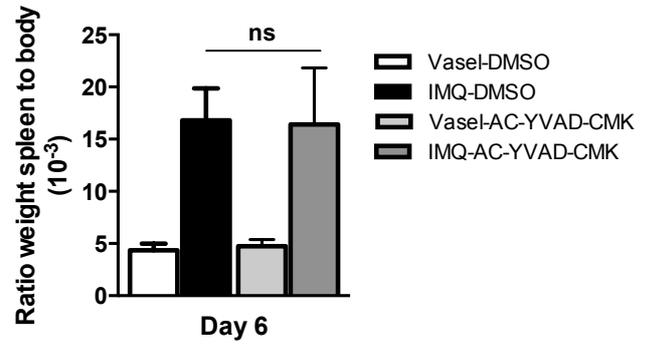
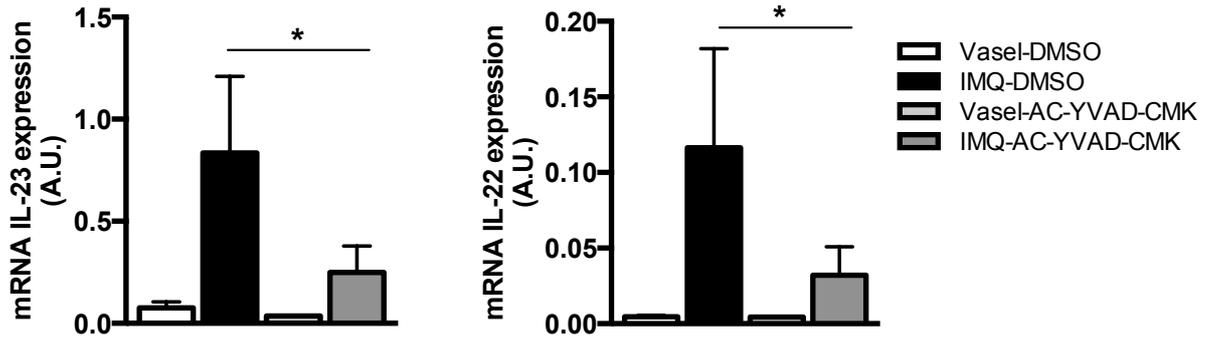
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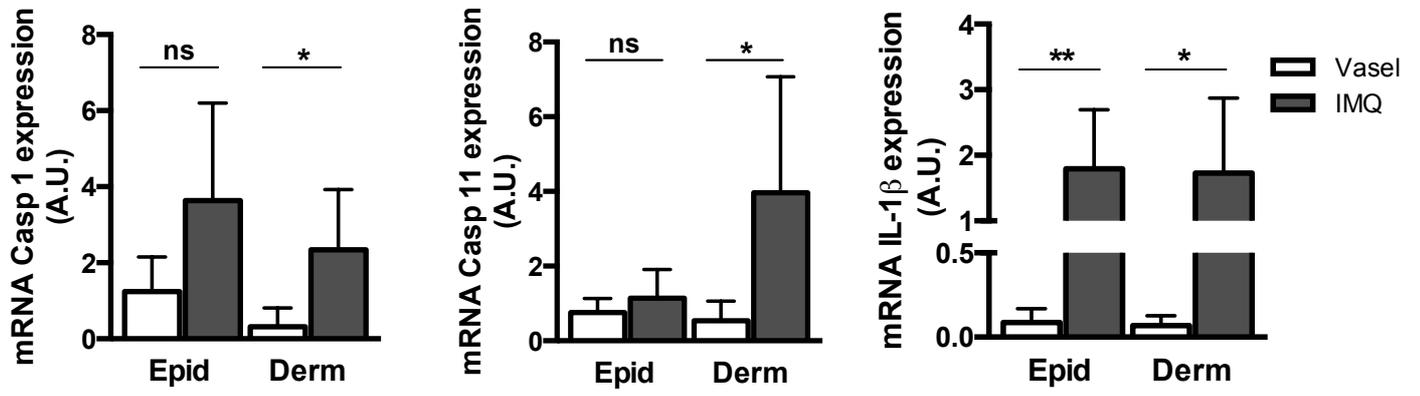


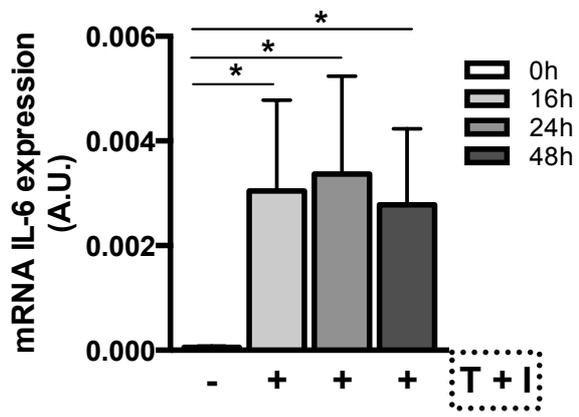
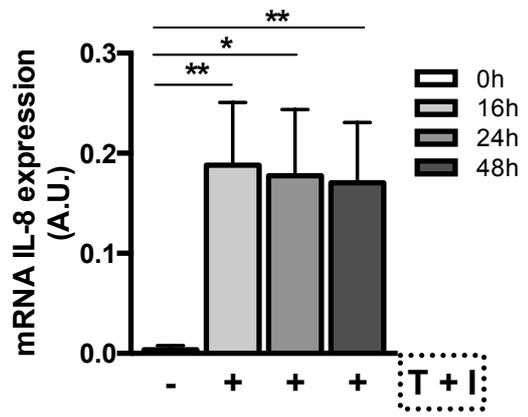
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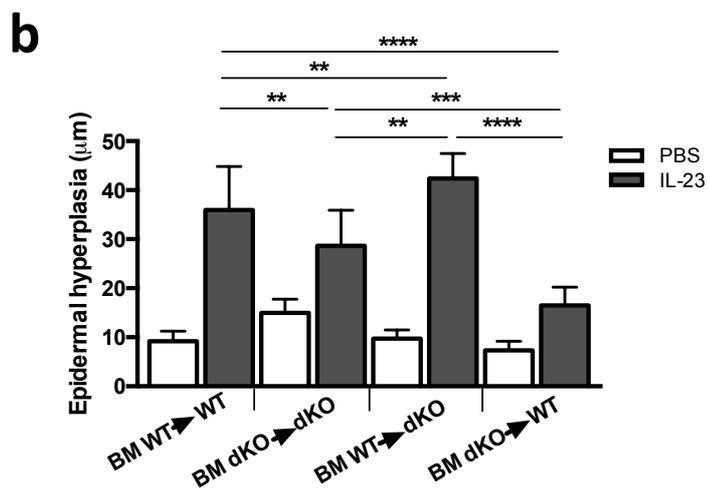
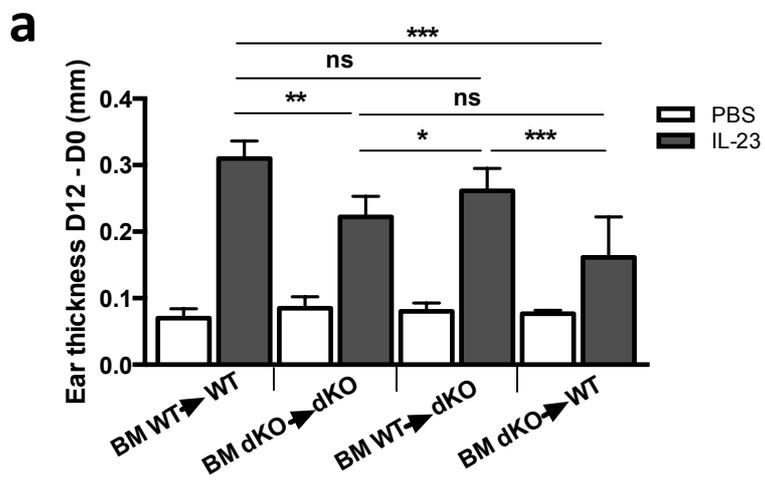




**a****b****c**



**a****b**



## **RESUME ARTICLE 3:**

### ***Lyn tyrosine kinase: a pivotal factor in psoriasis pathogenesis***

Psoriasis is a chronic T cell-mediated inflammatory disease with a relative high prevalence worldwide. Albeit a large number of studies have been performed the etiology and pathogenicity of this cutaneous diseases is not fully understood. Psoriasis is characterized by an intense dialogue between keratinocytes and the immune cell, and it has been well accepted the key role of T cells as well as pro-inflammatory cytokines and chemokines in the development and maintenance of this disease. In fact several drugs against T cells and pro-inflammatory cytokines have been approved for psoriasis treatment, and although clinical remission has been observed in most patients, psoriasis does not get cured.

Activation of the Src family kinases, a family of proteins with a key role in the control of various cellular processes such as differentiation, migration and survival has been observed in lesional skin biopsies from psoriasis patients. Moreover, it has recently been shown that the overexpression in mice of a particular form of Lyn, one of the eight members of the Src family kinases, leads to a skin inflammatory syndrome, which has several features in common with human psoriasis, indicating that Lyn can play an important function in psoriasis pathogenesis. Based on these results, we wanted to know whether Lyn had a role in this autoimmune disease.

First, we analyzed the expression and activation of Lyn in lesional human skin biopsies from psoriasis patients and we found an increase in Lyn expression compared to non lesional psoriasis skin biopsies and healthy donors that was not observed for several other members of SFK. These results were corroborated in two mouse models of psoriasis-like disease (IMQ and IL-23 models), where an increase in Lyn expression was observed at both mRNA and protein levels.

It is well known that psoriasis immune cell infiltrate is composed by dendritic cells, neutrophils, macrophages, NK cells and to a lesser extent by B cells. All these cells have in common that Lyn is an important mediator of their signaling process. Thus, the increase in Lyn expression observed in psoriasis could be explained by the recruitment of immune cells. However, keratinocytes are the principal cells in the epidermis and certainly in the injured skin. Therefore, we investigated what kind of cells was involved in this modulation. In this context, we observed that Lyn expression was increased both in the dermis and epidermis in human and mice, indicating that the recruitment of immune cells within the injured skin but also the modulation of Lyn in

keratinocytes were both involved. Interestingly, an increase in Lyn expression and activation was observed in human keratinocytes stimulated *in vitro* with TNF- $\alpha$  and IL-17A, two of the principal cytokines implicated in psoriasis pathogenesis, suggesting a role of Lyn in keratinocyte proliferation during psoriasis pathogenesis. To confirm this hypothesis, we induced a psoriasis-like phenotype in Lyn deficient mice by IMQ application, and a significant reduction was observed as compared to WT treated mice, indicating that Lyn was necessary for the continuous development of the disease. Moreover, by bone marrow transfer experiments we highlighted that the expression of Lyn in keratinocytes was enough to obtain a fully inflammatory response.

Altogether, these results show that Lyn is an important regulator in the maintaining of psoriasis, presenting a pro-inflammatory role in this disease and validating this Src family protein kinase as a potential target for psoriasis treatment.

The manuscript presented herein contains all the results I obtained during my thesis in the form of a manuscript in preparation. The discussion of this work is grouped in the discussion part of my thesis. It put forward in particular the different experiments that we would want to finish before submitting this work for publication.

# **Lyn tyrosine kinase: a pivotal factor in psoriasis pathogenesis**

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## **Keywords**

Lyn tyrosine kinase, inflammatory response, pro-inflammatory cytokines, keratinocytes, human skin biopsies, psoriasis

## **Running title**

Implication of Lyn tyrosine in psoriasis

## **ABSTRACT**

Lyn, a Src tyrosine kinase, has a well-established function in hematopoietic cells; but recent studies have shown that Lyn can also control non-hematopoietic tissues. As we found Lyn expression in human skin, and that its expression was increased in lesional skin biopsies from psoriasis patients, we wanted to know whether Lyn plays a role in this disease. In two different psoriasis mouse models, an increase in Lyn expression was observed, which was not seen for Src and Fyn, two other members of the Src family kinase. Interestingly, we observed that the expression of Lyn was increased both in the dermis and the epidermis in human and mice, suggesting that altogether the recruitment of immune cells, where Lyn is highly expressed, and the modulation of its expression in keratinocytes were part of this observation. In vitro experiments on primary human keratinocytes confirmed that Lyn expression was increased upon stimulation with TNF and IL17, two principal cytokines implicated in psoriasis pathogenesis. Finally, when psoriasis-like phenotype was induced in Lyn deficient mice, a significant reduction was observed as compared to WT mice. Interestingly, Lyn deficiency in keratinocytes/fibroblast was sufficient for a full development of the psoriasis-like phenotype, whereas its absence in immune cells had no or little impact. Altogether, these results show that Lyn could be a crucial regulator of psoriasis pathogenesis and highlight an important and new aspect of Lyn function in keratinocytes.

## INTRODUCTION

Psoriasis is an immune-mediated and chronic inflammatory skin disease of unknown etiology (Chiricozzi *et al*, 2018), whose prevalence worldwide is around 3% (Danielsen *et al*, 2013). The principal phenotype of this skin pathology is the development of a chronic erythematous and scaly plaque, generally well demarcated and covered with silvery scales (Raychaudhuri *et al*, 2014); leading to the development of psoriasis plaques, which is the main manifestation of this disease, affecting 90% of all psoriasis patients (Lowes *et al*, 2014). There are three main characteristics of psoriasis: thickness of the epidermis (acanthosis) histologically defined by elongated rete ridges into the dermis (epidermal hyperplasia) producing desquamation (scaling), dilated and prominent blood vessels in the dermis (erythema) and epidermal and dermal immune cell infiltration (Ogawa *et al*, 2018).

Several immune cell populations are implicated in psoriasis pathogenesis. T helper (Th), cytotoxic and  $\gamma\delta$  T cells as well as dendritic cells, mast cells, neutrophils, natural killer (NK) and innate lymphoid cells have been involved in psoriasis plaque development (Boehncke and Schon, 2015), leading to postulate psoriasis as a result of a key dialogue between the aberrant proliferation and differentiation of keratinocytes with the huge recruitment of immune cells. This dialogue is made through the release of different pro-inflammatory cytokines, acting as mediators, such as tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-17, -23, -6 and IL-1 $\beta$  (Ayala-Fontanez *et al*, 2016) that lead to the activation of several signaling pathways (Wu *et al*, 2017; Ali *et al*, 2016).

The Src family protein of tyrosine kinase (SFK) is a family of proto-oncogene proteins composed of eight members with similar structure and molecular weight, which has been classified in two subgroups according to the similarities in their amino acids sequences: Src-related proteins (Src, Fyn, Yes and Fgr) and Lyn-related members (Lyn, Lck, Hck and Blk) (Parsons and Parsons, 2004). All members of this family have been implicated in a large amount of cellular processes such as cell growth, differentiation, adhesion, migration, survival as well as apoptosis, gene transcription and angiogenesis (Espada and Martin-Perez, 2017; Maa and Leu, 2016; Roskoski, 2015). SFK are activated by a wide variety of cell surface receptors, such as antigen receptors, growth factor receptors as well as interleukin receptors (Mitchell *et al*, 2018). In the particular case of psoriasis, it has been demonstrated an activation of SFK in lesional skin (Ayli *et al*, 2008), suggesting a possible role of family in psoriasis pathogenesis.

The Lyn tyrosine kinase has a pivotal role in hematopoietic cell homeostasis presenting a dual role with a positive and negative function (Xu *et al*, 2005). In fact, deregulations in the expression level and in the activity of this protein have been related with a large number of blood malignancies (Ingleby, 2012), and Lyn deficient mice develop a lupus-related syndrome characterized by a huge increase in autoantibodies-secreting plasma cells population

(Gutierrez *et al*, 2010). However, although in the first studies related with this protein it was described as a specific hematopoietic kinase, nowadays it is fully accepted that Lyn is expressed in many others tissues (Liu *et al*, 2017; Roseweir *et al*, 2016; Mello *et al*, 2015; Bundela *et al*, 2014; Ingley, 2012).

Anti- and pro-inflammatory functions have been described for Lyn. Indeed, its protective role has been evidenced through the susceptibility observed in Lyn deficient mice to develop endotoxin-induced lung inflammation (Gao *et al*, 2015) and to dextran sulfate sodium (DSS)-induced colitis (Roberts *et al*, 2014). On the other hand, the pro-inflammatory function of Lyn has been observed in diabetes. In fact, a decrease in Lyn signalization was associated with a decrease in reactive oxygen species (ROS)-mediated inflammation preventing high glucose-induced innate immune response (Wang *et al*, 2017). Moreover, it was previously reported that the overexpression of a particular form of Lyn in mice led to the development of a skin inflammatory syndrome resembling human psoriasis (Marchetti *et al*, 2009), suggesting an implication of this protein in psoriasis pathogenesis.

Thus, taking in consideration that Lyn can exhibit a pro-inflammatory role in the new scenario of psoriasis; we focused our work on the mechanism by which Lyn could participate in psoriasis pathogenesis. Here, we show that Lyn is increased and activated in lesional skin biopsies from psoriasis patients and after induction of psoriasis-like phenotype in mice, demonstrating a significant modulation of Lyn expression and activation in cytokine-stimulated keratinocytes and evidencing that Lyn deficient mice present a delay in the psoriasis-like disease. Finally, our results highlight a new aspect of Lyn function in the context of psoriasis that is related to its role in keratinocytes.

## **MATERIAL AND METHODS**

### **Study approval.**

This study was conducted on biopsies from 170 patients enrolled in a study registered in clinicaltrials.gov under the identifier NCT01538342: Role of Tyrosine Kinase Lyn and Cleaved Form by Caspases in Psoriasis. This study was approved by the local ethics committee (Comité de Protection des Personnes Sud-Méditerranée V, n° 2011-A00786-35.065), and was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practices guidelines. All patients and healthy donors included signed informed consent before their inclusion in the study. The ethics review board from the French Minister for the High Education and Research approved all animal studies together with the Institutional Animal Care and Use Committee of the Centre Méditerranéen de Médecine Moléculaire (C3M), Nice, France (INSERM U1065).

**Biopsies.** Four-millimeter punch biopsies were collected from healthy donors and psoriasis patients. In healthy donors, biopsies were taken in the upper thigh and in psoriasis patients, were taken from the center of a chronic plaque (lesional biopsy) and from non-lesional skin. All biopsies were frozen immediately in liquid nitrogen and then stored at -80 °C.

**Mice.** Mice were housed under specific pathogen-free conditions and fed with standard laboratory food. All mice (WT, LynKO) were on a C57BL/6J genetic background and were used at 8-10 weeks of age, except for adoptive transfer, where mice were irradiated and reconstituted at six weeks. Both males and females were used in this study. WT mice (C57Bl6/J) were purchased from Envigo and LynKO deficient mice were a gift from Dr Nicolas Charles (INSERM U1149, Centre de Recherche sur l'Inflammation, Paris, France).

### **Experimental protocols**

To induce psoriasis-like phenotype, mice were treated either with Aldara cream corresponding to 3,125 mg of IMQ (daily for six days) in the backs after shaving and depilation or with mouse recombinant IL-23 (500 ng diluted in PBS) by intradermal injection in the ears (each other day for 12 days). The severity of inflammation in the back skin through erythema (redness) and scaling (desquamation) was assessed by an adapted version of the clinical PASI score, 0: none; 1: slight; 2: moderated; 3: strong; 4: very strong phenotype. Moreover, a cumulative score, resulting from the sum of erythema and scaling, was calculated. The severity of inflammation in the ears was measured through the thickness of the ears using a caliper Mitutoyo. The change from baseline was determined.

For the adoptive transfer experiment, six weeks WT and LynKO deficient mice were lethality irradiated at 7 Gy and then reconstituted with  $5 \times 10^6$  bone marrow cells from donor mice. Donor bone marrow cells were harvested from the hind limbs (femur and tibia) and a single cell suspension of bone marrow cells was made. All recipient mice were left for 8 weeks to ensure recovery and then IMQ-protocol to induce psoriasis-like phenotype was performed.

**Reagents.** Aldara (5% IMQ) cream was purchased from MEDA Pharma. TRIzol reagent was acquired from Life Technologies. All TaqMan reagents were bought from Thermo Fisher Scientific. Dispase enzyme was obtained from Sigma-Aldrich. Human recombinant proteins IL-17A and TNF- $\alpha$ , for keratinocyte stimulation, were purchased from PeproTech. siRNA were obtained from Invitrogen (siLyn: 305588F09, siFyn: 305588G01).

**Antibodies.** Rabbit polyclonal antibodies against Lyn (CS-2732, CS-2796), Src (CS-2123), Fyn (CS-4023), P-Src (CS-6943), STAT1 (CS-9172), Lamin A/C (CS-2032), LAMP2 (CS-49067) and ERK (CS-4695) were purchased from Cell Signaling Technology and rabbit polyclonal antibody against P-Src (Ab-46660) was purchased from Abcam. Rabbit polyclonal antibody against keratin 10 (PRB-159P) and keratin 6 (PRB-169P-100) were bought to Covance. Mouse monoclonal antibody against IL-1 $\beta$  (CS-12242) was obtained from Cell Signaling Technology. Mouse monoclonal antibodies against Lyn (SC-7274), Lck (SC-433), Hck (SC-101428) and HSP-90 (sc-69703) were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibody against Actin (A2228) was obtained from Sigma-Aldrich. Mouse monoclonal antibody against Ioricrin (PRB-145P) was bought to Covance. Purified rabbit antibodies against CD45 (304002) and laminin (L9393) were obtained from Biolegend and Sigma, respectively.

**H&E staining.** Skin biopsies were fixed in 10% neutral-buffered formalin and then include in paraffin. Five-micrometer thick sections were cut and stained with H&E (Sigma-Aldrich). To quantify epidermal hyperplasia, 14 assessments were made in both portions of the tissue. Histopathological examinations were performed on a Nikon light microscope.

**RNA extractions and qPCR assessments.** Total RNA was extracted from skin tissues using TRIzol reagent and reverse transcribed using random primer from Promega and SuperScript II reverse transcriptase from ThermoScientific. For qPCR analysis, different TaqMan sondes were used (Thermo Fisher Scientific), (Mouse Taqman, RPLP0: Mm00725448, IL-6: Mm00446190\_m1, IL-22: Mm01226722\_g1, IL-23: Mm00518984\_m1, IL-1 $\beta$ : Mm00434228\_m1, Lyn: Mm01217488\_m1 and Mm01217484\_g1; Human Taqman, RPLP0: Hs99999902\_m1, Lyn: Hs00176719\_m1, Src: Hs01082246\_m1; Fyn: Hs00941600\_m1.

**FACS.** Two 8-millimeter punches were taken and single-cell suspension of back skin was obtained as described previously (Riol-Blanco *et al*, 2014). Briefly, for the back skin digestion, all hypodermis was mechanically eliminated. Then, a digestion for 90 minutes at 37°C in gentleMACS tubes (Miltenyi) with gentle agitation in freshly prepared digestion mix consisting of RPMI (Gibco) supplemented with Collagenase IV (Sigma) was produced. After enzymatic digestion, the mixture was processed using a gentleMACS homogenizer (Miltenyi) in order to obtain a cell suspension, which was then filtered through a 70 $\mu$ M cell strainer

(BD). Cells were then resuspended in FACS buffer for analysis (PBS with 2mM EDTA and 2% FCS (GIBCO)).

For FACS staining, the following antibodies were used: FITC-conjugated anti-CD19 (Biolegend), Pacific blue-conjugated anti-CD3 (Biolegend), Alexa647-conjugated anti-CD11b (cloneM1/70; Biolegend) and APC-conjugated anti-CD45 (cloneM5/114.15.2; Biolegend). For analysis, cells were acquired on a MACSQuant FACS (Miltenyi) and analyzed using the MACSQuantify Software (Miltenyi).

**Tissue dissociation.** Mouse tissues and psoriasis patient skin biopsies were homogenized with a CK14 Soft Tissue Homogenizing Kit (VWR) in a Precellys 24 (Bertin Corporation) and cells were lysed in buffer containing 50 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM PMSF, 10 mg/ml leupeptin, 5 mg/ml aprotinin and 1% Triton X-100. Supernatants were harvested, dosed and stored immediately at -80 °C.

**Dermis-Epidermis dissociation.** Two 8-millimeter punches were obtained from mice backs and after removing hypodermis, punches were put, dermis face down, in a dish containing 1% dispase for 1h30 at 37 °C. Then, epidermis and dermis were mechanically separated and immediately frozen for total RNA isolation.

**Western blotting.** Proteins were separated by SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, Millipore). After blocking nonspecific binding sites, the membranes were incubated with specific antibodies. Then, membranes were washed and incubated further with horseradish peroxidase conjugated antibody. The immunoblots were visualized using an enhanced chemiluminescence detection kit (Pierce).

**Keratinocyte culture.** Normal Human Keratinocytes were a gift from Dr Karine Bille and Dr Corine Bertolotto (C3M, Nice, France). NHK were culture in presence of irradiated fibroblasts and a special medium consisting of 60% DMEM, 30% F-12 NutMix and 10% FCS supplemented with Cholera toxin 0,1 nM, Adenine 0,18 mmol/L, Insulin 5 ug/mL, Hydrocortisone 0,4 ug/ml, Triiodothyronine 2nmol/L and EGF 10 ng/ml.

These cells were stimulated at different time points with both IL-17A (200 ng/ml) and TNF- $\alpha$  (10 ng/ml) cytokines and in presence of basal KGM medium plus supplemented solutions (PromoCell).

**Statistics.** The number of animals used in each experiment is described in the figure legends. All statistical analyses were performed in GraphPad Prism software (GraphPad, version 6) and results are represented as mean  $\pm$  SD. To compare means between two groups, the unpaired two-tailed Student t test was used. To compare means between three or more groups, the one-way ANOVA with the uncorrected Fisher's Least Significance Difference (LSD) multiple comparison test was applied. (\*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant).

## RESULTS

### **Increased Lyn expression is confirmed in lesional samples from psoriasis patients**

An activation of SFK was previously shown in patients suffering psoriasis (Ayli *et al*, 2008). Moreover, an increase in Lyn expression was observed in six lesional samples compared to non-lesional one from psoriasis patients (Marchetti *et al*, 2009). Here, we decided to confirm these results on a large cohort of psoriasis patients. Western blot analysis on skin protein extracts showed a significant increase in Lyn expression (Figure 1a, b) associated to an increase in SFK activation (Figure 1c) in lesional samples compared to non-lesional biopsies in all assessed psoriasis patients. Surprisingly, no increase either in Src, Fyn, Lck or Hck expression, several other SFK members, was observed in lesional samples with respect to non-lesional one (Figure S1a, b), indicating that Lyn seems to be the only SFK modulated in this chronic disease.

To decipher if this Lyn modulation is specific to psoriasis, we then decided to analyze Lyn expression in atopic dermatitis, a chronic relapsing inflammatory skin disease, whose principal difference from psoriasis is the crucial Th2 immune response (Gittler *et al*, 2013; Gittler *et al*, 2012). Interestingly, no Lyn modulation was observed between lesional and non-lesional skin biopsies of atopic dermatitis patients (Figure 1c, d). Altogether, these data confirm that Lyn protein tyrosine kinase is increased in skin suffering psoriasis plaques and identify that Lyn modulation is not a general mechanism of skin disease.

### **Lyn is augmented after induction of psoriasis-like phenotype in mice**

Next, we wanted to confirm the results observed in psoriasis patients in two different mouse psoriasis-like models: the imiquimod (IMQ) model (van der Fits *et al*, 2009) and the IL-23 model (Hedrick *et al*, 2009). We first observed in the IMQ treated mice (Figure S2a) a significant increase in Lyn mRNA (Figure S2b) and protein (Figure 2a) expression as well as in SFK activation (Figure 2a) as compared to control mice. In the case of IL-23-dependent psoriasis-like model (Figure S2c), similar results were obtained (Figure S2d). Then, we wanted to know whether the increase in Lyn expression was dependent of the development of a well-marked psoriasis-like phenotype. To address this, we analyzed the expression of Lyn along the IMQ induction of psoriasis disease: after two days (slight psoriasis-like phenotype), four days (moderate phenotype) and six days (highly marked phenotype) of IMQ application. Thus, as is shown in figure 2b, the increase in Lyn expression and SFK activation is observed starting from day four, reaching its maximum level at day six (Figure 2b; S2e), which demonstrates that the modulation in Lyn expression and activation requires the development and establishment of psoriasis-like disease.

It is well known that two phases of psoriasis disease are developed in patients: an early onset (acute phase) and a late stable phase (chronic phase) (Ayala-Fontanez *et al*, 2016), in which the immune cell population is transiently increased in both dermis and epidermis (Terhorst *et al*, 2015). Thus, to know whether Lyn modulation was maintained once psoriasis plaques were already established, a biphasic model of psoriasis-like phenotype was induced in WT mice (Figure S2f). Interestingly, a significant increase in Lyn mRNA (Figure S2g) and protein (Figure 2c) expression was observed in the chronic phase of psoriasis-like disease compared to control mice, indicating that Lyn expression is maintained in an established lesional skin although the numbers of different immune cell populations might be transiently modified.

Two main groups of cells are involved in psoriasis pathogenesis: the inflammatory immune cell, principally into the dermis, and keratinocytes in the epidermis (Ogawa *et al*, 2018). To elucidate which compartment is responsible for Lyn modulation observed by global analysis of skin extract, dermis/epidermis dissociation experiment was done from IMQ-treated skin. Surprisingly, although an increase in Lyn expression was observed in the dermis due to huge amount of immune cell infiltration, a significant increase in Lyn mRNA (Figure S2h) and protein (Figure 2d, e) expression was found in the epidermis; suggesting a modulation of Lyn tyrosine kinase in the keratinocytes, the principal cellular type found in this compartment.

### **An increase in Lyn expression is observed in human keratinocytes**

To validate the results obtained in mice, immunofluorescence-staining approaches were made in non-lesional and lesional skin biopsies from psoriasis patients. As it is shown in figure 3a, Lyn expression is augmented in lesional skin with respect to non-lesional skin in both dermis and epidermis, which was also observed in a large cohort of psoriasis patients (Figure 3b). Outstandingly, when a co-staining of CD45 and Lyn was performed, three populations were observed in lesional skin biopsies (Figure 3c): i) CD45<sup>+</sup> Lyn<sup>+</sup>, indicating myeloid and B cells (empty arrowhead); ii) CD45<sup>+</sup> Lyn<sup>-</sup>, showing T cells (full arrowhead) and iii) CD45<sup>-</sup> Lyn<sup>+</sup> (dotted arrowhead), completely present in the epidermis, suggesting a significant modulation of Lyn expression in keratinocytes.

Altogether, these results highlight a modulation of Lyn protein expression in the epidermis of both IMQ-treated mice and lesional skin biopsies of psoriasis patients, suggesting a specific modulation of this SFK member on keratinocytes.

### **Lyn tyrosine kinase is modulated in cytokine-stimulated keratinocytes**

To clarify whether a Lyn modulation can be found in keratinocytes, immunofluorescence-staining approaches were performed in normal human keratinocytes stimulated *in vitro* with recombinant human IL-17A and TNF- $\alpha$ , two cytokines implicated in psoriasis pathogenesis

(Greb *et al*, 2016). An enhanced expression of Lyn was seen in treated keratinocytes compared to control cells (Figure 4a), which was principally observed in the nucleus and associated to granules. Similar results were obtained when a subcellular fractionation was made on these cells (Figure S3a), suggesting that Lyn modulation in keratinocytes is linked to an increased expression in the microsome and nuclear fraction. Moreover, the high levels in Lyn expression (Figure 4b) was associated to an increase in Pan-P-Src activation at different time points of stimulation (Figure 3b), correlating with a significant increase in Lyn mRNA expression over time (Figure 4c).

Different studies have elucidated the crucial role of Fyn in keratinocyte differentiation (Fenton and Denning, 2015) and transformation (Fenton *et al*, 2015). Moreover, Src has been also implicated in keratinocyte differentiation (Szalmas *et al*, 2013) and migration (Sophors *et al*, 2016). Nevertheless, few things are known about the role of these proteins in mimicking psoriasis conditions of keratinocyte proliferation. Thus, although these two members of the SFK were not augmented in total skin from psoriasis patients, we wanted to determine whether a modulation in these proteins could be observed in stimulated keratinocytes. No modulation of either Src or Fyn mRNA (Figure S3b) and protein (Figure S3c) expression was observed in our mimicking in vitro psoriasis conditions, suggesting that these two SFK members are not involved in keratinocyte behavior through pro-inflammatory cytokine stimulation.

To determine whether the SFK activation observed in stimulated keratinocytes was related to Lyn tyrosine kinase activation, immunoprecipitations experiments were performed. Interestingly, Lyn tyrosine kinase is activated in cytokine-stimulated human keratinocytes (Figure 4d, e; S3d), reinforcing the idea that Lyn could have a key role in psoriasis pathogenesis.

Several cytokines are secreted by stimulated keratinocytes in psoriasis pathogenesis (Chiricozzi *et al*, 2018). So next, we analyzed which of these cytokines were modulated in our mimicking psoriasis condition and a significant increase in IL-1 $\beta$  expression (Figure S3e) as well as IL-6 and IL-8 (data do not shown) expression was found. To go deeper in details, we decided silencing Lyn expression to determine whether this effect could impact cytokine production. Strikingly, the silencing of Lyn expression, but not those from Fyn protein leads to a decrease in protein (Figure 4f) and mRNA (Figure 4g) expression of IL-1 $\beta$ ; suggesting that the role of Lyn in psoriasis development could be related to the favoring of pro-inflammatory cytokine secretion such as IL-1 $\beta$  by keratinocytes.

Finally, we decided to analyze whether Lyn could be implicated in keratinocyte differentiation. Curiously, no modulation in Lyn expression was appreciated through keratinocyte differentiation (Figure S3f), thus Lyn would play a role only in keratinocyte proliferation.

### **Lyn deficiency impairs the IMQ induced psoriasis-like disease phenotype**

To confirm the contribution of Lyn tyrosine kinase in psoriasis pathogenesis, we induced the psoriasis-like phenotype in WT and Lyn deficient (hereafter KO) mice. The absence of Lyn delayed the onset of the clinical score measured. Indeed, erythema, scaling and cumulative scores were quantified after induction of psoriasis-like phenotype (Figure 5a) and a significantly decrease was observed in KO mice compared to WT mice. Further, H&E staining of skin sections revealed a decrease in epidermal hyperplasia (Figure 5b, c) along with a reduction in the immune cell infiltration (Figure 5d) in KO mice skin. A more precise analysis showed that the significant decrease in the number of CD45.2<sup>+</sup> immune cells into the Lyn KO mice skin was related to a diminution in T cell and myeloid cell populations (Figure 5d). Additionally, pro-inflammatory cytokine mRNA expression (Figure 5e) was significantly decreased in Lyn KO mice with respect to WT mice. Altogether, these data indicate that Lyn protein is required for the establishment of the disease to reach the maximal inflammation, corroborating our previous results obtained in figure 2b and c, where it is showed that the increase in Lyn expression is observed after day 4 of IMQ application.

### **Lyn activation in keratinocytes is sufficient for the development of a complete psoriasis-like disease in mice**

As we showed that 1) Lyn activation is necessary to obtain a fully psoriasis-like phenotype, and 2) Lyn expression/activation can take place both in immune cells and keratinocytes, we decided to perform bone marrow transplantation experiments to elucidate in which cell type Lyn protein is responsible for the psoriasis pathogenesis. As expected, Lyn KO mice receiving Lyn KO bone marrow showed a significant decrease in the adapted PASI score (Figure 6a), compared to WT mice transplanted with WT bone marrow. After IMQ treatment, WT mice transplanted with Lyn KO bone marrow showed a phenotype close to WT mice transplanted with WT bone marrow, indicating that the presence of Lyn in immune cells is not related to the phenotype development. However, in Lyn KO mice reconstituted with WT bone marrow a partial reduction in the erythema and scaling score was observed compared to WT mice transplanted with WT bone marrow, phenotype that was similar to the one obtained in Lyn KO mice receiving Lyn KO bone marrow, suggesting that the presence of Lyn in keratinocytes is crucial in psoriasis pathogenesis. These results provided evidence of a pivotal role of the expression and activation of Lyn tyrosine kinase activation in keratinocytes to trigger a psoriasis-like inflammatory response in mice.

**Conflict of interest**

There is no conflict of interest declared by the authors.

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**Author contributions**

LEA obtained the majority of data presented herein and were assisted by DG, CRB, PLO, FL, AJ, GR, BS, JC, VE and PC. JPO, JPL and TP included patients and performed clinical examination. SM and LEA wrote and edit the manuscript. JER and PA provided intellectual input and edit the paper. SM conceived the project, supervised the study and obtained funding. All authors reviewed and approved the final version of the manuscript.

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## FIGURE LEGENDS

### **Figure 1: Increased Lyn expression is observed in lesional skin biopsies from psoriasis patients.**

Western Blot analysis from skin biopsy total extracts of healthy donors (HD) as well as non-lesional (NL) and lesional (L) psoriasis **(a)** and atopic dermatitis patients **(c)**; **(b, d)**: Densitometry quantification of Lyn expression **(b)** HD: 3 patients, L and NL: 45 psoriasis patients; **(d)** L and NL: 10 atopic dermatitis patients.

### **Figure 2: Lyn is augmented after induction of psoriasis-like phenotype in mice.**

WT were treated daily with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream and after sacrificed them back skin was harvested and total protein was prepared and Western Blot analyses were performed. **(a)**: Mice were treated for 6 days to induce the phenotype; **(b)**: A kinetic assay was made to assess Lyn expression at several time points; **(c)**: A biphasic model of psoriasis-like disease was obtained treating mice daily for 12 days with Vasel or IMQ; **(d)**: A dermis/epidermis dissociation experiment was made (T: total skin, E: epidermis, D: dermis); **(e)**: Densitometry quantification of Lyn expression (T: total skin, Ep: epidermis, De: dermis) (Vasel: n=2, IMQ: n=4).

### **Figure 3: An increased Lyn expression is observed in human keratinocytes.**

Immunofluorescence approaches were used in lesional and non-lesional human skin biopsies from psoriasis patients. **(a)**: Immunostaining of DAPI (blue), laminin (green,  $\lambda=498$  nm) to separate dermis and epidermis as well as Lyn (violet,  $\lambda=647$  nm); **(b)**: Total of analyzed patients showing in how many patients an increase in Lyn expression in lesional sample compared to non-lesional sample was observed; **(c)**: Immunostaining of DAPI (blue), laminin (green), CD45 (red,  $\lambda=594$  nm) and Lyn (violet,  $\lambda=647$  nm). HD: Healthy donors, NL: Non-lesional skin sample, L: Lesional skin biopsy.

### **Figure 4: Lyn tyrosine kinase is modulated in cytokine-stimulated keratinocytes**

**(a)**: Primary human keratinocytes were stimulated with TNF- $\alpha$  (10 ng/ml) and IL-17A (200 ng/ml) for 24h and Lyn expression was assessed by immunofluorescence approach; **(b, c)**: Keratinocytes were stimulated at different time points. **(b)**: Cells were lysed and Western Blot analyses were performed, (n=5), **(c)**: Total RNA isolation was made and Lyn gene expression was determined by qPCR, (n=5). **(d, e)**: Immunoprecipitation experiments were done. **(d)**: P-Src expression was blotted after IP-Lyn plus densitometry quantification; **(e)**: Lyn expression was blotted after IP-P-Src. **(f, g)**: Normal human keratinocytes were silenced for Lyn (siRNA Lyn) and Fyn (siRNA Fyn) and then stimulated for 24h, siLuc was used as

control. **(f)**: Cells were lysed and Western Blot analyses were performed, (n=3); **(g)**: total RNA isolation was made and IL-1 $\beta$  gene expression was determined by qPCR, (n = 3).

AU: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , T: TNF- $\alpha$  (10 ng/ml), I: IL-17A (200 ng/ml).

### **Figure 5: Lyn deficiency impairs the IMQ induced psoriasis-like disease phenotype**

WT and Lyn deficient mice (KO mice) were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream and after sacrificed them back skin was harvested. **(a)**: Severity of IMQ-induced psoriasis like phenotype represented by Erythema, Scaling and Cumulative score measurement; **(b)**: Representative histological sections stained with haematoxylin and eosin (20X), scale bars 50  $\mu$ m; **(c)**: Epidermal hyperplasia quantification through haematoxylin and eosin staining; **(d)**: The number of immune cell populations was quantified by FACS; **(e)**: Skin was processed for total RNA isolation and gene expression was determined by qPCR. (Vasel, n=6; IMQ-WT, n=12; IMQ-KO, n=16).

AU: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , \*\*\*\*  $p \leq 0.0001$ , ns: non-significant.

### **Figure 6: Lyn activation in keratinocytes is sufficient for the development of a complete psoriasis-like disease in mice**

WT and Lyn deficient (KO) mice were irradiated and then reconstituted with bone marrow from WT (WT $\rightarrow$ WT and WT $\rightarrow$ KO) and KO (KO $\rightarrow$ KO and KO $\rightarrow$ WT) mice, which were left to recuperate for 8 weeks. Then, the four groups of mice were treated daily for six days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream. After treatment back skin was harvested. **(a)**: Severity of IMQ-induced psoriasis like phenotype represented by Erythema, Scaling and Cumulative score measurement, (WT $\rightarrow$ WT and KO $\rightarrow$ KO n=7; WT $\rightarrow$ KO and KO $\rightarrow$ WT n=6).

AU: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ , ns: non-significant.

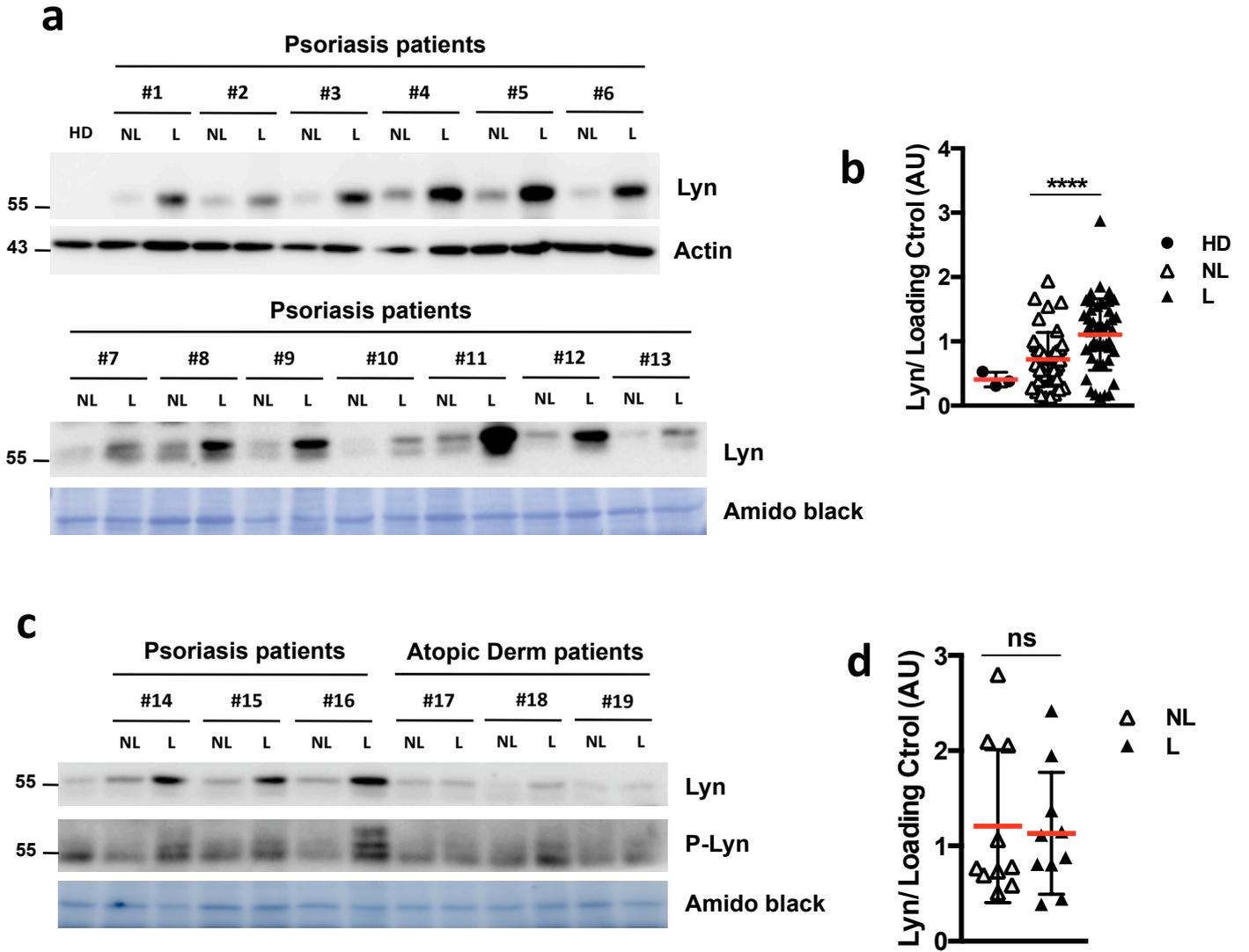


Figure 1, Aira-Lyn Pso

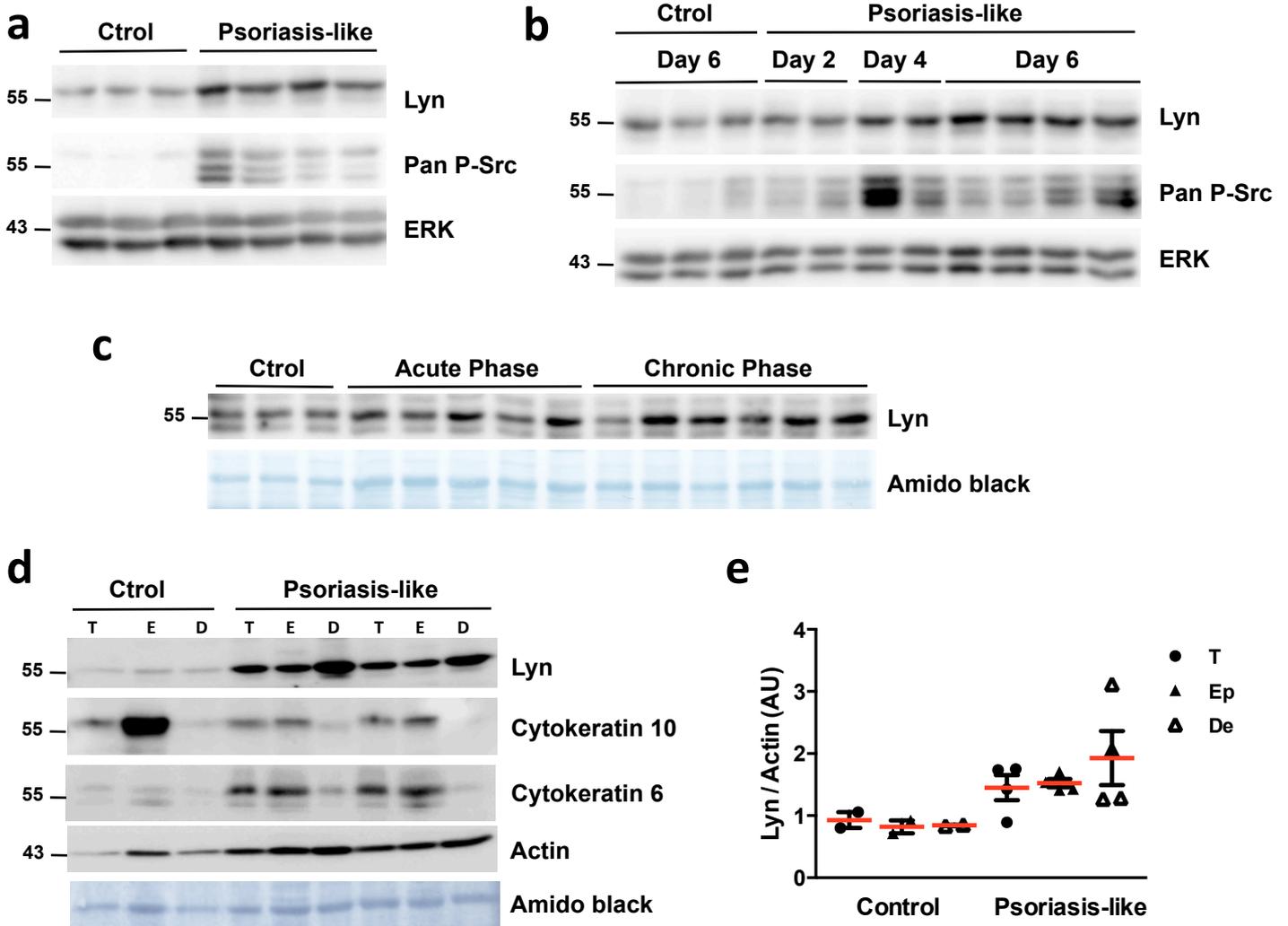
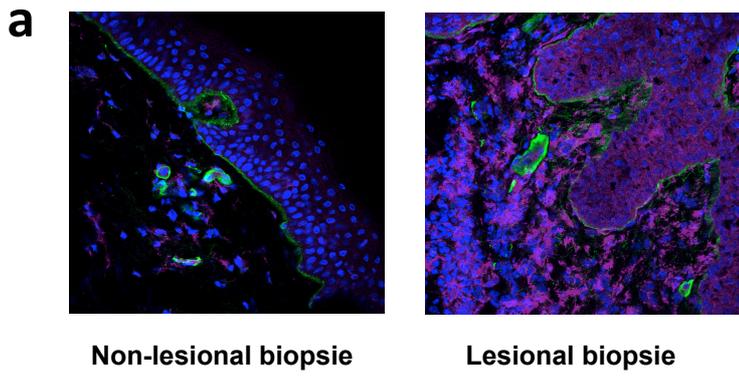


Figure 2, Aira-Lyn Pso



**b**

	HD	NL	L
	3/3	14/14	14/14
Lyn expression	+	+	++

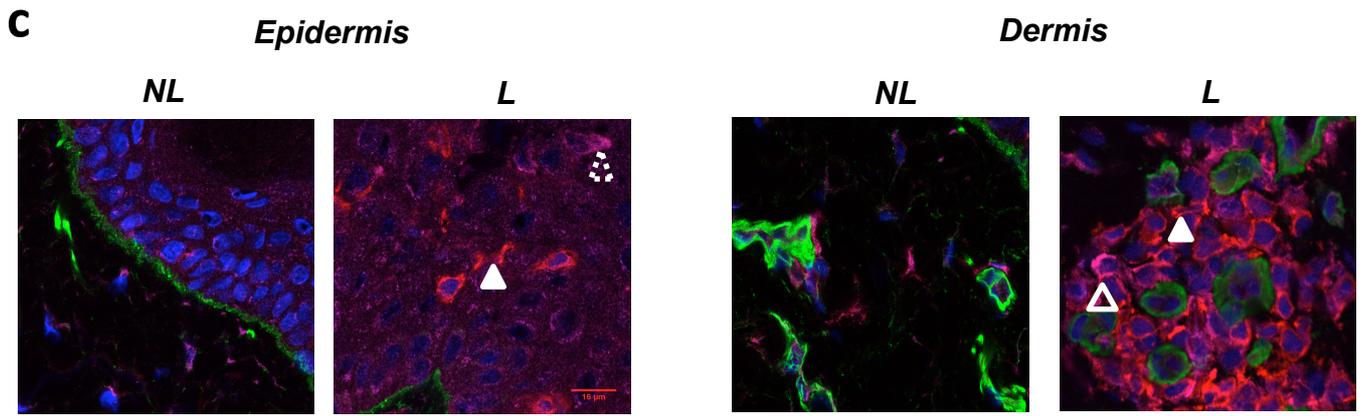


Figure 3, Aira-Lyn Pso

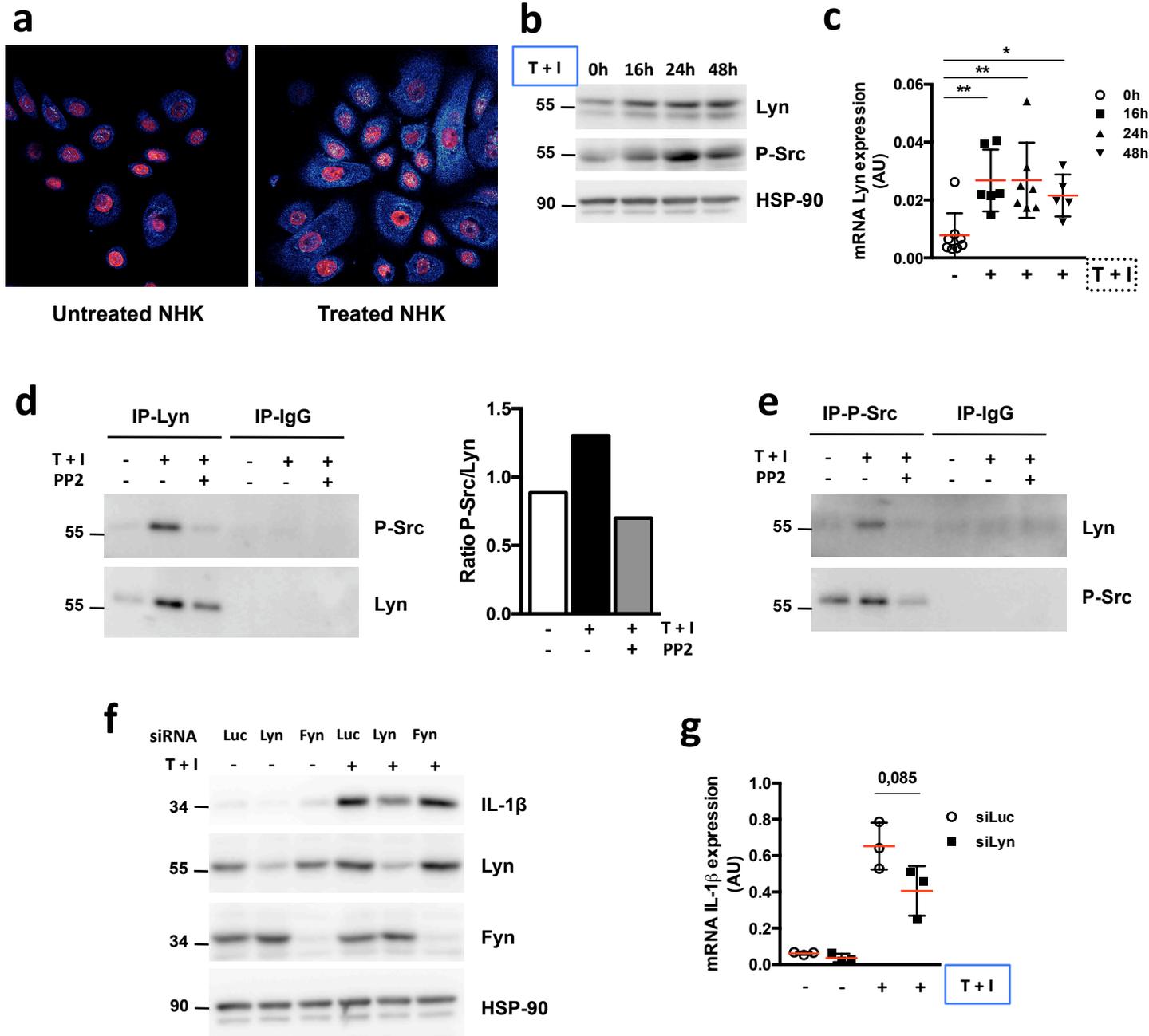


Figure 4, Aira-Lyn Pso

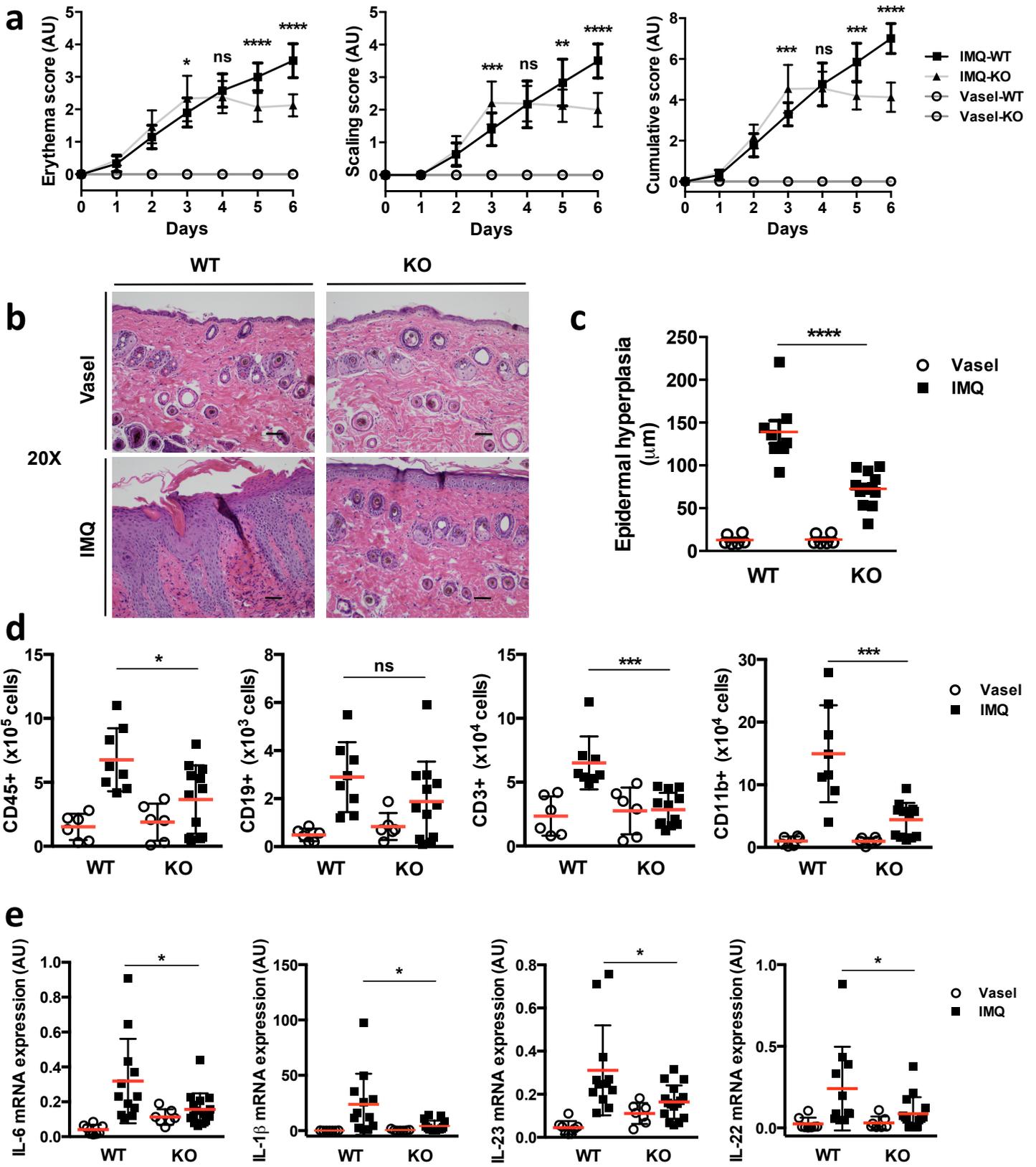


Figure 5, Aira-Lyn Pso

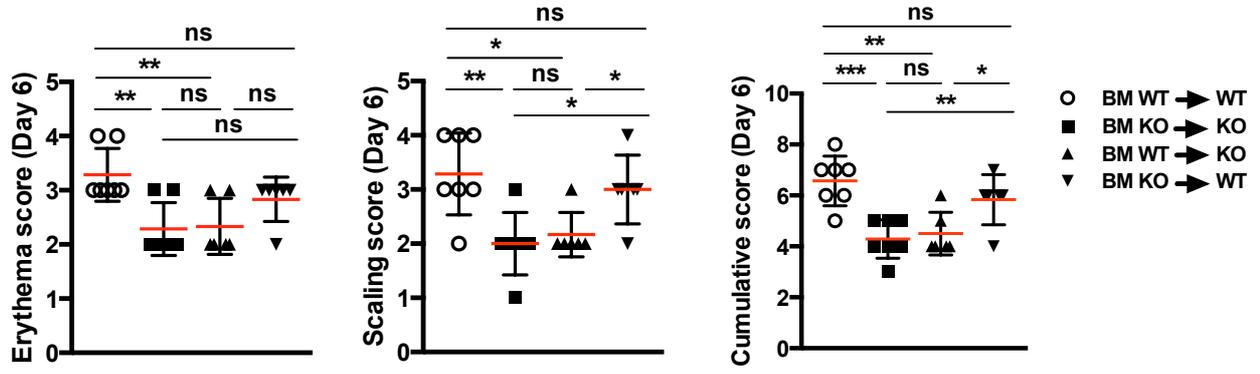


Figure 6, Aira-Lyn Pso

### Supplementary Figure 1:

**(a):** Western Blot analysis from skin biopsy total extracts of healthy donors (HD) as well as non-lesional (NL) and lesional (L) psoriasis patients **(b):** Densitometry quantification.

### Supplementary Figure 2:

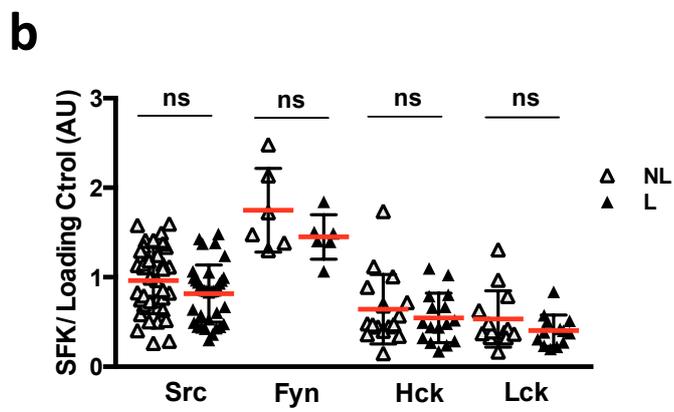
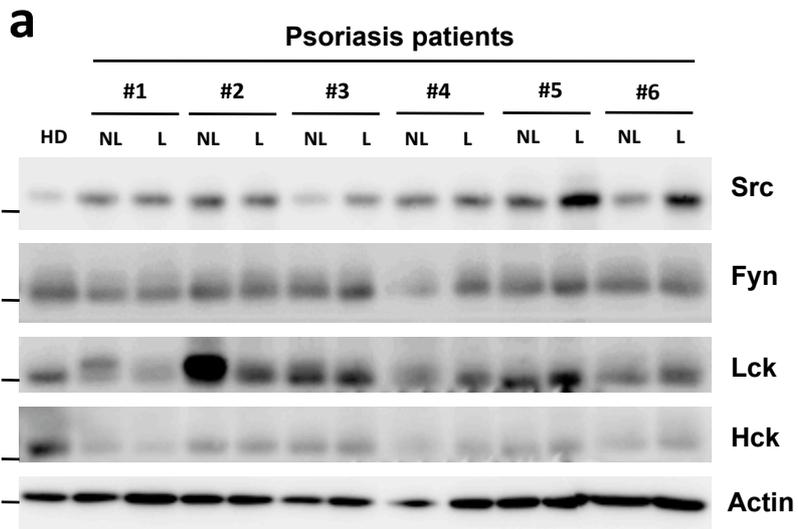
**(a, b):** WT were treated daily for 6 days with vehicle (Vaseline (Vasel)) or imiquimod (IMQ) cream. **(a):** A cumulative score (Erythema plus Scaling) was evaluated, **(b):** Skin was processed for total RNA extraction and gene expression was determined by qPCR (Vasel n=3, IMQ n=4); **(c, d):** WT were treated daily for 8 days with vehicle (phosphate buffered saline, PBS) or mouse recombinant protein IL-23. **(c):** Increase in ear thickness at the end of the study (PBS n=4, IL-23 n=8); **(d):** Ear skin was harvested and total protein was prepared for Western Blot analysis; **(e):** WT were treated daily for 6 days with Vasel or IMQ and a kinetic assay was made. Skin was harvested at different time points and then processed for total RNA extraction. Gene expression was determined by qPCR; **(f, g):** A biphasic model of psoriasis-like disease was obtained treating mice daily for 12 days with Vasel or IMQ. **(f):** A cumulative score (Erythema plus Scaling) was evaluated, **(g):** Skin was processed for total RNA extraction and gene expression was determined by qPCR (Vasel n=3, IMQ day 2 n=2, IMQ day 4 n=2, IMQ days 6 n=4); **(h):** A dermis/epidermis dissociation experiment was made and then processed for total RNA extraction. Lyn gene expression was determined by qPCR (Vasel n=6, IMQ n=12).

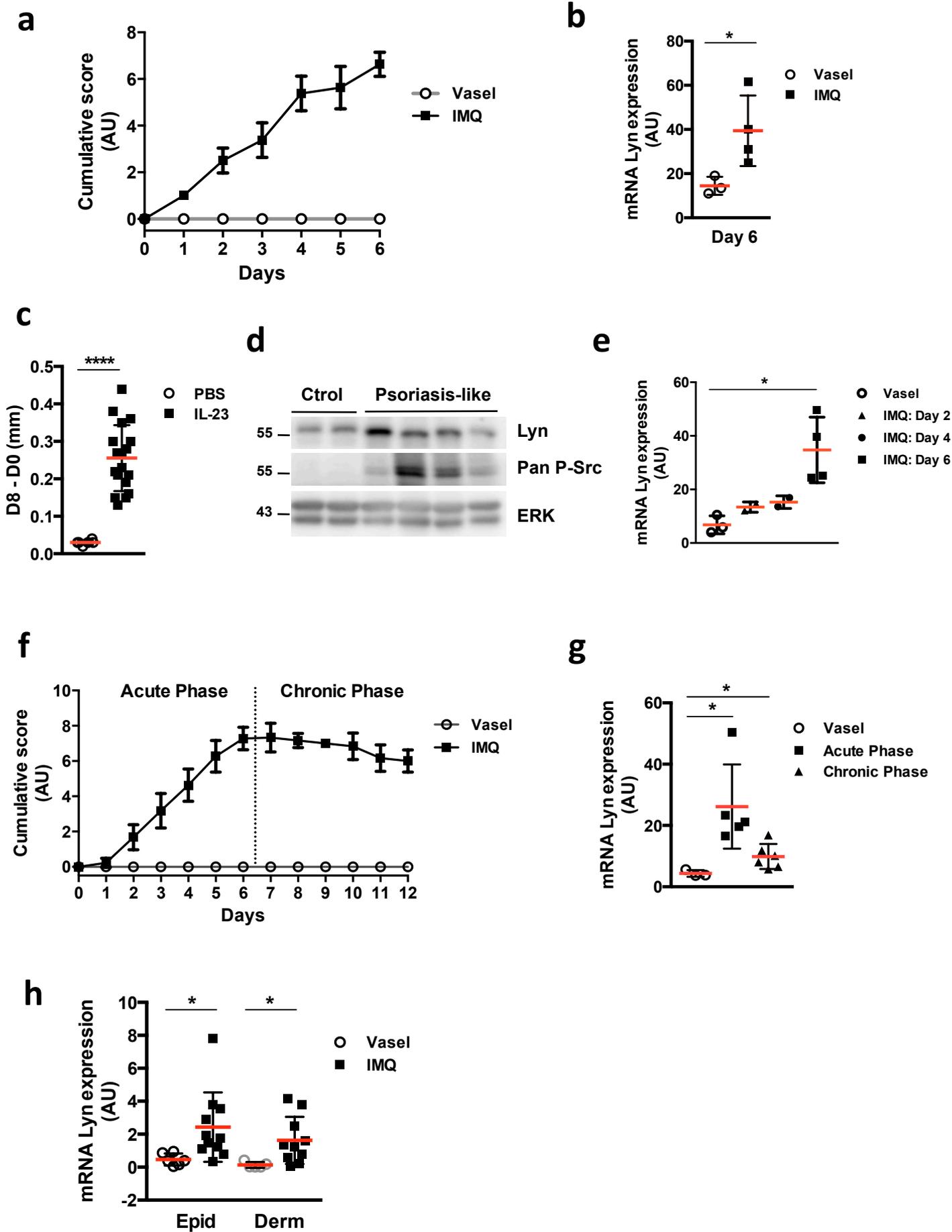
AU: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, \*  $p \leq 0.05$ , \*\*\*\*  $p \leq 0.0001$ .

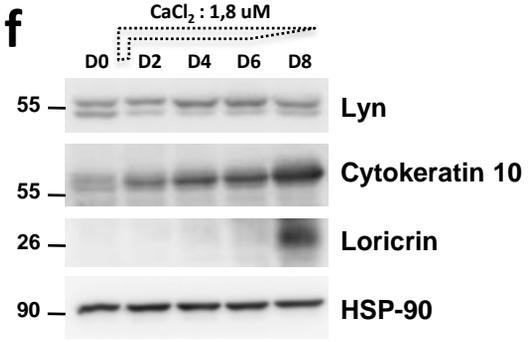
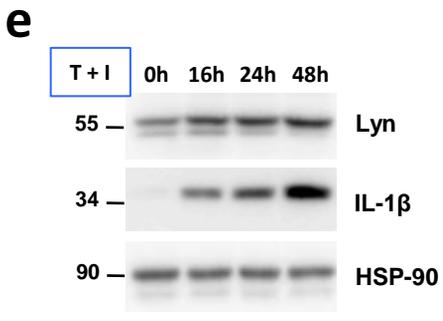
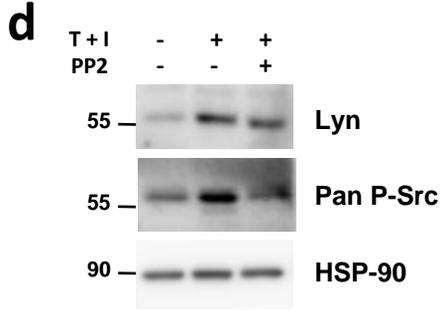
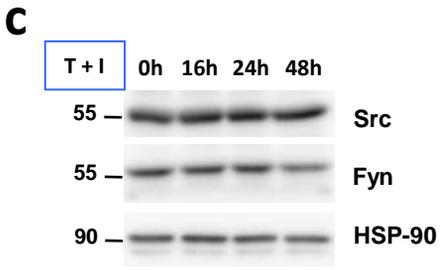
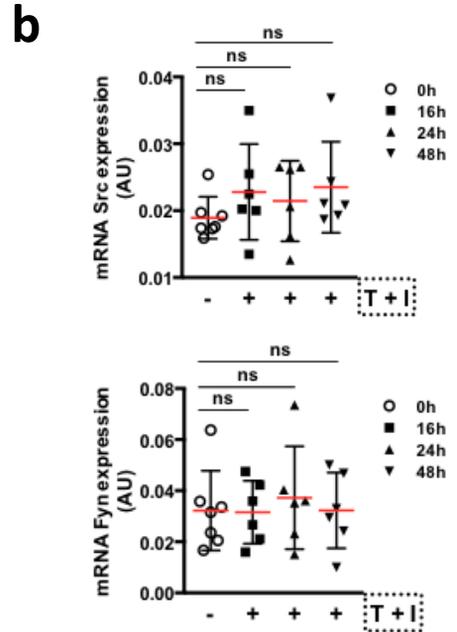
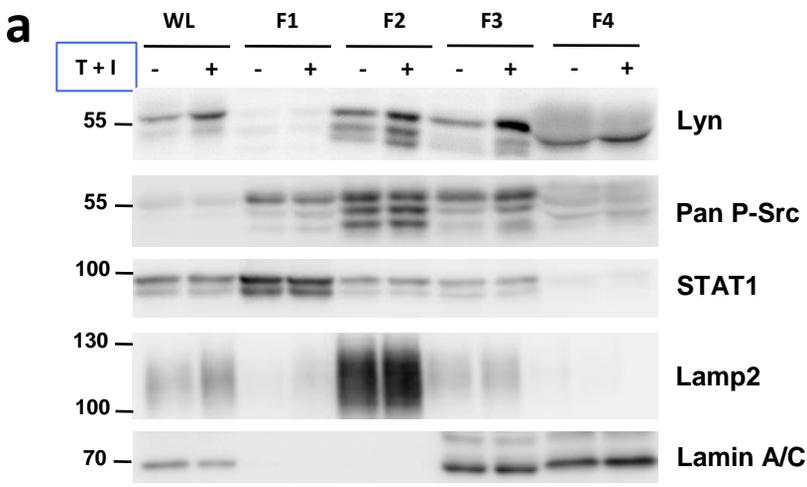
### Supplementary Figure 3:

**(a):** Primary human keratinocytes were stimulated with TNF- $\alpha$  (10 ng/ml) and IL-17A (200 ng/ml) for 24h and a fractionation experiment was made; **(b, c, e):** Keratinocytes were stimulated at different time points. **(b):** Total RNA isolation was made and gene expression was determined by qPCR, (n=5); **(c, e):** Cells were lysed and Western Blot analyses were performed, (n=5); **(d):** Input from Figure 4d, e; **(f):** Differentiation process was induced in normal human keratinocytes through culture in high concentration of calcium chloride. Then cells were lysed and Western Blot analyses were performed, (n=2).

AU: arbitrary units. Errors bars represent mean  $\pm$  SD. Student t test, ns: non-significant. T: TNF- $\alpha$  (10 ng/ml), I: IL-17A (200 ng/ml).









## **DISCUSSION AND PERSPECTIVES**



## Role of the Lyn tyrosine kinase in cell death

Mitochondria produce most of the cellular energy ATP that cells need for several reactions through OXPHOX pathway, thus their critical role in both health and disease has been hugely documented. In fact, mitochondrial dysfunction has been related to many types of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Ischemic stroke ([Ghasemi et al, 2018](#); [Akbar et al, 2016](#)), and its physiological functions include processes such as pyruvate conversion to other metabolites through the Krebs cycle, urea metabolism and steroid synthesis pathways as well as regulation of the membrane potential and the programmed cell death signaling called apoptosis ([Pickles et al, 2018](#)).

Two signaling pathways have been linked to apoptosis: the extrinsic and the intrinsic pathways. While the extrinsic pathway is produced by the stimulation of cell death receptors, the intrinsic pathway is mediated by extracellular and developmental stimulus leading to MOMP, release of apoptogenic proteins and caspase activation ([Kapoor et al, 2013](#)). All these processes are tightly regulated by the members of the Bcl-2 family, which are divided in anti-apoptotic and pro-apoptotic members ([Green and Llambi, 2015](#)); being the pro-apoptotic members, such as the BH-3 only protein Bim, implicated in the induction of MOMP via Bax/Bak activation once "stress signaling" are detected in cells ([Shukla et al, 2017](#)); leading to apoptosis. Therefore, an unbalance driving to an increase in the anti-apoptotic members or to a decrease in the pro-apoptotic factors is one of the main causes of deregulated mitochondrial apoptosis and has been related with the survival of different cancer cells ([Yip and Reed, 2008](#)), being considered evasion of apoptosis as a hallmark of cancer development ([Hanahan and Weinberg, 2011](#)). Indeed, downregulation of Bim has been linked to defects in apoptotic pathways driving in tumor growth and conferring resistance to several cancer drugs ([Chen et al, 2014](#)); which has proposed Bim as a targeted chemotherapy ([Shukla et al, 2017](#)).

It has been demonstrated that the overexpression and activity of the Lyn protein tyrosine kinase are associated to imatinib resistance in some CML cell lines and patients ([Kimura et al, 2005](#); [Dai et al, 2004](#); [Donato et al, 2003](#)). Interestingly, my thesis laboratory previously shown that cLyn had an anti-apoptotic role through IgM engagement to BCR in immature B cells ([Luciano et al, 2003](#)) and after imatinib treatment in CML cells ([Gamas et al, 2009](#)). Thus, the hypothesis of our work was that the apoptotic suppressor effects evidenced by cLyn can be explained by its interaction with the Bcl-2 pro-apoptotic member Bim. In fact, we demonstrated that Lyn protein tyrosine kinase is enabled to interact with BimE1 and BimL leading to an impairment of

the pro-apoptotic function of Bim. Indeed, we showed that this interaction seems specific for Lyn, taking in consideration that no interaction was evidenced with Fyn, another member of SFK for which anti-apoptotic effects have been also reported ([Ricci et al. 2001](#)). However, it is worth noting that SFK are composed of eight members, thus it would be interesting to elucidate whether other members of this family are able to interact with Bim or other BH-3 only proteins, leading to the same outcome.

Bim is one of the most potent inducers of apoptotic pathways directly activating Bax/Bak-mediated cytochrome c release and initiating the apoptotic cascade ([Doerflinger et al. 2015](#)), thus Bim expression and stability processes are strictly regulated ([Shukla et al. 2017](#)) to prevent cells to undergo apoptosis. One of these Bim regulatory mechanisms is the post-translational regulation by phosphorylation. In fact, it has been demonstrated that ERK1/2-mediated phosphorylation on Ser residues produces Bim ubiquitylation leading to its degradation by the proteasome, reducing the interaction with pro-survival or multi-domains pro-apoptotic members ([Wiggins et al. 2011](#); [Luciano et al. 2003](#)); thus, negatively regulating the pro-apoptotic function of Bim. Here, we reported for the first time in the literature Bim phosphorylation process on Tyr residues. Indeed, only Bim phosphorylation on Ser and Thr residues have been previously described, but in this study we demonstrated that Lyn can phosphorylate Bim on Tyr residues impairing its pro-apoptotic function. Two tyrosine residues were identified as target of cLyn in Bim protein and this phosphorylation process was abrogated either in the presence of an inactive form of cLyn or Lyn inhibitors; indicating that Bim tyrosine phosphorylation was a Lyn dependent process. However, for other BH3-only protein tyrosine phosphorylation has been already seen. In fact, PUMA can be tyrosine phosphorylated by HER2 in breast cancer cells leading to a destabilization expression of the protein ([Carpenter et al. 2013](#)), which could be explain the HER2-mediated growth and survival of these cancer cells, and a tyrosine phosphorylation process is needed in Bak inactivation to avoid apoptotic cascade ([Fox et al. 2010](#)), although the tyrosine kinase implicated in this event is still unknown. Therefore, it will be interesting to elucidate whether Lyn or other member of the SFK could be involved in Bak tyrosine phosphorylation process or on the tyrosine phosphorylation from other BH3-only proteins. In fact, it was already demonstrated that Src can inhibit apoptosis through indirectly induction of the BH-3 only Bik protein degradation ([Lopez et al. 2012](#)).

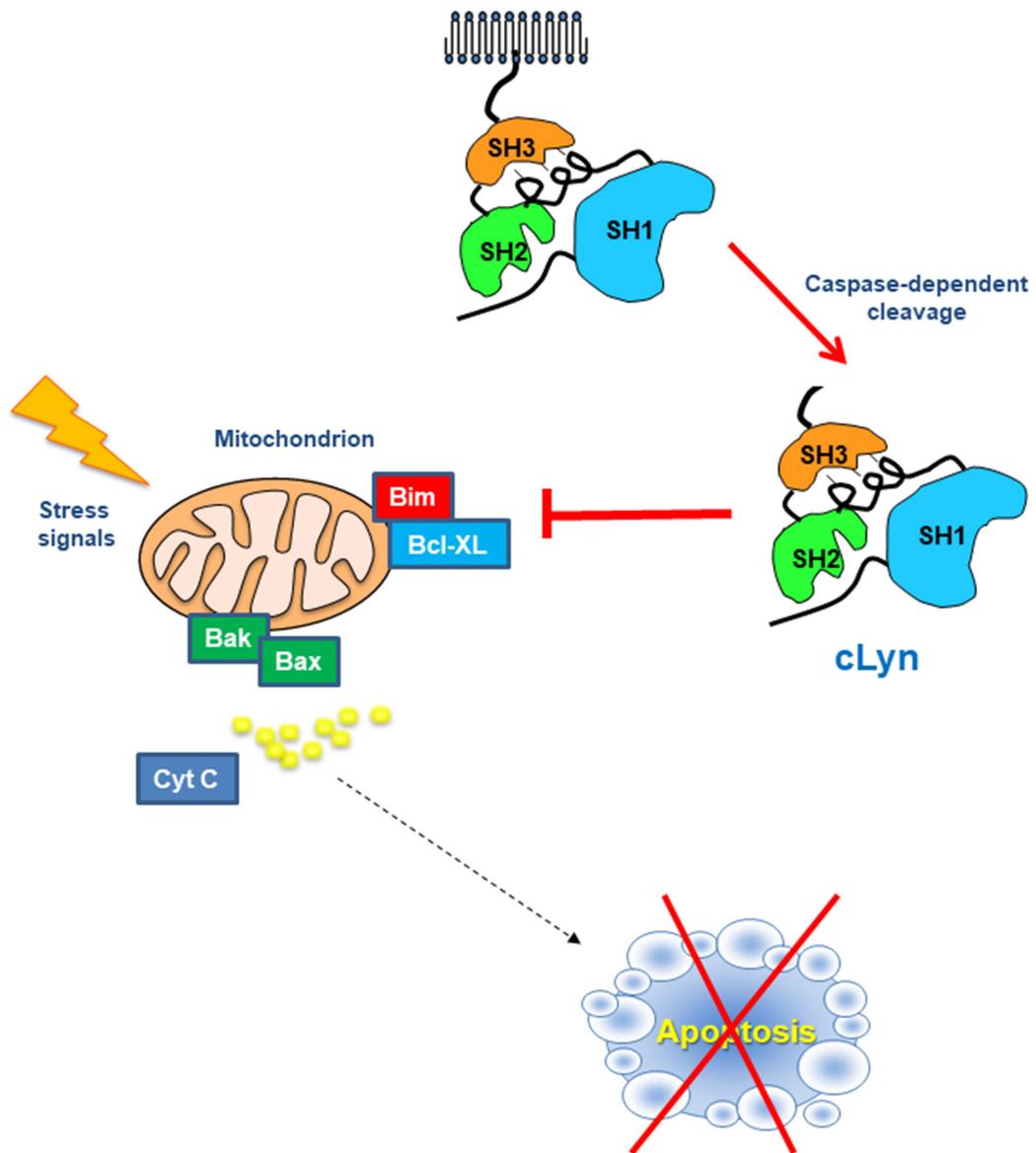
Lyn WT is able to phosphorylate BimEL inhibiting Bim pro-apoptotic function, but at much lower levels than cLyn. Moreover, a mutated full-length form of Lyn in its anchored-lipid regions has the same effect of cLyn inhibiting Bim pro-apoptotic function, which indicates that the cytosolic localization of Lyn has the key role acting as

suppressor of the apoptosis. However, the overexpression of a mutated full-length form of Src in its UD significantly reduces the cell invasiveness capacity compared to the overexpression of the WT form of this protein ([Arbesu et al. 2017](#)), demonstrating that in this case the WT form has a protective role, although it is noteworthy that the UD of each SFK is sensitive to specific input signals.

In our study we showed that Bim is more stable once it is phosphorylated by Lyn, avoiding apoptosis. However, quite of contrary to our results in B cells isolated from Lyn deficient mice an increase in Bim expression is observed ([Gross et al. 2011](#)), and stabilization of Bim expression has been related to an increase in its pro-apoptotic function ([Luciano et al. 2003](#)). Thus, this result indicates that phosphorylation status is the key point impairing pro-apoptotic function though, maybe, strong interaction with anti-apoptotic Bcl-2 family members such as Bcl-XL. Next, more experiments are needed to explore how tyrosine phosphorylation can stabilize Bim and also is other survival members of Bcl-2 family such as Mcl-1 or Bcl-2 can be involved in apoptosis protection.

Due to the strong capacity of Bim inducing cell death it is not possible to analyze the effect of its overexpression in mice (cells would undergo quickly to apoptosis and mice phenotype will be totally lethal) to then analyzed the interaction with cLyn in preventing apoptosis, reason for which a phosphomimetic construction was employed in our study. However, to understand the physiological role of this interaction in vivo, a mouse model is totally required. Thus, a knock-in mouse model of this protein is required, although taking in consideration that two Tyr residues were identified in our work, a double knock-in mouse model will be necessary to real mimic the double tyrosine phosphorylation in Bim protein by Lyn, preventing apoptosis. However, another experiment to confirm our results would be working with cellular models where Bim protein is not expressed and to analyze if after cell death induction cLyn overexpression in these cells could impair apoptosis, albeit a question would be present related whether the absence of Bim in these cells would allow a normal apoptosis process.

Altogether these results showed a new mechanism involved in the negative regulation of apoptosis by Lyn (**Figure 23**), evidencing an interaction between a member of the SFK and another one of the Bcl-2 family through direct tyrosine phosphorylation, indicating a new explanation related with chemoresistance, which could be an important target in the development of new treatment in liquid but also in solid cancers.



**Figure 23:** Recapitulative schema of the anti-apoptotic role of Lyn

Several stress signals activate Bim, which immediately bind to pro-survival members such as Bcl-2, Mcl-1, Bcl-XL leading to Bax/Bak oligomerization, MOMP and release of cytochrome C to initiate the cell death process by apoptosis. However, in these conditions Lyn tyrosine kinase can be cleaved by apoptotic caspases impairing the pro-apoptotic function of Bim and preventing apoptosis.

## Role of pro-inflammatory caspases in Psoriasis

Caspases are proteases involved in the propagation of signaling events leading to cell death either by apoptosis (apoptotic caspases) or pyroptosis (pro-inflammatory caspases). Specifically, pro-inflammatory caspases are implicated in the maturation and activation of IL-18 and IL-1 $\beta$  pro-inflammatory cytokines, leading to a lytic cell death and the recruitment of monocytes/macrophages to the inflammation site ([Ramirez and Salvesen, 2018](#)). However, it is noteworthy that cell death is not absolutely required for the caspase-mediated IL-1 $\beta$  and IL-18 activation and secretion ([Conos \*et al.\*, 2016](#)), although the mechanism through which these cytokines are released remains unknown. Two cellular pathways have been described for pro-inflammatory caspase activation. The first one, known as canonical pathway, works in the recognition of PAMP through TLR leading to the activation of caspase 1 in an inflammasome-dependent manner, while the second one, called non-canonical pathway, produce the activation of caspase 11 (caspase 4 and 5) and integrates cytoplasmic pathogen recognition in an inflammasome-independent manner ([Gonzalez Ramirez \*et al.\*, 2018](#)). Nevertheless, activation of inflammatory caspases has not been only related to infections. In fact, an important role of these proteases has been evidenced in sterile inflammation, showing a crucial function in intestinal inflammation ([Dupaul-Chicoine \*et al.\*, 2010](#)) and metabolic disorders ([Wen \*et al.\*, 2012](#)). Moreover, although some aspects about their role in skin inflammation and particularly psoriasis have been elucidated, their implication in this disease remains controversial. Indeed, activation of caspase 1 ([Marchetti \*et al.\*, 2009](#)) and caspase 5 expression ([Salskov-Iversen \*et al.\*, 2011](#)) have been reported in lesional psoriasis skin; but opposite results have been described in the literature regarding the impact of pro-inflammatory caspases in psoriasis pathogenesis.

In our work, we showed in three different models of psoriasis-like disease that the invalidation or the pharmacology inhibition of pro-inflammatory caspases leads to a significant reduction in the induced inflammatory skin phenotype, demonstrating that activation of pro-inflammatory caspases is involved in the development of psoriasis pathogenesis. However, similar and totally different results have been previously obtained.

First, a study where caspase 1 deficient mice (after demonstrated that were also deficient for caspase 11) were treated with IL-17 and IL-22 intradermal injections, a significant decrease in the induced psoriasis-like disease was achieved, outcome that was also observed when the same mice were treated with IMQ ([Cho \*et al.\*, 2012](#)), demonstrating that signaling through Th17 cell activation produces IL-1 $\beta$  secretion in a

NLRP-3-Caspase-1 activation pathway. However, a second study evidenced that although the deficiency of IL-1 $\alpha$ , IL-1 $\beta$  and IL-1R1 was enough to decrease the IMQ-induced psoriasis-like phenotype, the invalidation of caspase 1 did not produce the rescue of the phenotype ([Rabeony et al, 2015](#)). Nevertheless, it is worth noting that the fact that IL-1 $\alpha$  and IL-1 $\beta$  deficiency has a strong impact on psoriasis development indicates that IL-1 family plays an important role in this disease, suggesting an indirectly role of pro-inflammatory caspases taking in consideration that IL-1 $\beta$  maturation and secretion is dependent on these caspases, although it should be noted that other proteases present in the immune cell infiltration of psoriasis disease ([Clancy et al, 2017](#)) and caspase 8 ([Gurung and Kanneganti, 2015](#)) can cleave this cytokine. Moreover, here we must highlight that although a redundant role for IL-1 $\alpha$  and IL-1 $\beta$  in psoriasis development has been previously described ([Rabeony et al, 2015](#)), in our study only a significant decrease in IL-1 $\beta$  protein expression was appreciated, confirming the role of pro-inflammatory caspases in the development of the skin phenotype.

Furthermore, in another study with chronic proliferative dermatitis mutation (cpdm) mice; resembling atopic dermatitis and psoriasis diseases in humans, the crossing with caspase 1/11 deficient mice leads to a significant decrease in the skin pathology ([Douglas et al, 2015](#)). Near to the clinical endpoint to sacrifice cpdm mice, in cpdm crossed with caspase 1/11 deficient mice, no lesions and no epidermal hyperplasia were observed, demonstrating the main role of pro-inflammatory caspases in the induction of dermatitis in these mice and the function of inflammasome activation as an initiating signal in cpdm mice. In general, in cpdm mice crossed with pro-inflammatory caspase deficient mice, a delay in the appearance of dermatitis was seen, which is congruent with the delay observed in our Lyn $\Delta$ N dKO mice, showing the improvement in psoriasis-like phenotype through the invalidation of pro-inflammatory caspases. It is worth to note that while genetic ablation of TNFR1 from cpdm mice produced a complete rescue of the phenotype, the generation of cpdm IL-1R1 deficient mice showed a delay in the inflammation symptoms ([Rickard et al, 2014](#)), demonstrating that while signaling through TNFR1 is one of the principal pathway to induce skin inflammation, signaling through IL1R is key to maintain and exacerbate the disease. Previously, our group established that crossing of Lyn $\Delta$ N mice with TNFR1 deficient mice completely rescue the psoriasis-like phenotype ([Marchetti et al, 2009](#)), thus the delay observed in the onset of the inflammatory phenotype in Lyn $\Delta$ N dKO mice and in the IMQ and IL-23 treated dKO mice in our study prompted us to hypothesize that the invalidation of pro-inflammatory caspases leads to a decrease in IL-18 and IL-1 $\beta$  maturation and secretion. Therefore, the signaling through IL1R1 is

reduced in the psoriasis-like phenotype, leading to a significant diminution in general inflammation. Indeed, this hypothesis is reinforced by the fact that IL-1R enhances the initiated IMQ-induced chemokine expression, being a crucial factor in the recruitment of IL-17-producing  $\gamma\delta$  T cells ([Cai et al., 2011](#)), and in IL1R1 deficient mice this response is totally abolished ([Alvarez and Jensen, 2016](#); [Uribe-Herranz et al., 2013](#)). Moreover, in IL1R deficient mice and in caspase 1 and caspase 11 deficient mice, a significant decrease in the numbers of tumors and delayed tumor incidence after the induction of epithelial skin carcinogenesis was observed compared to WT mice, demonstrating that tumor formation and general inflammation were associated to IL-1 signaling ([Drexler et al., 2012](#)).

It has been evidenced that type I IFN production is a central point in the negative regulation of caspase-1 activation and IL-1 $\beta$  secretion ([Guarda et al., 2011](#)), leading to a decrease in NLRP-3 inflammasome activation and to an increase in IL-10 production via STAT1. In our study, we checked neither type I IFN production nor IL-10 secretion, but this could indirectly explain the reduced inflammation phenotype observed in caspase 1/11 deficient mice. In fact, in DNA virus infection models with caspase 1 deficient macrophages, an increase in type I IFN has been found, showing that deficiency in inflammasome components resulted in a strong protection against DNA virus infection ([Wang et al., 2017c](#)). Additionally, our levels of psoriasis-like phenotype inhibition in the absence of pro-inflammatory caspases are consistent with other studies where other important components for psoriasis disease development, like T cells, are deleted ([van der Fits et al., 2009](#)).

Caspase 1 is activated and processed through inflammasome complex such as NLRP-3 inflammasome. However, it has been demonstrated that deficiency in NLRP3 inflammasome has no impact in the rescue of the IMQ-induced psoriasis-like phenotype ([Rabeony et al., 2015](#)), which could be explained either by the fact that other inflammasome such as NLRP-1, NLRP-6 and AIM-2 could be involved in the activation of caspases 1 or by the role of caspase 11 in this disease taking in consideration that this specific caspase does not require inflammasome-dependent activation. In our study we showed that NLRP-3, NLRP-1 and AIM-2 inflammasomes are significantly reduced and although all of them are upstream caspase activation, an explanation for that might be the significant decrease obtained in general inflammation from dKO mice compared to WT mice. Moreover, it will be interesting to determine the specific role of each pro-inflammatory caspase in psoriasis development through induction of psoriasis-like phenotype either in caspase 1 deficient mice or caspase 11 deficient mice.

Although it was previously demonstrated that cleaved-form of caspase-5 is present in lesional skin biopsies from one psoriasis patient ([Zwicker et al, 2017](#)), here we confirmed with a huge amount of lesional and non-lesional biopsies from psoriasis patients that cleaved forms of caspase-1, 4 and 5 are found in all lesional samples. Moreover, cleaved forms of proinflammatory caspases were associated with an increase in the NLRP-3 and AIM-2 inflammasomes, found in the same assessed patients. In fact, AIM-2 inflammasome has been observed in psoriasis keratinocytes related to an increase in cytosolic DNA leading to an AIM-2-dependent IL-1 $\beta$  production ([Dombrowski et al, 2011](#)).

Psoriasis is a chronic inflammatory immune-mediated skin disease with an unpredictable course ([van de Kerkhof, 2018](#)), which affects around 2-3% of worldwide population ([Danielsen et al, 2013](#)). At present, despite all therapies developed to cure this disease, no treatment is effective to guarantee the absolute remission of psoriasis plaques, but rather the palliative treatment of the main clinical symptoms of the disease has been achieved. And although the biological drugs have represented a new target more specific for the cure of the disease ([Kim et al, 2012](#)), various adverse events have occurred in the treated patients and no long-term treatments have been established. Caspase inhibitors have been evaluated in clinical trials, specifically with the small molecule inhibitors VX-740 and VX-765, also known as Pralnacasan and Belnacasan, respectively; which are potent and selective inhibitors of caspase 1 and caspase 4 (Vertex Pharmaceuticals). Psoriasis patients were treated with Belnacasan, which progressed into a phase II clinical trial (<http://clinicaltrial.gov> Identifier: NCT00205465) and although in mouse models of skin inflammation the compound showed a significant reduction in disease severity and in IL-1 $\beta$  and IL-18 secretion ([Wannamaker et al, 2007](#)), in treated patients the objective response was not achieved; which could be explained because this compound did not affect the secretion of IL-6, IL-8 and TNF- $\alpha$ . In our study, we co-treated mice with IMQ and the selective caspase-1 inhibitor AC-YVAD-CMK and a significant decrease in the erythema, scaling and pro-inflammatory cytokine production was found, reinforcing the idea that pharmacological inhibition of pro-inflammatory caspases could be a potential target to treat psoriasis disease. Indeed in a model of acute gastric injury, mice were pre-treated with AC-YVAD-CMK and an inhibition in the production of IL-1 $\beta$  was observed leading to the protection of these mice, which was correlated with the impairment of NLRP-3 inflammasome activity ([Zhang et al, 2016a](#)). Moreover, like in our AC-YVAD-CMK treated mice, Zhang et al found a strong diminution in other pro-inflammatory cytokines like IL-6, IL-8 and TNF- $\alpha$ . Therefore, it would be interesting to analyze the therapeutical effect of AC-YVAD-CMK compound. In fact, in our study we administered this drug concomitantly

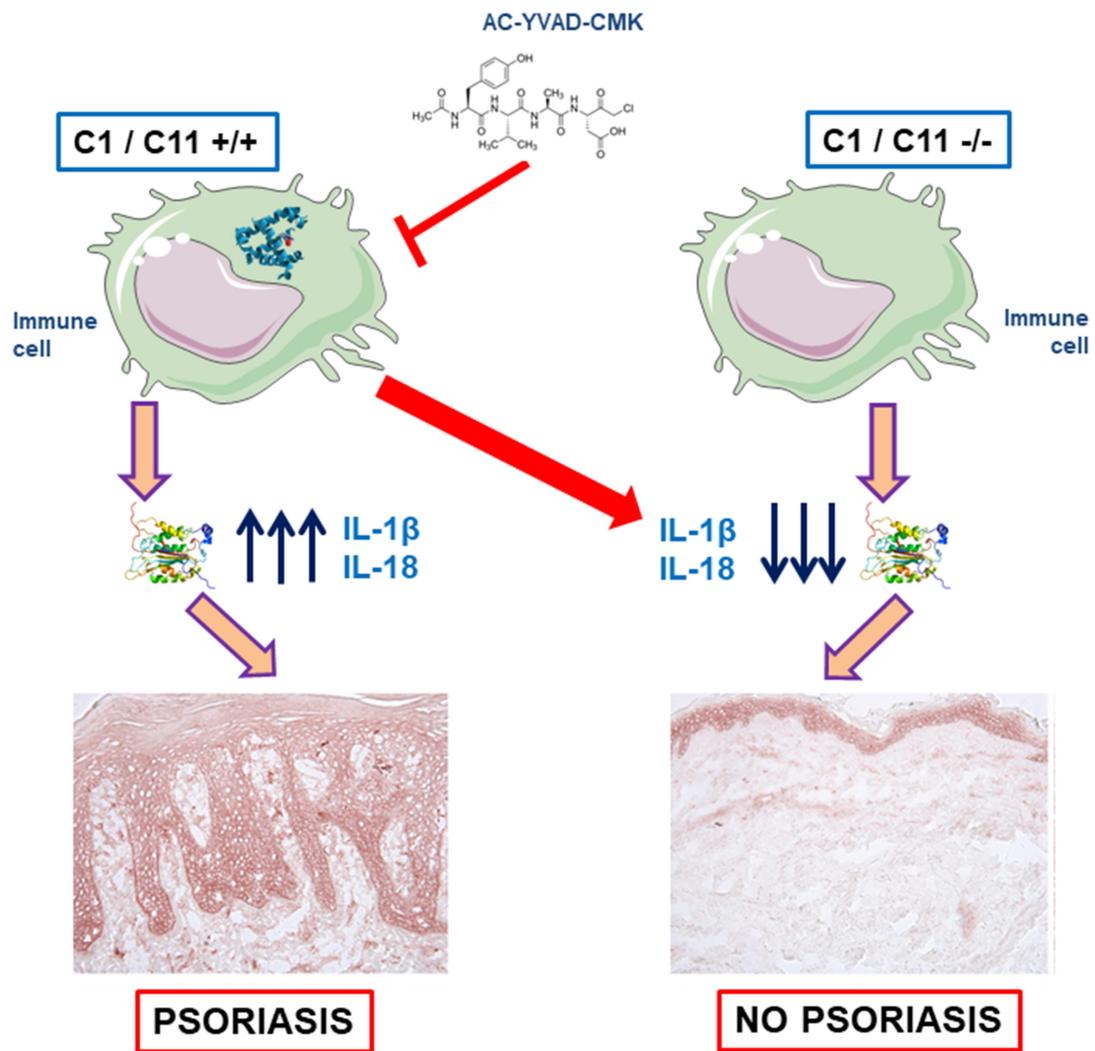
with IMQ, but future experiments should be planned to decipher the role of this drug once psoriasis plaques have been developed and also to test this compound not only by systemic administration, but through subcutaneous injections and topical application. Furthermore, actually not only caspase inhibitors are evaluated as potential drugs to treat psoriasis disease, but IL-1, IL-18 and inflammasome inhibition could be possible targets to treat inflammatory skin diseases ([Fenini et al, 2017](#)). In fact, psoriasis patients were treated in an open-label clinical trial with the fully human monoclonal antibody against IL-1 $\alpha$ , MABp1, and an encouraging clinical response was achieved that must be improved with an increase in dose/frequency ([Coleman et al, 2015](#)). Additionally, another compound that inhibits NF- $\kappa$ B pathway, leading to a significant decrease in the NLRP-3 inflammasome pathway, was able to decrease IMQ-induced psoriasis-like phenotype ([Irrera et al, 2017](#)) and a significant reduction in the secretion of several pro-inflammatory cytokines was observed.

In our work, we showed that keratinocytes are primed to secrete IL-1 $\beta$  once inflammasomes are activated into these cells. Moreover, we showed that an activation of pro-inflammatory caspases is observed after stimulation with two of the principal pro-inflammatory cytokines involved in psoriasis pathogenesis. Recently, it has been demonstrated that the stimulation of keratinocytes only with TNF- $\alpha$  or with IL-17A did not produce caspase activation ([Zwicker et al, 2017](#)), but it is necessary the combination of at least two pro-inflammatory cytokines ([Cho et al, 2012](#)). We stimulated keratinocytes with a combined treatment of TNF- $\alpha$  and IL-17, but future approaches are required to analyze the activation of pro-inflammatory caspases in presence of several pro-inflammatory cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-17 and IL-22), which would be closely related to what happens in the site of the disease, where keratinocytes are in direct contact with a huge amount of pro-inflammatory cytokines secreted from the recruited immune cells and from themselves.

Our data showing that human keratinocytes are primed to secrete IL-1 $\beta$  through the activation of inflammasome and caspase 5, reinforcing the idea that keratinocytes are immunological active cells. Therefore, we decided to analyze whether the impact of pro-inflammatory caspases in the development of psoriasis-like disease observed in our study was more dependent on their activation either in keratinocytes or in the recruited immune cells. We demonstrated that activation of pro-inflammatory caspases in immune cells is sufficient to induce a fully inflammatory response, because of a significant reduction in psoriasis-like phenotype was observed when pro-inflammatory caspases were not present in these cells, phenotype that is quite similar to that obtained in mice either when T cells are not present through the induction of psoriasis-like disease in Rag deficient mice ([Hedrick et al, 2009](#)) or when mice are pre-treated

with anti-CD3 depleting antibodies ([van der Fits et al, 2009](#)). Thus, next it will be captivating determine in which specific immune cell population from the IMQ model caspase activation is produced. However, although no impact of caspase invalidation in keratinocytes was associated to an improvement in psoriasis development, it is worth noting that it was recently demonstrated that expression of inflammasome proteins in murine keratinocytes is very low or even undetectable ([Sand et al, 2018](#)), suggesting that immune functions in murine psoriasis-like phenotype is carried out by professional immune cells.

Altogether, our results confirm that pro-inflammatory caspases are involved in psoriasis pathogenesis and their absence or inhibition leads to a delay in psoriasis development (**Figure 24**), reinforcing the idea that control of pro-inflammatory caspase activation and IL-1 $\beta$  maturation and secretion is essential for the improvement of chronic inflammatory disease. Nevertheless, it will be interesting to determine the implication of pro-inflammatory caspases in other immune-mediated skin pathologies.



**Figure 24:** Recapitulative schema of the role of pro-inflammatory caspases in psoriasis

Activation of pro-inflammatory caspases in immune cells is sufficient to induce a psoriasis-like phenotype. However, the invalidation or the pharmacological inhibition of these pro-inflammatory caspases leads to a reduced psoriasis-like disease.

## Role of Lyn tyrosine kinase in Psoriasis development

Lyn protein tyrosine kinase has been well characterized in the context of B lymphocyte function regulation and myeloid cell homeostasis ([Scapini et al, 2009](#); [Xu et al, 2005](#)), presenting a positive and negative role through the activation or inhibition by phosphorylation of down-stream pathways. In fact, deregulation in Lyn expression and activity has been linked to several hematological malignancies, including AML, CML, B-ALL and B-CLL ([Ingley, 2012](#)). However, published studies in the last years have demonstrated that this tyrosine kinase can also have a pivotal function in other non-hematopoietic tissues, as the oncogenic role that has been evidenced in several solid cancers such as prostate, basal breast, colon cancer and glioblastoma, among others ([Ingley, 2012](#)). Thus, several are the signaling pathways regulated by Lyn after receptors or integrin-initiated networks, which has led to address the role of this tyrosine kinase both in homeostasis and in conditions of malignancy.

Notwithstanding, Lyn deregulation has not only been related with cancer development but also with autoimmune diseases. In fact, Lyn functions have been described in allergy and asthma ([Wang et al, 2017b](#)). The control of innate immune signaling by Lyn along the TLR-MyD88 pathways in dendritic cells and B cells has been linked to inhibition of autoimmunity ([Ban et al, 2016](#); [Lamagna et al, 2014](#)) and Lyn deficient mice develop plasma cell accumulation, B cell hyperactivation, myeloproliferation and glomerulonephritis, leading to an autoimmune syndrome resembling human SLE ([Gutierrez et al, 2010](#)). Moreover, an increase in the activation of the SFK has been observed in lesional skin biopsies from psoriasis patients ([Ayli et al, 2008](#)), and an augmentation in Lyn mRNA expression has been observed in lesional skin from psoriasis patients (GEO Data). Indeed, the overexpression of cLyn in mice leads to the development of a psoriasis-like phenotype ([Marchetti et al, 2009](#)), associated to an epidermal hyperplasia, increase in the immune cell infiltration and expression of several pro-inflammatory cytokines, indicating an important role of Lyn tyrosine kinase in psoriasis development.

In the third part of my thesis work, we demonstrated that Lyn could have a crucial role in psoriasis development. An increase in Lyn mRNA and protein expression as well as in its activity was shown in lesional human skin biopsies and in psoriasis-like treated mouse skin compared to non-lesional human samples and control mouse skin, respectively. It is worth noting that although an activation of SFK has been observed in lesional skin ([Ayli et al, 2008](#)), in our study no modulation in other SFK members such as the Src-related Src and Fyn and the Lyn-related Lck and Hck was appreciated in psoriasis skin biopsies, indicating that Lyn could be the only SFK related with psoriasis

pathogenesis. However, to confirm this hypothesis some experiments are needed to determine the expression of Yes, Fgr and Blk; SFK members that were not analyzed in this study and of which is known that they are expressed in epithelial cells. In fact, Yes expression has been observed in melanocytes and it constitutes a pivotal factor in enhancing malignant properties of melanoma cells ([Hamamura et al, 2011](#)). Moreover, the anticancer property of Bakuchiol, a natural drug used for the treatment of skin cancer, is by targeting Blk in the EGF-induced skin carcinogenesis ([Kim et al, 2016b](#)).

Surprisingly, our study elucidated that the increase in Lyn protein expression was specific of psoriasis because no Lyn modulation was observed in atopic dermatitis patients, a disease that although the molecular mechanism of pathogenicity is different from psoriasis; the patient clinical pictures are quite similar ([Gavrilova, 2018](#)).

It is noteworthy that although the basis of the project was that the overexpression of cLyn in mice leads to the development of a psoriasis-like phenotype ([Marchetti et al, 2009](#)), in our study no caspase-cleavage of Lyn was found either in both models of psoriasis-like phenotype (IMQ and IL-23) or in human skin biopsies. In fact, Lyn deficient mice ([Gutierrez et al, 2010](#)) or mice with a sustained activation of Lyn ([Hibbs et al, 2002](#)) are both involved with autoimmune disease development, but no defect in the skin have been previously reported. Notwithstanding, the cleaved-form of Lyn can be present in our study, but it could be quickly degraded or to be present in small proportions, that are not detected by our WB conditions, but with an important function in the disease. The cleavage of Lyn has been shown in presence of pro-apoptotic caspases, which, with the exception of caspase 7 ([Marchetti et al, 2009](#)), are not activated in lesional psoriasis skin (data not shown). However, pro-inflammatory caspases are increased and activated in lesional skin biopsies, but Lyn is not a substrate of this kind of caspases. Nevertheless, other proteases such as calpains ([Hossain et al, 2013](#)) have been demonstrated that can cleave SFK in their N-terminal UD, which could justify Lyn cleavage in inflammatory conditions. Nonetheless, lipid binding regions different from those implicated in myristoylation and palmitoylation processes have been found in SFK ([Perez et al, 2013](#)), being implicated in the regulation of these proteins. Indeed, lipid binding to the unique lipid binding regions (ULBR) in Src protein modifies the protein conferring access to new substrates without to be detached from the plasma membrane. Thus, one hypothesis to explain the role of the WT form of Lyn in psoriasis could be though lipid modifications in the ULBR of the protein that allows interact with new substrates in inflammatory conditions. Moreover, immunofluorescence approaches in psoriasis patients suggest an intracellular localization of Lyn in the epidermis, thus another hypothesis could be that the WT form of Lyn is delocalized in inflammatory conditions producing a different regulation

process and interacting with different substrates, which would indicate that the importance of Lyn in psoriasis is given by its cytosolic localization.

Lyn mRNA and protein expression were not only found increased in the induced psoriasis-like disease but also in stable phase of this phenotype, indicating that Lyn could be required for the maintaining of psoriasis pathogenesis, process that is mediated by an intense dialogue between the immune cell infiltration and keratinocytes through the release of pro-inflammatory cytokines ([Ayala-Fontanez et al, 2016](#)). In fact, our mouse dermis/epidermis experiments and our immunofluorescence approaches with patient biopsies demonstrated that Lyn is increased in both compartments of the skin, indicating a regulation of its expression in keratinocytes and a huge number of infiltrating Lyn positive immune cells. However, it will be interesting to know in what specific immune cell population Lyn expression would be increased and to confirm its modulation in keratinocytes. To do that, co-staining experiments analyzing Lyn expression alongside keratinocytes, fibroblasts and immune cells markers would be done by FACS after IMQ-induced psoriasis-like phenotype in mice or from fresh lesional and non-lesional biopsies obtained from psoriasis patients. Among the different immune cell population special emphasis should be made on macrophages, neutrophils and dendritic cells, which are the most important Lyn positive immune cells found in psoriasis skin.

To go deeper in details about the Lyn modulation in keratinocytes we next performed experiments with normal human keratinocytes, which were stimulated with TNF- $\alpha$  and IL-17A, two of the principal cytokines implicated in psoriasis pathogenesis ([Masalha et al, 2018](#)). A significant increase in Lyn mRNA and protein expression was seen in these cells confirming previous results observed with total skin extracts obtained from psoriasis patients and after psoriasis-like induction in mice. No expression of Lck and Hck was detected in these cells (data not shown), but no modulation in Src and Fyn was not observed either. However, it was possible determine by immunoprecipitations experiments an increase in Lyn activation, although to confirm that Lyn is the only SFK activated on stimulated keratinocytes in mimicking psoriasis conditions, other experiments to assess Src and Fyn activation are required. Further, it would be important to determine Lyn modulation in keratinocytes stimulated with several cytokines at the same time such as IFN- $\alpha$ , IFN- $\gamma$ , IL-22 in addition to TNF- $\alpha$  and IL-17A, with the goal to be in conditions more similar to those of the real disease and perhaps a more important modulation in Lyn expression and activation could be found. Moreover, Lyn modulation can be evaluated in co-culture experiments between activated T-cells or dendritic cells with keratinocytes to ensure that pro-inflammatory cytokines are going to be released by the activated cells.

Several cytokines such as IL-6, IL-8 and IL-1 $\beta$  are secreted by stimulated keratinocytes ([Harden et al, 2015](#)). Thus, next we wanted to know whether the modulation of Lyn in stimulated keratinocytes could be related with a modulation in the expression of these cytokines. Strikingly, the increase in Lyn expression at different time points of stimulation was associated to an increase in IL-1 $\beta$  expression and, surprisingly, Lyn silencing led to a marked decrease in IL-1 $\beta$  levels that was not observed when other SFK, like Fyn, was silenced, suggesting that the positive role in psoriasis disease could be linked to enhance pro-inflammatory cytokine release by keratinocytes. However, more experiments are required to assess the effect of Lyn on the rest of keratinocyte-secreted cytokines. Moreover, a more generalized study analyzing several cytokines at the same time through the Cytokine Array Method would be performed with supernatant obtained from cytokine-stimulated normal human keratinocytes in the presence or absence of Lyn tyrosine kinase.

It has been demonstrated that IL-1 $\beta$  leading to the translocation of p65 subunit into the nucleus driving in the activation of NF- $\kappa$ B signaling pathway ([Wuertz et al, 2011](#)). In fact, it is well known that NF- $\kappa$ B pathway is implicated in the regulation of IL-1 $\beta$  production and moreover, it has been reported an activation of this pathway in lesional biopsies from psoriasis patients ([Goldminz et al, 2013](#)). Nevertheless, cLyn mice have an inhibition in the NF- $\kappa$ B, but increased levels of IL-1 $\beta$  are observed ([Marchetti et al, 2009](#)). Therefore, it would be interesting to confirm the effect of Lyn modulation in NF- $\kappa$ B pathway to elucidate whether an impact of Lyn on NF- $\kappa$ B pathway could explain the direct relationship between the silencing of Lyn and the significant decrease in IL-1 $\beta$  pro-inflammatory cytokine levels, taking in consideration that it was also described that NF- $\kappa$ B might have a negative regulator role on IL-1 $\beta$  secretion ([Greten et al, 2007](#)).

Once we demonstrated that Lyn was modulated in psoriasis skin, we wanted to know if Lyn was implicated in the development and pathogenesis of this skin pathology. Strikingly, the IMQ-induced psoriasis-like phenotype was impaired in Lyn deficient mice, indicating a pivotal role of this tyrosine kinase in the pathogenicity of psoriasis that was associated with a significant decrease in the recruitment of myeloid cells and the pro-inflammatory cytokine production compared to WT mice. It is worth noting that although Lyn deficient and WT mice developed a similar phenotype in the first days of IMQ application, after the day three the psoriasis-like phenotype was delayed in Lyn deficient mice, which is the time point were Lyn expression and activity begin to be increased in the induced psoriasis-like disease. Next, our preliminary results of adoptive transfer experiments allowed us to determine that the absence of Lyn in keratinocytes and not in the recruited immune cells seems to be essential in the

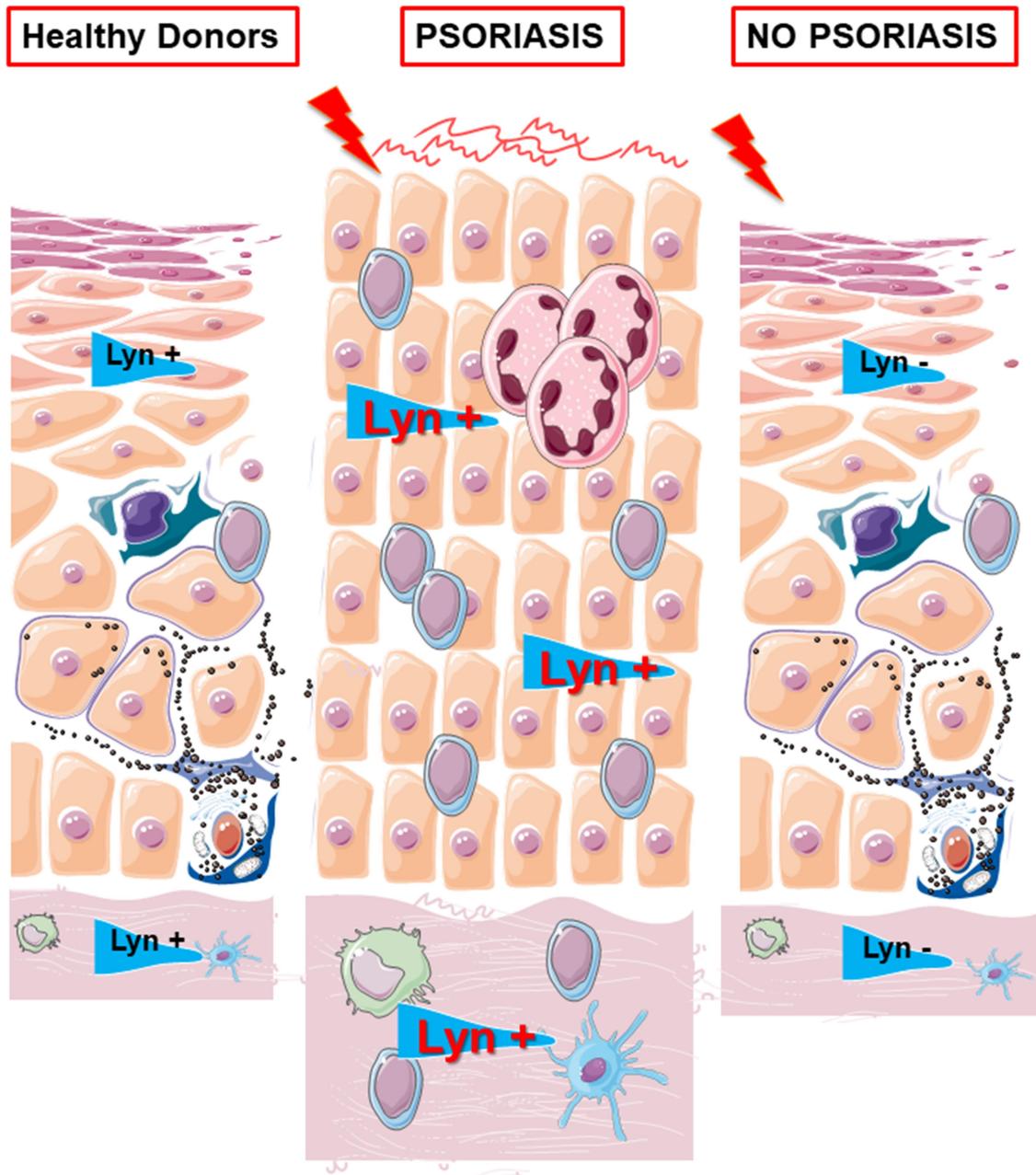
impairment of psoriasis-like phenotype, although more experiments are needed to confirm this outcome. One hypothesis to explain this could be that Lyn is implicated in the secretome of activated keratinocytes leading to a positive feedback loop, thus Lyn deficiency impairs the secretion pattern decreasing the feedback loop and the continuous proliferation of keratinocytes. Further, to validate this hypothesis a conditional knock-in K14 mouse model (Lyn deficiency only in keratinocytes) might be developed.

Albeit our preliminary results indicate that Lyn deficiency in immune cells is not involved in psoriasis phenotype development, it would be interesting to develop different mouse models to determine the impact of Lyn deficiency in psoriasis development only in B cells, in macrophages, in dendritic cells or in granulocytes; which are the most important Lyn positive immune cells recruited to the skin. For example, quite the contrary to our results, it has been reported that CD19 deficient mice develop a stronger psoriasis-like disease compared to WT mice ([Yanaba et al., 2013](#)), which is explained by the loss of IL-10-producing regulatory B cells in the skin. Therefore, although in a general context of Lyn deficiency it resembles that the presence of Lyn in immune cells is not important in psoriasis development; perhaps its presence in a specific type of these cells would be more important than in another one.

It was previously described that no real cure exists for psoriasis disease, only palliative treatments have been obtained, which has led to outbreaks of the disease and to the development of resistance in treated patients. Thus, although a large numbers of biological drugs and small inhibitor molecules are in the clinic or under development ([Greb et al., 2016](#)), new targets are still required for the treatment development of this immune-mediated disease. In this context our results confirm the idea that Lyn could be a potential target for psoriasis treatment. Dasatinib is a reversible BCR/Abl and SFK inhibitor that has been approved in the treatment of CML and ALL patients ([Li and Zhang, 2016](#)) and though its uses has been principally focused in blood malignancies, IMQ-treated mice could be treated with this drug at the same concentration that it is employed in patients to assess the impact of the pharmacological inhibition of Lyn in psoriasis development. However, dasatinib is a pan-SFK inhibitor, thus more specific Lyn inhibitors such as saracatinib ([McGivern et al., 2018](#); [Harrach et al., 2017](#)) and bafetinib ([Zhang et al., 2016c](#)) might be also evaluated in IMQ-treated mice.

Altogether, our results confirm that Lyn protein tyrosine kinase is implicated in psoriasis pathogenesis and their absence leads to impairment in the induced psoriasis-like phenotype (**Figure 25**), enhancing a new function of Lyn in the context of psoriasis

and more specifically about its role in keratinocytes; validating this protein as a potential target in the treatment of psoriasis.



**Figure 25:** Recapitulative schema of the role of Lyn tyrosine kinase in psoriasis

In normal skin Lyn is expressed in keratinocytes and resident immune cells. When psoriasis is developed an increase in Lyn expression is observed in both compartments, linked to an increase in its expression in recruited immune cells and keratinocytes. However, an invalidation of Lyn impairs the induced psoriasis-like phenotype.



## **CONCLUSION**



One of the principal characteristics of cancer cells is their resistance to cell death by apoptosis, producing a chemoresistance to different drugs. Here, we determined that Lyn tyrosine kinase, and particularly its cytosolic form, acts as an apoptosis suppressor through impairment of the pro-apoptotic function of the BH-3 only member, Bim. This study represents the first approach directly linking the one member of the SFK and another one of the Bcl-2 family in the tumor resistance to apoptosis.

On the other hand, although pro-inflammatory caspases were previously associated to psoriasis pathogenesis, here we demonstrated that pro-inflammatory caspases are activated in lesional samples from psoriasis patients and its expression in immune cells is sufficient to develop a psoriasis-like phenotype.

We identified a key function of Lyn in psoriasis development and a pivotal regulation of Lyn in keratinocytes under psoriasis conditions, confirming the idea that this kinase can control several biological functions in non-hematopoietic cells.

Altogether, these results show that Lyn has an important function preventing apoptosis in cancer cells as well as pro-inflammatory caspases and Lyn are crucial factors in psoriasis pathogenesis, thus they could be used as new targets to develop new treatments in eliminating tumor cells and treating psoriasis patients.



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## **ANNEXES**



## ARTICLE 4

### Low protein diet induces IRE1 $\alpha$ -dependent anticancer immunosurveillance

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## **Abstract**

Dietary restriction (DR) prevents the growth of certain types of tumors. However, DR is not suitable for most cancer patients. In addition, how DR limits cancer progression remains largely unknown. Here, we demonstrate that feeding mice a low-protein (Low PROT) isocaloric diet but not a low-carbohydrate (Low CHO) diet prevents tumor growth in two independent mouse models. Surprisingly, this effect relies on anticancer immunosurveillance, as depleting CD8<sup>+</sup> cells or using immunodeficient mice prevented the beneficial effect of the diet. Mechanistically, we established that a Low PROT diet induces the unfolded protein response (UPR) in tumor cells and demonstrated that the activation of IRE1 $\alpha$  signaling induces cytokine production, thus mounting an efficient anticancer immune response. Collectively, our data suggest that a Low PROT diet induces an IRE1 $\alpha$ -dependent UPR in cancer cells, enhancing a CD8-mediated T-cell response against tumors.

## **Keywords**

Dietary restriction, cancer, immunosurveillance, ER stress, IRE1 $\alpha$

This article was accepted for publication in Cell Metabolism Journal

## ARTICLE 5

### **Lysosomal cholesterol hydrolysis couples efferocytosis to anti-inflammatory oxysterol production**

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## Abstract

**Rationale-** Macrophages face a substantial amount of cholesterol following the ingestion of apoptotic cells and the lysosomal acid lipase (LIPA) has a major role in hydrolyzing cholesteryl esters in the endocytic compartment.

**Objective-** Here, we directly investigated the role of LIPA-mediated clearance of apoptotic cells both *in vitro* and *in vivo*.

**Methods and Results-** We show that LIPA inhibition causes a defective efferocytic response due to impaired generation of 25-OHC and 27-OHC. Reduced synthesis of 25-OHC after LIPA inhibition contributed to defective mitochondria associated membrane (MAM) leading to mitochondrial oxidative stress-induced NLRP3 inflammasome activation and caspase 1-dependent Rac1 degradation. A secondary event consisting of failure to appropriately activate liver X receptor-mediated pathways led to mitigation of cholesterol efflux and apoptotic cell clearance. In mice, LIPA inhibition caused defective clearance of apoptotic lymphocytes and stressed erythrocytes by hepatic and splenic macrophages, culminating in splenomegaly and splenic iron accumulation under hypercholesterolemia.

**Conclusions-** Our findings position lysosomal cholesterol hydrolysis as a critical process that prevents metabolic inflammation by enabling efficient macrophage apoptotic cell clearance.

## Keywords:

Lysosomal acid lipase (LIPA), oxysterols, efferocytosis, inflammation

This article was accepted for publication in Circulation Research Journal

## ARTICLE 6

### **ATP-competitive Plk1 inhibitors induce caspase 3-mediated Plk1 cleavage and activation in hematopoietic cell lines.**

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## **Abstract**

Polo-like kinases (Plks) define a highly conserved family of Ser/Thr kinases with crucial roles in the regulation of cell division. Here we show that Plk1 is cleaved by caspase 3, but not by other caspases in different hematopoietic cell lines treated with competitive inhibitors of the ATP-binding pocket of Plk1. Intriguingly, Plk1 was not cleaved in cells treated with Rigosertib, a non-competitive inhibitor of Plk1, suggesting that binding of the inhibitor to the ATP binding pocket of Plk1 triggers a conformational change and unmasks a cryptic caspase 3 cleavage site on the protein. Cleavage occurs after Asp-404 in a DYSD/K sequence and separates the kinase domain from the two PBDs of Plk1. All Plk1 inhibitors triggered G2/M arrest, activation of caspases 2 and 3, polyploidy, multiple nuclei and mitotic catastrophe, albeit at higher concentrations in the case of Rigosertib. Upon BI-2536 treatment, Plk1 cleavage occurred only in the cytosolic fraction and cleaved Plk1 accumulated in this subcellular compartment. Importantly, the cleaved N-Terminal fragment of Plk1 exhibited a higher enzymatic activity than its non-cleaved counterpart and accumulated into the cytoplasm conversely to the full length and the C-Terminal Plk1 fragments that were found essentially into the nucleus. Finally, the DYSD/K cleavage site was highly conserved during evolution from *c. elegans* to human. In conclusion, we described herein for the first time a specific cleavage of Plk1 by caspase 3 following treatment of cancer cells with ATP-competitive inhibitors of Plk1.

### **Keywords:**

*Plk1; caspases; hematopoietic cells; G2M arrest; mitotic catastrophe*

This article was accepted for publication in Oncotarget

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### **Bourse de thèse**

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## **FORMATION / PROFESSIONNELLE SCIENTIFIQUE**

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## **PRESENTATION / CONFERENCES**

25<sup>th</sup> European Cell Death Organization (ECDO) conference

Leuven, Belgium 27-29/09/2017

Cell Death and Immunity in Disease: from molecules to translational medicine

**Caspase 1/11 deficiency in mice leads to a decrease in the induced psoriasis-like phenotype**

**AIRA LE**, Gonçalves D, Colosetti P, Rubio-Patino C, Ricci JE, Ortonne JP, Lacour JP, Auberger P, Marchetti S

ECDO Best Poster Presentation Award

Labex-SIGNALIFE: Meeting-2017

Nice, France

09-10/05/2017

**Caspase 1/11 deficiency in mice leads to a decrease in the induced psoriasis-like phenotype**

**AIRA LE**, Gonçalves D, Colosetti P, Rubio-Patino C, Ricci JE, Ortonne JP, Lacour JP, Auberger P, Marchetti S

Poster presentation

1<sup>st</sup> Symposium Inflammation and Disease, C3M Nice, France 02-05/10/2016

**Role of the Lyn tyrosine kinase in the regulation of Psoriasis**

**AIRA LE**, Gonçalves D, Colosetti P, Ortonne JP, Lacour JP, Ricci JE, Auberger P, Marchetti S

Poster presentation

3<sup>rd</sup> International Mouse Models of Skin Cancer Symposium

Nice, France 02-05/10/2016

**Role of the Lyn tyrosine kinase in the regulation of cutaneous inflammation**

**AIRA LE**, Gonçalves D, Colosetti P, Ortonne JP, Lacour JP, Ricci JE, Auberger P, Marchetti S

Poster presentation

Labex-SIGNALIFE: Meeting-2015

Nice, France

09-10/11/2015

**Role of inflammatory caspases in Psoriasis**

**AIRA LE**, Gonçalves D, Colosetti P, Ricci JE, Ortonne JP, Lacour JP, Auberge P, Marchetti S

Poster presentation

Latinpharma-2013

La Havane, Cuba

21-25/10/2013

**Preliminary results of immunological biomarkers in patients with Rheumatoid Arthritis treated with Itolizumab**

**AIRA LE**, Pineda I, Frometa M, Urquiza A, Perez T, Falcon L, Crombet T, Mazorra Z, Hernandez P

Poster presentation

Immunotherapy-2012

La Havane, Cuba

12-16/11/2012

« Therapeutic manipulation of the inflammatory microenvironment »

**Preliminary results of immunological biomarkers in patients with Psoriasis treated with Itolizumab**

**AIRA LE**, Pineda I, Frometa M, Urquiza A, Perez T, Falcon L, Crombet T, Mazorra Z, Hernandez P

Poster presentation

## **PRIX**

**Prix du meilleur poster** : ECDO-meeting, Leuven, Belgique

septembre-2017

**Prix National de l'Académie de Sciences de Cuba** : La Havane

2015

# LISTE DES PUBLICATIONS

## MANUSCRITS PUBLIES

### **Lysosomal Cholesterol Hydrolysis Couples Efferocytosis to Anti-Inflammatory Oxysterol Production.**

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### **The oncogenic tyrosine kinase Lyn impairs the pro-apoptotic function of Bim.**

**Aira LE**, Villa E, Colosetti P, Gamas P, Signetti L, Obba S, Proics E, Gautier F, Bailly-Maitre B, Jacquell A, Robert G, Luciano F, Juin PP, Ricci JE, Auburger P, Marchetti S.

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**Type 1 diabetes associated and tissue transglutaminase autoantibodies in patients without type 1 diabetes and coeliac disease with confirmed viral infections.**

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**MANUSCRITS ACCEPTES**

**Low protein diet induces IRE1 $\alpha$ -dependent anticancer immunosurveillance**

Camila Rubio-Patiño<sup>1,5</sup>, Jozef P. Bossowski<sup>1,5</sup>, Gian Marco De Donatis<sup>1</sup>, Laura Mondragón<sup>1</sup>, Elodie Villa<sup>1</sup>, **Lazaro E. Aira**<sup>1</sup>, Johanna Chiche<sup>1</sup>, Rana Mhaidly<sup>1</sup>, Cynthia Lebeau<sup>1</sup>, Sandrine Marchetti<sup>1</sup>, Thierry Passeron<sup>1</sup>, John Patterson<sup>2</sup>, Els Verhoeyen<sup>1</sup>, Béatrice Bailly-Maitre<sup>1</sup>, Eric Chevet<sup>3,4</sup> and Jean-Ehrland Ricci<sup>1\*</sup>

*Accepté dans le Journal Cell Metabolism*

**MANUSCRITS SOUMIS**

**Caspase 1/11 deficiency or pharmacological inhibition mitigates psoriasis-like phenotype in mice**

**Aira LE**, Gonçalves D, Rubio-Patino C, Chiche J, Mondragon L, Lecuq-Ottavi P, Obba S, Colosetti P, Luciano F, Bailly-Maitre B, Boyer L, Jacquelin A, Robert G, Ricci JE, Ortonne JP, Passeron T, Lacour JP, Auberger P, Marchetti S

*En revisión dans le Journal of Investigative Dermatology*

**MANUSCRITS EN PREPARATION**

**Lyn tyrosine kinase: a pivotal factor in psoriasis pathogenesis**

**Aira LE**, Gonçalves D, Rubio-Patino C, Chiche J, Lecuq-Ottavi P, Colosetti P, Luciano F, Jacquelin A, Robert G, Boulakirba S, Chiche J, Verhoeyen E, Ricci JE, Ortonne JP, Passeron T, Lacour JP, Auberger P, Marchetti S



## **SUMMARY**



The Src family kinase, of which Lyn is one member, plays a key role in controlling many biological processes. Lyn has a well-established function in hematopoietic cells, presenting an important role in the regulation of hematopoietic abnormalities. In fact, Lyn plays a key role in maintaining several kind of leukemia, and furthermore its expression is altered in solid tumors. Different studies have shown that Lyn has an anti-apoptotic role. Lyn can be cleaved by caspases, cysteine proteases involved in apoptosis and inflammation, giving a new protein with a cytosolic location, different from the WT and membrane-anchored form. Thus, cLyn could have access to new substrates that would explain its role as suppressor of apoptosis. We have shown that the cytosolic form of Lyn (cLyn) regulated Bim, a pro-apoptotic member of the Bcl-2 family, involved in the control of mitochondrial apoptosis. We have identified that Bim is phosphorylated on tyrosine 92 and 161 by Lyn, resulting in an inhibition of its pro-apoptotic function, increasing its interaction with anti-apoptotic members such as Bcl-XL, thus limiting the permeabilization of mitochondrial outer membrane and impairing cell apoptosis.

Lyn also has a pro-inflammatory role. We have previously shown that overexpression of the caspase-cleaved form of Lyn, in mice, leads to an inflammatory skin syndrome, resembling human psoriasis. Based on this result, we wanted to know if Lyn played a role in this chronic skin disease, which is characterized by an abnormal differentiation of keratinocytes and an increase in immune infiltrate, leading to the formation of psoriasis plaques, the main clinical feature of the disease. Analysis of Lyn's expression in psoriasis patients showed that Lyn was overexpressed in lesional skin compared to non-lesional or healthy skin, which was subsequently confirmed in two mouse models of psoriasis disease. Interestingly, we have shown that the increase in Lyn expression is in both the dermis and the epidermis in humans and in mice, indicating that recruitment of immune cells into lesional skin but also the modulation of Lyn in keratinocytes are involved. Moreover, an increase in Lyn expression was observed in human keratinocytes stimulated by TNF- $\alpha$  and IL-17A, two important cytokines in psoriasis pathogenesis. To determine the role of Lyn in this skin disease we induced a psoriasis-like phenotype in Lyn deficient mice. A significant reduction in cutaneous phenotype was observed in LynKO mice compared to WT mice, identifying Lyn as a new player in the pathogenesis of psoriasis. In addition, our results established that Lyn expression in keratinocytes seemed crucial and sufficient for the maintenance of the psoriasis phenotype, indicating a new role of Lyn in the regulation of keratinocytes.

During this work, we observed that inflammatory caspases were activated in lesional skin from psoriasis patients. Inflammatory caspases, following their activation within the inflammasome, will cleave IL-1 $\beta$  and IL-18, leading to their maturation. They play an important role in the control of inflammation in response to a pathogen but also contribute to the pathogenesis of many inflammatory diseases, such as diabetes and obesity. We then wanted to know if inflammatory caspases participated in the development of psoriasis. We were able to show that when we induced a psoriasis-like disease in mice, the invalidation of inflammatory caspases or its pharmacological inhibition significantly reduced the development of the disease compared to WT mice. Although immune cells and keratinocytes were able to secrete IL-1 $\beta$  via activation of the inflammasome, our data established that only activation of inflammatory caspases in the immune system seemed necessary for a complete inflammatory response.

In summary, my thesis shows a molecular mechanism by which Lyn tyrosine kinase negatively regulates the mitochondrial apoptotic pathway, which may contribute to the transformation and/or chemotherapeutic resistance of cancer cells. On the other hand, our results show that Lyn could be an important regulator of psoriasis and our study indicates that inflammatory caspases activated in immune cells are involved in the pathogenesis of psoriasis. To date, although several treatments have been developed for psoriasis, the disease remains unresolved, so the development of therapeutic targets against Lyn and inflammatory caspases could be of interest for the treatment of the disease.



## **RESUME**



La famille des kinases Src, dont Lyn fait partie, joue un rôle clé dans le contrôle de nombreux processus biologiques. Lyn a une fonction bien établie dans les cellules hématopoïétiques, jouant un rôle important dans la régulation des désordres hématopoïétiques. Lyn est notamment impliqué dans le maintien de différentes leucémies et son expression protéique est altérée dans les tumeurs solides. Plusieurs études ont mis en évidence qu'elle avait un rôle anti-apoptotique. Lyn peut être clivée par les caspases, protéases à cystéine impliquées dans l'apoptose et l'inflammation, ce qui donne une protéine tronquée avec une localisation subcellulaire, cytosolique, différente de la forme entière membranaire. Ainsi, elle pourra avoir accès à de nouveaux substrats qui expliqueraient son rôle de suppresseur de la mort cellulaire par apoptose. Nous avons ainsi montré que Lyn cytosolique (cLyn) régulait Bim, un membre pro-apoptotique de la famille Bcl-2, impliqué dans le contrôle de l'apoptose mitochondriale en le phosphorylant sur les tyrosines 92 et 161. Cette modification post-traductionnelle entraîne une inhibition de la fonction pro-apoptotique de Bim, en augmentant son interaction avec les membres anti-apoptotiques tels que Bcl-XL, limitant ainsi la perméabilisation de la membrane externe mitochondriale et l'apoptose des cellules.

Lyn possède également un rôle pro-inflammatoire. Nous avons préalablement montré que la surexpression de cLyn, chez la souris, conduit à un syndrome inflammatoire de la peau, ressemblant au psoriasis humain. Sur la base de ce résultat, nous avons voulu savoir si la tyrosine kinase Lyn jouait un rôle dans cette maladie chronique de la peau, qui est caractérisée par une différenciation anormale des kératinocytes et une augmentation de l'infiltrat immunitaire, conduisant à la formation de plaques de psoriasis, la principale caractéristique clinique de la maladie. L'analyse de l'expression de Lyn chez des patients souffrant de psoriasis a montré que Lyn était surexprimée dans la peau lésionnelle par rapport à la peau non lésionnelle ou saine, résultats confirmés dans deux modèles de psoriasis chez la souris. De façon intéressante, nous avons montré que l'augmentation de l'expression de Lyn se situe à la fois dans le derme et dans l'épiderme chez l'homme et chez la souris, indiquant que le recrutement de cellules immunitaires dans la peau lésionnelle mais aussi la modulation de Lyn dans les kératinocytes sont impliqués. Par ailleurs, une augmentation de l'expression de Lyn a été observée dans les kératinocytes humains stimulés par le TNF- $\alpha$  et l'IL-17A, deux cytokines importantes dans le psoriasis. Afin de déterminer le rôle de Lyn dans cette maladie cutanée nous l'avons induit chez des souris déficientes pour Lyn. Une réduction significative du phénotype cutané a été observée dans les souris LynKO par rapport aux souris WT, identifiant Lyn comme un nouvel acteur dans la pathogénie du psoriasis. De plus, nos résultats ont établi que l'expression de Lyn dans les kératinocytes semblait cruciale et suffisant pour le maintien du phénotype psoriasique, indiquant un nouveau rôle de Lyn dans la régulation des kératinocytes.

Au cours de ce travail, nous avons observé que les caspases inflammatoires étaient activées dans la peau lésionnelle de patients atteints du psoriasis. Les caspases inflammatoires, suite à leur activation au sein de l'inflammasome, vont cliver l'IL-1 $\beta$  et l'IL-18, ce qui conduit à leur maturation. Elles jouent donc un rôle important dans le contrôle de l'inflammation en réponse à un agent pathogène mais participent également à la pathogénie de nombreuses maladies inflammatoires, comme le diabète et l'obésité. Nous avons alors voulu savoir si les caspases participaient au développement du psoriasis. Nous avons pu montrer que lorsque nous induisions une maladie semblable au psoriasis chez des souris, l'inactivation des caspases inflammatoires ou son inhibition pharmacologique réduisait de façon significative le développement de la maladie par rapport aux souris WT. Bien que les cellules immunitaires et les kératinocytes soient capables de sécréter de l'IL-1 $\beta$  via l'activation de l'inflammasome, nos données ont établi que seule l'activation des caspases inflammatoires dans le système immunitaire semblait nécessaire pour une réponse inflammatoire complète.

En résumé, l'ensemble de mon travail de thèse a permis de montrer un mécanisme moléculaire par lequel la kinase Lyn régule négativement la voie apoptotique mitochondriale, ce qui peut contribuer à la transformation et/ou la résistance des cellules cancéreuses. D'autre part, nos résultats montrent que Lyn pourrait être un régulateur important du psoriasis, et notre étude indique que les caspases inflammatoires activées dans les cellules immunitaires sont impliquées dans la pathogénie du psoriasis. A ce jour, bien que plusieurs traitements aient été développés pour le psoriasis, la maladie reste non résolue, donc le développement de cibles thérapeutiques contre Lyn et les caspases inflammatoires pourraient être intéressants pour le traitement de la maladie.