



# Identification and characterization of novel molecular causes of primary immunodeficiency : RELA mutations are associated to common variable immunodeficiency and systemic lupus erythematosus

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# Université Paris Descartes

Ecole doctorale HOB 561

*Spécialité Hématologie*

## **Identification and characterization of novel molecular causes of primary immunodeficiency:**

***RELA* mutations are associated to common variable  
immunodeficiency and systemic lupus  
erythematosus**

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*“Science makes people reach selflessly for truth and objectivity; it teaches people to accept reality, with wonder and admiration, not to mention the deep awe and joy that the natural order of things brings to the true scientist.” – Lise Meitner*

*“Diplôme: signe de science. Ne prouve rien.” – Gustave Flaubert*

**Al Mutanabi** – “ لكل داء دواء يستطاب به الا حماقه اعيت من يداويها ”

*This work is dedicated to the offsprings of my friends*

*Who were born during my thesis*

*With all my love*

*Nawfel, Naël, Fouad, Sami & Leïla, Rafael, Mathilde, Milo & Charlie, Giulio*

*We aim to deeper understand our world*

*And make it a better place*

*I wish you a happy life and a bright future*

# 1 Abstract

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## 1.1 English abstract

Beyond the clinical benefit for diagnosis, the study of patients with primary immunodeficiency (PID) has also largely contributed to the deciphering of the complex molecular mechanisms involved in the human adaptive response against pathogens. Still, a large number of PIDs, especially common variable immunodeficiency (CVID), are genetically not defined. During my thesis, I aimed to identify and characterize novel molecular causes of PIDs based on human natural mutants as a research model (1). By whole-exome sequencing of DNA from patients presenting either with pediatric or familial form of CVID and Systemic Lupus Erythematosus (SLE), we identified three distinct heterozygous single nucleotide variations predicted deleterious in a CVID patient (*RELA*<sup>Y306X</sup>), a pediatric SLE patient (*RELA*<sup>R329X</sup>) and familial SLE patients (*RELA*<sup>H86N</sup>). To better understand how the identified mutations may impact the role of RELA in the NF-κB pathway, we confirmed that the two nonsense *RELA* mutations led to the expression of truncated forms of the protein, while the missense mutation led to the expression of mutated forms of the protein. By immunoblotting of nuclear protein extracts and cellular immunofluorescence, we demonstrated that the two truncated forms of RELA can translocate into the nucleus. Then, using a labeled NF-κB consensus oligonucleotide, we demonstrated that the two truncated forms of RELA were able to bind to DNA. All three mutated RELA proteins, when expressed ectopically, had an impaired transcriptional activity. Finally, we showed by immunoprecipitation that all three ectopically expressed mutated RELA proteins are able to interact with protein partners and form homodimers.

As a whole, our results indicate that mutations affecting the transcription factor RELA can be associated with CVID or SLE. Given the previous cases associating RELA haploinsufficiency to autoimmune lymphoproliferative syndrome with autoimmune cytopenia and to TNF-dependent mucocutaneous ulceration and inflammatory intestinal disease, our work widens the spectrum of disease and clinical phenotypes associated with RELA dysfunction and suggests that different *RELA* mutations lead to different functional consequences.

## 1.2 French abstract

Au-delà du bénéfice clinique du diagnostic, l'étude des patients atteints de déficits immunitaires héréditaires a aussi largement contribué à la compréhension des mécanismes moléculaires complexes impliqués dans la réponse adaptative humaine contre les pathogènes. Cependant, un grand nombre d'immunodéficiences primaires n'a pas encore été génétiquement défini, en particulier le déficit immunitaire commun variable (ou CVID en anglais). Au cours de ma thèse, j'ai cherché à identifier et caractériser de nouvelles causes moléculaires aux immunodéficiences primaires en me basant sur des mutants naturels humains comme modèle de recherche. Par séquençage entier de l'ADN de patients présentant une forme pédiatrique ou familiale de lupus érythémateux disséminé (ou SLE en anglais) et CVID, nous avons identifié trois variations hétérozygotes distinctes prédites comme délétères chez un patient atteint de CVID ( $RELA^{WT/Y306X}$ ), un patient pédiatrique SLE ( $RELA^{WT/R329X}$ ) et les patients atteints de SLE ( $RELA^{WT/H86N}$ ). Afin de comprendre comment les mutations identifiées peuvent affecter le rôle de RELA dans la voie NF- $\kappa$ B, nous avons confirmé que les deux mutations non-sens de *RELA* entraînent l'expression de formes tronquées de la protéine, tandis que la mutation faux-sens menait à l'expression de formes mutées de la protéine. Par immunoblot des protéines nucléaires et par immunofluorescence cellulaire, nous avons démontré que les deux formes tronquées de RELA peuvent entrer dans le noyau. Ensuite, en utilisant un oligonucléotide consensus NF- $\kappa$ B marqué, nous avons démontré que les deux formes tronquées de RELA étaient capables de se lier à l'ADN. Les trois protéines RELA mutées, lorsqu'elles étaient exprimées de manière ectopique, présentaient une altération de l'activité transcriptionnelle. Enfin, nous avons montré par co-immunoprécipitation que les trois protéines RELA mutées exprimées de manière ectopique sont capables d'interagir avec ses partenaires protéiques et de former des homodimères.

En conclusion, nos résultats indiquent que des mutations affectant le facteur de transcription RELA peuvent être associées à des CVID ou des SLE. Étant donnés les cas précédents décrivant des haploinsuffisances de RELA liées à un syndrome lymphoprolifératif avec auto-immunité associé à une cytopénie auto-immune ainsi qu'aux ulcérations cutanéomuqueuses TNF-dépendantes associées à des inflammations intestinales, notre travail élargit le spectre des maladies et des phénotypes cliniques liés à un dysfonctionnement de la protéine RELA et suggère que différentes mutations du gène *RELA* entraînent diverses conséquences fonctionnelles.

## 2 Abbreviations

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ALPS: Autoimmune Lymphoproliferative Syndrome  
APC: Antigen presenting cell  
BCR: B cell receptor  
CD: Cluster of differentiation  
CID: combined immunodeficiency  
CVID: Common variable immunodeficiency  
DNA: Deoxyribonucleic acid  
EMSA: Electrophoretic mobility shift assays  
EV: Empty vector  
HEK293T: Human embryonic kidney cells 293T  
HSC: Hematopoietic stem cell  
Ig CSR: Immunoglobulin class-switch recombination  
IKK: I $\kappa$ B kinase  
I $\kappa$ B: NF- $\kappa$ B inhibitor  
INF: Interferon  
IRF: Interferon regulatory factors  
MHC: Major histocompatibility complex  
NF- $\kappa$ B: nuclear factor kappa-light-chain-enhancer of activated B cells  
PAD: Primary antibody deficiency  
PID: Primary immunodeficiency  
PMA: phorbol-12-myristate-13-acetate  
RHD: Rel-homology domain  
RNA: Ribonucleic acid  
SCID: Severe combined immunodeficiency  
SHM: Somatic hypermutation  
SLE: Systemic Lupus Erythematosus  
SNV: Single-nucleotide variant  
TCR: T cell receptor  
TLR: Toll-like receptor  
TNF: Tumor necrosis factor  
V(D)J: variable, diversity, joining  
WES: Whole-exome sequencing  
WGS: Whole-genome sequencing  
WT: Wild type  
9AATAD: nine amino acid transactivation domain

## 3 Introduction

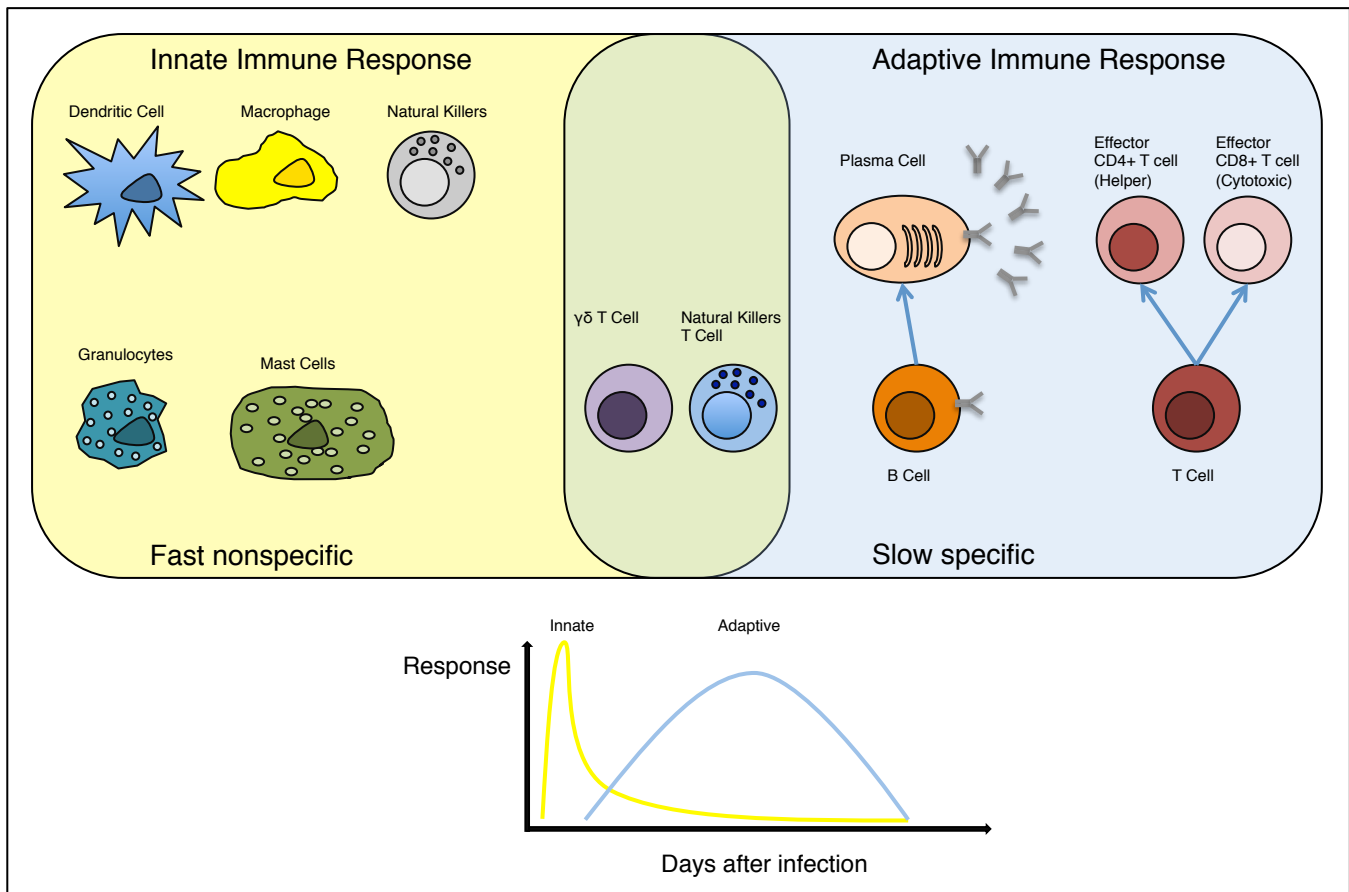
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### 3.1 Overview of the adaptive immune response

To defend the human body against infections, the innate immune response is first in line (1). It recognizes predetermined particular molecular patterns (antigens) of pathogens, making it a nonspecific defense. When continuously accumulating and replicating pathogens overwhelm the innate immune response, the adaptive immune response is consequently triggered. It allows the effective defense against an exceptionally vast range of pathogens. Indeed, the innate immune response is overwhelmed by most pathogens, rendering the adaptive immune response essential (**figure 1**). Thus, immunodeficiency syndromes can be associated to failures of specific parts of the adaptive immune response. The concept of non-self-antigens recognition is the basis of the adaptive immune response; it has evolved to recognize a countless variety of different antigens. Exaptation is theorized to be the essential thriving forces for innovation of genes involved in the adaptive immune response (2, 3). Its evolution, common to jawed vertebrates, resulted to a clonally distinct repertoire in which lymphocytes (B cells and T cells) produce an exclusive antigen-recognizing molecule. This immune response is mediated by immunoglobulins (antibody-mediated immune response by B cells) and cell-to-cell activations (cell-mediated immune response by T cells). These cells are highly specialized by undergoing different types of somatic genetic recombinations on genes coding for the binding sites of T cell receptors and immunoglobulins, allowing these expressed antigen-recognizing molecules able to bind to a unique antigen from a pathogen. In humans, B cells and T cells hold a key role in the immunological memory to pathogens (4). Lymphocytes originates and achieve development in primary organs (bone marrow and thymus), and first-encounter their matching pathogen in secondary lymphoid organs (such as lymph nodes, the spleen, Peyer's patches). Key genetic events and cellular mechanisms regulate and guide lymphocytes throughout their journey to become effector cells, thus any defects controlling their maturation can lead to impaired adaptive immune responses.



**Figure 1. Overview of the immune response.**



Schematic representation of the innate immune response (yellow box) and the adaptive immune response (blue box). In between, cells that play a role in both adaptive and innate immune response (green box). Bottom graph is a schematic representation of the response kinetics of both the innate (yellow curve) and adaptive (blue curve) immune systems. The adaptive immune response is triggered in delay.

## 3.2 Cell-mediated immunity

T cells hold the central role of the cell-mediated immunity, involving cytokines production and cell-to-cell physical contact. These actions lead to regulation of the immune response or to cytotoxic action on infected cells (5).

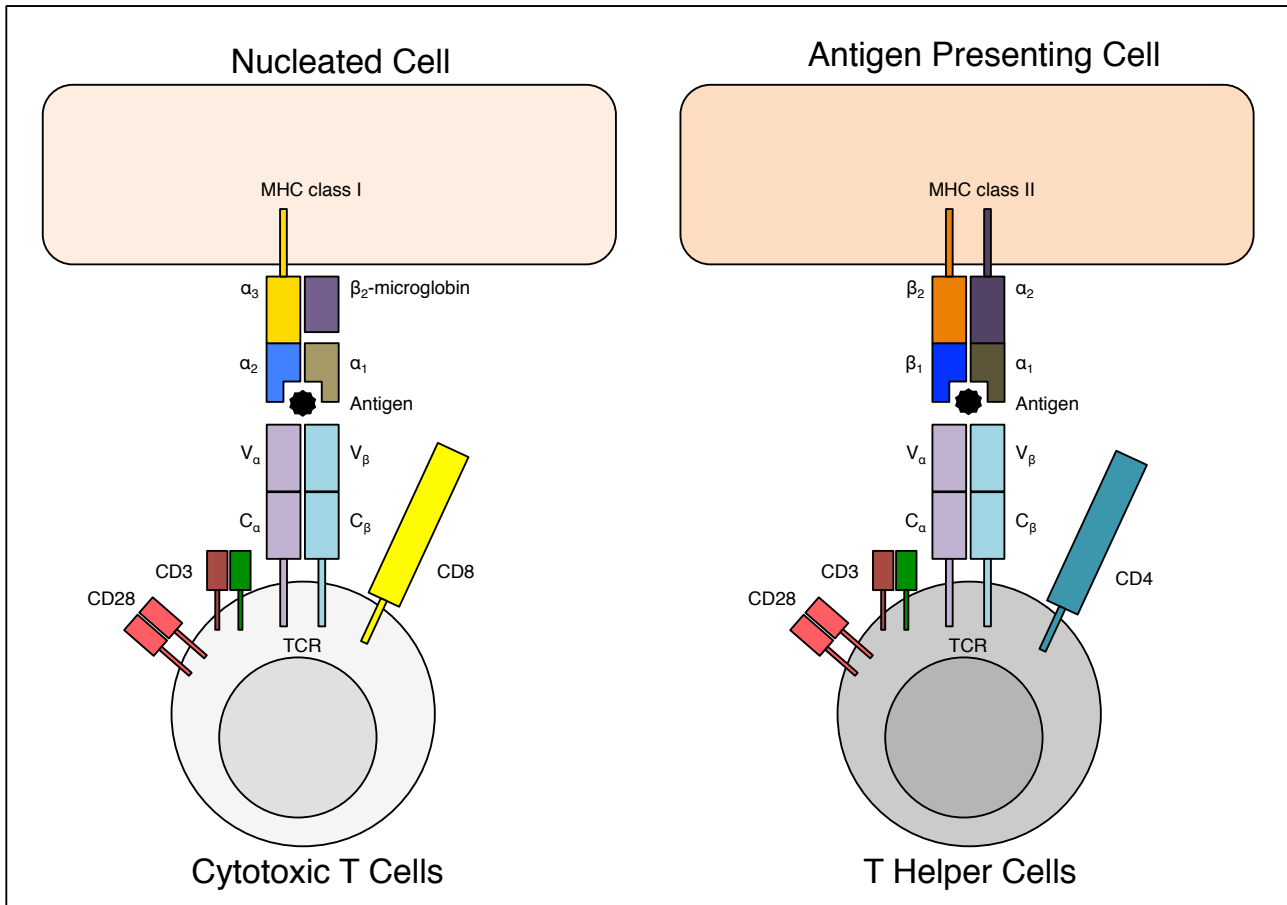
### ***3.2.1 Antigen recognition and formation of the immune synapse***

For T-cells, antigen-recognition molecules consist exclusively of membrane bound proteins and function to signal the T cells for activation (6). They uniquely recognize foreign antigens that are present on the membrane surfaces of body's cells. This recognition is based on the immunological synapse, which consists in an MHC molecule, T cell receptor, and the antigen in between (**figure 2**).

Antigens are presented at the cell surface by the MHC molecule, which is a specialized glycoprotein (7). The genes encoding this molecule have been identified by their role on the immune system following tissue transplantation. Consequently, the complex of genes was named major histocompatibility complex and the peptide-binding glycoprotein was termed MHC molecule. Due to the numerous alleles and combinations possible, the MHC gene complex is always different between individuals (except for monozygotic twins). MHC molecules are instable proteins, and are stabilized only by having an exclusive interaction with a specific antigen capable to establish ionic bonds and hydrogen bonds with the amino acid residues within the cavity of the antigen-binding site (or peptide-binding cleft).

There are four classes of MHC molecules, and we shall discuss only the mainly expressed two first classes. The two classes of MHC molecules are expressed according to the cell type. Both classes have distinct subunit composition but similar three-dimensional structures. The two classes are highly polymorphic at their peptide-binding site, allowing the specificity of each site. MHC class I molecules are expressed by all nucleated cells of the body and present intracellular peptides. Antigens are obtained when the proteasome catalyzes the pathogen or the foreign molecule in the cytosol into small peptides. Then the antigen is linked to the MHC molecule in the endoplasmic reticulum and the resulting complex is externalized to the cell surface. Whereas MHC class II molecules are expressed by antigen-presenting cells (such as B cells, dendritic cells, monocytes, macrophages, epithelial cells of the thymus and microglia cells of the brain) and present extracellular peptides.

A T cell receptor (TCR) binds to an antigen in a form of a complex of a MHC molecule and a foreign peptide (8). The TCR is a heterodimer polypeptide chain composed of either  $\alpha\beta$  chains or rarely by  $\gamma\delta$  chains. Subsequently,  $\alpha\beta$  TCR will be referred as TCR, unless indicated otherwise. Each chain has an extracellular constant domain that spans the cell membrane and an extracellular variable domain that contains the binding site for the foreign peptide. The variable domain gives the TCR diversity thanks to the genetic somatic recombination sustained by the genes segments (V: variable, D: diversity and J: joining) encoding the variable domain. This genetic event is named the V(D)J recombination, a random genetic rearrangement of coding regions for the antigen-binding part of T cell receptors (and of immunoglobulin). The TCR by itself does not trigger cellular signals; it forms a TCR complex with other co-receptors, such as CD3, CD28, CD4 or CD8. All T cells express the CD3 co-receptor, which is responsible of downstream intracellular cell signaling. Both CD4 and CD8 co-receptors are responsible for increasing the specificity and the binding strength of the immune synapse. CD4 is the specific co-receptor for the MHC II molecule and is expressed by helper T cells and regulatory T cells. CD8 is the specific co-receptor of the MHC I molecule for cytotoxic T cells.

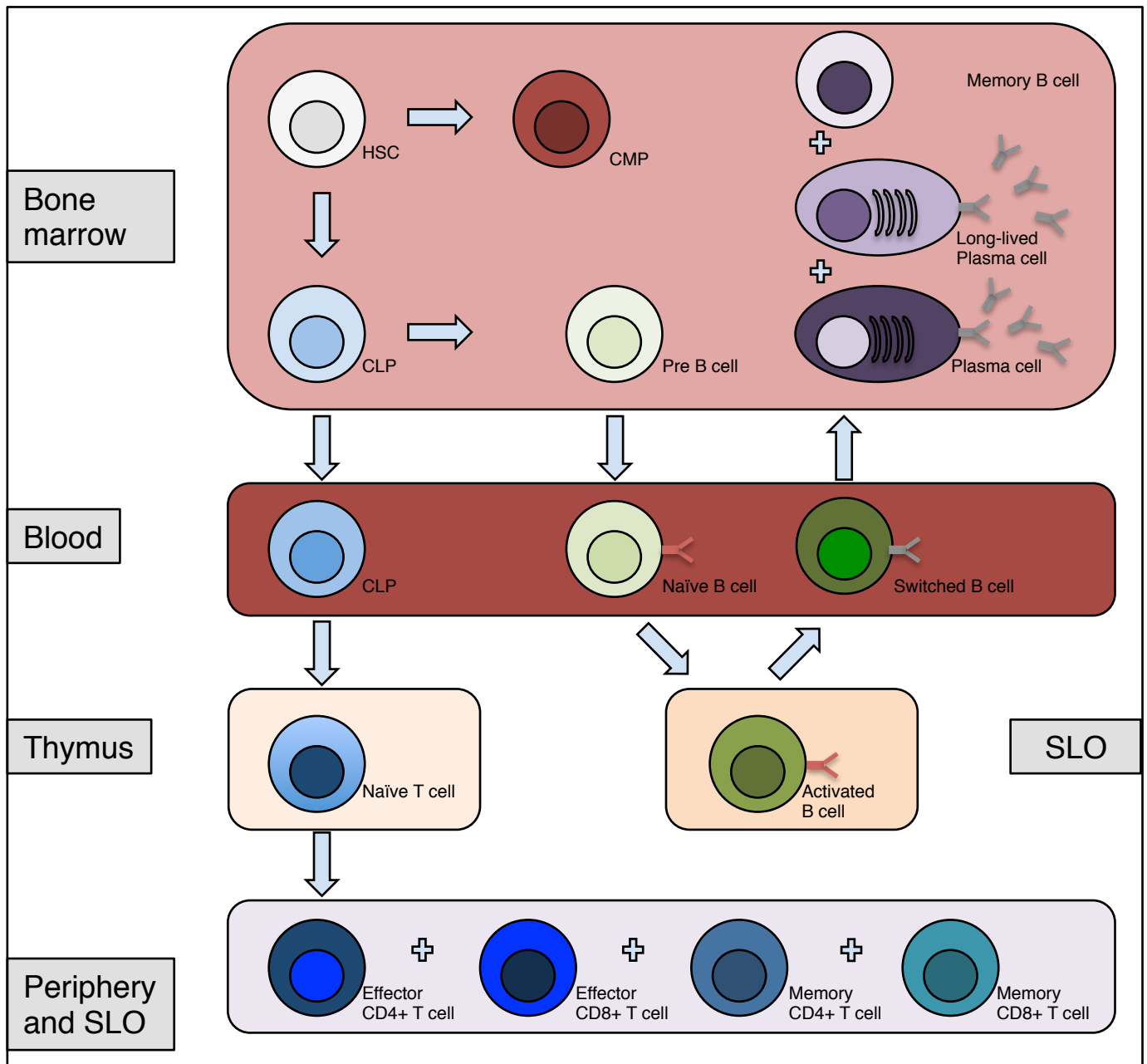


**Figure 2. The immunological synapse.**

The MHC class I molecule is composed of two polypeptide chains, one  $\alpha$ -chain and one  $\beta_2$ -microglobulin (with only the  $\alpha$ -chain being anchored to the cell membrane and encoded by the MHC gene complex). The  $\alpha$ -chain is composed of three domains –  $\alpha_1$ ,  $\alpha_2$  and  $\alpha_3$ . The  $\alpha_1$  and  $\alpha_2$  form together a cavity (the peptide-binding site) where binds the antigen. The MHC class II molecule is composed of a  $\alpha$ -chain and one  $\beta$ -chain, both encoded in the MHC gene complex. The two  $\alpha$  and  $\beta$ -chains spans the cell membrane, and both domains  $\alpha_1$  and one  $\beta_1$  form the peptide-binding site. The TCR is composed of a  $\alpha$ : $\beta$  polypeptide chains, containing each a variable domain V with the antigen-binding site or a constant domain C that spans the cell membrane. MHC and TCR genes are only found in jawed vertebrates. The co-receptors CD3 and CD28 bind to the TCR in order to transduce intracellular signaling. Co-receptors CD4 and CD8 enhance the stability of the complex for stronger signaling.

### **3.2.2 T cell development**

All cells of the immune response are generated from pluripotent hematopoietic stem cells located first in the fetal liver and then in the bone marrow. A common lymphoid progenitor is generated from these stem cells, which can produce three kinds of lymphocytes (T cell, B cell and NK cell) (**Figure 3**). In order to generate T cells, the common lymphoid progenitor migrates to the thymus to generate CD3 positive (CD3+) thymocytes (T cell progenitor). Thymocytes mature into double negative cells (CD3+ CD4- CD8-), then into double positive cells (CD3+ CD4+ CD8+) (9). Double negative and double positive Thymocytes express RAG-1 and RAG-2 protein in order to generate the genetic diversity of the antigen-binding sites through V(D)J recombination. Cells expressing receptors for self-antigens (auto-reactivity) and cells that are hypo-activated by their specific antigen (clonal anergy) receive death signals (negative selection). Cells reacting appropriately to MHC molecules receive survival signals (positive selection). The vast majority of thymocytes are eliminated by negative selection. The thymic differentiation is continued by becoming CD8+ T cytotoxic cells (loss of CD4 expression) or becoming CD4+ helper T cell ( $T_H$  cells) or regulatory T cells ( $T_{reg}$  cells) (loss of CD8 expression for  $T_H$  cells and  $T_{reg}$  cells). Once a mature T cell achieves its development in the thymus, it enters the bloodstream. At that point, the mature T cell has yet not encountered an antigen and is called a naïve T cell. Naïve T cells migrate to peripheral lymphoid organs, where foreign antigens presented by antigen-presenting cells activate them. In humans, recent thymic emigrants of naïve CD4+ helper T cells can be identified by CD31 expression.



**Figure 3. Lymphocyte development.**

Lymphocytes originate from HSC cells that generate progenitors of both B and T cells. T cells mature in the thymus before rejoining the periphery to meet its specific foreign antigen. B cells further mature in germinal centers (spleen and lymph nodes), after meeting their specific foreign antigen when entering these secondary lymphoid organs. After sustaining somatic hypermutation and immunoglobulin class switch recombination in the germinal centers, switched B cells exit the SLO. Finally, B cells reenter the bone marrow for antibody secretion as plasma cells. To note, memory B cells are found also in SLO and in blood. HSC= Hematopoietic stem cell; CLP= Common lymphoid progenitor; SLO= Secondary lymphoid organs.

### **3.2.3 Effector T cells**

In order to trigger the activation of T cells, the co-receptors (CD3, CD28, and either CD4 or CD8) allow the TCR-antigen-MHC complex to induce downstream intracellular signaling pathways. Upon activation, naïve CD4+ T cells triggers several signaling pathways (e.g.; the mitogen-activated protein kinases (MAPK) pathway, the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor family.

When activated by an antigen, both naïve CD4+ and CD8+ T cells produce the cytokines that allow their differentiation and proliferation into effector T cells and memory T cells. These cytokines are essential for effector T cells survival and inducing clonal expansion. Activated effector T cells rapidly undergo apoptosis in absence of cytokines.

Effector CD4+ T cells (also named T<sub>H</sub> Cells, for helper T cell) hold a central role in the regulation and organization of the immune response. Activated CD4+ T cells produce a variety of cytokines – locally or at distance – to correctly direct and modulate the immune response according to the type of infections the body is dealing with (10). They differentiate into subsets that are defined by the type of cytokine they produce. T<sub>H</sub>1 cells activate macrophages against bacteria, regulate the elimination of cells containing intracellular bacteria, and modulate antigen-activated B cells production of antibodies. T<sub>H</sub>2 cells regulate the antibody response against extracellular pathogens and induce naïve B cells to undergo antibody class switching (11). T<sub>H</sub>17 cell is a recently described subset with pro-inflammatory properties. They are involved in microbial defense of mucosal surfaces and regulation of other T cells (12). CD4+ effector T cells need to be regulated and the main role of this regulation is held by the T<sub>reg</sub> Cells. T<sub>reg</sub> cells are known for regulating CD4+ T cells by inhibiting them most of the time, thus having a necessary immunosuppressive role (13). They are generated either during differentiation in the thymus or directly from peripheral naïve CD4+ T cell (14).

Effector CD8<sup>+</sup> T cells (also named cytotoxic T cells) are cell killers (15). By the recognition of MHC I molecules displaying antigens on the surface of nucleated cells, they specialize in the elimination of virus-infected cells, tumorous cells and damaged cells. Once the immune synapse is established and the decision made to kill the infected or abnormal cell, they eliminate cells by inducing apoptosis. Cytotoxic T cells have two ways to provoke cell death. First mechanism is based on the secretion of cytotoxins into the targeted cell, which induces the apoptosis in any type of cell. The two main cytotoxins are Granzymes that activates apoptosis once in the cytoplasm, and Perforin that allows the delivery of Granzymes in the cytoplasm. The second mechanism consists on activating Fas receptors expressed on the surface of targeted cells, which triggers the caspase pathway for apoptosis. The main purpose of the second mechanism is to regulate other immune cells by reducing their cell numbers (16).

### **3.2.4 Memory T cells**

Memory T cells are fundamental for fighting against previously ulterior identical infection (**figure 4**). They represent the highest proportion of lymphocytes (17). The complexity of memory T cells subtypes is still being deciphered, and new subpopulations are discovered and discussed. Another layer of complexity is from which cell-type they originate (18). We shall discuss very briefly three main subsets of memory T cells. Memory T cells may be CD4<sup>+</sup> or CD8<sup>+</sup>.

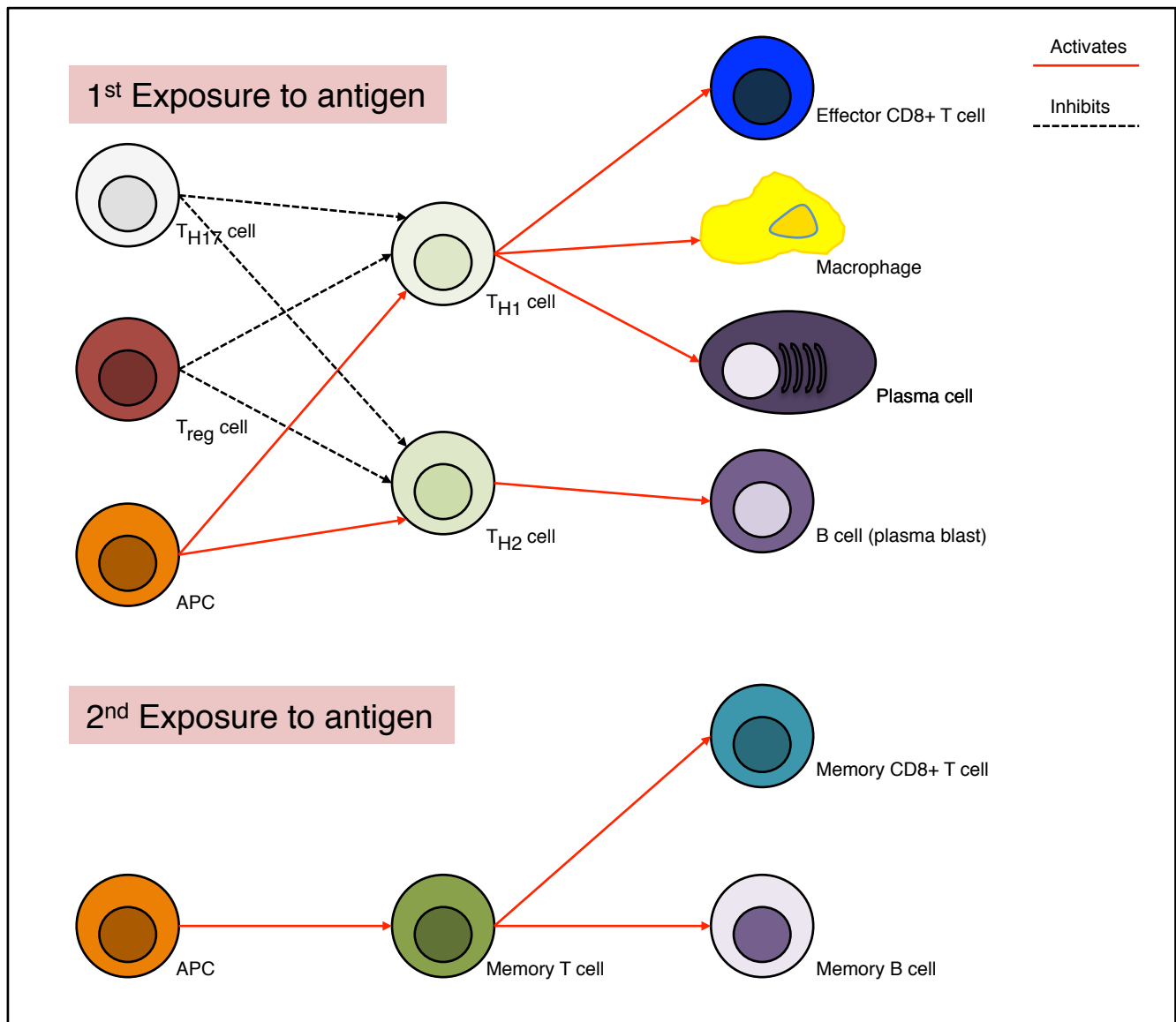
The central memory T cell ( $T_{CM}$ ) is a subset circulating mainly in the lymph nodes and in the periphery (19). They express at their surface CD45RO, C-C chemokine receptor type 7 (CCR7), CD44 and L-selectin (CD62L).

The effector memory T cells ( $T_{EM}$ ) are found mainly in the peripheral circulation and in tissues (19).  $T_{EM}$  cells express CD45RO and CD44 surface makers but do not express CCR7 and L-selectin, which makes them unable to settle in lymph nodes. When  $T_{EM}$  cells lose their CD45RO maker and re-express CD45RA (a naïve T cell marker), they become  $T_{EMRA}$ .



The resident memory T cell ( $T_{RM}$ ) is non-recirculating cell that populates tissues (lung, gastrointestinal tract, skin, etc.).  $T_{RM}$  cells express CD103 (integrin  $\alpha\epsilon\beta7$ ) for tissue homing. As  $T_{RM}$  cells have been associated to a major role in protective immunity against pathogens, defects in  $T_{RM}$  cells can lead to autoimmunity (e.g., psoriasis, rheumatoid arthritis, inflammatory bowel disease) (20).

A common principle of memory T cells is that they are rapidly inducible, which allow a quick clonal expansion. When antigen-presenting cells present the previously encountered antigen, memory helper T cells reactivates memory cytotoxic T cells and memory B cells. Therefore, the second exposure to the foreign antigen triggers a considerably faster adaptive immune response than the first exposure. The immune memory is a central paradigm of the adaptive immune response.



**Figure 4. First and secondary exposure to antigen.**

During a first exposure to a foreign antigen, APC present the antigen and activates effector T<sub>H1</sub> and T<sub>H2</sub> cells. Subsequently, T<sub>H</sub> cells activate and regulate effector CD8+ T cells, macrophages, B cells and Plasma cells. T<sub>reg</sub> cells modulate T<sub>H1</sub> and T<sub>H2</sub> cells. After second exposure to the same foreign antigen, APC cells present the same antigen to the corresponding memory T cell that reactivates memory CD8+ T cells and memory B cells. APC= antigen presenting cell; T<sub>H</sub>= helper T cell; T<sub>reg</sub>= regulatory T cell.

### 3.3 Humoral immunity

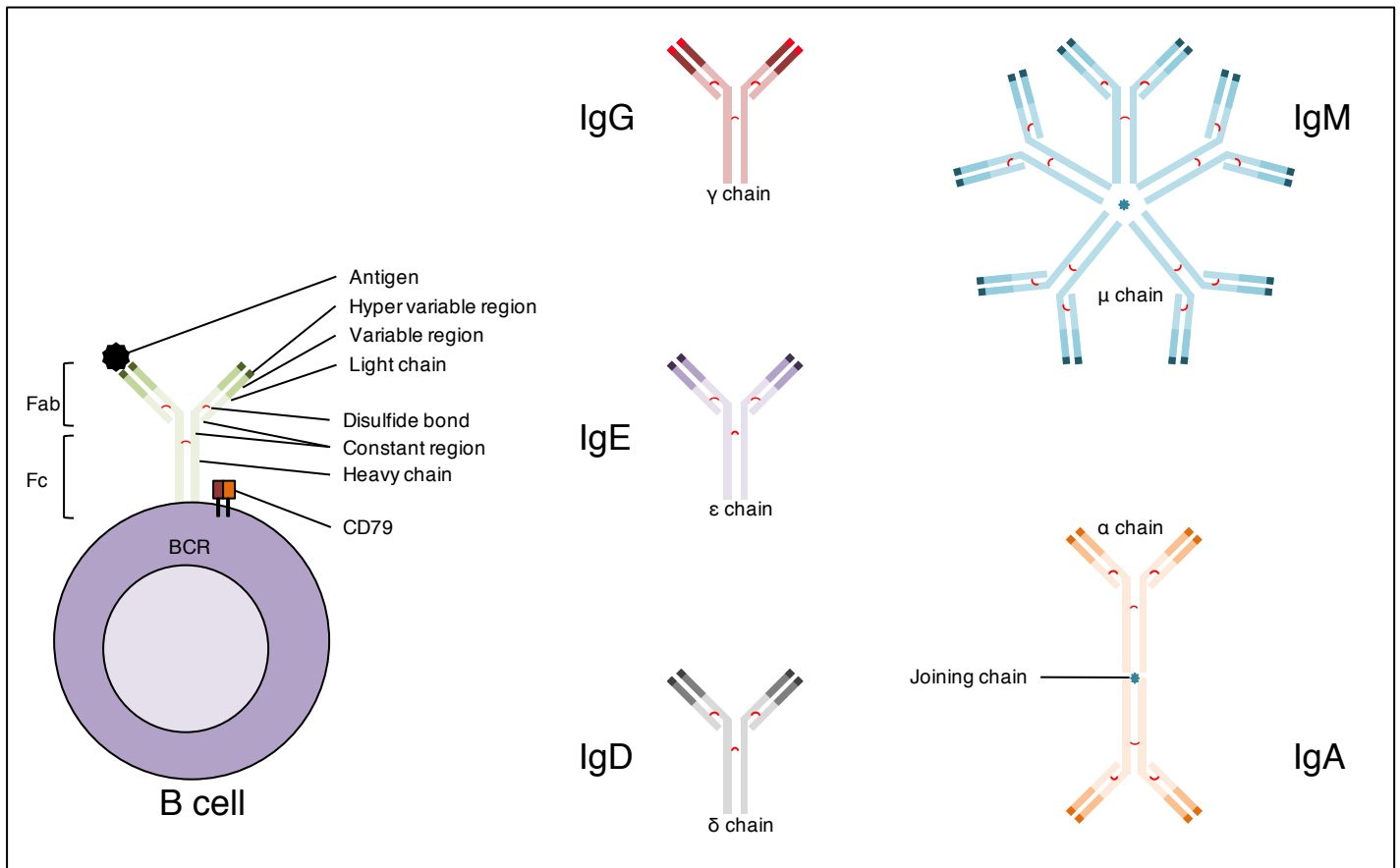
B cells are responsible of the antibody-mediated response during an infection. B cell maturation was first described in poultry, thus the letter B stands for "bursa-derived" referring to the specialized organ bursa of Fabricius in birds (21). This organ does not exist in mammals. In mammals, B cells originate and act from the bone marrow. Their final differentiation state results in the production of immunoglobulin. For clarity purposes, the term "antibody" will refer to secreted soluble immunoglobulin and "B cell receptor" (BCR) will refer to the membrane-bound immunoglobulin. An antibody has two distinctive roles. First, it has to bind to a unique antigen from the pathogen that triggered the immune response. And second, it has to bind to effector cells (mast cells, macrophages, granulocytes) to phagocyte opsonized-pathogens.

#### ***3.3.1 Immunoglobulin: structures and functions***

An immunoglobulin is a Y-shaped glycoprotein composed of two heavy chains and two light chains (22). The two chains are made of a variable region and a constant region. The variable region is generated by the V(D)J recombination of their genes, allowing recognition of a unique antigen. This genetic recombination is mediated as for T cells by RAG-1 and RAG-2 proteins. Plus, the variable region sustains another genetic event known as somatic hypermutation (SHM), a programmed process of mutation affecting the variable region and thus allowing the selection of an enhanced affinity of immunoglobulins for antigens. The Fab fragments are the arms of the Y-shaped molecule and are dedicated to the antigen recognition function. The Fc fragment is the base of the Y-shaped molecule. Fab and Fc fragments have been discovered by cleavage of the hinge regions of an antibody via the papain enzyme. For the B cell receptor, it interacts (upon binding of the antigen to the Fab) with the co-receptor heterodimer for cell signaling. For an antibody, it determines for the antibody its isotype and effector functions.

Different antibody isotypes are created by the genetic rearrangement of the constant region genes corresponding to the Fc fragment (**figure 5**). This rearrangement is called immunoglobulin class switch recombination (Ig CSR), and is the final genetic modifying event to achieve antibody maturation (23). Each antibody isotype has different half-lives, localizations and functions (24). IgM is the first antibody secreted as a soluble pentamer in reaction to a new antigen, and has not yet sustained Ig CSR. IgD is a poorly understood immunoglobulin. It is found mainly as a plasma membrane protein during B cell development, and can also be found in a secreted form as an allergy associated immunoglobulin. IgA is secreted as a dimer and is involved in mucosal immunity. IgG is the most abundant antibody in the serum, has a very large spectrum of functions, and is the only antibody that can go through the placenta. IgE, involved in immunity to parasites and the response to allergens, is the least abundant antibody but triggers the strongest inflammation reactions. IgE is responsible for allergy reactions. Only mammals produce IgE.

The B cell receptor (BCR) constitutes the central key in the cell fate, maturation and activation of the B cell for its life course towards an antibody-producing plasma cell (25). It constitutes a complex with the CD79 heterodimer (CD79A and CD79B), which triggers cell signaling upon binding with an antigen. Similar to TCR activation, downstream signaling pathways (e.g.; MAPK, PI3K, NF- $\kappa$ B) are activated upon BCR complex crosslinking. Specific immunoglobulin isotype and co-receptors form the BCR-complex that determines the strength and duration of downstream intracellular signal. The BCR-complex induced-signals control the highly specialized functions and fate of the B cell.



**Figure 5. The immunoglobulin, an antibody and a cell receptor.**

An immunoglobulin structure is comprised of two heavy chain and two light chain. Both light chain and heavy chain are linked by a disulfide bond, giving the Y shape to the immunoglobulin. Each chain has a constant region and a variable and hyper variable region at the Y extremities that binds to an antigen. The membrane-bound form of an immunoglobulin forms the BCR, with CD79 heterodimer transducing the intracellular signal upon BCR-antigen binding. The soluble form of an immunoglobulin is the antibody, comprising five types: IgM (pentamer), IgA (dimer), IgD (monomer), IgG (monomer) and IgE (monomer).

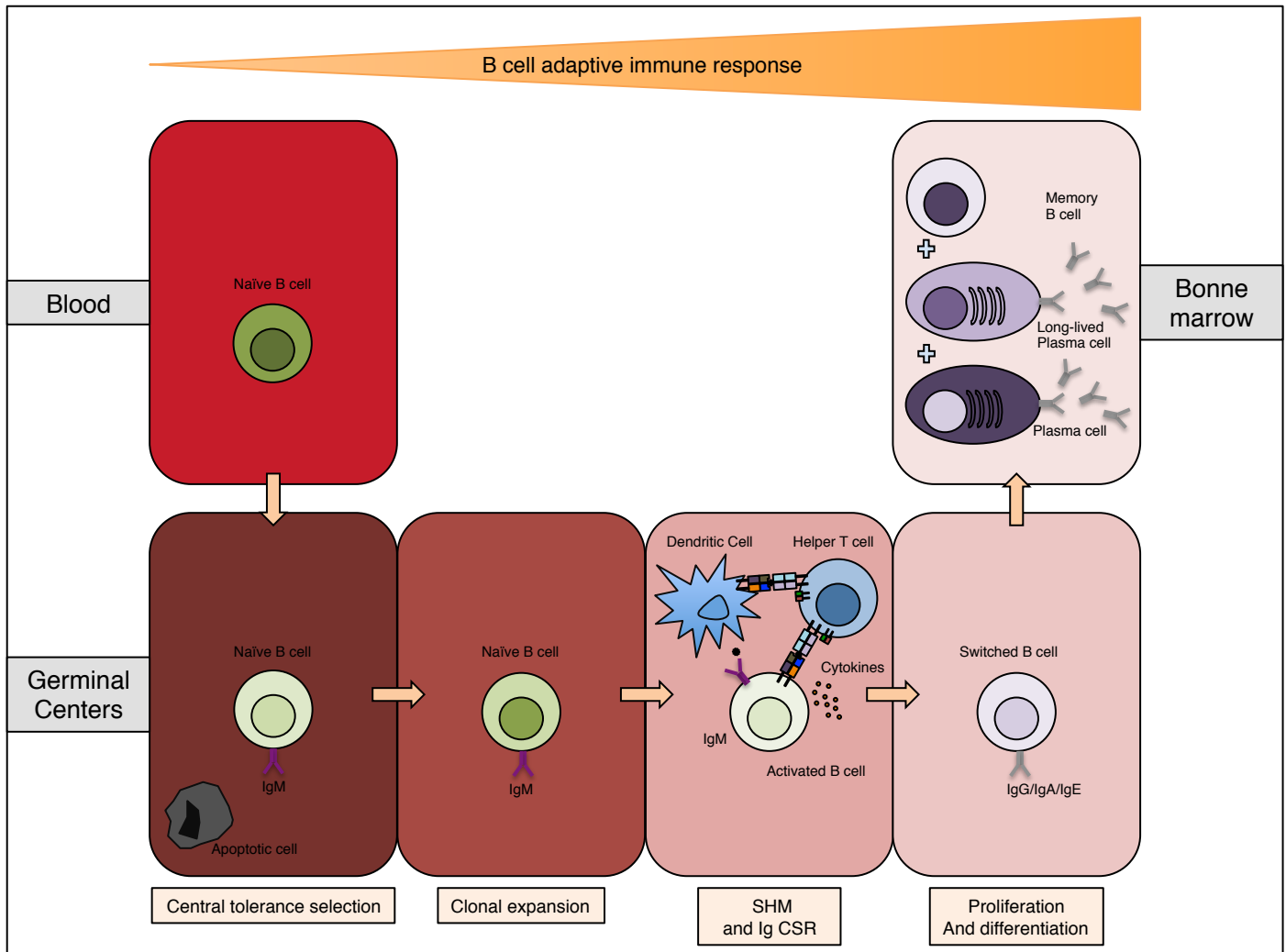
### **3.3.2 B cell maturation and activation**

As T cells, B cells derive from a common lymphoid progenitor. Once a cell is committed into the B-lineage, it expresses the CD19 co-receptor throughout its entire development and ceases expressing it once it achieves its terminal differentiation as a Plasma cell (26). During development, B cells express a variety of surface protein markers that define each stage of maturation. The maturing B cell has to go through

checkpoints allowing negative selection of auto-reactivity and clonal anergy, and thus resulting in a pool of mature B cells for future positive selection of antibody-producing clones (27). The negative selection of both B and T cells defines the central tolerance, a vital mechanism that prohibits the immune system to not react to self-antigen. V(D)J recombination of the heavy and light chains occurs during the pre- and pro- B cell stages. Once the recombination successful, the B cell expresses at its surface the BCR of the IgM class. Then, these IgM expressing B cells enters a transitional stage. Once in the blood stream, these transitional B cells express at their surface  $CD21^{low}CD24^{high}CD38^{high}CD43^{+}CD10^{+}CD5^{+}IgM^{high}IgD^{+}$  (28). Transitional B cells circulate in the blood stream and become mature B cells before entering secondary lymphoid organs. Mature B cells (also named naïve B cells) migrate from the blood stream into the germinal centers located in the spleen or in lymph nodes where they encounter their antigen to achieve their maturation. In the germinal centers, dendritic cells and B cells present foreign antigens to  $CD4^{+}$  T cells. These activated  $CD4^{+}$  T cells will activate B cells in return. During a T cell dependent activation, the B cell internalizes and processes the antigen in order to present it via its MHC class II complex to the  $CD4^{+}$  T cell, creating an immune synapse. Once the synapse established,  $CD4^{+}$  T cells produces cytokines (such as IL-4 and IL-21) and expresses a ligand (CD40L) to the B cell co-receptor CD40. Subsequently, B cells undergo SHM and Ig CSR to increase antibody affinity and enrich the isotype repertoire. Following these somatic genetic modifications, the B cell undergoes clonal expansion and exists the germinal center as switched B cells. Mature B cell can also be activated independently from T cells. Bacterial polysaccharides and unmethylated CpG DNA binds to Toll like receptors (TLRs) such as TLR4 or TLR9 (29), which activates B cells that are also located in the spleen and lymph nodes but not in germinal centers. These B cells that do not enter germinal centers do not undergo neither SHM nor Ig CSR and become Plasmablast and non-switched memory B cells. This rapid form of activation leads to the production of solely IgM antibodies, with less affinity and specificity to antigens.

Once switched B cells from the germinal centers exit the spleen and lymph nodes, they migrate back to the bone marrow. These switched B cells and Plasmablast further differentiate into Plasma cells (30) (also known as Plasmocytes,

antibody producing and secreting cells) or into memory B cells (long-lived cells that proliferate rapidly upon recall antigenic stimulation). (**figure 6**).



**Figure 6. B cell maturation.**

Naïve B cell enters germinal centers for central tolerance selection, followed by clonal expansion, then undergoes genetic modifying events (SHM and Ig CSR) when stimulated by dendritic cells and helper T cells. Finally, proliferation and differentiation in plasma cells or memory B cells that re-migrates to the bone marrow. B cell differentiation phases are governed by sequential gene expression and stochastic immunoglobulin gene rearrangements. SHM= somatic hypermutation; Ig CSR= immunoglobulin class switch recombination.

### **3.3.3 Memory B cells**

Once generated in the germinal centers, memory B cells are located in secondary lymphoid organs and in blood. Reactivated memory CD4<sup>+</sup> T cells rapidly reactivate memory B cells. Then the B cell undergoes massive clonal expansion, creating again plasma cells and memory B cells (31). To note, non-switched memory B cells can migrate back to germinal centers to undergo further maturation that they did not sustain prior to their memory stage.

Switched memory B cells and non-switched memory B cells express CD19 and CD27 surface markers. Moreover, non-switched memory B cells are still IgD<sup>+</sup>, whereas switched memory B cells lost the IgM and IgD expression due to the Ig CSR process.

Similar to memory T cells, memory B cells are vital when the organism faces a previous encountered pathogen. And similarly to memory T cells, their subtypes (defined by the immunoglobulin isotype and maturation pattern) and reactivation mechanisms are complex, necessitate more in-depth research and will not be further discussed.

## **3.4 Overview on primary immunodeficiency diseases**

### **3.4.1 Brief history**

In beginning 1950's, the first allegedly description of a human primary immunodeficiency (PID) was in X-linked agammaglobulinemia patients (no antibody production). As many great breakthroughs, the discovery was occurred by serendipity, as reported by Ogden Bruton and Charles Janeway (32). Several years later, it has been proven that those were not X-linked agammaglobulinemia cases but non-hereditary early onset of hypogammaglobulinemia (normal or high levels of IgM, and low or absence of IgG, IgA or IgE). Still in the 1950's, Robert Good



demonstrated the autosomal recessive inheritance of patients with chronic granulomatous disease (CGD) and hypergammaglobulinemia. The work led to description of multiple cases of hyper IgM syndromes that were X-linked. By the late 1960's, severe combined immunodeficiency disorders (SCID) were described when patients with drastic lymphopenia (lack of both T cell and B cell) and agammaglobulinemia.

Sequencing the human genome brought an era of genomics as a central tool for identifying and characterizing molecular causes of PIDs. By WES, linking a single-nucleotide variant (SNV) to a rare immunological disorder paved the way to a new extensive classification of PIDs. In a recent study, an estimation of six million people in the world may be affected by a PID, with between 27,000 and 60,000 individuals have been identified in 2013 (33).

### ***3.4.2 Primary immunodeficiencies diagnosis and etiology***

PIDs are hereditary immunological defects that constitute a heterogeneous group of diseases with a broad spectrum of susceptibility to recurrent infections, malignancies, allergies, autoimmune and inflammatory afflictions. PIDs can feature mild symptoms, debilitating outcomes up to early morbidity and mortality. Disease-associated gene mutations lead to developmental and/or functional impairment of both the innate and adaptive immune system. Although PIDs are mainly considered to occur during infancy and childhood, the adult onset is likely to be underestimated(33).

Based on clinical presentations, physicians and laboratories use typical testing to determine a PID diagnosis (34). They initiate the patient study with a complete blood count and serum levels of IgM, IgG, IgA and IgE, which they compare to age-matched healthy donors. With extracellular markers, it is fundamental to evaluate by flow-cytometer the number of T cells (CD3, CD4, CD8, TCR- $\alpha\beta$ , TCR- $\delta\gamma$ ), B cells (CD19, CD20, CD21, Ig), NK cells (CD16, CD56), monocytes (CD15); and activation

markers for B cells (HLA-DR, CD25, CD69, CD80, CD86) and for T cells (CD25, CD40L, CCR7, CD69, CD154).

They evaluate T cell functions by assessing:

- Cell proliferation response to mitogens (anti-CD3, phytohemagglutinin (PHA), concanavalin A),
- Delayed hypersensitivity skin tests (Candida, histoplasmin, and tetanus toxoid)
- Allogeneic cells (mixed lymphocyte response)
- Cytokine levels (serum and cell culture)

B cells functions are evaluated by assessing:

- Physiologically acquired antibodies
- IgG subclass levels
- Auto-antibodies
- Immunization response to protein and carbohydrate antigens

Phagocytes functions are assessed by:

- Reduction of nitroblue tetrazolium
- Chemotaxis assays
- Bactericidal activity

Based on these evaluations, further characterization to establish T and B cell subtypes (naïve cells, memory cells, effector cells, etc.) is necessary to fine-tune the analysis of immune deficiency.

Until recently, over three hundred genes have been associated to PIDs (35), and new genes are still being identified and reported yearly. A single gene mutation can potentially lead to significantly variable phenotypes between affected family members (e.g. STAT1 (36). Reversely, several gene defects can cause comparable phenotypes, such as SCIDs caused by both T cell and B cell defects (37) or familial hemophagocytic lymphohistiocytosis (FHL) (38). A SCID can be caused by several genes independently (e.g. *ADA*, *IL2RG*, *ZAP70*, *RAG1*, *RAG2*, etc.). PIDs features significantly overlap between all the different classification groups.

Primary antibody deficiencies (PADs) represent the major part of human PIDs (39). They result of either an intrinsic defect of the B cell or an extrinsic defect caused by cells that are fundamental for the development and function of B cells. The identification and characterization of the underlying causes leading to PADs has allowed unraveling the molecular mechanisms that govern B cell development and antibody maturation. Our laboratory focuses on deficiencies leading to PADs. Therefore, we study patients with immunological and clinical features such as hyper IgM syndromes and Ig CSR deficiencies, which lead to an impaired antibody response.

Until very recently, the monogenic cause has been the main approach to PIDs, and it allowed identifying and characterizing many candidate genes. However, more and more studies are discussing the multifactorial factors (other single-nucleotide variations, environment, etc.) that can better explain phenotypes variabilities or even specificities.

The etiology of PIDs differs from autoimmune diseases (AIDs) (40), as AIDs are defined by a multifactorial polygenic origin in which environmental triggers are essential in their pathogenesis. Currently, more studies are linking the etiologies of PIDs and AIDs (41). Focus will be made on common variable immunodeficiency (CVID) and systemic lupus erythematosus (SLE) associated to PID, as the patients that will be studied in this work suffers either of CVID or SLE. To note, CVID and SLE have been associated in several studies (42-44)

### **3.4.3 Common variable immunodeficiency**

Common variable immunodeficiencies (CVIDs) account the majority of PAD patients (45). Most patients diagnosed with CVID are adults, but it can be diagnosed at a juvenile-onset as well. The disease can go through alternations of acute phases and temporary remissions. Clinical features of this affliction are:

- Recurrent bacterial infections (particularly the upper and lower respiratory tract, but also skin, eyes, etc.), commonly with *staphylococcus aureus*, *streptococcus pneumoniae* or *haemophilus*

*influenzae*. These infections lead to frequent sinusitis, otitis, bronchitis and pneumonia.

- Recurrent fever
- Tissue infiltration by lymphocytes leading to their enlargement of inflammation, such as hepatomegaly for the liver, splenomegaly for the spleen (30% of patients), lymphadenopathy for lymph nodes, recurrent granulomas formation for skin, lungs and bone marrow.
- Lymphoproliferation or lymphopenia of specific cell subsets.
- possible growth retardation if occurring of the disease in an early onset.
- autoimmune manifestations (20 % of patients (46) such as idiopathic thrombocytopenic purpura (47), rheumatoid arthritis (48), SLE (49) ,etc.).
- transient or persistent diarrhea
- malignancies (e.g. non-Hodgkin's lymphoma, stomach carcinoma possibly due to chronic gastritis associated with *helicobacter pylori*).

The main immunological features of CVIDs are Hypogammaglobulinemia (low levels of IgG and IgA, normal or low levels of IgM) and defect in response to vaccine or common infections (European Society for Immunodeficiencies (ESID) - CVID diagnosis criteria). Because of the variability of phenotypes, patients may have normal B and T cell levels or they can feature lymphoproliferation or lymphopenia. Lymphocytes can also have developmental and differentiation defects. Therefore, defects in specific subsets of B or T cells can explain the phenotype (e.g. lower levels of switched B cells, low levels of CD4+ T cells). Immunoglobulin replacement therapy, mainly with IgGs, allows a robust improvement of symptoms.

Even though CVID are the most common PAD, and PADs the most common PIDs, the primary causes of CVIDs remain mostly unknown. Some monogenic causes have been identified as causative of CVID with mutations in *ICOS*, *TNFRSF13B*, *TNFRSF13C*, *MS4A1*, *CR2*, *CD80*, *CTLA4* and *PLCG2* (50). Nevertheless, multiple genetic factors leading to antibody production defect is becoming central prism of view in research. Therefore, single candidate genes in humans and mice single knockouts may not be relevant in the study of CVIDs.

### **3.4.4 Systemic Lupus Erythematosus associated to PID**

Systemic Lupus Erythematosus (SLE) is a chronic autoimmune disease with potential lethal outcome. The variable severity and heterogeneous features of the disease spreads from indolent to life threatening (**table 1**). Although any gender and ages can be affected, the clinical manifestations occur mainly in female patients between puberty and menopause. Currently, there is no cure to SLE but therapies allow remission of the disease. It is well described that the disease is caused by the production of auto-antibody, mainly anti-nuclear antibodies that targets several different components of the cell's nucleus (e.g. anti-DNA auto-antibody, anti-Smith au-antibody, anti-phospholipid auto-antibody). Therefore, about all the tissues of the human body are potentially affected by autoimmunity and inflammation. This self-targeting leads to the aberrant production of type 1 IFNs. Initial manifestations identified in a patient are commonly fever, arthritis and malar rashes, with unexplained remissions and relapses. Late complication can reach to severe cardiac, renal and neurological symptoms.

Type of manifestation	Symptoms
Constitutional (70%)	Fever, lethargy, weight variations
Hematologic (50%)	Auto-antibody, anemia, lymphopenia, leukopenia, trompopenia
Dermatologic	Malar rash, oral and nasal ulcer, alopecia (hair loss), photosensitivity
Musculoskeletal (50%)	Arthritis (joints), avascular necrosis (necrotic bone tissue by hypovascularisation), myalgia (muscle pain)
Renal (30%)	Glomerulonephritis, proteinuria (protein in urine), hematuria (blood in urine), sclerosis, renal failure

Neurological (70%)	Headaches, seizures, neuropsychiatric syndromes (in severe cases)
Pulmonary (40%)	Pleurisy, pneumonitis, etc.
Gastrointestinal (50%)	Peritonitis
Cardiac	Pericarditis, myocarditis, endocarditis

**Table 1. Criteria for classification as SLE.**

Non-exhaustive list of symptoms for each type of manifestation. Indicated-percentages of SLE complications in patients presenting the type of manifestation are based on *Kaul et al, 2016*.

SLE occurrence depends on multifactorial genetic background and environmental triggering (e.g. Ultraviolet (UV) lights are well described to trigger SLE). Rare single mutations have been linked to SLE, such as mutations in genes coding for proteins of the complement system (51). Research of SNV's that favor SLE manifestation has known extensive research. For example, several genetic variants associated to SLE have been identified in increased type I IFNs production and response (*IRF5*, *STAT4*, *DNASE1*, *TREX1*, *DNASE1L3*), in altered antigen presentation (*HLA-DR2* and *HLA-DR3*), in defects in the adaptive immune system such as altered T cell signaling (*PTPN22*, *BLK*, and *BANK1*), in defects in B and T cell lymphocyte differentiation (*PRDM1* and *TNFSF4*) (52-54).

As previously mentioned in section 1.4.2, SLE and CVID have been associated in several studies. Several groups of PID have been associated with SLE (55). The following deficiencies have been categorized in *Picard et al, 2015*:

- Defects in proteins of the Complement system (C1q, C1r, C1s, C4, and C2) have been associated to PIDs and SLE (56).
- Chronic granulomatous disease (CGD) is associated to SLE and PIDs with genes such as *CYBB*, *CYBA*, *NCF1*, *NCF2* and *NCF4* (55).
- Wiskott–Aldrich syndrome (WAS) (57).
- Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (58).
- Autoimmune lymphoproliferative syndrome (ALPS) (59).
- Idiopathic CD4+ lymphocytopenia (ICL) (60).
- Partial T cell immunodeficiency and hyper-immune dysregulation (61).

More specifically in the context of this thesis, SLE-related SNVs can also lead to considering SLE in the context of a PID. For several PADs few cases of SLE have been reported suggesting the pathomechanisms underlying these PADs might possibly predispose to the development of autoimmune diseases (e.g.; rheumatoid arthritis and SLE) (**table 2**) (62). To note, use of anti-TNF drugs for arthritis symptoms can lead to drug-induced SLE (63, 64), thus rendering a search for genetic causes problematic.

PAD associated to SLE	Main Clinical Manifestations	Mutated gene and Inheritance
Common variable immunodeficiency	Hypogammaglobulinemia, recurrent infections, poor response to immunization, rheumatoid arthritis, anemia, splenomegaly, hepatomegaly, granulomas	<i>ICOS</i> - AR <i>CD19</i> - AR <i>CD81</i> - AR <i>MS4A1</i> - AR <i>TNFRSF13B</i> - AD or AR <i>TNFRSF13C</i> - AR
Hyper IgM syndrome	Decreased IgG, normal to elevated IgM, recurrent sinopulmonary infections, rheumatoid arthritis, uveitis	<i>CD40LG</i> - XL <i>CD40</i> - AR <i>AICDA</i> - AR <i>UNG</i> - AR
Isolated IgG subclass deficiency	Decrease in one or more IgG subclass, mainly asymptomatic; possible poor immunization response to specific antigens and recurrent viral/bacterial infections	Unknown
Selective IgA deficiency	Mainly asymptomatic (Yel. L et al, 2010), recurrent infections with poor antibody responses to carbohydrate antigens, rheumatoid arthritis	Unknown
Selective IgM deficiency	Recurrent respiratory tract infections, asthma	Unknown

**Table 2. Primary antibody deficiencies possibly predisposing to SLE and other autoimmune diseases.**

Information collected from *Picard C et al 2015* and *Errante P.R. et al 2015*. AR= autosomal recessive; AD= autosomal dominant; XL= x-linked.

## 3.5 NFκB

### 3.5.1 *Brief history*

In 1986, Ranjan Sen and David Baltimore from the Massachusetts Institute of Technology (Cambridge, USA) were the first to describe evidence for the existence of an NF-κB signaling pathway (65). By using electrophoretic mobility shift assays (EMSA) to study protein-DNA complexes on nuclear extracts from different tissues, Sen discovered three proteins that were binding to specific enhancer sequences of immunoglobulin heavy chain and light chain. Thus they defined the protein binding specifically to the light chain enhancer sequences in B cell nuclear extracts as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). From there, Baltimore and Sen published a paper showing that this transcription factor was constitutively expressed, however binding to DNA only after lipopolysaccharides (LPS) stimulation (66), suggesting the existence of proteins that sequestered the NF-κB protein until activation of the pathway. Since that initial paper, other proteins were discovered and associated to the NF-κB transcription factor family. Indeed, these proteins have been described to interact to promoters and enhancers of thousands of genes. Therefore, NF-κB family is involved in the inducing of genes coding for cytokines, receptors, transcription factors, cell survival factors, cell stress, development and more (67). Being a pillar pathway in immunity, the extensive research on this pathway showed that the NF-κB is involved in most cellular tissues. Genes of this pathway are found conserved in higher eukaryotic organisms, such as the determination of embryologic axes or immune regulation in drosophila (68). NF-κB is considered as an ancient and ubiquitous signaling system that adapted to a pivotal role in the immune system in vertebrate and insect lineages by convergent evolution (69). Thus, the pathway has revealed to be an intricate knot of convergent stimuli leading to diverse outcomes, all in a fine-tuned, orderly and sequentially response.



### **3.5.2 The NF- $\kappa$ B family proteins, their inhibitors (I $\kappa$ B proteins) and activators (IKK complex)**

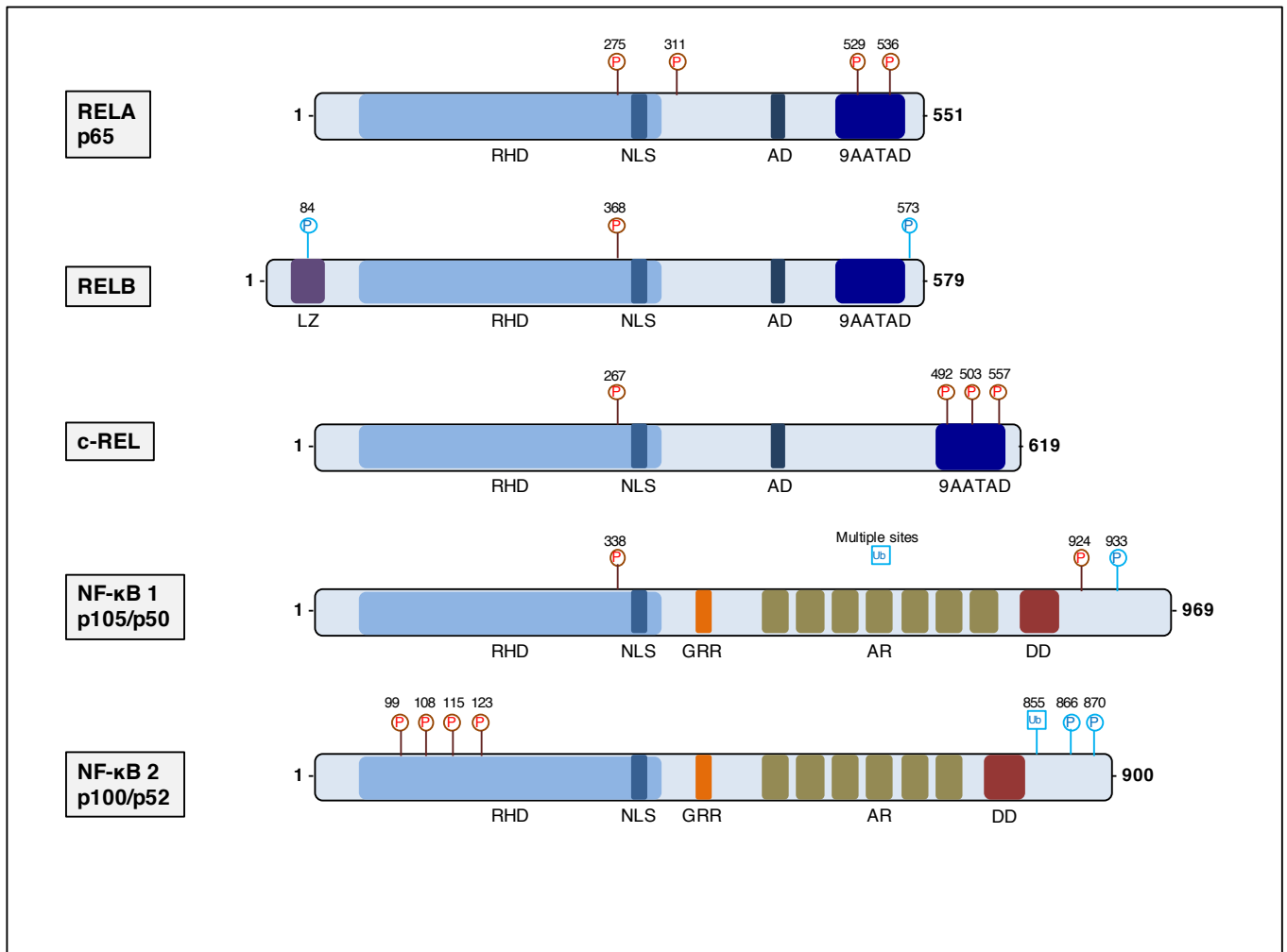
#### ***NF- $\kappa$ B family***

In mammals, five proteins compose the NF- $\kappa$ B transcription factor family: p65 (RELA), RELB, c-REL, p50 (NF- $\kappa$ B-1) and p52 (NF- $\kappa$ B-2) (70). The encoding genes are respectively *RELA*, *RELB*, *REL*, *NFKB1* and *NFKB2*. These transcription factors are direct positive or negative regulators of nearly a thousand target genes affecting various cell mechanisms. Dimerization and nuclear activity of these five proteins are highly dependent on key post-translational modifications (activating or inhibiting phosphorylation or ubiquitination) on the different protein domains

They all have in common a highly conserved N-terminal Rel-homology domain (RHD), structured as an immunoglobulin-like beta barrel that has multiple functions. It allows the homo-dimerization or hetero-dimerization of the protein between the members of the NF- $\kappa$ B family. Up to 15 potential homodimers or heterodimers can be formed (71). Still, the main dimer is p65-p50 for the canonical NF- $\kappa$ B pathway and RELB-p52 for the alternative NF- $\kappa$ B pathway. The N-terminal part of the RHD also confers ability for the protein to bind to the DNA molecule (72) (**figure 8**). The binding occurs on promoter or enhancer regions (73) that are sequence-specific to each protein with the following pattern: 5'-GGGRNWYYCC-3' (N, any base; R, purine; W, adenine or thymine; Y, pyrimidine) (74). Finally, the C-terminal part of the RHD contains the nuclear localization sequence (NLS) for nuclear translocation and the I $\kappa$ B interaction site for inhibition of the protein by I $\kappa$ B proteins masking the NLS sequence. Additionally, phosphorylation of the C-terminus of the RHD is a crucial phase for liberating the protein from sequestering I $\kappa$ B proteins for subsequent activation. Therefore, in most unstimulated cells of the organism, NF- $\kappa$ B proteins are sequestered in the cytoplasm, hence making the pathway inactive.

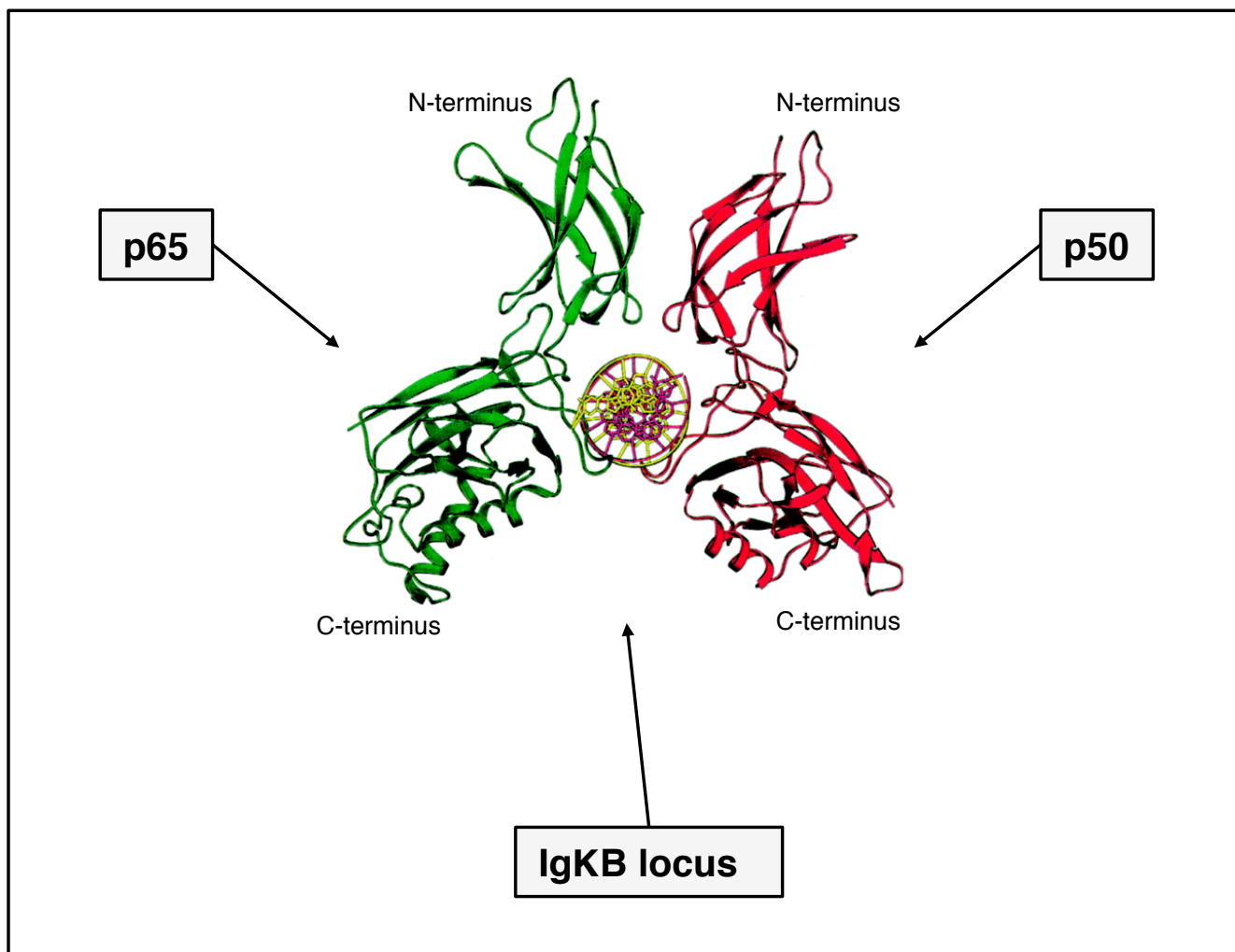
RELA, RELB and c-REL have in common a C-terminal nine amino acid transactivation domain (9AATAD) that recruits co-activators such as TAF9, TFIIB and CBP-p300 in order to initiate transcription of the target gene. This domain makes them essentially transcription activators, and requires phosphorylations for being active. Whereas p50 and p52 are devoid of such ability, making them essentially transcription repressors. RELB differs from the two other Rel proteins by its leucine-zipper (LZ) motif, and is inhibited by phosphorylation of the LZ and 9AATAD region (**figure 7**).

NF- $\kappa$ B-1 and NF- $\kappa$ B-2 are located as cytosolic precursors (respectively p105 and p100). These precursors possess at their C-terminus a death domain (DD) and six to seven Ankyrin repeats. The C-terminus of p105 and p100 is removed by proteolytic cleavage, resulting into the final form of nuclear proteins p50 and p52 respectively. These Ankyrin repeats are very similar to those of the I $\kappa$ B proteins and are involved in interacting with I $\kappa$ B proteins but especially to prevent NF- $\kappa$ B dimers to translocate to the nucleus by binding to RHDs (75). Thus, p50 and p52 active forms are obtained by cleavage at their glycine rich region (GRR) of p105 and p100 respectively. This processing allows nuclear translocation and DNA binding of p50 and p52 (**Figure 7**).



**Figure 7. The five members of the mammalian NF-κB family.**

p65 (RELA), RELB, c-REL, p50 (NF-κB-1) and p52 (NF-κB-2) are the five members of the NF-κB family. They all have in common a highly conserved RHD which contains the dimerization site, the NLS and the DNA binding site. Size of the human protein is indicated on the right side. The p50 and p52 proteins differ by their GRR, AR and DD regions and the absence of an AD and a 9AATAD. RELB has an additional LZ domain. Red P= activating phosphorylation; Blue P= inhibiting phosphorylation; Blue Ub= inhibiting ubiquitination; RHD= Rel-homology domain; NLS= Nuclear localization sequence; AD= activation domain; 9AATAD= nice amino acid transactivation domain; LZ= leucine zipper; GRR= glycine rich region; AR= Ankyrin repeats; DD= death domain.



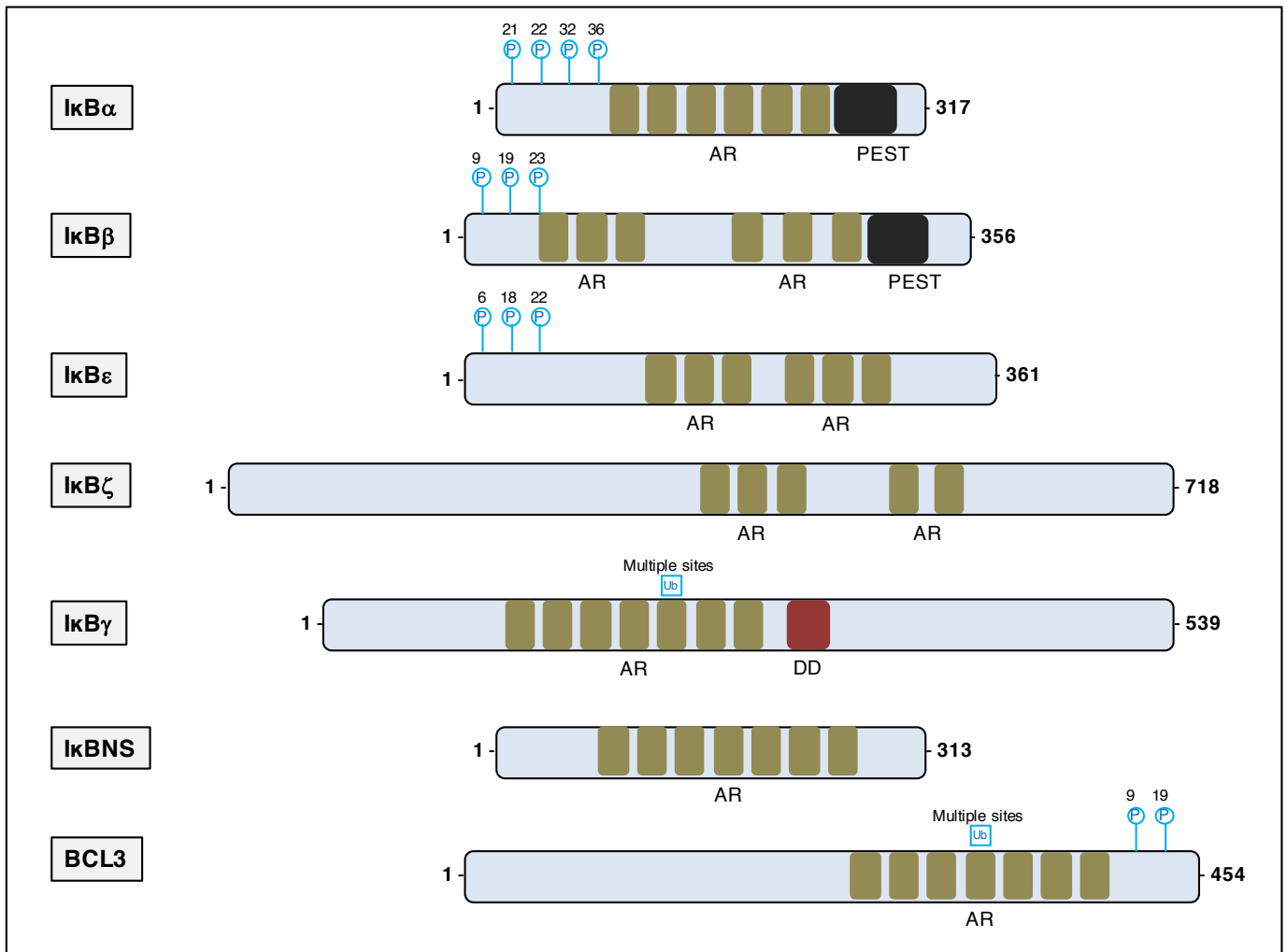
**Figure 8. The p65-p50 heterodimer 3D structure bound to the Ig  $\kappa$ B locus.**

In red and green respectively, the p65 and p50 molecules with the N-terminal on top and the C-terminal in the bottom. Ig  $\kappa$ B locus is represented in the center (76).

### ***I $\kappa$ B family***

The I $\kappa$ B protein family is the main actor for disabling NF- $\kappa$ B proteins functions. Primarily, they sequester NF- $\kappa$ B by masking their NLS. Also, they are involved in nucleus-to-cytoplasm shuttling of NF- $\kappa$ B dimers. I $\kappa$ B degradation is necessary for releasing NF- $\kappa$ B dimers. Several I $\kappa$ B proteins have been identified so far (70): I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\zeta$ , I $\kappa$ BNS, Bcl-3 and their respective encoding gene *NFKBIA*, *NFKBIB*, *NFKBIE*, *NFKBI*, *NFKBZ*, *NFKBID* and *BCL3* (**figure 9**). They are mainly composed of five to seven Ankyrin repeats that mediates binding to NF- $\kappa$ B dimer. The typical and most described members are I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$  and I $\kappa$ B $\epsilon$ . I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$

have a PEST domain, which is associated with proteins with rapid degradation (77). It has been described that  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice die shortly after birth and that  $\text{I}\kappa\text{B}\alpha^{-/-}$  mice with  $\text{I}\kappa\text{B}\beta$  knock-in totally have a restored phenotype, suggesting redundancy between these two proteins (78). Nonetheless,  $\text{I}\kappa\text{B}\alpha$ ,  $\text{I}\kappa\text{B}\beta$  and  $\text{I}\kappa\text{B}\epsilon$  have also distinct biochemical activities.  $\text{I}\kappa\text{B}\alpha$  is the main member of the  $\text{I}\kappa\text{B}$  protein family expressed in all cells, and contains (as well as  $\text{I}\kappa\text{B}\epsilon$ ) both a NLS and a nuclear export signal (NES) (79). After synthesis,  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\epsilon$  enter the nucleus in order to bind to  $\text{NF-}\kappa\text{B}$  dimers and expel them out of the nucleus.  $\text{I}\kappa\text{B}\alpha$  and  $\text{I}\kappa\text{B}\epsilon$  are directly synthesized following a  $\text{NF-}\kappa\text{B}$  activation, thus creating a negative regulatory loop. However,  $\text{I}\kappa\text{B}\epsilon$  has a much slower re-synthesis process. Consequently,  $\text{I}\kappa\text{B}\alpha$  is responsible of a fast transient  $\text{NF-}\kappa\text{B}$  activation. Whereas  $\text{I}\kappa\text{B}\beta$  has no NES sequence and is associated to  $\text{NF-}\kappa\text{B}$  sequestration in the cytoplasm and to prolonging  $\text{NF-}\kappa\text{B}$  action in the nucleus. The atypical  $\text{I}\kappa\text{B}$  members are  $\text{I}\kappa\text{B}\gamma$ ,  $\text{I}\kappa\text{B}\zeta$ ,  $\text{I}\kappa\text{BNS}$  and  $\text{Bcl-3}$ . Little is known about  $\text{I}\kappa\text{B}\gamma$ , which was discovered in the mouse as an alternative transcript of *NFKB1*.  $\text{Bcl-3}$  is misleadingly classified as member of the  $\text{I}\kappa\text{B}$  family, when it interacts exclusively with p50 and p52 homodimers and has co-activator effect (80).  $\text{I}\kappa\text{B}$  proteins are phosphorylated by the  $\text{I}\kappa\text{B}$  kinase (IKK) protein family, which is followed by the poly-ubiquitination of the  $\text{I}\kappa\text{B}$  proteins for degradation by the proteasome, subsequently leading to  $\text{NF-}\kappa\text{B}$  nuclear translocation.



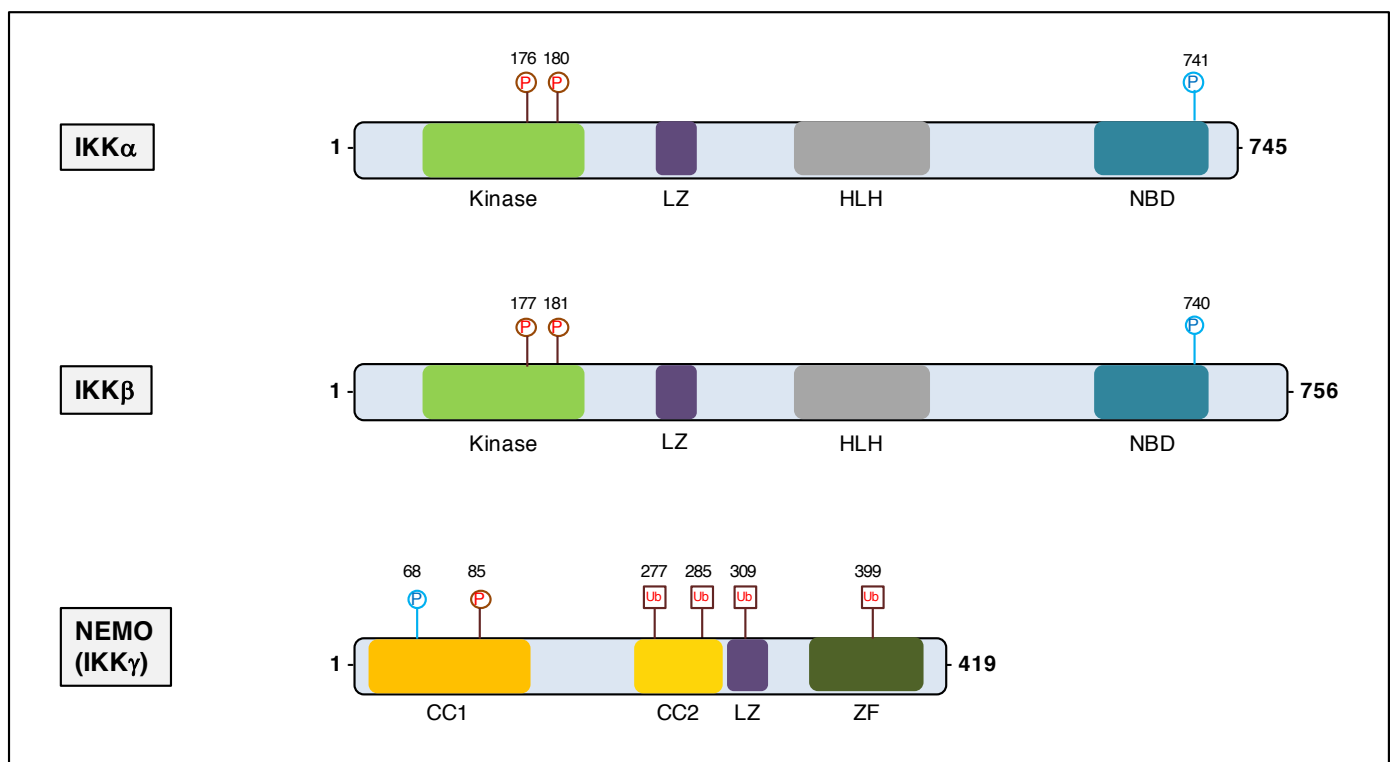
**Figure 9. The mammalian inhibitors of NF-κB family.**

The IκB family is characterized by their tandem of ankyrin repeats, which allows the binding to NF-κB proteins. IκBα, IκBβ and IκBε are phosphorylated in their N-terminal residues, leading to their rapid ubiquitination and fast degradation. Blue P= inhibiting phosphorylation; Blue Ub= inhibiting ubiquitination; AR= ankyrin repeats; PEST= proline, glutamic acid, serine and threonine rich regions; DD= death domain.

### ***IKK family***

IKKs are the bottleneck activation proteins of the NF-κB pathway (81). IKKs function together as a complex named the IKK complex. Upon phosphorylation by upstream signaling pathways, the IKK complex in turn phosphorylates IκBs, upon which sustains poly-ubiquitination for subsequent degradation.

The IKK complex is formed by the 3 members of the IKK family: IKK- $\alpha$  (or IKK1), IKK- $\beta$  (or IKK2) and nuclear factor  $\kappa$ B essential modulator (NEMO or IKK- $\gamma$ ) encoded respectively by *CHUK*, *IKBKB*, *IKBKG* (82) (**figure 10**). IKK- $\alpha$  and IKK- $\beta$  are biochemically very similar kinases but have specific biological functions. Both proteins have a N-terminal kinase domain that is activated by phosphorylation, a leucine zipper (LZ) domain and helix-loop-helix (HLH) domain for IKK- $\alpha$  and IKK- $\beta$  for hetero or homo-dimerization, and a C-terminal NEMO-binding domain that allows binding to NEMO. NEMO is the non-enzymatic regulatory subunit of the complex and is composed of two coiled coil (CC1 and CC2) domains, an LZ domain, and a zinc finger (ZF) domain. The N terminal part of NEMO is dedicated to dimerization with IKK- $\alpha$  and IKK- $\beta$ , whereas the C-terminal ZF domain is the docking site for upstream activating proteins (83). The IKKs are pivotal in activating the canonical NF- $\kappa$ B pathway and the alternative NF- $\kappa$ B pathway.



**Figure 10. The three members of the IKK complex.**

The IKK complex consist in one structural protein (NEMO) and two kinases (IKK- $\alpha$  and IKK- $\beta$ ). LZ= leucine-zipper motif; HLH= helix-loop-helix region; NBD= NEMO-binding domain; CC1= coiled-coil domain 1; CC2= coiled-coil domain 2

### ***Alternative Pathway***

The alternative NF- $\kappa$ B pathway is essential for dendritic cells and B cells, operating on B cells development (84) and function. There is no IKK complex as such in the alternative pathway. Initiation of the pathway goes through activated NF- $\kappa$ B-inducing kinase (NIK) that phosphorylates an IKK- $\alpha$  dimer (85). To note, NIK is constantly degraded, and is stabilized by upstream signaling when the alternative pathway is triggered. Then the IKK- $\alpha$  dimer phosphorylates a p100 protein that is sequestering a RELB protein. Phosphorylation of p100 is followed by its ubiquitination and its degradation into p52. The new liberated p52-RELB dimer then enters the nucleus and binds to its specific DNA targets. The p52-RELB DNA binding is terminated by newly synthesized p100 that enters the nucleus, binds to the dimer and transport it out to the cytoplasm. Responding mainly in a subset of TNFR signals, the alternative NF- $\kappa$ B pathway is a slow and persistent gene inducer, that depends on the synthesis of protein of the pathway to be induced.

### ***Canonical Pathway***

The canonical NF- $\kappa$ B pathway in most cell types goes through an activation of the IKK complex by phosphorylation. The activated complex then phosphorylates through the subunit IKK- $\beta$  mainly I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  proteins that are sequestering NF- $\kappa$ B dimers (p65-p50 in most cell type). Once the I $\kappa$ B protein is degraded by the proteasome, the NF- $\kappa$ B dimer is translocated to the nucleus, binds to a target promoter or enhancer and interact with other transcription factors such as TFIIB and CPB in order to initiate transcription via RNA Polymerase II. In order to terminate the activation, subsequently newly synthesized non-phosphorylated I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  proteins enter the nucleus to translocate NF- $\kappa$ B dimers back to the cytoplasm. The balance of cytoplasmic-nuclear shuttling via I $\kappa$ B $\alpha$  and I $\kappa$ B $\epsilon$  of NF- $\kappa$ B dimers contributes to the concept of latency of the canonical NF- $\kappa$ B pathway, for rapid induction and resolution of the signal. Acetylation of p65 is also a mechanism to lower the affinity of p65-p50 dimers for DNA and separate the complex (86).



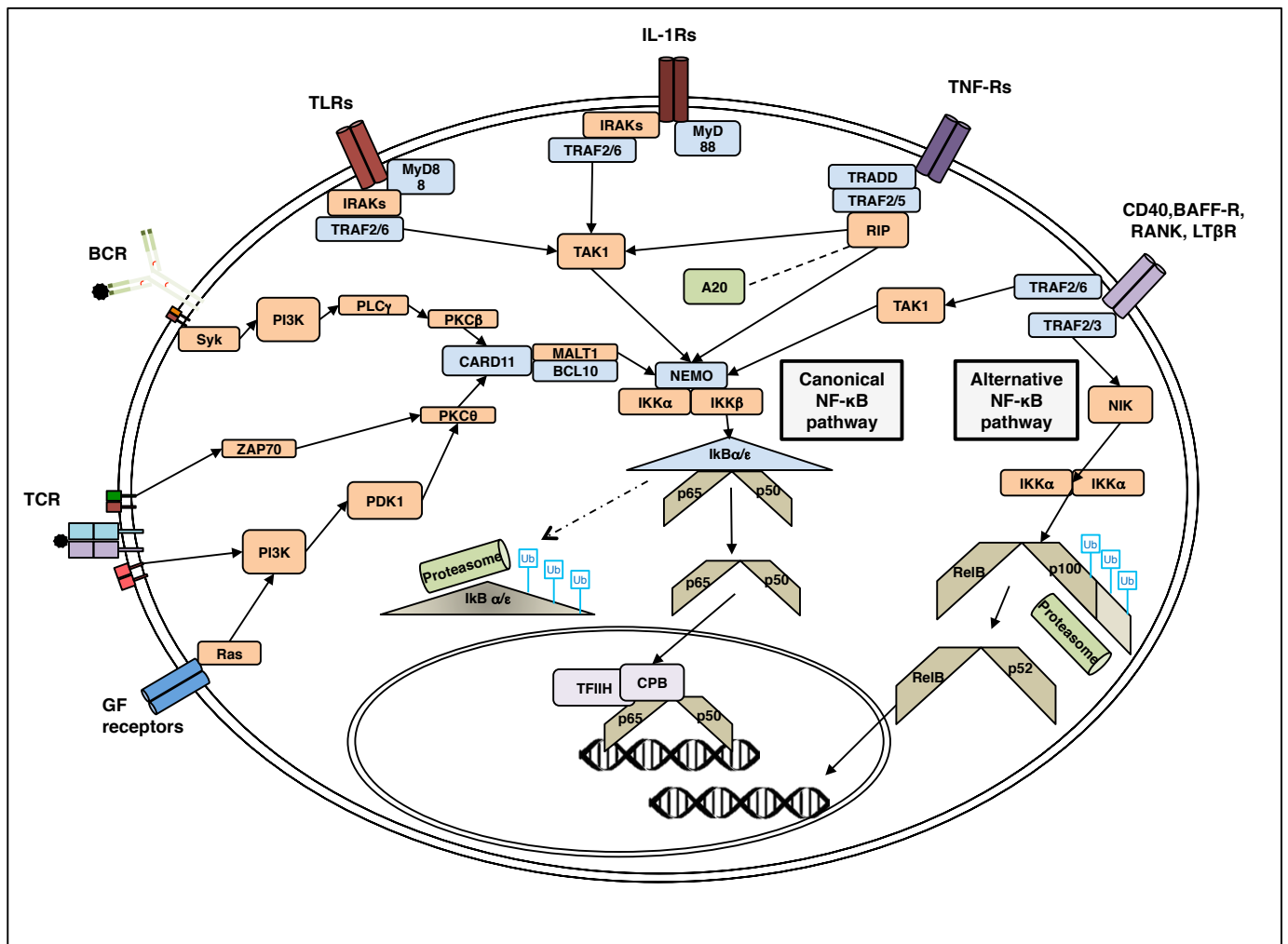
Responding to numerous types of cell receptors for diverse functions, the canonical NF- $\kappa$ B pathway is a rapid and transient gene inducer due to its independence from protein synthesis.

Each protein member of the NF- $\kappa$ B, I $\kappa$ B and IKK families features redundant and specific roles and functions. More studies are revealing the various possibilities of NF- $\kappa$ B dimers formation as well as diverse interaction with I $\kappa$ Bs and IKKs. This showcases the level of complexity leading to adapt each extracellular signal and environmental stimuli to a fine-tuned cellular response through specific targeted gene activations. Indeed, cell signaling cannot be considered separately but as highly interconnected.

### ***3.5.3 A complex pathway, nexus of cell signaling***

The complex activation of roughly a thousand genes by up to 15 potential dimers of the NF- $\kappa$ B protein family is already a great deal to envision. Nonetheless, a whole new layer of complexity is the signaling pathway leading to the bottleneck activation of the IKKs. This complexity starts by the various signals and stimuli that induce cell receptors; and followed by the cascade of adapter proteins balancing and tuning the signal (**figure 11**).

To trigger the canonical NF- $\kappa$ B pathway, a wide range of extracellular biochemical signals (such as cytokines, growth factors, foreign molecules) and chemical or mechanical stimuli (stress such as UVs, reactive oxygen species, ischemia and mechanical tissue damages) can induce several types of receptors. Chemical and mechanical stimuli induce the NF- $\kappa$ B pathway through JNK (87) or p38 for ubiquitination of I $\kappa$ Bs (88), and will not be discussed further.



**Figure 11. A synthetic view of the canonical and alternative NF-κB activation pathways in a mammalian cell.**

Several types of receptors and pathways can lead to NF-κB activation. Crosstalks can occur between upstream triggering pathways. The NF-κB pathway leads to cell survival, cell proliferation, inflammation and immune regulation. A20 is a inhibitor of the TNFR pathway by ubiquitination of RIP1 protein for degradation. Newly synthesized IκBα/ε proteins translocate in the nucleus, binds to p65-p50 dimers and expel them from the nucleus. Proteins in pale orange are kinases. Blue Ub= ubiquitination signal for degradation.

## ***Cytokine receptors***

The main cytokines receptors are the super family of the interleukin-1 receptors (IL-1Rs) and the super family of the tumor necrosis factor receptors (TNFRs) and their corresponding signal transducing adapter protein.

IL1-Rs are membrane bound proteins. They bind to the interleukin-1 molecules, a set of 11 different cytokines that modulate the development and function innate and adaptive immune cells. Once triggered, IL-1Rs recruit the adapter protein myeloid differentiation primary response 88 (MYD88), that transmits in turn the signal to an interleukin-1 receptor-associated kinase (IRAK) and its partner adapter protein TNF receptor associated factor 2 and 6 (TRAF2 and TRAF6). IRAK phosphorylates the adaptor transforming growth factor beta-activated kinase 1 (TAK1), which phosphorylates the IKK complex (89). To note, A20 is rapidly induced after a TNF response, allowing the newly synthesized A20 protein to mediate RIP1 and TRAFs ubiquitination, thus terminating NF- $\kappa$ B activation (90).

TNFRs are numerous super family of evolutionarily conserved membrane receptors that controls inflammation, cell proliferation, survival, and differentiation by rapid activation of the NF- $\kappa$ B pathway (91). They bind to the TNF cytokine super family. Once TNFRs initiate the intracellular signaling by recruiting the adapter protein tumor necrosis factor receptor type1-associated death domain (TRADD). Then TRADD recruits either FADD for apoptosis or TRAF2 and TRAF5 proteins and receptor-interacting protein-1 (RIP1) kinase for canonical NF- $\kappa$ B activation (92). TRAF proteins recruit then the IKK complex to be phosphorylated by RIP1.

Some TNFRs can also be inducers of the alternative NF- $\kappa$ B pathway (93). The B-cell activating factor receptor (BAFF-R also known as TNFRSF13C) recognizes BAFF ligand, a key cytokine modulating B cell development, differentiation, proliferation and activation. While stroma cells express the lymphotoxin- $\beta$  receptor (LT $\beta$ R) from the TNFR super family, T cells express the LT $\alpha\beta$  heterodimer. LT $\alpha\beta$ -LT $\beta$ R interaction facilitates lymphocyte trafficking and migration in tissues (94). The

CD40 receptors are expressed in antigen-presenting cells (such as B cells and Dendritic cells) as a co-stimulatory receptor. Although CD40 belongs to the TNFR super family, it does not bind to cytokines but to a ligand expressed mainly by T cells, CD40L. Receptor activator of NF- $\kappa$ B (RANK or TNFRSF11A) is more known for osteoclast differentiation and activation but also for being expressed in T cells. It binds RANK ligand (RANKL). The TNF receptor superfamily member Fn14 (TNFRSF12A) is the only receptor for the pro-inflammatory cytokine TWEAK (TNFSF12) (95).

In order to activate the alternative NF- $\kappa$ B pathway, these receptors directly recruit TRAFs (TRAF2 and TRAF3) without going through TRADD. They activate the NIK kinase with TRAF3 protein.

### ***Foreign molecules receptors***

In the innate immune response, TLRs activates the NF- $\kappa$ B pathway. They share a great structural homology to IL-1Rs and have similar adapter proteins to transduce the intracellular signal to the IKK complex. They are pattern-recognition receptors that bind to bacterial cell-wall molecules (such as LPS), viral single-strand DNA (ssDNA), viral double-stranded DNA (ds DNA) and CpG DNA. The intracellular signal goes through MyD88, IRAK, TRAF6 and TAK1 to activate the IKK complex, alike IL-Rs intracellular signaling.

In the adaptive immune response, the TCR and the BCR are the main receptors to activate NF- $\kappa$ B pathway following antigen binding. The TCR and the BCR are unable to transduce a cell signal by themselves, hence they form a complex with co-receptors CD3-CD28 (96) and CD79 heterodimer respectively (97).

In T cells, the TCR complex initiates a recruitment of several adapter proteins. Through the CD28 co-receptor, the PI3K pathway is triggered, which leads to the activation of pyruvate dehydrogenase lipoamide kinase isozyme 1 (PDK1). Then PDK1 phosphorylates the protein kinase C $\theta$  (PKC $\theta$ ). Through the CD3 co-receptor, the ZAP70 pathway is activated, which leads also to the phosphorylation of PKC $\theta$ . Activated PKC $\theta$  leads to phosphorylation of the caspase recruitment domain-

containing protein 11 (CARD11, also known as Carma1). CARD11 is a scaffold protein that assembles two other proteins, B-cell lymphoma 10 (BCL10) and mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1). The complex CARD11-BCL10-MALT1 phosphorylates the IKK complex for subsequent canonical NF- $\kappa$ B activation.

In B cells, the BCR-antigen complex first recruits the adapter protein spleen tyrosine kinase (Syk) that transduces the signal to PI3K, which leads to the phosphorylation of phospholipase C $\gamma$  (PLC $\gamma$ ). Then PLC $\gamma$  allows the phosphorylation of PKC $\beta$ , which in turn phosphorylates the IKK complex for subsequent canonical NF- $\kappa$ B activation.

### ***Growth factors receptors***

Growth factors receptors (GFR) are also able to trigger the NF- $\kappa$ B pathway. The first GRF to be described was the epidermal growth factor receptor (EGFR) that binds EGF (98). Other growth factors have been described to trigger NF- $\kappa$ B e.g., Bone morphogenetic protein (BMP), human growth hormone (HGH), nerve growth factor (NGF), transforming growth factor  $\alpha$  (TGF- $\alpha$ ) and insulin. The GRF recruit the protein rat sarcoma (RAS) that triggers the PI3K signaling pathway, leading to the activation of the canonical NF- $\kappa$ B pathway.

To note, more and more studies and reviews are unraveling the level of crosstalk and intricacies leading to the activation of the alternative and the canonical NF- $\kappa$ B. This indicates the necessity of fine-tuning the activation process for a specific cell response.

### ***3.5.4 NF- $\kappa$ B in immunology***

Though the NF- $\kappa$ B pathway is involved in most tissues of the body, it is fundamental the development and the physiological functions of the immune system (99). It also plays a role in the development, structuring and function of primary (100) and secondary lymphoid organs (101) (102). It has a fundamental role in the development of the innate immune response and inflammation, notably through

PRRs and TLRs. Indeed, the NF- $\kappa$ B pathway governs dendritic cells development and maturation (103), which are pivotal in initiating the adaptive immune response by mediating foreign antigen presentation. Focus will be made here on the role of the NF- $\kappa$ B pathway in the development and function of the adaptive immune response, in normal and pathological context. During hematopoiesis, and specifically lymphopoiesis, developing and differentiating cells are submitted to a rapid turnover. While B and T cells can feature great longevity (e.g., memory cells), their selection throughout the bone marrow and the thymus is mainly characterized by greater apoptosis. Hence, genes related to survival, proliferation and apoptosis are highly regulated by the NF- $\kappa$ B pathway (104).

### ***In Mouse models***

To better understand the roles of the NF- $\kappa$ B pathway in immunology, genetic single knockouts have been studied in animal models and *in vitro* cellular knockouts (105) (106) (107). Both NF- $\kappa$ B pathways have been demonstrated to have an early role in the immune system by regulating the development of secondary lymphoid organs. Indeed, *It $\beta$ <sup>-/-</sup>* mice have affects expression of organogenic chemokines (such as CXCL12, CXCL13, CCL19 and CCL21 regulated by the alternative pathway) and adhesion molecules (such as VCAM1 regulated by the canonical pathway), which leads to absence of lymph nodes and Peyer's patches (108) (109). Similar defect is seen in *rela<sup>-/-</sup> tnfr1<sup>-/-</sup>* (canonical pathway) with additional spleen and GC disorganization (101). Also, *nik<sup>-/-</sup>* mice survive to adult stage but lacks lymph nodes, Peyer's patches and have abnormal spleen and thymus architecture (110). Alterations in the development of lymphoid structures prevent the initiation of an adaptive immune response.

Single knockout of upstream signaling components in mice impairs a wide range of immunological features.

In B cells, the alternative pathway inducer *nik* is necessary for B cell maturation, activation and survival (111). CD40<sup>-/-</sup> mice have in Ig CSR deficiency, memory B cell development defect, and germinal center formation defect (112). The

*nfkb2*<sup>-/-</sup> mice have defects in their germinal centers and splenic architecture (113). Although, *relb*<sup>-/-</sup> have B cell proliferation defects but still undergo Ig CSR, thus showing that the alternative pathway is not essential for Ig CSR (114). But more recent studies have demonstrated that the alternative pathway is critical for naïve B cell survival, Ig CSR and plasma cell survival (115).

In T cells, *pkcθ* is necessary for T cell activation through TCR by recruiting the canonical pathway (116). Interestingly, mice knockouts in *card11*, *bcl10* or *malt1* results in T cell activation defects but does not affect their development (117) (118) (119). But a more recent study revealed that CD8<sup>+</sup> T cell could still be activated with a robust TCR stimulation (120).

For the NF-κB family, only *rela* knockout is embryonic lethal due to lack of survival signal leading to TNF-dependent liver apoptosis. The four other members do not feature development defects (121). However, they were all presenting immune deficiencies. The *c-rel*<sup>-/-</sup> mice had lymphocyte and macrophage dysfunctions, and *c-rel* is important for T<sub>H1</sub> differentiation and INFγ production (122). When T cells differentiate into TH1 or TH2 cells, they repress IL-2 NF-κB-dependent expression and starts IL-4 and INFγ production (123). *relb*<sup>-/-</sup> mice die after birth from general inflammation and featured Dendritic cell defects. They also show defects in B cell maturation and splenic architecture (124). The *nfkb1*<sup>-/-</sup> mice have lymphocyte activation defects, pre-aging and chronic inflammation (125). Comparably to the *relb*<sup>-/-</sup> mice, the *nfkb2*<sup>-/-</sup> mice have lymphocyte activation defects, absence of mature B cells and spleen architecture (113). Interestingly, *ikba*<sup>-/-</sup> *nfkb1*<sup>-/-</sup> mice display an attenuated phenotype than *nfkb1*<sup>-/-</sup> mice. *ikka*<sup>-/-</sup> mice feature developmental defects and no mature B cell, and *ikba*<sup>-/-</sup> *ikbe*<sup>-/-</sup> mice have lymphocyte survival defect with no T or B cells (126). The *ikkb*<sup>-/-</sup> mouse is embryonic lethal by liver apoptosis, unless combined to a *tnfr1* knockout, then shows NF-κB activation defect. The single knockouts in the NF-κB family, IKK family and IκB family showcase their importance in T cell and B cell survival.

More and more studies are deciphering how the NF-κB pathway is involved and triggers lymphocytes to become memory lymphocytes and how they are

reactivated. Initially, the canonical pathway was identified in the maturation and establishment of memory T cells through a *ikkb<sup>-/-</sup>* murine model (127). Then, the alternative pathway has been identified as being also involved through NIK signaling in CD4<sup>+</sup> and CD8<sup>+</sup> T cells differentiation into memory cells (128). As for B cells, MCL-1 has been shown to regulate maturation into memory B cells (129), and MCL-1 is regulated by the NF-κB pathway (130).

Although both NF-κB pathways have a pro-inflammatory action by favoring proliferation and activation of immune cells, it has been shown that T<sub>reg</sub> cells are driven by the NF-κB pathway in order to negatively regulate the adaptive immune response (131).

### ***Human mutations***

Currently, most human mutations affecting the NF-κB pathway have been identified through skin diseases or immunodeficiencies (132). But the implication of such mutations is described also in human cancer, as a direct or indirect etiology (133). For clarity purposes, only mutations in genes mentioned in the previous chapters will be briefly described.

Eighteen years ago, the first ever-described human mutations were in the encoding gene of the NEMO protein in skin disorders. *IKBKG* is an essential gene in humans located on the X chromosome. Consequently, males with null or amorphic mutations are embryonic lethal, whereas heterozygous females would survive and suffer from incontinentia pigmenti (IP) (134). A mouse model with the same phenotype was published the same year (135). *IKBKG* heterozygous hypomorphic mutations have an impact on human immunology in patients with anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) (136). Surprisingly, these patients have normal presence of T and B cells but low Ig levels and suffer of severe microbial infection. Patient's cells have defective canonical pathway NF-κB activation. On one hand, some heterozygous hypomorphic mutations of *IKBKG* that affects protein structural integrity lead to immunodeficiency without EDA. On another hand, heterozygous hypomorphic mutations with ubiquitination defect lead to



keratinocyte differentiation impairment. Thus, *IKBKG* mutations leading to loss-of-functions are more severe for immune cell functions, and mutations with defective post-translational modification affect both skin cell biology and immune cell functions in humans.

Autosomal recessive *CHUCK* (gene coding for IKK $\alpha$ ) mutations are embryonic lethal and have been detected in deceased human fetuses (137). To note, *ikka*<sup>-/-</sup> mice die soon after birth. Patients with *IKK $\beta$*  mutations are born normal but they develop a SCID on an early onset with either hypogammaglobulinemia or agammaglobulinemia. They also feature normal T and B cell count, but with no T<sub>reg</sub> cells nor  $\gamma\delta$  T cells (138). To note, *ikk $\beta$* <sup>-/-</sup> mice are embryonic lethal. Despite the normal count of T and B cell of patients, they were blocked at a naïve state and failed to differentiate to memory or effector cells upon activation (139). Surprisingly, *IKBKB* mutations have a far less severe phenotype than *CHUCK* mutations, even though IKK $\beta$  has a more pivotal role in the canonical NF- $\kappa$ B pathway than IKK $\alpha$ , as it can still form homodimers when interacting with NEMO. Also, in these studies, they noticed a decrease of expression of NEMO and IKK $\alpha$  RNAs and proteins, suggesting either a stabilization role of IKK $\beta$  on the IKK complex in humans (not seen in *ikk $\beta$* <sup>-/-</sup> mice). These studies demonstrate that IKK $\beta$  has unique roles in specific receptor signals that are not compensated by IKK $\alpha$ .

Several *CARD11* mutations have been described since 2012. Two publications associated different gain-of-function autosomal dominant mutations to B cell lymphocytosis with hyper-responsively of B cells to NF- $\kappa$ B triggering, also named B cell expansion with NF- $\kappa$ B and T cell Anergy (BENTA) disorder (140, 141). Another other studies associated a loss-of-function autosomal recessive mutations in *CARD11* to SCID with reduced antigen-receptor responsiveness of the NF- $\kappa$ B pathway due to absence of I $\kappa$ B $\alpha$  degradation (142).

MALT1 deficiencies have been identified in patients with CID, hypogammaglobulinemia with a paradoxal hyper-IgE, poor lymphocyte activation response, with decreased BCL10 cleavage and I $\kappa$ B $\alpha$  degradation (143, 144).

BCL10 deficiency is comparable to MALT1 deficiencies; with autoimmunity and CID featuring normal T and B cell count with activation defects (145). Patient cells showed decreased NF- $\kappa$ B translocation and cytokine production.

*TNFAIP3* (*A20*) heterozygous mutation leads to early-onset of systemic auto-inflammation characterized by oral and genital ulcers, arthritis, and ocular inflammation (146). The mutation leads to abnormal increase of I $\kappa$ B $\alpha$  degradation and increase of nuclear translocation of RELA protein.

*NFKBIA* mutations in humans are all of autosomal dominance inheritance, leading to gain-of-function dominant-negative effect (147-149). First described human mutation of the gene encoding the I $\kappa$ B $\alpha$  protein causes similar skin and immunological phenotypes as human *NEMO* mutations, and a comparable poor T cell antigen activation as *IKK $\beta$* . The mutated I $\kappa$ B $\alpha$  protein sequesters the NF- $\kappa$ B proteins, thus disabling the canonical pathway.

*NFKB1* mutations have been associated to common variable immunodeficiency (CVID) (150, 151). Mutations lead to haploinsufficiency with a dominant-negative effect since the wild-type allele does not compensate in terms of protein expression. At least seven mutations have been reported for *NFKB2*, all affecting the C terminal part of p100. The mutated p100 proteins end up not being processed into a p52 protein (152). Autosomal dominant mutations of *NFKB2* are also associated to CVID, with a mutation associated to autoimmune complications (153, 154).

Human homozygous loss-of-function in *NIK* features lymphopenia, B cell maturation and activation defect and memory T cell and T<sub>H</sub> cell defect as the murine *nik<sup>-/-</sup>* (155). However, the patients did not feature second lymphoid organ defects as the murine model. In patients' cells, NIK had defective kinase activity on IKK $\alpha$ .

Two different *RELB* mutations have been described in a cohort of combined immunodeficient (CID) patients. The first publication was about a homozygous mutation that leads to absence of RELB protein. Patients had normal levels of T and B cells with activation defects and absence of immunization response (156). The second paper concerned other autoimmune and CID patients with an autosomal recessive mutation on *RELB* (157). Patients featured a specific clonal enrichment of memory T cells and no memory B cells, with lymphocyte activation defects.

*RELA* mutations will be discussed in a following chapter and will also be the scientific results of this thesis.

To note, mutations in the genes encoding for TRAFF6 protein and TAK1 protein have been published but have no reported immunological defects.

## **3.6 RELA in genetic diseases**

We identified a human *RELA* mutation in a CVID patient and three SLE patients. Therefore, we initiated the characterization of these patients and of the different *RELA* mutants when still any published studies of human diseases related to a *RELA* mutation. After a year of investigation, two major papers were published.

### **3.6.1 *RELA* in chronic mucocutaneous ulcerations**

The first paper was named “*Human RELA haploinsufficiency results in autosomal-dominant chronic mucocutaneous ulceration*” and was published the 9<sup>th</sup> of June 2017 in the Journal of Experimental Medicine by Youssef Badran, Fatma Edeoglu and Janet Chou (158). They described a familial case of chronic mucocutaneous ulceration. The family included a healthy father and four members (mother and her three proband, with a focus on a 5-years-old female proband) suffering of early onset of mucocutaneous lesions. The proband of interest presented recurrent oral ulcers, abdominal pain and fever. She responded to infliximab treatment (a TNF inhibitor). The proband featured neither infections nor auto-antibodies, despite elevated inflammatory markers, with no inflammatory bowel disease. She presented with normal immunological features (B and T cell counts and subtype proportions were normal). The proband of interest featured a more severe phenotype than her siblings and her mother. By WES, they identified a common *RELA* mutation in the affected patients, on the assumption that *RELA* is known to counteract TNF toxicity (159). They describe an “alternative splicing of a cryptic splice site within exon 6 to the canonical acceptor splice site before exon 7 deletes 73 nucleotides at the 3’ of exon 6 and introduces a premature stop codon at residue 174”. Thus the mutation results with a haploinsufficiency of *RELA* RNA and *RELA*

protein. Functional assays revealed that patients' fibroblasts had higher cell death and peripheral blood mononuclear cells (PBMCs) had impaired interleukin-6 (IL-6) response when both cell types were stimulated with TNF. Also, PBMCs had a normal IL-10 response to RELA-independent LPS stimulation. These results suggest an NF- $\kappa$ B activation defect through RELA in fibroblasts and caspase 8 sensitivity to TNF (cell death response). Subsequently, they showed a decreased transcription of NF- $\kappa$ B activated genes in patients' fibroblasts, notably anti-apoptotic related genes and cytokines. Finally, they showed that *RELA*<sup>-/+</sup> mice featured severe cutaneous ulceration after TNF exposure, which was partially corrected either by infliximab treatment or when injecting *RELA*<sup>+/+</sup> bone marrow cells in the *RELA* haploinsufficient mice.

This mutation does not generate a truncated protein, but a haploinsufficiency of RELA. The patients did not feature immunological defects. The authors suggest that RELA haploinsufficiency leads to cutaneous diseases in mice and humans.

### ***3.6.2 RELA in CD4 lymphoproliferative disease with autoimmune cytopenias***

This second study by William Comrie and Michael Lenardo was named "*RELA haploinsufficiency in CD4 lymphoproliferative disease with autoimmune cytopenias*" and accepted in Journal of Allergy and Clinical Immunology the 10<sup>th</sup> of November 2017 (160). They reported a pediatric case of autoimmune lymphoproliferative syndrome (ALPS) with refractory immune thrombocytopenic purpura (ITP), anemia, neutropenia and splenomegaly, with mild lymphadenopathy. The patient did not feature abnormal levels of auto-antibody. They hypothesized that an augmented proportion of IFN $\gamma$ -producing effector T cell largely drove the disease. By WES, they determined that the patient carried a heterozygous nonsense *de novo* mutation R246X, with an observed a nonsense-mediated decay of RNA and a haploinsufficiency.

This mutation does not lead neither to a truncated protein, but a RELA haploinsufficiency. Interestingly, the patients had cutaneous defects. Hence, the authors explain these contrasting clinical manifestations between their study and Badran's findings as due to genetic and/or environmental context as key modifying factors.

## **3.7 Overview on whole exome sequencing and identification of deleterious single nucleotide variant detections**

### ***3.7.1 Concepts***

Giant leaps have been made since the discovery of the structure of the DNA molecule in 1953 (161), to the achievement of the human genome project in 2003 and the use of next generation sequencing technologies for clinical diagnosis (162). Although DNA sequencing has had a considerable reduction of cost and dramatic improvement in terms of rapidity and precision, the necessity to develop new tools for genetic diagnosis has become inexorable. Indeed, sequencing 3.2 billion base pairs of the human genome in the beginning of the decade was not compatible with quick identification of rare genetic variants. Thus, the advent of next-generation sequencing (NGS, also known as massive parallel sequencing) generated numerous high-throughput technologies that enhanced the search of genetic etiologies in human cohorts (163). NGS is based on a high-throughput parallelized sequencing process, contrary to chain-terminator sequencing of single reactions (Sanger sequencing). Since less than 1.5 percent of the human genome corresponds to the coding sequence of proteins, the strategy to use high-throughput sequencing solely on exons revealed to be a decisive strategy. Detecting rare genetic variants in the exome could elucidate the vast majority of unexplained inherited disorders (164). Ideally, access to the analysis of the whole human genome is evidently drastically consequential and exhaustive to identify deleterious genetic variants in both coding

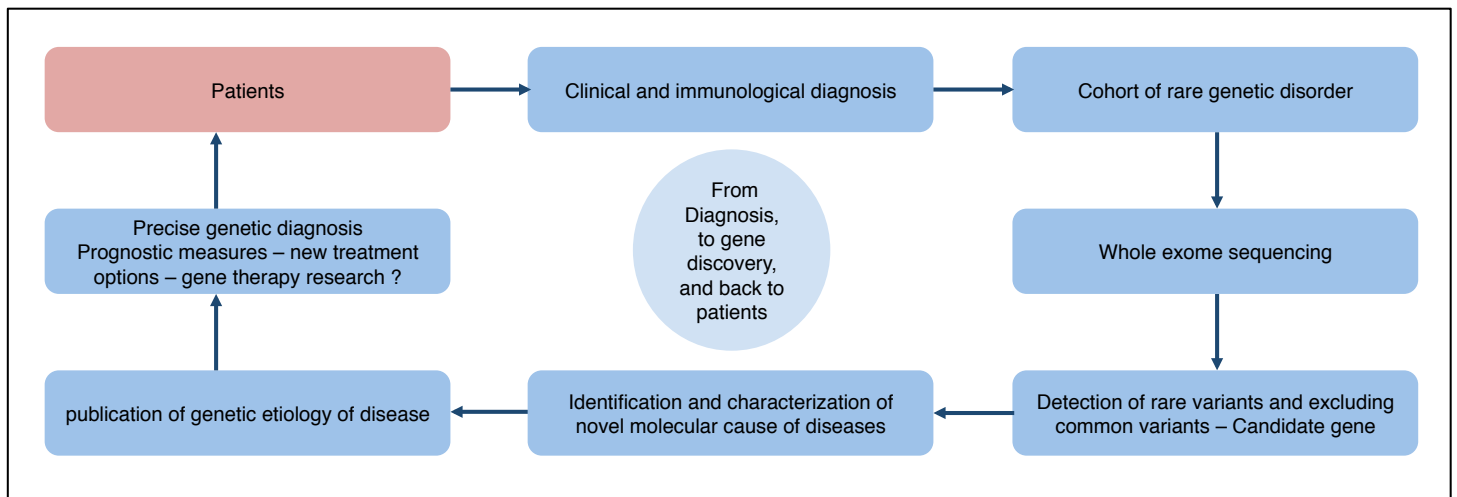
and non-coding. A recent study demonstrated that whole-genome sequencing (WGS) can potentially detect around 3% more significant SNVs in exomes than whole-exome sequencing (WES) (165). However, the use of WGS for identification of disease-causing genetic variants generates a colossal amount of data, making the data approach to identify new rare genetics variants significantly complex. Additionally, WGS methods are still very expensive to use on cohorts. Therefore, WES is a robust step until the improvement of WGS cost-efficiency as a diagnosis tool (166).

By the end of 2009 and beginning 2010, the two first mendelian disorders were identified by using WES (167, 168). Soon after, the use WES has been extended to other primary diseases. The increase and pooling of sequenced exomes data led to identifying more and more single nucleotide variants (SNVs) as disease-causing rare genetic variants. Consequently, the development of robust statistical metrics was needed to distinguish false positives from true positives and to clarify causative heterogeneity of phenotypes. Nevertheless, establishing proof of concepts by functional studies and animal and cellular models remains unavoidable in order to validate candidate genes. These revolutions have paved the way for the use of whole-exome sequencing to identify genetic causes of rare inherited diseases.

### ***3.7.2 WES and search for a candidate gene***

WES is based on target-enrichment by using exome capture technics. First technic to be developed is the array-based where DNA samples are fragmented, hybridized to high-density microarrays then amplified and sequenced. Time and genomic in-depth is limited by the use of arrays. Second technic is the solution-based that differs from array-based by the use of biotinylated oligonucleotide probes for selecting target regions (exons). The solution-based is the main exon-capture technology used nowadays, although it has been shown that array-based is efficient in GC rich regions (169). After exon-capture, sequencing is performed by NGS

instruments (such as Roche 454 sequencer, Life Technologies SOLiD systems or Ion Torrent, and Illumina's Illumina Genome Analyzer or Illumina MiSeq, HiSeq, and NovaSeq series). Collected data are studied by bioinformatic platforms who use algorithms for principale component analysis, homozygous rates and familial linkage (170). Finally, researchers exploit the data by the use of in-house virtual platforms for candidate gene selection.



**Figure 12. Identifying a molecular cause of a rare genetic disorder.**

Clinicians form as homogeneous possible cohorts based on clinical features. WES is performed on genomic DNA. Bioinformatics analysis allows detection of rare SNVs and consequently study of a candidate gene. Basic research is performed to associate the candidate gene to the disease. Identification and characterization of the molecular cause allows better future diagnosis, prognosis, and future treatment research.

The establishment of phenotypically homogeneous cohorts can seem an obvious basis for a candidate gene approach. Still, polygenic diseases increase significantly the complexity of finding potential causative SNVs (171). Particularly in CVIDs and SLEs, epistasis and environmental factors can mislead the designation of candidate genes. The first bias is the constitution of the cohort based on size and genetic complex traits. Once the cohort constituted and the WES performed, the data is pooled and analyzed by an in-house virtual platform.

In exons, several types of variants can occur. Nonsense mutations are a premature termination of the amino-acid sequence occurring at a very low frequency

and predicted damageable. Missense mutations occur at a low frequency and lead to a substitution of an amino acid on the protein sequence, potentially very damageable if the new amino-acid has different chemical properties. Insertion/deletions occur at a low frequency and lead to a frameshift in the exon with a very high risk on protein integrity. Thereby, variants can be related to phenotypes according to their nature, frequencies in the genome and impact prediction on protein integrity. Thus, a SNV of interest is vetted by searching in databases their unicity (e.g., IMAGINE institute in-house variant database, Exome Aggregation Consortium (ExAC), 1000 genome project databases, human genome variation society (HGVS), ClinVar, dbSNP, etc.).

Finding a gene candidate will depend on various strategies such as how the gene function is related to the biological defect or how severe is the SNV on the protein fate. A crucial step in the selection is the score given by functional-damage predictive algorithms, which calculates potential deleterious outcome of an SNV on the structure and function of a human protein, based on structural and evolutionary considerations, such as PolyPhen-2 (polymorphism phenotyping v2) (172), SIFT (sorting intolerant from tolerant) (173), CADD (combined annotation dependent depletion) (174) or PROVEAN (protein variation effect analyzer) (175).

Finally, the selected strategy (or combined strategies) greatly depend on the disease and on the considered gene (176). Nevertheless, the main vulnerability of a candidate gene approach remains to exclude a false negative or to work on a false positive candidate gene. Through the course of my PhD work, I have studied multiple candidate genes, which some were false positives. Others had to be aborted due to the limits of the available functional tests and cellular models in order to demonstrate the deleterious character of those SNVs. Also, we may have excluded some false negative candidate genes. Unfortunately, these are the risks of gene prioritization.



## 4 Aim of the project

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Throughout my doctoral studies, I have worked on the analysis and characterization of several PID patients. In the course of my third year, we characterized a family with a complex CVID history and we identified in one of the patients *RELA* as a candidate gene. By the end of my third year, I initiated collaboration with the laboratory of immunogenetics of pediatric autoimmune diseases headed by Frédéric Rieux-Laucat within our institute who characterized the clinical phenotype of several cases of SLE and identified *RELA* as a possible candidate gene. Thus, I extended my research by adding to my ongoing project two SLE families, which had different *RELA* mutations as potential candidate genes. We aimed to understand the impact of the mutations on the protein expression. Then, we worked to investigate if these mutant proteins are still able to fulfill their role in the NF- $\kappa$ B pathway as nuclear transcription factors by studying their cellular localization and their transcriptional activity. Because *RELA* works as a dimer and interacts with several proteins, I investigated how the mutations can affect *RELA* protein-protein partnering. This PhD work brings evidence that truncated forms of *RELA* and nonsense mutations on *RELA* can be associated to distinct clinical manifestations, specifically here CVID and SLE. During the course of our study on *RELA* as a candidate gene, two distinct publications associated different *RELA* mutations to skin disorders and autoimmune lymphoproliferative disorders. Therefore, I will discuss the potential molecular scenarios due to *RELA* defect, as well as the broad different phenotypes that the defects can generate.

## 5 Results

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### 5.1 Investigating RELA mutations

#### *5.1.1 Patients clinical and immunological features*

##### *CVID patients*

The patient of interest is F3-II-2 and belongs to a family with a strong history of immunodeficiency with hypogammaglobulinemia. Although we do not have immunological data from the father (F3-I-1), physicians have reported a history of IgA deficiency with recurrent infections, diarrheas and adult asthma. Also reported are a paternal uncle and a deceased paternal grandmother with the same kind of recurrent infection. The mother F3-II-1 is reported as healthy with no history of recurrent infections or diseases. The patient F3-II-2 has one healthy sibling and two siblings with CVID. The CVID of the two other siblings differs from the one of F3-II-2. Indeed, F3-II-3 and F3-II-4 have less recurrent infections. Both siblings presented with normal count of T cells and B cells. F3-II-3 has an IgA deficiency, not treated with Ig replacements and extremely low proportion of class-switched memory B cells. F3-II-3 is under IgG replacement, low IgA, episodes of upper respiratory tract infections and extremely low proportion of class-switched memory B cells. The main defect in these two siblings is the lack of switched memory B cells and a high proportion of naïve B cells, hence their hypogammaglobulinemia.

On the clinical part (**table 3**), F3-II-2 is a female born in 1986 with no developmental disorder. Her first infection episode started at first week of life with otitis and bronchitis, which were recurrent during her first year of life. Physicians then reported eczema (1-year-old), lymphocytic meningitis (2-year-old), persistent upper tract infections (4-year-old) and hemophilus meningitis (8-year-old), which lead to the detection of a hypogammaglobulinemia (low IgA and IgG2). Since then, she has been on immunoglobulin replacements (IgG) and constant antibiotic treatment. Growth from childhood to adulthood was reported normal. At age 16, low neutropenia at several time points have been reported, physicians suggesting a possible

autoimmune etiology (autoimmune cytopenia). Still from age 16, she has been diagnosed with recurrent mild splenomegaly (still perceivable at age 32) and recurrent conjunctivitis. Long term antibiotic treatments and IgG replacement have been used until present time.

On the immunological part (**table 3**), F3-II-2 had her B cell count (CD19+) decreased since 8 years of age until almost depletion of her B cells once she reached adulthood. Her last evaluation at 32 years of age showed 7 B cells/ $\mu$ l. The B cell count was so low that we could not detect memory B cells. Still, the major proportion of B cells were transitional B cells (53.3%). Former laboratory investigation reported that her B cells responded BAFF stimulation (activator of the alternative NF- $\kappa$ B pathway). The patient slowly deteriorated from a hypogammaglobulinemia to an agammaglobulinemia (still with IgG replacement). Indeed, she had no IgA or IgM in her blood at age 32. T cells have fluctuated between normal count and fairly below minimal threshold. At the last evaluation, she was under the minimal threshold in total CD3+ T cells (820 cells/ $\mu$ l), in CD4+ T cells (367 cells/ $\mu$ l), in CD8+ T cells (350 cells/ $\mu$ l). Also, the proportions of her subset were severely disturbed with low proportions for naïve CD4+ T cells (11%), naïve CD8+ T cells (9%) and central memory CD8+ (3%). The major proportion of T cells was effector memory CD8+ T cells (59%), which is abnormally very high. The patient also displayed a NK lymphopenia (17 cells/ $\mu$ l). Additionally, no cell response was observed when PBMCs were stimulated with CpG DNA (TLR activator, canonical NF- $\kappa$ B pathway).

Clinical features		
	F3-II-2 (31-years-old)	
Respiratory features	Chronic URT and LRT infections	
Lymphoproliferation	Persisting splenomegaly	
Autoimmunity	Eczema, possible autoimmune neutropenia	
Viral infection	Lymphocytic meningitis Haemophilus meningitis	
Immunological features		
	F3-II-2 (31-years-old)	Control values (age matched)
IgG g/L (age in yr)	Replacement	5.3–10.1

IgA g/L (age in yr)	0	0.34–0.78
IgM g/L (age in yr)	0	0.54–1.06
Immune phenotype at last evaluation		
	F3-II-2 (31-years-old)	Control values (age matched)
T cells/ $\mu$ l	820	1000 - 2200
CD4 <sup>+</sup> T cells/ $\mu$ l	367	530 - 1300
CD8 <sup>+</sup> T cells/ $\mu$ l	350	330 - 920
Naïve CD4 <sup>+</sup> (%)	11	43 - 55
Naïve CD8 <sup>+</sup> (%)	9	52 - 68
Central Memory CD8 <sup>+</sup> (%)	3	3 - 4
Effector Memory CD8 <sup>+</sup> (%)	59	11 - 20
TEMRA CD8 <sup>+</sup> (%)	29	16 - 28
B cells/ $\mu$ l	7	110 - 570
Transitional B cells (%)	53.3	
Memory B cells (%)	ND	12.6 - 25.2
Class-switched Memory B cells (%)	ND	6,5 - 14.2
Natural killer cells	17	70-480

**Table 3. CVID patient F3-II-2 clinical and immunological features.**

URT= upper respiratory tract; LRT= lower respiratory tract; naïve CD4<sup>+</sup> T cells= CD31+ CD45RA+/CD4<sup>+</sup>; naïve CD8<sup>+</sup> T cells= CCR7+ CD45RA+ /CD8<sup>+</sup>; transitional B cells= CD21+ CD24++/CD19<sup>+</sup>; memory B cells= CD27+ /CD19<sup>+</sup>; class-switched memory B cells= IgM- IgD- /CD27+ CD19<sup>+</sup>; natural killer cells= CD16+ CD56<sup>+</sup>; ND= not determined.

F3-II-2 CVID differs clinically and immunologically from the rest of the family CVID. Moreover, we were unable to determine a significant SNV common to the three CVID siblings. Our attention was driven by a *RELA* mutation. We detected *RELA*<sup>WT/Y306X</sup> mutation in F3-II-2 daughter (F3-III-2, 2-years-old) and not in her son. The daughter was diagnosed only with atonic eczema and does not have infections. Unfortunately, we have very few medical data on F3-III-2. Also, were not able to sequence F3-III-1 DNA and we have no medical data on him.

### **SLE Patients**

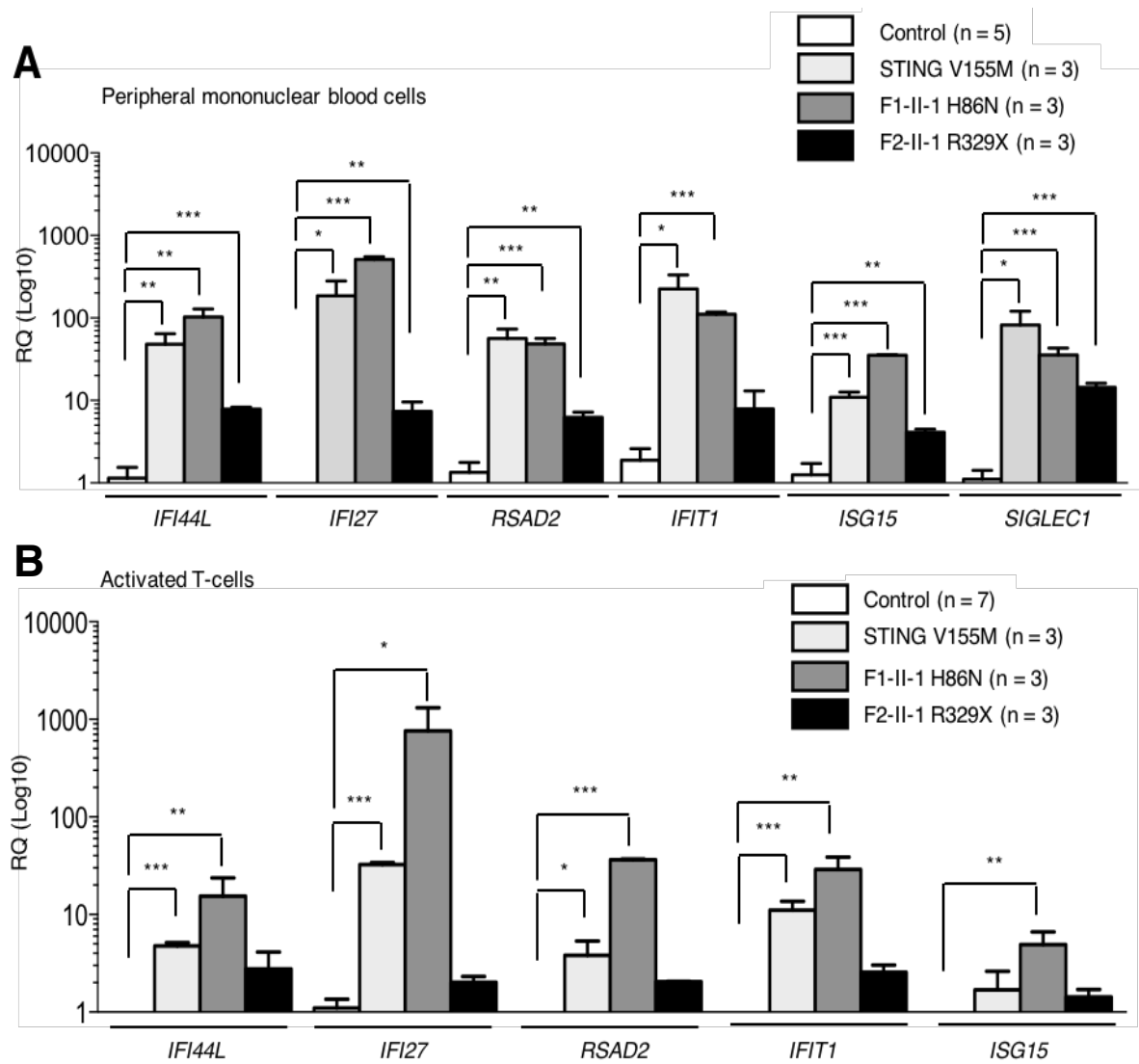
Family 1 has two North-African patients with SLE, F1-I-2 (mother) and F1-II-2 (daughter). The parents (F1-I-1 and F1-I-2) originate from the same north-African village, with no consanguinity to our knowledge. The mother's SLE onset was at age

33, as she manifested as severe non-erosive polyarthritis, pericarditis and class IV nephritis associated with positive antinuclear antibodies (ANA) (anti-double-stranded DNA). For complement component values in the blood, she featured normal C3 and low C4. At 48 years of age, F1-I-2 still did not have cutaneous manifestation aside non-scarring alopecia. The daughter (F1-II-2) presented a much severe and early SLE at age of 1 year and half old. F1-II-2 manifested malar rash, oral ulcers, polyarthralgia, alopecia and recurrent fevers. Blood analyses revealed normal C3 values and low C4 values, increased levels of antinuclear antibodies (anti-double-stranded DNA, anti-SSa, and anti-SSb) and rheumatoid factor. At 12 years of age, she developed severe skin ulcers of toes, fingers and ears. All attempts at decreasing corticosteroids were associated with severe cutaneous flares and fever, despite the association of mycophenolate mofetyl or rituximab. At 18 years of age, corticosteroids were still required. The patient developed a prolonged hypogammaglobulinemia and B cell lymphopenia after rituximab infusion. Before treatment the patient's globulin level was elevated (IgG: 19 g/L, IgA: 2.26 g/L and IgM: 2.53 g/L). The IgG levels dropped to 5g/L and the IgM were undetectable 15 months, and 3 years after initiation of treatment. Of note, the IgA level remained within normal values. The use of rituximab has caused controversy in regards of the recent results of its use on SLE patients (177, 178). A month after the first rituximab delivery, CD19+ B cells were undetectable, and monitoring showed that the B cell pool is still absent three years after first delivery. Consequently, F1-II-2 was administrated under immunoglobulin replacement. Despite the treatment, she manifested chronic suppurative otitis associated to *Hemophilus Influenzae* infections.

Family 2 is French European, with both parents being healthy and non-related. At the age of 9, the son (F2-II-1) presented a lupus rash, oral and skin ulcers, polyarthritis, recurrent fever, class IV lupus nephritis, and pericarditis. Lupus anticoagulant, anti-2- $\beta$ -glycoprotein1 and anti-double-stranded DNA was detected in the blood. The patient had normal C3 and C4 values. He was then treated with hydroxychloroquine in combination with a low-dose prednisone (10 mg/d). Afterward, he suffered of type IV lupus glomerulonephritis and pericarditis. At age of

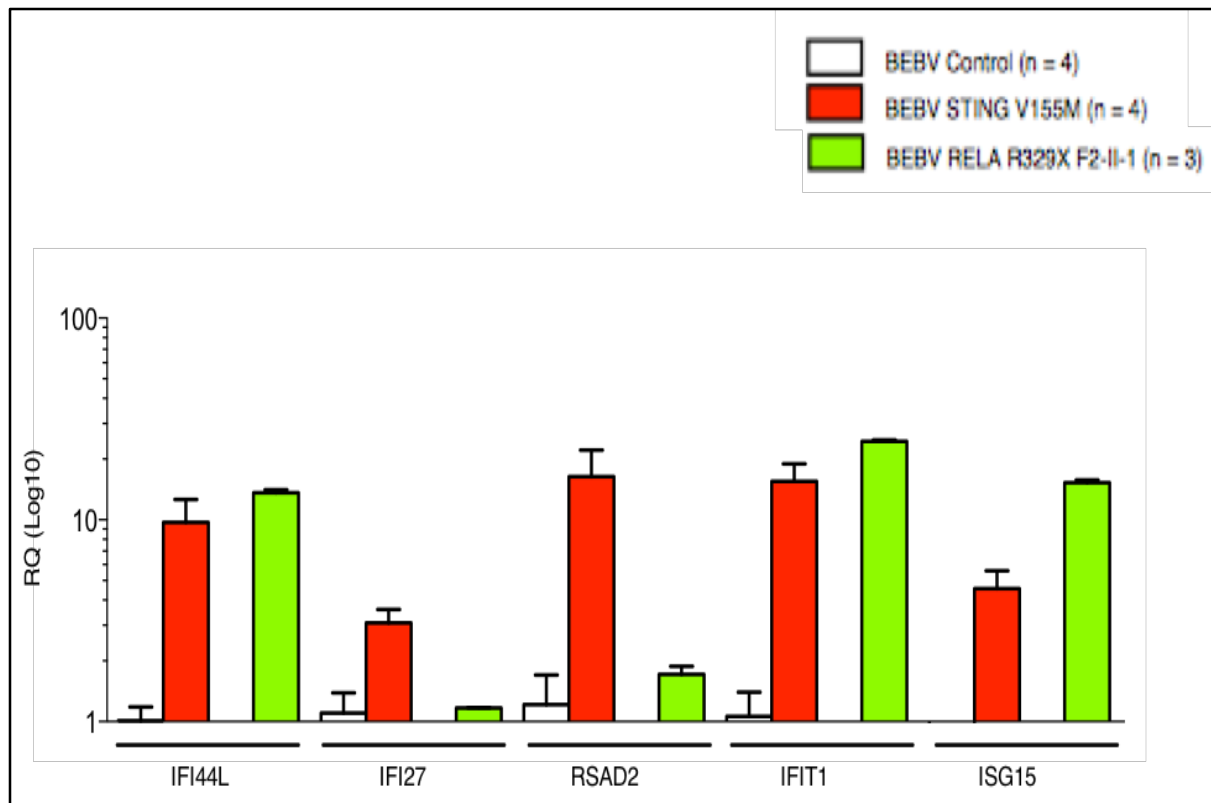
17, the patient was in clinical and biological remission with mycophenolate mofetil treatment.

SLE is an autoimmune-based disease, which has been associated to type-I IFN production (179, 180). Consequently, the study of an interferon-stimulated genes (ISG) signature by quantitative PCR has been performed by using previously published primers IFI44L, IFI27, RSAD2, IFIT1, ISG15 and SIGLEC1 (181). The mRNA used was extracted either from PBMCs or Epstein-Barr virus EBV-immortalized B cell lines (EBV-B cells). Healthy donors were used as negative controls because they do not have an ISG signature. Whereas STING patients (carrying an activating V155M TMEM173/STING mutation (182, 183)) cells were used as positive control because they feature a strong production of type-I IFN and high ISG signature. In PBMCs (**figure 13A**), patient F1-II-1 and patient F2-II-1 featured a significantly increased ISG signature compared to the healthy donor. Also, F1-II-1 has a fairly comparable ISG signature to the STING patient high control (between 50-fold and 110-fold increase), which is consistent with her severe SLE clinical features. We observed in T cell blasts an ISG signature, which was significant in F1-II-1 (above 10-fold increase for IFI44L, IFI27, RSAD2, IFIT1 and ISG15) (**figure 13B**). As ISG signature on PBMCs can be biased by an ongoing infection at the time of the blood sample collection, EBV-B cells were tested from F2-II-1. Therefore, the ISG signature was confirmed significantly increased for three genes (above 10-fold increase for IF44L, IFIT1 and ISG15) (**figure 14**).



**Figure 13: Increased Interferon Stimulated Genes expression in PBMCs and T cells from SLE patients.**

Quantitative PCR analysis of ISG mRNA expression (**A**) in peripheral blood mononuclear cells (PBMC) and (**B**) in activated T-cells derived from PBMC. Primers IFI44L, IFI27, RSAD2, IFIT1, ISG15 and SIGLEC1 were used as ISG signature marker. Student's t-test, \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$  mean  $\pm$  s.e.m



**Figure 14: Increased Interferon Stimulated Genes expression in B-EBV cells from SLE patient.**

Representative quantitative RT-PCR analysis results of IFN type I signatures in EBV+ B cell-lines derived from primary B-lymphocytes immortalized by Epstein-Barr virus. Primers IFI44L, IFI27, RSAD2, IFIT1 and ISG15 were used as ISG signature marker.

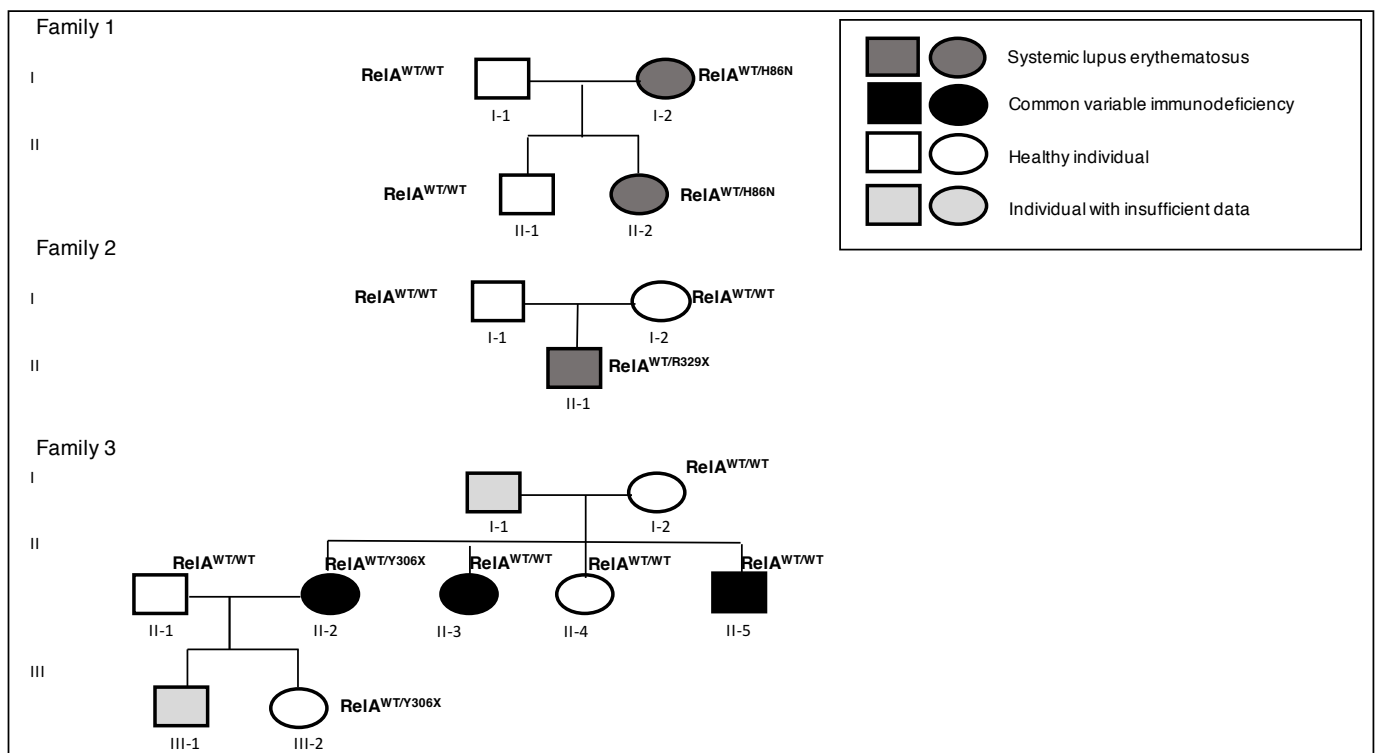
### 5.1.2 WES of CVID and SLE patients

The study is based on three distinct families (**figure 15**). CVID patients were brought to our attention due to a strong familial history of hypogammaglobulinemia. SLE patients were part of a cohort studied by the laboratory of immunogenetics of pediatric autoimmune diseases in our institute. Genomic DNA was extracted from total blood samples and used for after exome-capture. Then, Illumina WES was performed to identify the underlying deleterious SNV causing the diseases. Data were analyzed with Polyweb, in-house software for variant selection. We detected in F3-II-2 and F3-III-2 a heterozygous T to G nonsense variation at cDNA position 918 (GRCh37; NM\_021975.3 c.918 T>G) of the gene *RELA* (exon9), with a predicted stop instead of the tyrosine at the amino acid position 306 *RELA*<sup>WT/Y306X</sup> (RHD of *RELA* ends at 304). In the DNA from F1-I-2 and F1-II-2, our collaborators detected a



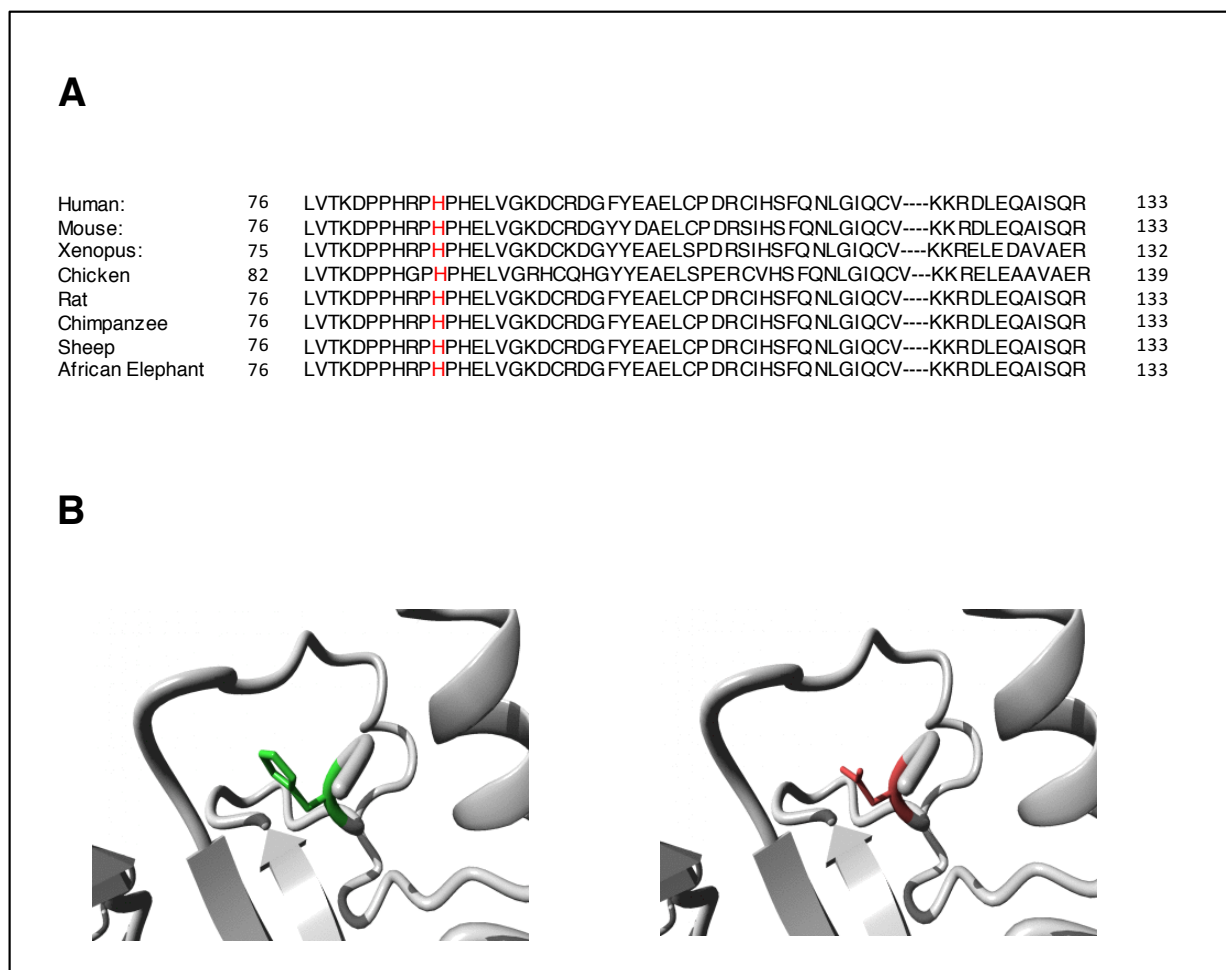
heterozygous C to A variation at cDNA position 256 (GRCh37; NM\_021975.3 C.256 C>A) in exon 4 of *RELA*, predicted highly damageable by PolyPhen (0.997 on scale 0 to 1) and a CADD score of 28.3. The SNV led to the substitution of a histidine to an asparagine at the amino acid position 86 *RELA*<sup>WT/H86N</sup> located in the RHD of *RELA*. Interestingly, the histidine residue at the position 86 is evolutionarily highly conserved (**figure 16**). They also detected a *de novo* heterozygous C to T nonsense variation at cDNA position 985 (GRCh37; NM\_021975.3 c.985 C>T) the *RELA* gene (exon 10) in F2-II-1, with a predicted stop instead of the arginine at the amino acid position 329 *RELA*<sup>WT/R329X</sup>. Sanger sequencing confirmed SNV of interest in genomic DNA of all patients and its absence in healthy family members and healthy donors.

At these genomic positions, no annotations of the discovered SNVs were contained in our in-house variant database (Polyweb) or in the exome sequencing project (ESP) and exome aggregation consortium (ExAC) databases. Finally, all three mutations we detected in *RELA* were predicted very damageable by SIFT, PolyPhen and CADD algorithm.



**Figure 15. Pedigree of all three studied families.**

Family 1 and Family 2 contain SLE patients. Family 3 presents with a strong history of CVID. Roman numbers on the left represent generation. Squares represent male patients; circles represent female patients.



**Figure 16: Evolutionary conservation analysis of the RHD of RELA protein at the histidine substitution position and protein crystallography of RELA p.H86N mutation.**

Alignment shows that the histidine residue at the amino-acid substitution at the Rel-homology domain (red H) is highly evolutionarily conserved among vertebrates (A). RELA<sup>WT</sup> 3D protein structure (green) and mutant RELA p.H86N 3D protein structure (red) (B). Natural and mutant residues differ in hydrogen bond linking.

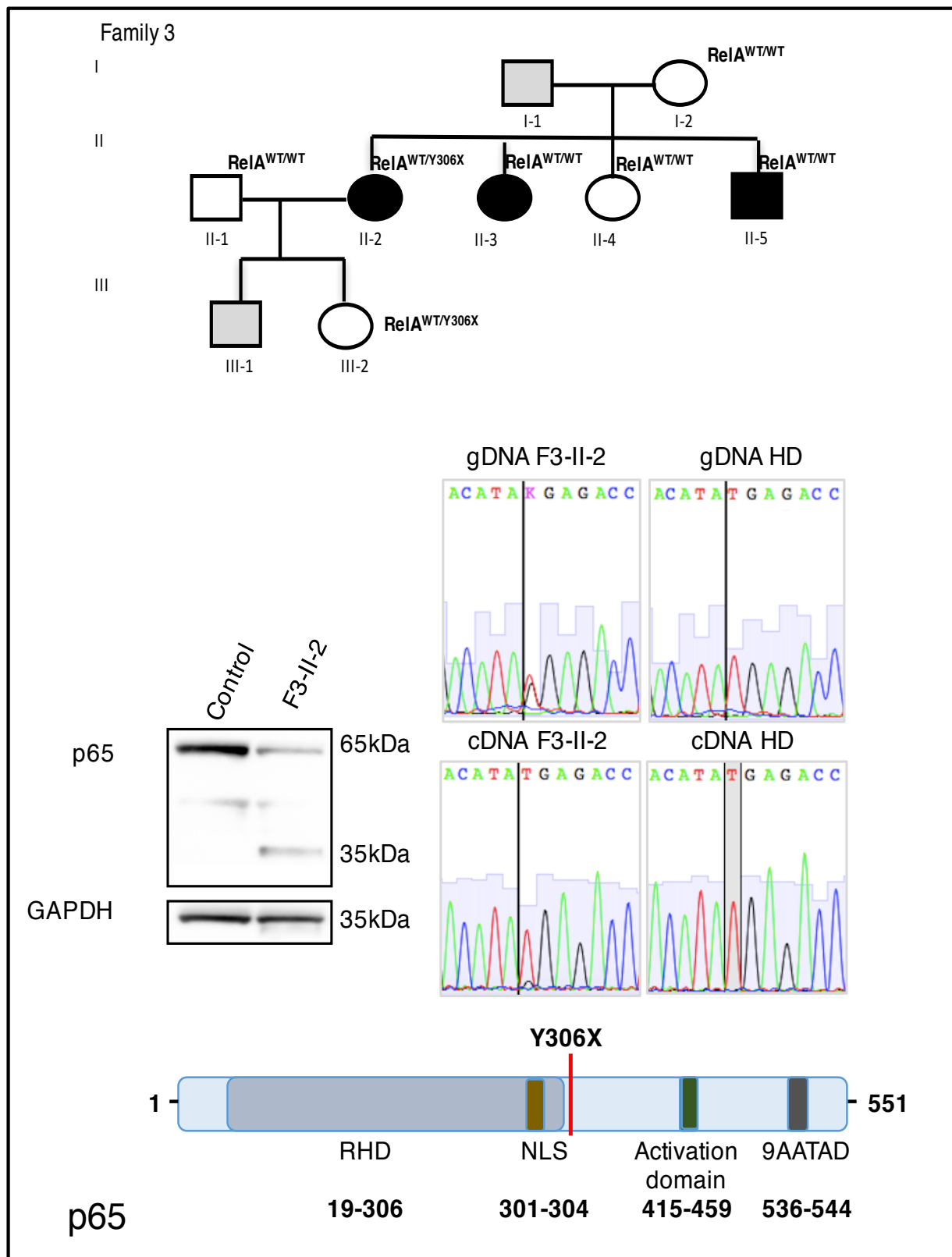
### 5.1.3 Mutated *RELA* expression

To assess the expression of the *RELA*<sup>Y306X</sup>, *RELA*<sup>H86N</sup> and *RELA*<sup>R329X</sup> mutations, we extracted mRNA from activated T-cells derived from PBMCs of the patients. We extracted the mRNA from the cells and performed reverse transcription to obtain cDNA. Finally, we performed Sanger-sequencing on the patient and healthy control's cDNA.

In order to study the mutations at the protein level, we performed immunoblotting assays on protein extract from the activated T-cells lysates derived from PBMCs. We detected *RELA* by using an anti-p65 antibody directed to the N-terminal part of the protein.

#### ***CVID patient***

At the cDNA level of F3-II-2, we confirmed the substitution T to G at position 918 of *RELA* and we noticed a weak signal in sequencing chromatogram of the mutated allele compared to chromatogram of the wild-type allele, which suggests a nonsense-mediated decay of the mRNA carrying the mutation (**figure 17**). At the protein level, we detected two distinct bands in the patient's protein extract. The band at 65-kDa is the expected band for the wild type form of *RELA* as in the control, and the expression seems half reduced compared to the control. Additionally, we observed an approximately 35-kDa band, which is the expected band for a protein synthesized with 306 amino acid residues. Despite nonsense-mediated mRNA decay of *RELA*<sup>Y306X</sup>, a truncated *RELA*<sup>Y306X</sup> protein is detectable in the patient's cells corresponding to solely the RHD domain alongside the wild type form. In the CVID patient cells with *RELA*<sup>WT/Y306X</sup>, there is an equivalent ratio of expression between the wild type form of *RELA* and the truncated form of *RELA*. The sum of intensity of both bands in the patient match the intensity of the control's *RELA* protein band.



**Figure 17:  $RELA^{WT/Y306X}$  mutation associated with common variable immunodeficiency.**

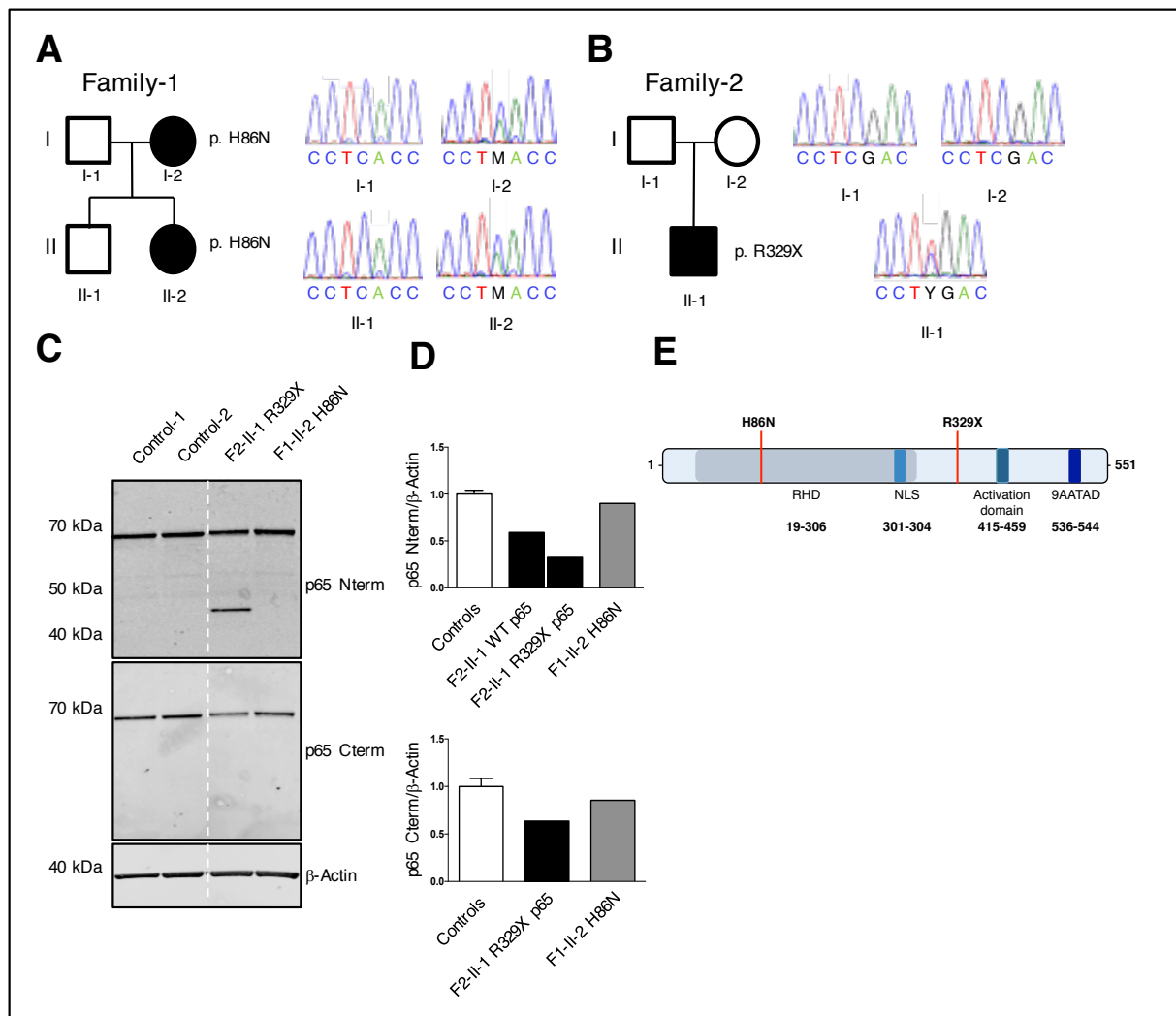
Pedigree of family 3 carrying the  $RELA$  p. Y306XN heterozygous mutation. Sequencing chromatogram of cDNA shows the heterozygous site mutation CDS 918 T > G leading to a premature stop codon TAG. Immunoblot representing N-terminal anti-p65 protein levels in lysates from activated T-cells derived from peripheral blood mononuclear cells (PBMCs) of F3-II-2 and healthy donor. Anti-GADPH

was used as internal control. RELA protein structure with indicated mutation position. RHD = REL-homology domain; NLS = Nuclear localization signal; 9AATAD = 9 amino acid transactivation domain.

### ***SLE Patients***

In F1-II-1 cDNA, we confirmed a heterozygous C to A variation at the position 256 of *RELA* (**figure 18A**). When immunoblotting protein extract from F1-II-1 cells, we could detect a 65-kDa band at the same size and signal intensity as the controls. *RELA*<sup>H86N</sup> protein seems stable in activated T cells (**figure 18C and 18D**).

In F2-II-1 cDNA, we observe a heterozygous C to T variation at the 985 position of *RELA* (**figure 18B**). We revealed in F2-II-1 protein extract a band at 65-kDa corresponding to the wild type form of RELA and an approximately 35-kDa band, which is the expected band for a protein synthesized with 329 amino acid residues (**figure 18C**). The wild-type band of the patients is at half the abundance of that of the control. Additionally, we do not detect the truncated form when using an anti-p65 antibody directed to the C-terminal part of RELA. Similarly, to *RELA*<sup>WT/Y306X</sup>, we demonstrated that in the SLE patient with *RELA*<sup>WT/R329X</sup> mutation, the mutation leads to the expression of a truncated form of RELA slightly larger in size (23 amino-acid) than *RELA*<sup>Y306X</sup> protein. There is fairly a 1:1 ratio of expression between the wild type form of RELA and the truncated form of *RELA*<sup>R329X</sup> (**figure 18D**).



**Figure 18: *RELA* mutations associated with systemic lupus erythematosus.**

(A) Pedigree of the family-1 carrying the *RELA* p. H86N heterozygous mutation. Sequencing chromatogram of cDNA showing the heterozygous site mutation CDS 398 C > A leading to a missense mutation CAC > AAC. (B) Pedigree of the family-2 carrying the *RELA* p. R329X heterozygous mutation. Sequencing chromatogram of cDNA showing the heterozygous site mutation CDS 985 C > T leading to a premature STOP codon TGA. (C) Immunoblot representing N-terminal and C-terminal p65 protein levels in lysates from activated T-cells derived from peripheral blood mononuclear cells (PBMCs) of probands and healthy relative controls. Anti-beta-actin was used as internal control (D) Quantification of p65 protein levels. Protein levels were normalized by beta-actin and expressed relative to the average of the two controls. (E) *RELA* protein structure with indicated mutations positions. RHD = REL-homology domain; NLS = Nuclear localization signal; 9AATAD = 9 amino acid transactivation domain.

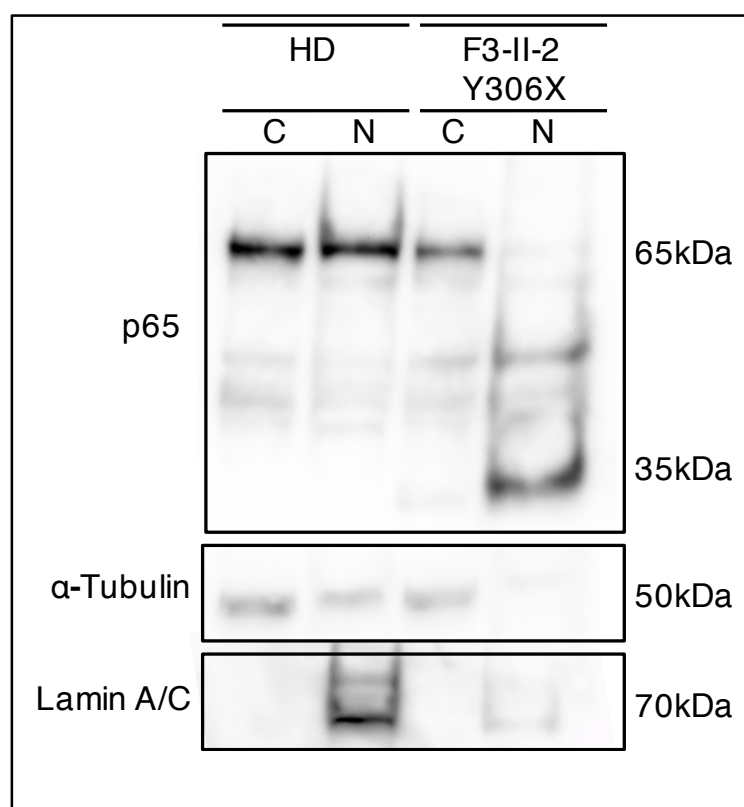
#### **5.1.4 Cellular localization of mutant RELA proteins**

RELA protein is mainly located in the cytoplasm when the canonical NF- $\kappa$ B pathway is inactive. After triggering of the pathway, RELA protein translocates in the nucleus. In order to evaluate the impact of the mutations on the RELA protein cellular localization, we performed immunoblotting on nuclear and cytoplasmic extracts of T cell blasts from patients. The T cell blasts were not stimulated (e.g., TNF- $\alpha$  or phorbol-12-myristate-13-acetate (PMA) with ionomycin), thus the NF- $\kappa$ B pathway is inactive.

Immunofluorescence assays were performed to further characterize cellular localization of the RELA protein. For F1-I-2 (H86N) and F2-II-1 (R329X), T cell blasts were stimulated or not with 15 or 30 minutes TNF- $\alpha$  or PMA-ionomycin. TNF- $\alpha$  triggers downstream signaling through TNFR, whereas PMA-ionomycin induces through TCR signaling. Next, the cells were fixated and stained using N-terminal anti-p65 antibody with a red fluorescent secondary antibody, C-terminal anti-p65 antibody with a green fluorescent antibody and DAPI (4'-6-diamidino-2-phenylindole) for nuclear DNA staining for confocal microscopy analysis. C-terminal anti-p65 antibody is used to bind full-length RELA proteins, thus not binding to truncated RELA proteins. Nuclear signal of N-terminal anti-p65 antibody was quantified.

#### ***CVID patient (RELA<sup>Y306X</sup>)***

In the immunoblotting of F3-II-2 unstimulated T cells protein extracts, we observed the expression of the truncated RELA<sup>Y306X</sup> protein mainly in the nuclear fraction in contrast to wild-type RELA protein, which was predominantly localized in the cytoplasm in patient cells (**figure 19**).



**Figure 19: RELA<sup>Y306X</sup> protein is detected mainly in the nuclear compartment of unstimulated T cells.**

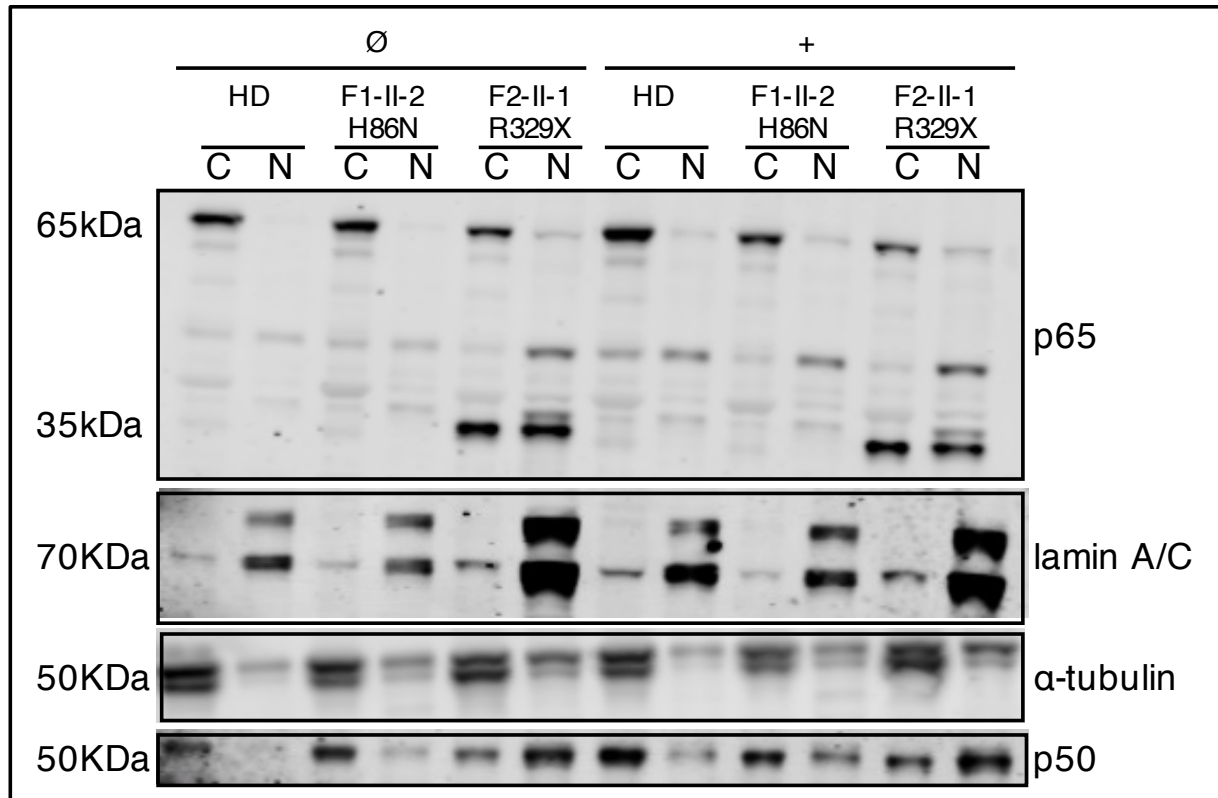
Immuno-blotting was performed on nuclear and cytoplasmic extracts from activated T-cells of F3-II-2 and healthy donor (HD). Protein expression was revealed by using anti-p65, anti-laminA/C (nuclear protein), anti-α-tubulin (cytoplasmic protein).

### ***SLE patients (RELA<sup>H86N</sup> and RELA<sup>R329X</sup>)***

In the immunoblotting of nuclear and cytoplasmic extracts of unstimulated T cell blasts (**figure 20**), we observed a comparable abundance of RELA protein in the cytoplasmic compartment between F1-II-2 and healthy donor and absence of presence of nuclear RELA protein in both conditions. Whereas in unstimulated F2-II-1 T cell blasts, we detected the truncated RELA<sup>R329X</sup> protein in both nuclear and cytoplasmic compartment. In stimulated T cell blasts, we detect wild-type RELA protein in the cytoplasm and a slight increase of wild-type RELA protein in the nucleus. To note, we were unable to distinguish between the mutant and wild-type form in F1-II-2. As for the truncated RELA<sup>R329X</sup> protein, we still detect it in both cytoplasmic and nuclear compartments. Furthermore, especially for the cell extract of



patient F1-II-2 we detected p50 in the cytoplasm and nucleus in unstimulated and activated T cells.



**Figure 20: RELA<sup>R329X</sup> protein in F1-II-2 and F2-II-1 is detected in both cytoplasmic and nuclear compartment in unstimulated and stimulated T cells.**

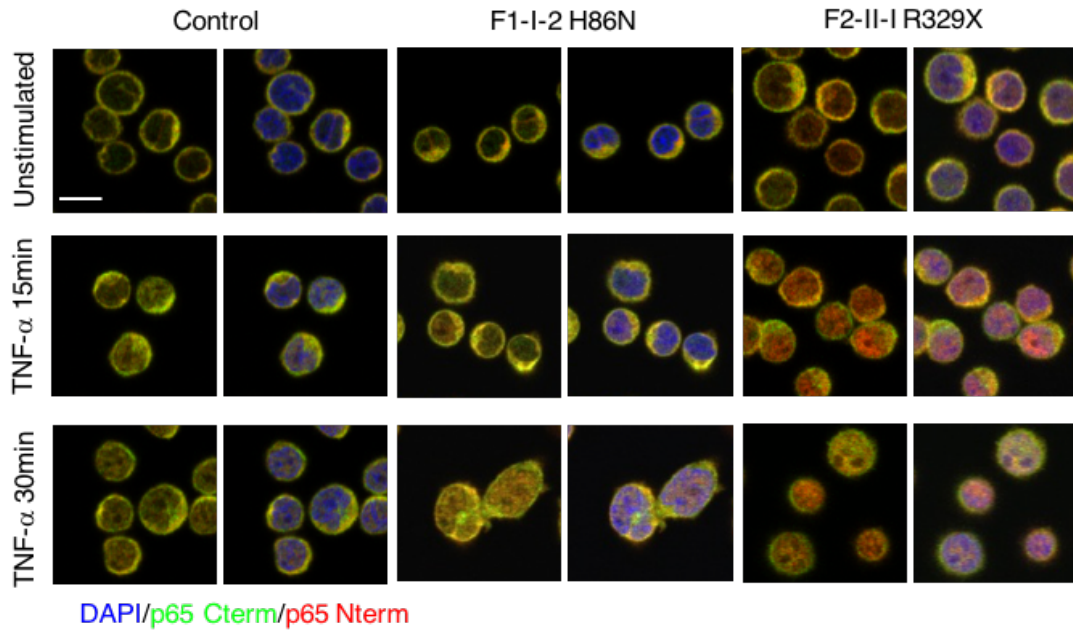
Immuno-blotting of nuclear and cytoplasmic extracts from activated T-cells of F1-II-2, F2-II-1 and healthy donor (HD) was performed with or without stimulation by 30 minutes of PMA-ionomycin. Protein expression was revealed by using anti-p65, anti-laminA/C (nuclear protein), anti-α-tubulin (cytoplasmic protein) and anti-p50 antibodies.

In the cellular immunofluorescence assay, the unstimulated condition of healthy donor, F1-1-2 and F2-II-1 revealed the co-localization of green fluorescence and red fluorescence in the cytoplasm of the cell (outer rim of a T cell), showing that RELA protein is mainly in the cytoplasm when the cell is in a steady state (**figure 21A**). Interestingly, there is a higher red signal in the nucleus of unstimulated patients' cells, especially in the T cells of F2-II-1 (R329X) (**figure 21B**). Knowing that a red signal indicates the presence of both wild-type and truncated form of RELA and that a green signal can originate only from the wild-type form of RELA, we confirmed

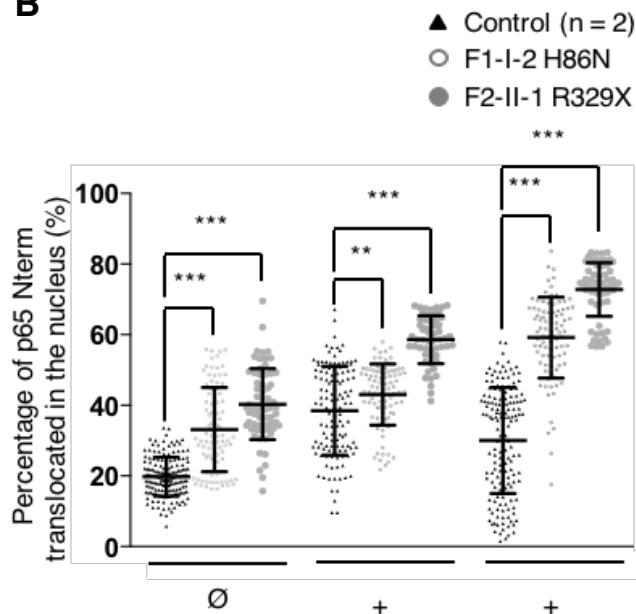
here the results of the immunoblotting assay where we detected the truncated form both in the cytoplasm and in the nucleus in unstimulated conditions.

After stimulation with TNF- $\alpha$ , all conditions featured red and green signal from both cytoplasm and nucleus after 15 minutes, confirming the translocation of RELA protein into the nucleus. To note, the maximum of protein entry in these condition was at 30 minutes. Whereas with PMA-ionocymmin stimulation, the strongest signal was detected at 15 minutes with a decrease after 30 minutes (**figure 22**). Also, F2-II-1 cells seem to have a slower decrease of the signal at 30 minutes PMA-ionomycin, suggesting a persistent presence of RELA proteins in the nucleus. In these conditions, we are not able to discriminate between RELA<sup>H86N</sup> and RELA<sup>WT</sup> proteins' translocation in the nucleus.

**A**



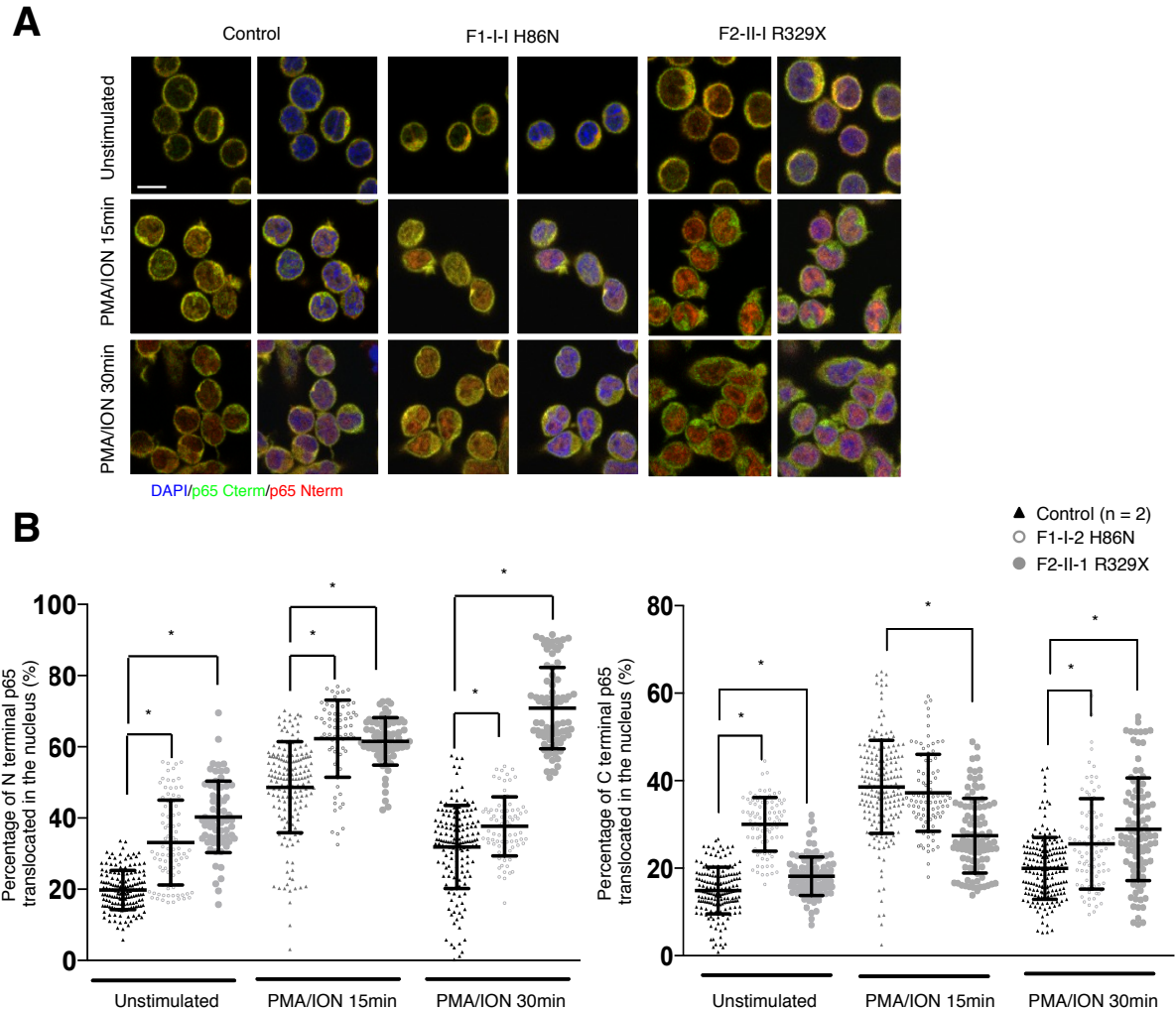
**B**



**Figure 21: Increased presence of RELA in the nuclear compartment of patients' T cell blasts with and without TNF- $\alpha$  stimulation.**

(A) Immunofluorescence of activated T-cells from F1-I-2 H86N, F2-II-1 R329X and healthy donor stimulated 15 or 30 minutes with TNF- $\alpha$  and analyzed by confocal microscopy using anti-N-term p65 (Red), anti-C-term p65 (Green) antibodies and DAPI (4'-6-diamidino-2-phenylindole). Scale bar =

5mm. (B) Quantification of signal from nuclear localization of p65. Statistical significance between conditions was evaluated using student's t-test, \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 mean  $\pm$  s.e.m



**Figure 22: Increased presence of RELA proteins in the nuclear compartment of patients' T cell blasts with and without PMA-ionomycin stimulation.**

(A) Immunofluorescence of activated T-cells from F1-I-2 H86N, F2-II-1 R329X and healthy donor stimulated 15 or 30 minutes with PMA-ionomycin and analyzed by confocal microscopy using anti-N-term p65 (Red), anti-C-term p65 (Green) antibodies and DAPI (4'-6-diamidino-2-phenylindole). Scale bar = 5mm. (B) Quantification of signal from nuclear localization of p65. Statistical significance between conditions was evaluated using student's t-test, \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 mean  $\pm$  s.e.m.

### **5.1.5 DNA binding of mutant *RELA* proteins**

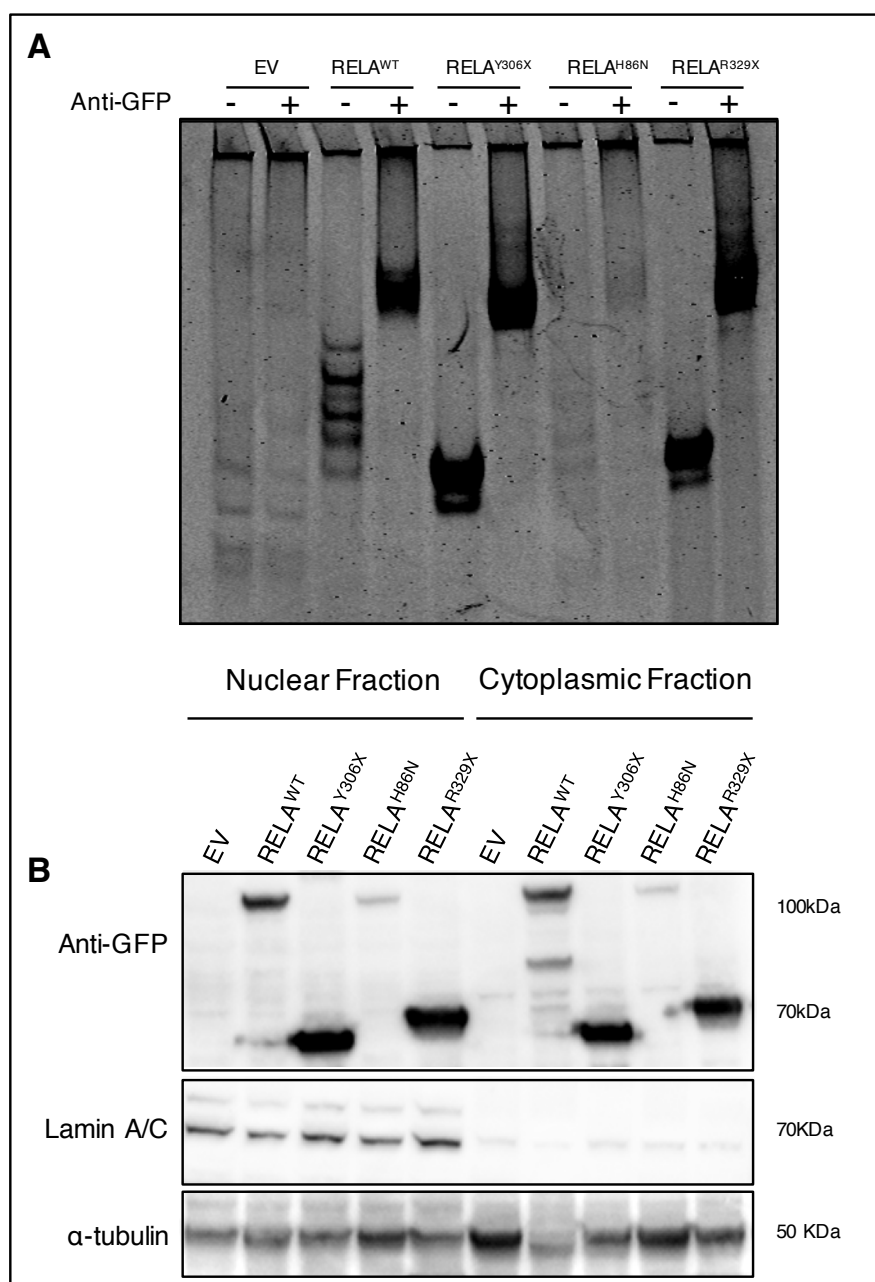
After observing that the mutant *RELA* proteins were able to translocate into the nucleus and because *RELA* binds to consensus sequences with their RHD domain, we investigated if the mutant *RELA* proteins can still bind to DNA. Indeed, the two truncated forms are mostly composed of the RHD, and the H86N mutation is a significant substitution at a highly conserved amino acid residue situated in the RHD. We used a fluorescent-tagged oligonucleotide NF- $\kappa$ B consensus sequences to perform an electrophoresis mobility shift assays (EMSA) on nuclear extracts. In order to assess the specificity of the protein-DNA complexes, we added to binding reaction an antibody targeting the *RELA* proteins in order to generate a super-shift. We first used HEK293T cell lines to test on over-expressed proteins, then we used patients and healthy donor's T cells blasts (with or without stimulation).

#### ***Ectopically expressed mutants***

Using constructs expressing wild type or mutant N-terminal GFP tagged *RELA*, we ectopically expressed *RELA* proteins in HEK293T cells and applied separation of the nuclear proteins and cytoplasmic proteins from the cell lysates. To produce super-shifts, we used an anti-GFP antibody.

Empty vector transfected cells feature a pattern of bands that corresponds to endogenous protein-DNA complexes, with no super-shift. In absence of anti-GFP antibody, Wild-type *GFP-RELA* HEK293T transfected cells featured several bands corresponding to shifts of protein-DNA complexes (**Figure 23-A**). In the presence of anti-GFP antibody, the aforementioned complexes super-shifted, indicating that GFP-*RELA* is one of the proteins in the observed proteins-DNA complexes. In HEK293T cells transfected with the truncated *RELA* expression, we observed two smaller bands. Then in the anti-GFP antibody condition, we observed a super-shift of both bands. These results indicate that the truncated protein is in the DNA-binding complex (super-shift). We observed faint bands of shift and a super-shift with *RELA*<sup>H86N</sup>, preventing us to formally conclude whether *RELA*<sup>H86N</sup> DNA binding properties are affected by the mutation.

To note, we performed the same experience with constructs expressing wild type or mutant N-terminal HA tagged RELA and using anti-HA antibody and we obtained similar results (data not shown).



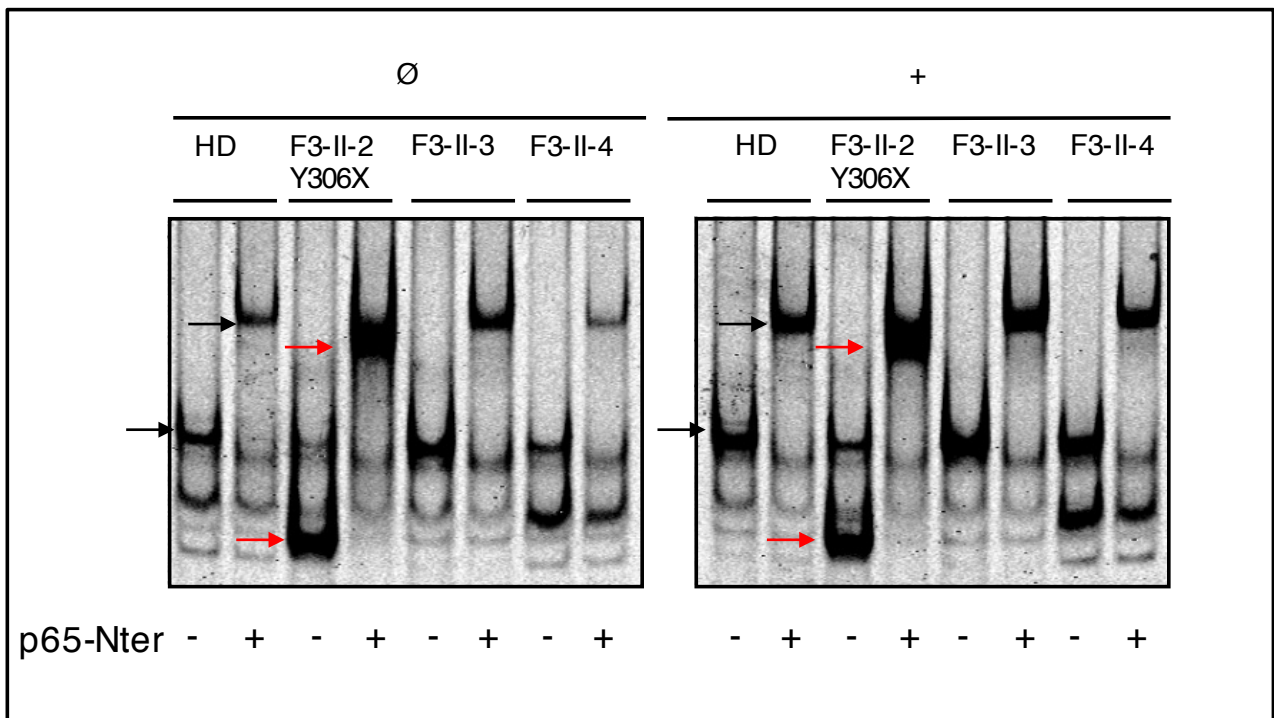
**Figure 23: Electrophoretic Mobility Super-shift assay of ectopically expressed RELA mutants.**

(A) Electrophoretic mobility shift assay using a luminescent labeled NF-κB consensus oligonucleotide for the shift and an anti-p65 antibody for the super shift was performed on nuclear extracts from HEK293T transfected with constructs expressing N-terminal HA tagged p65 (empty vector, wild-type or mutants indicated). EV= empty vector; WT= wild type. Anti-HA-p65, anti-LaminA/C and anti-α-tubulin antibodies were used to assess protein expression. (B) Immunoblotting of nuclear and cytoplasmic extracts from the same cells used in (A).

### ***CVID patient***

We investigated the RELA<sup>Y306X</sup> truncated protein-DNA binding by EMSA and super-shift on nuclear extracts of stimulated or unstimulated T cell blast. We observed comparable shifts and super-shifts between healthy donor and the siblings F3-II-3 and F3-II-4 (not carrying the mutation) (**figure 24**). Furthermore, the antibody specific to the N terminus of RELA super-shifted the bands, indicating that RELA proteins were part of the complexes bound on the DNA NF-κB consensus sequences. Also, the shifts and super-shifts of the bands migrated further in F3-II-2 than the controls, confirming that RELA<sup>Y306</sup> does bind to DNA and forms a lighter protein-DNA complex. In this experience, we already observed binding of RELA protein-DNA complexes in the unstimulated T cell blasts. The stimulation of T cell blast with PMA-ionomycin thus appeared to only slightly increase the abundance of RELA protein-DNA complexes. However, we reproduced the experiment on T cell blasts with or without OKT3 stimulation (**figure 25**). OKT3 is a monoclonal antibody that targets the CD3 co-receptor, subsequently triggering the TCR complex (184). We observed in the healthy control no shifts when not stimulated, and a shift if stimulated with 30 min of OKT3. Also, we used anti-p65-N-terminal antibody that induced a shift in the stimulated condition, or a anti-p65-C-terminal antibody that was unable to create a super-shift probably due to the unfavorable steric hinderance of the protein-DNA complex. Whereas the patient F3-II-2, we observe that she shifts and super-shifts in unstimulated and in stimulated conditions.

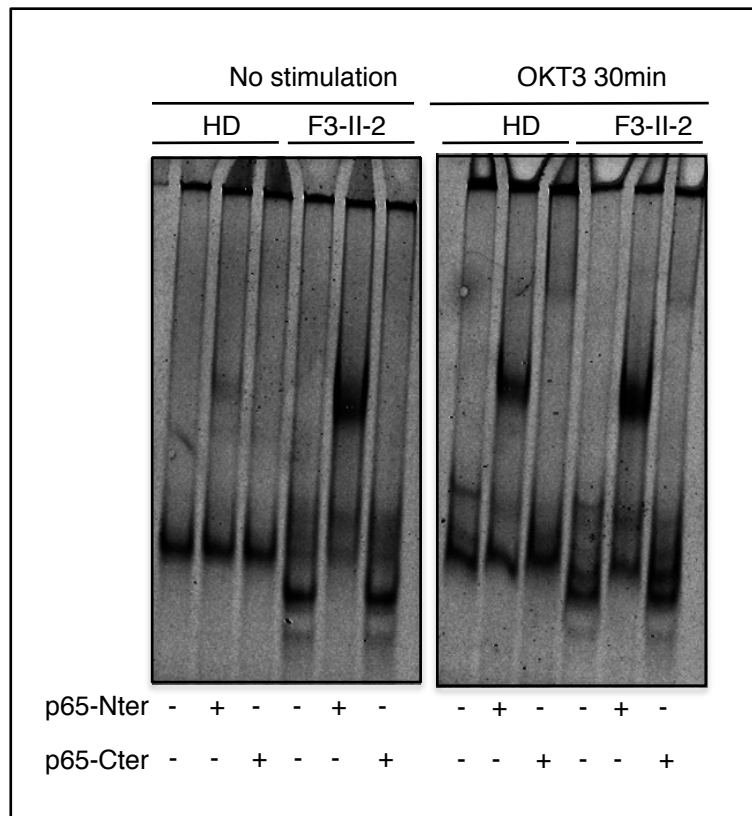
These results indicates that endogenous and ectopically expressed RELA<sup>Y306</sup> protein recognizes and binds NF-κB DNA target sequences.



**Figure 24: Nuclear RELA<sup>Y306</sup> binds to DNA NF-κB consensus sequence in patient cells stimulated with PMA-ionomycin.**

Electrophoretic mobility shift assay was performed on nuclear extracts from T cell blasts of healthy donor (HD), patient F3-II-2 and siblings F3-II-3 F3-II-4 using a luminescent-labeled NF-κB consensus oligonucleotide for the shift and an anti-p65 antibody for the super shift. T cell blasts were stimulated or not with 30 minutes PMA-ionomycin. Black arrow indicates a band formed by wild type RELA, whereas red arrow indicates the band formed by truncated RELA.





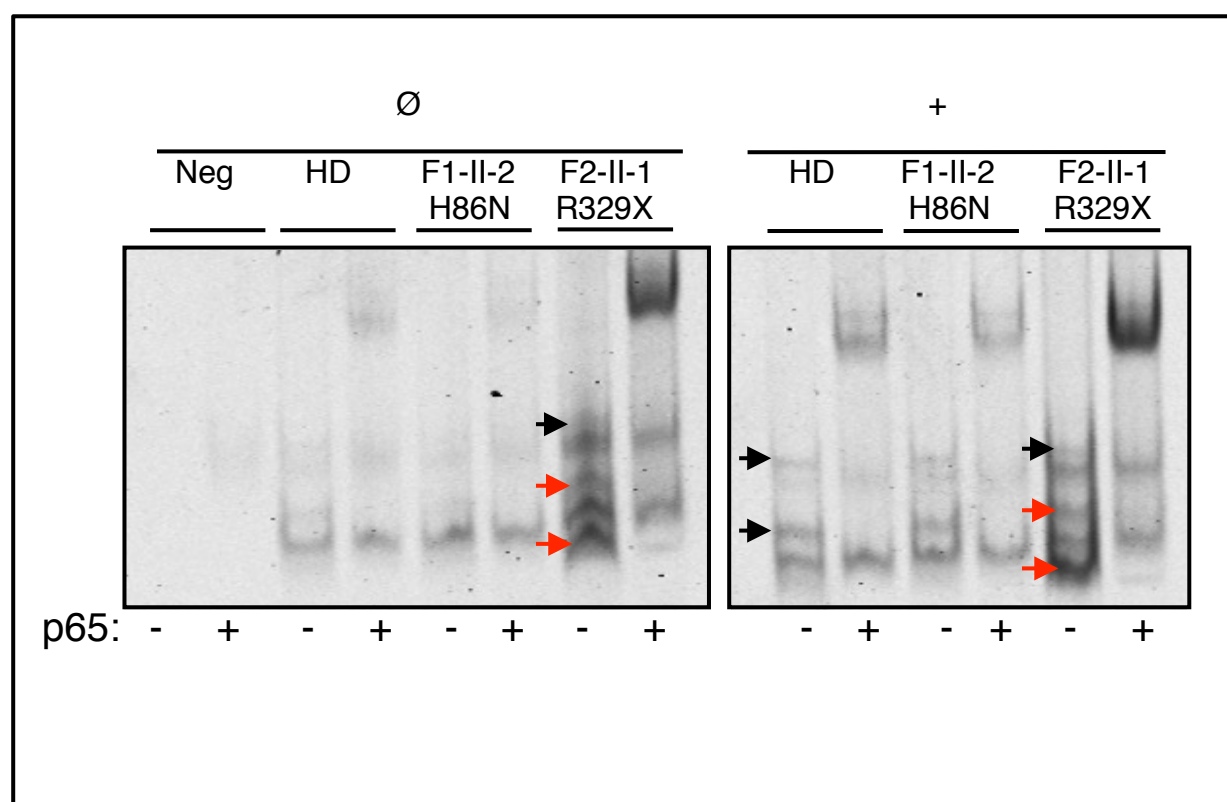
**Figure 25: Nuclear RELA<sup>Y306</sup> binds to DNA NF-κB consensus sequence in patient cells stimulated or not with OKT3.**

Electrophoretic mobility shift assay was performed on nuclear extracts from T cell blasts of healthy donor (HD) and patient F3-II-2 using a luminescent-labeled NF-κB consensus oligonucleotide for the shift and an anti-p65 antibody for the super shift. T cell blasts were stimulated or not with 30 minutes OKT3.

### ***SLE patients***

In order to determine if RELA<sup>H86N</sup> and RELA<sup>R329X</sup> mutant proteins can bind to DNA κB consensus sequences electrophoresis mobility shift assays (EMSA), we performed shift and super-shift assays on nuclear extracts of stimulated or unstimulated T cell blasts (**figure 26**). In nuclear extracts of control T cell blasts, we observed stronger bands of shifts and super-shifts (corresponding to wild type RELA complexes and RELA<sup>R329X</sup> complexes) in the PMA-ionomycin stimulated condition compared to unstimulated condition. Similar results are observed in nuclear extracts

of F1-II-2, although we are unable to differentiate between wild type RELA and mutant RELA<sup>H86N</sup> proteins. In F2-II-1, we observe specific shifts and super-shifts of the protein-DNA complexes formed by RELAR329X, with and without stimulation of patient's T cell blasts. These results indicate that REL<sup>R329X</sup> binds to DNA, and confirms previous results that RELA<sup>R329X</sup> is found in the nucleus without NF-κB activation. However, we are not able to conclude on RELA<sup>H86N</sup> DNA binding properties.



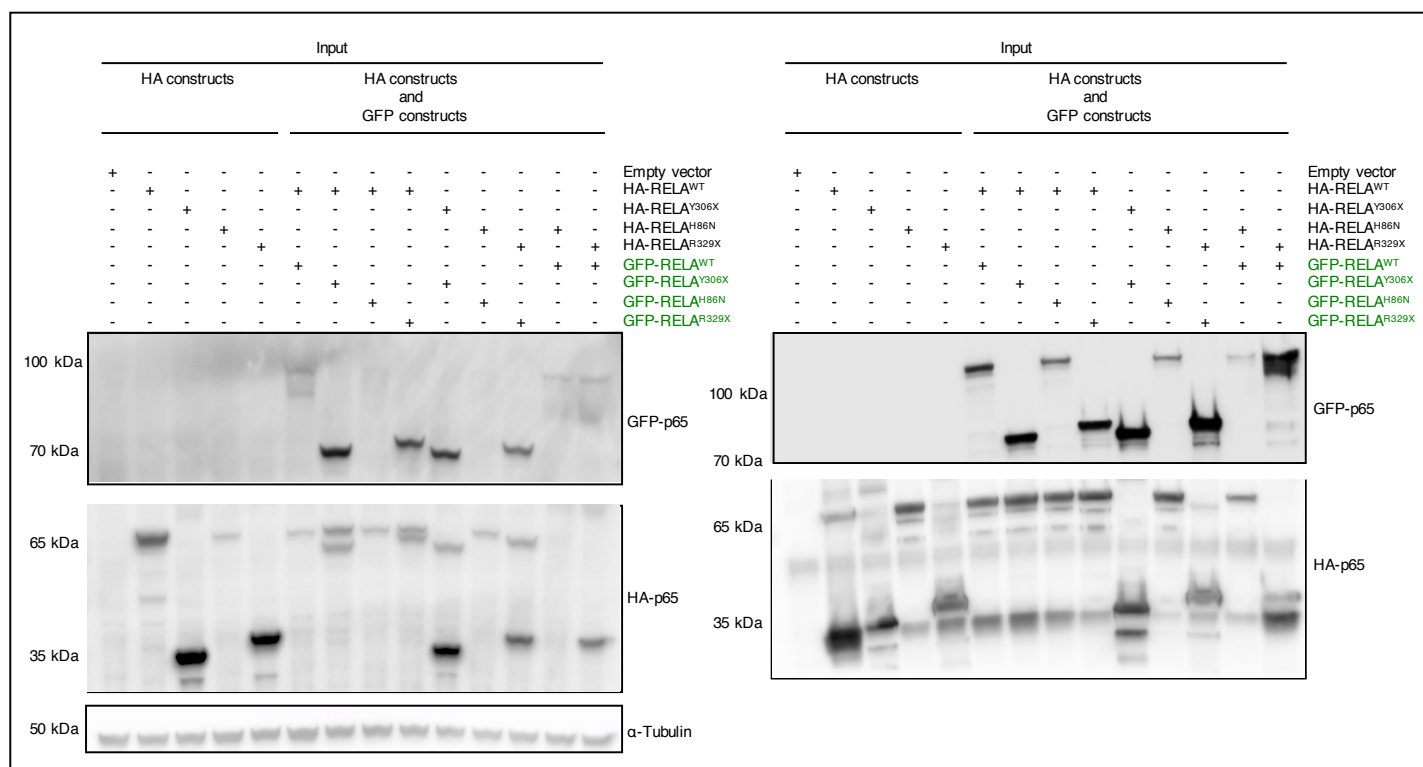
**Figure 26: Nuclear RELA<sup>R329X</sup> bind to DNA NF-κB consensus sequence in patients' T cells.**

Electrophoretic mobility shift assay was performed on nuclear extracts from T cell blasts of healthy donor (HD), patient F1-II-2 and patient F2-II-1 using a luminescent-labeled NF-κB consensus oligonucleotide for the shift and an anti-p65 antibody for the super shift. T cell blasts were stimulated or not with 30 minutes PMA-ionomycin. Black arrows indicate RELA<sup>WT</sup>-DNA complex shift and red arrows indicates RELA<sup>R329X</sup>-DNA complex shift.

### **5.1.6 Mutant RELA proteins interactions with partner proteins**

We have seen in chapter 3.1.5 that the shift and super shift of ectopically truncated RELA protein revealed different bands. Thus, the truncated proteins might potentially form different dimers for protein complexes. Also, the abundance of ectopically over-expressed RELA<sup>H86N</sup> led us to question its stability. Therefore, we investigated how the mutation could affect protein-protein interactions.

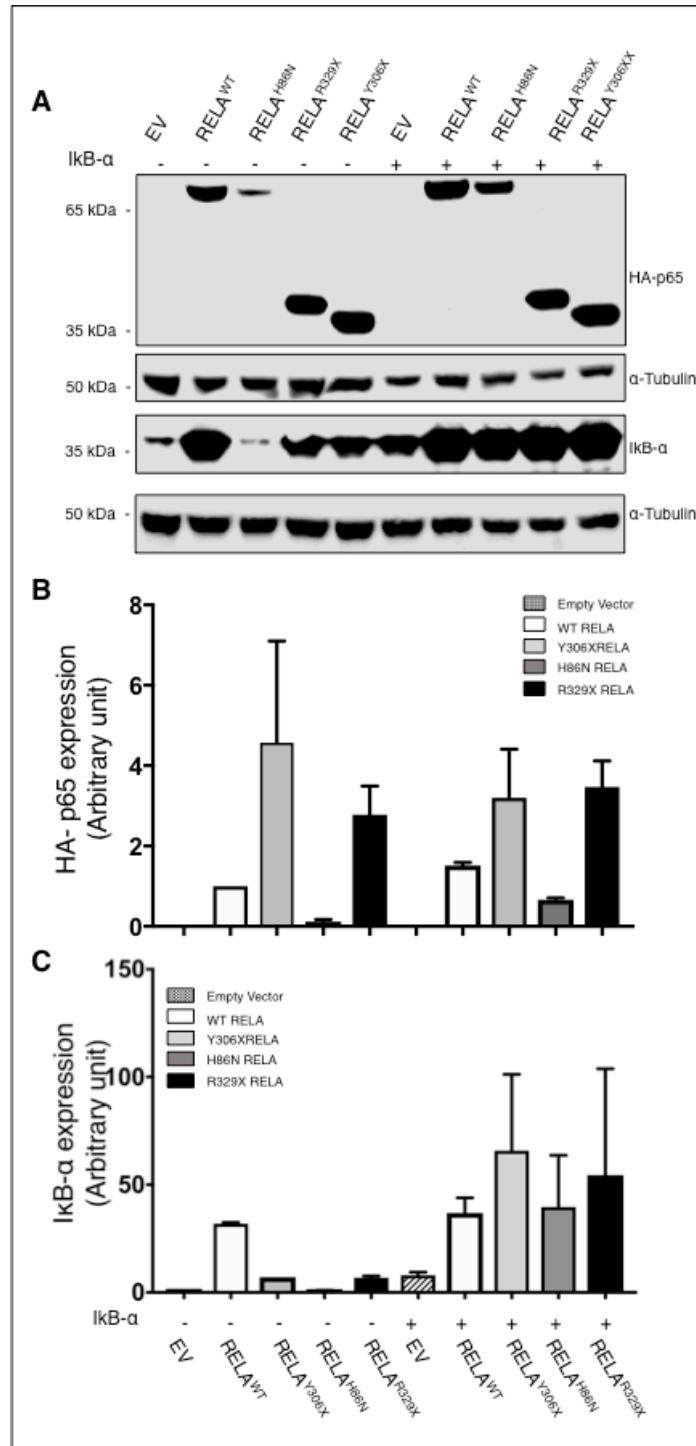
Because RELA proteins function as dimers and that we had at our disposal plasmid constructs with GFP or HA tagged RELA, we first performed immunoprecipitation assays to determine if it can form RELA<sup>WT</sup>-RELA<sup>MUTANT</sup> dimers or RELA<sup>MUTANT</sup>-RELA<sup>MUTANT</sup> (**figure 27**). Therefore, HEK293T cells were transfected with single constructs expressing HA tagged RELA or were co-transfected with combinations of a HA tagged RELA expression vector and a GFP tagged RELA expression vector. GFP tagged RELA has a size of 100 kDa for wild type and H86N forms, and approximately 70 kDa for truncated forms due to the additional 35 kDa of the GFP protein itself. We used HA antibody couple with magnetic beads to immunoprecipitate protein complexes. The immunoblotting of the immunoprecipitation revealed that all mutated RELA proteins could interact with RELA<sup>WT</sup>. Furthermore, we observed that all mutant RELA proteins could form homodimers.



**Figure 27: Ectopically expressed RELA mutant proteins are able to form heterodimers with RELA<sup>WT</sup> and form homodimers.**

Immunoblotting of cell lysates from single transfected or co-transfected HEK293T cells with HA-p65 constructs or GFP-p65 constructs was performed as indicated. Proteins were immuno-precipitated with anti-HA antibody. Initial lysates and immune-precipitated proteins were subjected to immunoblotting with anti-GFP, anti-HA and anti-  $\alpha$ -tubulin antibodies.

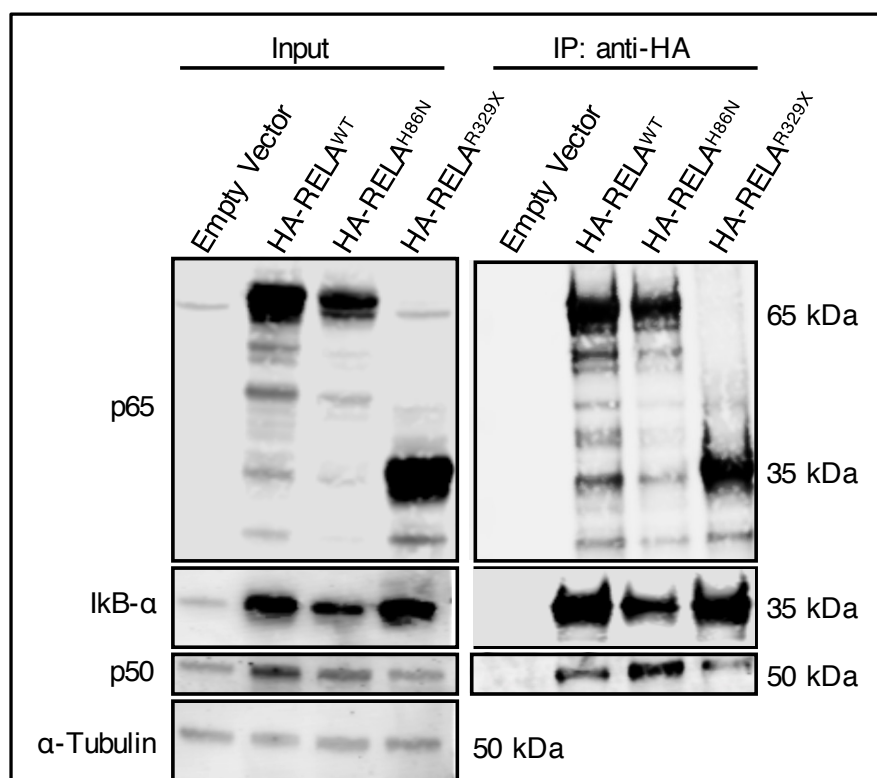
Still, we noticed that HA-RELA<sup>H86N</sup> and GFP-RELA<sup>H86N</sup> had significantly weaker bands than the other conditions, suggesting protein instability. Moreover, we suspected that the ectopically expressed RELA proteins are in an over-saturated abundance, which means there might not be enough endogenous I $\kappa$ B- $\alpha$  to interact. Therefore, we transfected HEK293T cells with the wild type and mutant HA-RELA expressing constructs and with or without I $\kappa$ B- $\alpha$  expressing constructs (**figure 28A**). The immunoblotting revealed that RELA<sup>H86N</sup> expression in cells with I $\kappa$ B- $\alpha$  co-transfection was significantly stronger than in cells solely transfected with a HA-RELA<sup>H86N</sup> construct (**figure 28B**), suggesting an interaction between I $\kappa$ B- $\alpha$  and RELA<sup>H86N</sup> and that I $\kappa$ B- $\alpha$  overexpression stabilizes the ectopically expressed RELA<sup>H86N</sup>.



**Figure 28: Increased abundance of mutated RELA proteins when co-expressed with IκBα.**

(A) Immuno-blotting of HEK293T cell lysates co-transfected with constructs expressing N-terminal HA tagged p65 and IκB-α with a ratio of 3:1. Anti-HA-p65, anti-IκB-α and anti-α-tubulin antibodies were used to assess protein expression. (B) Bar graph represents HA-p65 expression normalized to cells expressing only RELA<sup>WT</sup>. (C) Bar graph represents IκB-α expression normalized to cells transfected with only the empty vector (EV). Data depicted and are expressed as means of 2 independent experiments ± SD.

Next, we investigated by immunoprecipitation whether mutant RELA proteins can interact directly with partner proteins I $\kappa$ B- $\alpha$  and p50 (NF- $\kappa$ B1) in HEK293T cells. To avoid the bias of over-expression of HA-RELA proteins, we co-transfected all conditions with the I $\kappa$ B- $\alpha$  expression construct. We observed that, similarly to the wild type RELA protein, all mutant RELA proteins could interact with I $\kappa$ B- $\alpha$  and p50 (data not shown for RELA<sup>Y306X</sup>).



**Figure 29: Ectopically expressed P65 mutant proteins interact with I $\kappa$ B- $\alpha$  and p50 in cells co-transfected with I $\kappa$ B- $\alpha$ .**

Immunoblotting of cell lysates from co-transfected HEK293T cells with an I $\kappa$ B $\alpha$  construct and HA-p65 constructs performed as indicated. Proteins were immuno-precipitated with anti-HA antibody. Initial lysates and immune-precipitated proteins were subjected to immunoblotting with anti-p65, anti-I $\kappa$ B $\alpha$  and anti- $\alpha$ -tubulin antibodies. A representative experiment is depicted.

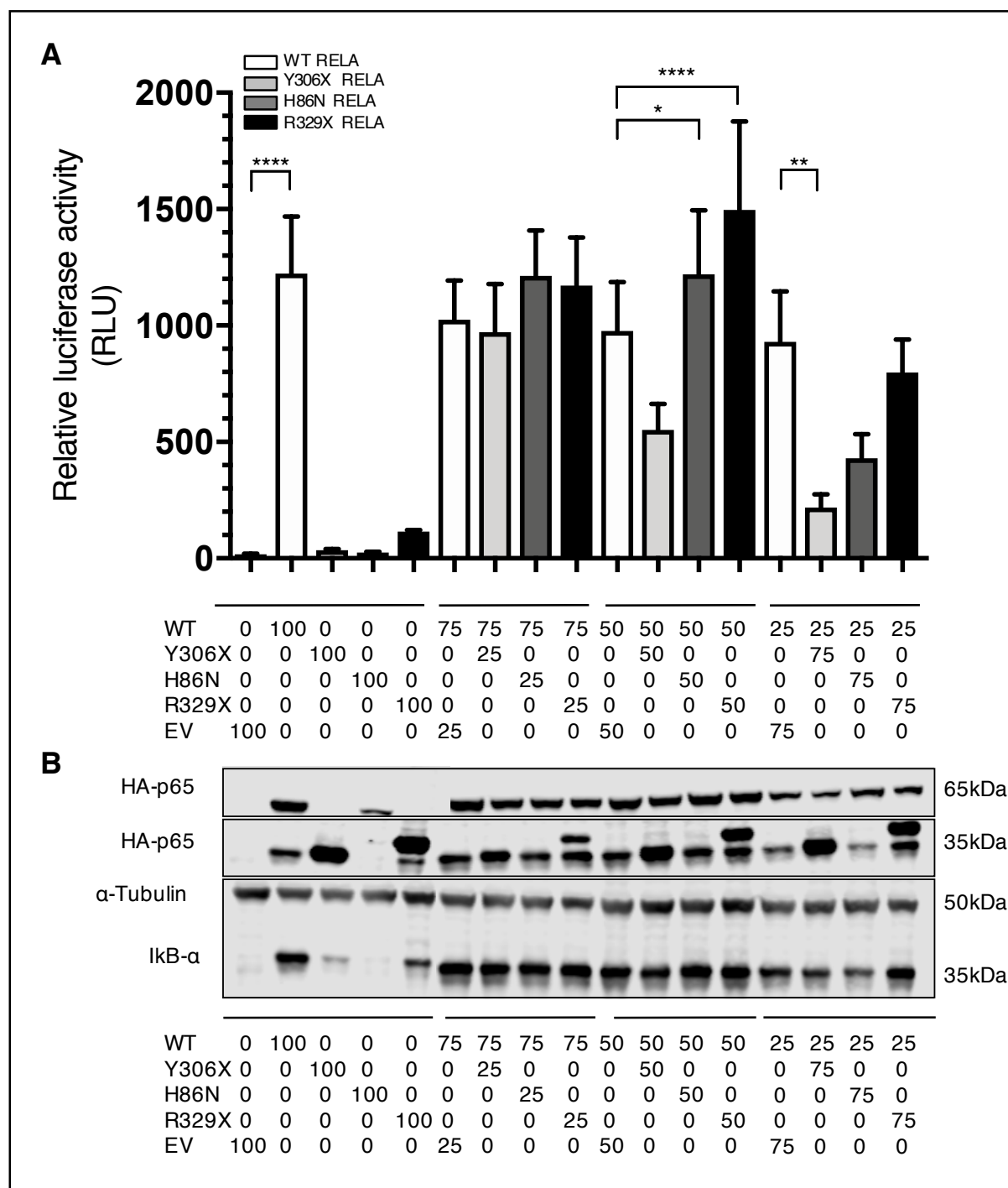
### 5.1.7 Mutant RELA proteins transcriptional activity

As we demonstrated that the mutant forms of RELA protein could enter the nucleus, bind to protein partners and bind to DNA, we investigated if the mutant proteins have a transcriptional activity. To note, the truncated forms do not possess

neither the activation domain nor the 9AATAD. We assessed the transcriptional activity by performing a luciferase activity reporter assay with an IgK-luciferase construct for NF- $\kappa$ B activation and a renilla construct for activity normalization. The IgK-luciferase construct encodes an immunoglobulin kappa light-chain motif (5'-GGGGACTTTCC-3') upstream of an INF- $\beta$  minimal promoter, which induces luciferase expression. HEK293T cells were co-transfected with the both luciferase and renilla constructs and HA-RELA constructs. Together to understand how the mutated RELA proteins can affect the function of the wild type RELA, we challenged wild type RELA proteins with mutant RELA proteins by increasing transfection percentages of mutant RELA constructs (or empty vector EV as control) and decreasing transfection percentages of wild type RELA protein (75% WT / 25% Mutant or EV; 50% WT / 50% Mutant or EV; 25% WT / 75% Mutant or EV; as indicated in the graph) (**figure 30A**).

We observed almost no luciferase activity in lysates from cells 100% transfected with mutant RELA constructs in comparison to lysates from cells transfected with wild type RELA. This result suggests that  $\text{RELA}^{\text{Y306X}}$ ,  $\text{RELA}^{\text{H86N}}$  and  $\text{RELA}^{\text{R329X}}$  mutant proteins were transcriptionally inactive on IgK target. Interestingly, there is a residual luciferase activity in the lysates from the 100%  $\text{RELA}^{\text{R329X}}$  transfected cells. Nevertheless, we noticed an increased expression of I $\kappa$ B- $\alpha$  in protein extracts from wild type and both truncated mutant RELA transfected cells used for the IgK luciferase reporter assay (**figure 30B**).

In regards of the challenging wild type RELA transcriptional activity with mutant RELA proteins, we observed a decreased luciferase activity in lysates from 50%  $\text{RELA}^{\text{Y306X}}$  transfected cells in comparison to 50%  $\text{RELA}^{\text{WT}}$  / 50% EV transfected cells. In contrast, we observed a significantly slight increase of luciferase activity for 50%  $\text{RELA}^{\text{H86N}}$  and 50%  $\text{RELA}^{\text{R329X}}$  transfected cells in comparison to 50%  $\text{RELA}^{\text{WT}}$  / 50% EV transfected cells. Also, in the condition of 25% WT / 75% Mutant or EV, we observe a decrease of luciferase activity for the condition with  $\text{RELA}^{\text{Y306}}$  and  $\text{RELA}^{\text{H86N}}$ , and approximately similar luciferase activity between 25%  $\text{RELA}^{\text{WT}}$  / 75% EV and 25% WT / 75%  $\text{RELA}^{\text{R329X}}$ .



**Figure 30: Ectopically expressed p65 mutants are transcriptionally inactive.**

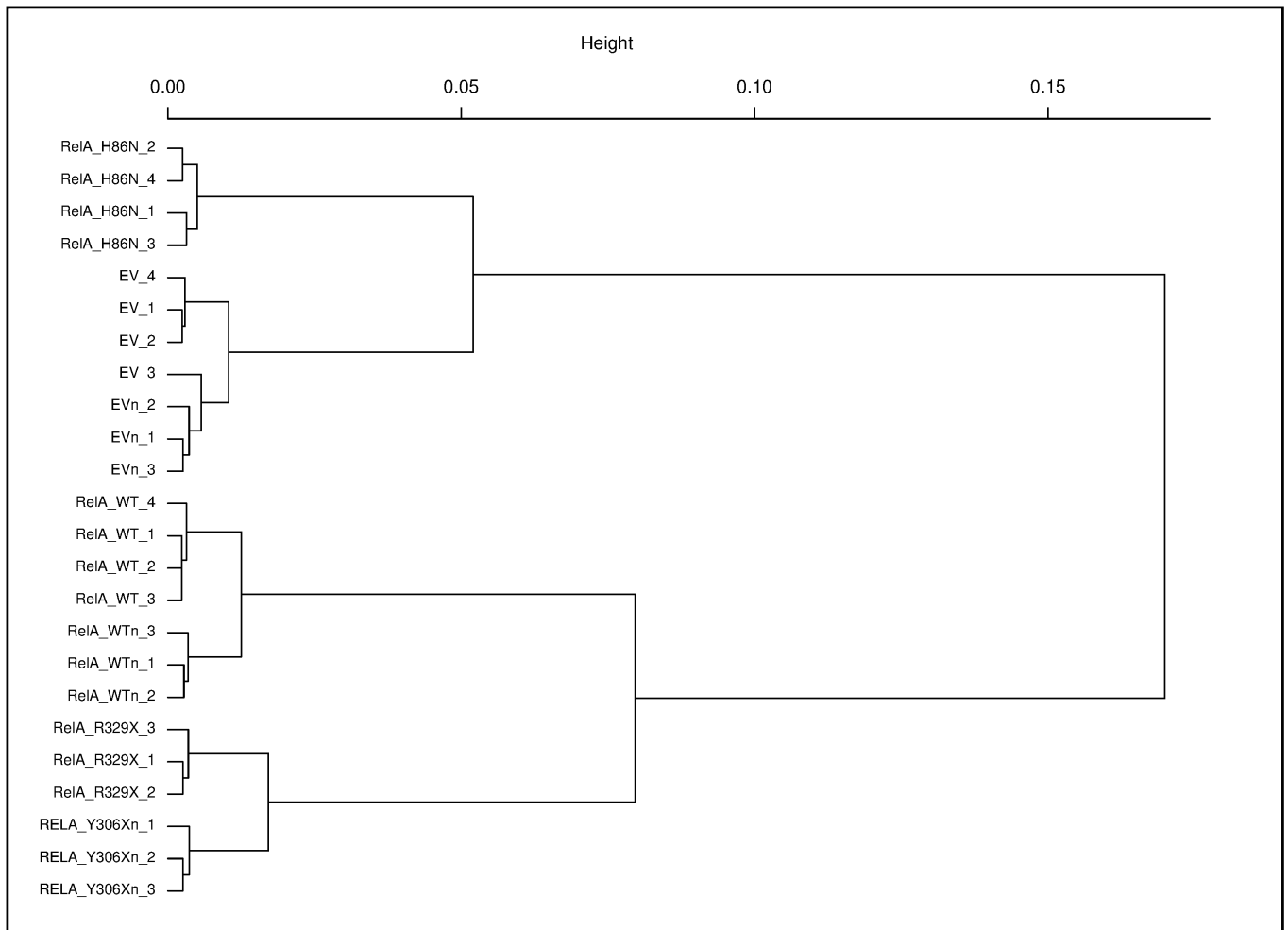
(A) Relative luciferase activity is the ratio of firefly over renilla activity measured on co-transfected HEK293T cell lysates with indicated transfection percentages of constructs expressing N-terminal HA-tagged p65, together with an IgK-luciferase reporter constructs. White bars represent cells transfected with RELA wild-type, grey bars with RELA H86N and black bars with RELA R329X expression constructs. Transfections were performed in triplicates and data from (A) expressed as mean of 4



independent experiments  $\pm$  SD; RLU= Relative luminescent unit; EV= Empty vector; WT= Wild-type; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; two-way Anova test. (B) Immuno-blotting of cell lysates from co-transfected cells used in (A) revealed with anti-HA-p65, anti-I $\kappa$ B- $\alpha$  and anti- $\alpha$ -tubulin antibodies. A representative experiment is depicted in figure (B).

Going further on the impact of the mutations on RELA transcriptional functions, we studied the mutated RELA proteins on a transcriptomic level. We opted to work on a homogeneous cell-line to avoid genetic background biases. In quadruplets condition, we transfected HEK293T cells with HA-RELA expressing constructs. We assessed effectiveness of transfection by immunoblotting the protein extracts from the cells with anti-HA antibody and the extracted RNA incurred thorough RNA quality check. Our genomic platform performed an RNA sequencing assay with 10-15 million reads per sample on the extracted RNA. We normalized the read counts and compared groups by using three independent and complementary Bioconductor software packages for differential expression analysis of digital gene expression data (Deseq2, edgeR, LimmaVoom). Consequently, the results of each package were compared and grouped (**Figure 31 and 32**). The results were then filtered at  $pvalue \leq 0.05$  and folds 2.

Then, we analyzed hierarchical clustering of the samples by using the Spearman correlation similarity measure and WardD2 linkage algorithm based on 14628 genes. We observed very close clustering of replicates from each samples according to the transfected expression constructs (**Figure 31**). The clustering reveals that the expression profiles of *RELA*<sup>H86N</sup> cells are close to EV transfected cells. Also, expression profiles of *RELA*<sup>WT</sup>, *RELA*<sup>Y306X</sup> and *RELA*<sup>R329X</sup> cells were significantly distant from *RELA*<sup>H86N</sup> and EV cells. Interestingly, the expression profiles of *RELA*<sup>Y306X</sup> and *RELA*<sup>R329X</sup> cells were clustering close from each other's.

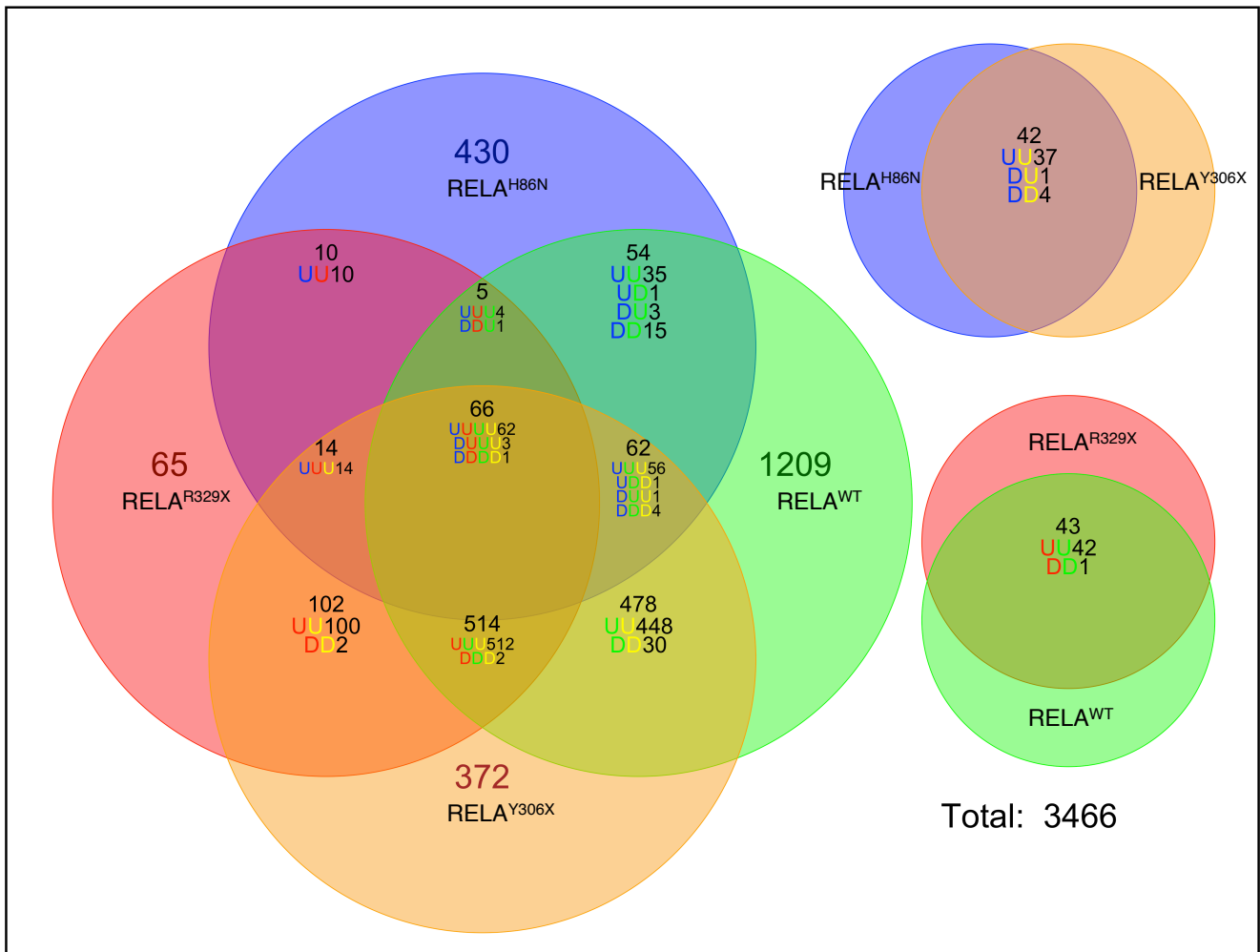


**Figure 31: Clustering of expression profiles of transfected HEK293 cells.**

RNA extracts from  $RELA^{WT}$  and EV transfected cells were sequenced twice, “n” signaling second run.  $RELA^{Y306X}$  transfect cells were analyzed with the second run and is also marked as “n”. Clustering was conducted by using WardD2 algorithm.

We generated Venn diagrams based on the comparison of each *RELA* condition to EV condition from the filtered list of differentially expressed genes (folds 2). Thereby, we obtained 3466 genes with a significant differential expression (**Figure 32**). A substantial 1209 of the 3466 differentially expressed genes are exclusively in  $RELA^{WT}$  transfected cells, displaying how an overexpression of *RELA* can strongly modulate genes expression. In contrast, the cells overexpressing the *RELA* mutant proteins showed fewer exclusively differentially expressed genes (65 genes for  $RELA^{Y306X}$ , 372 genes for  $RELA^{R329X}$  and 430 genes for  $RELA^{H86N}$ ).

Interestingly, there were more differentially expressed genes in common to  $RELA^{WT}$ ,  $RELA^{Y306X}$  and  $RELA^{R329X}$  (514 genes) than between  $RELA^{WT}$ ,  $RELA^{H86N}$  and  $RELA^{R329X}$  (5 genes). To note, a vast proportion of the differentially expressed genes in all condition are up regulated rather down regulated.



**Figure 32: Venn diagrams of folds2-filtered differentially expressed genes from HEK293T cells transfected cells.**

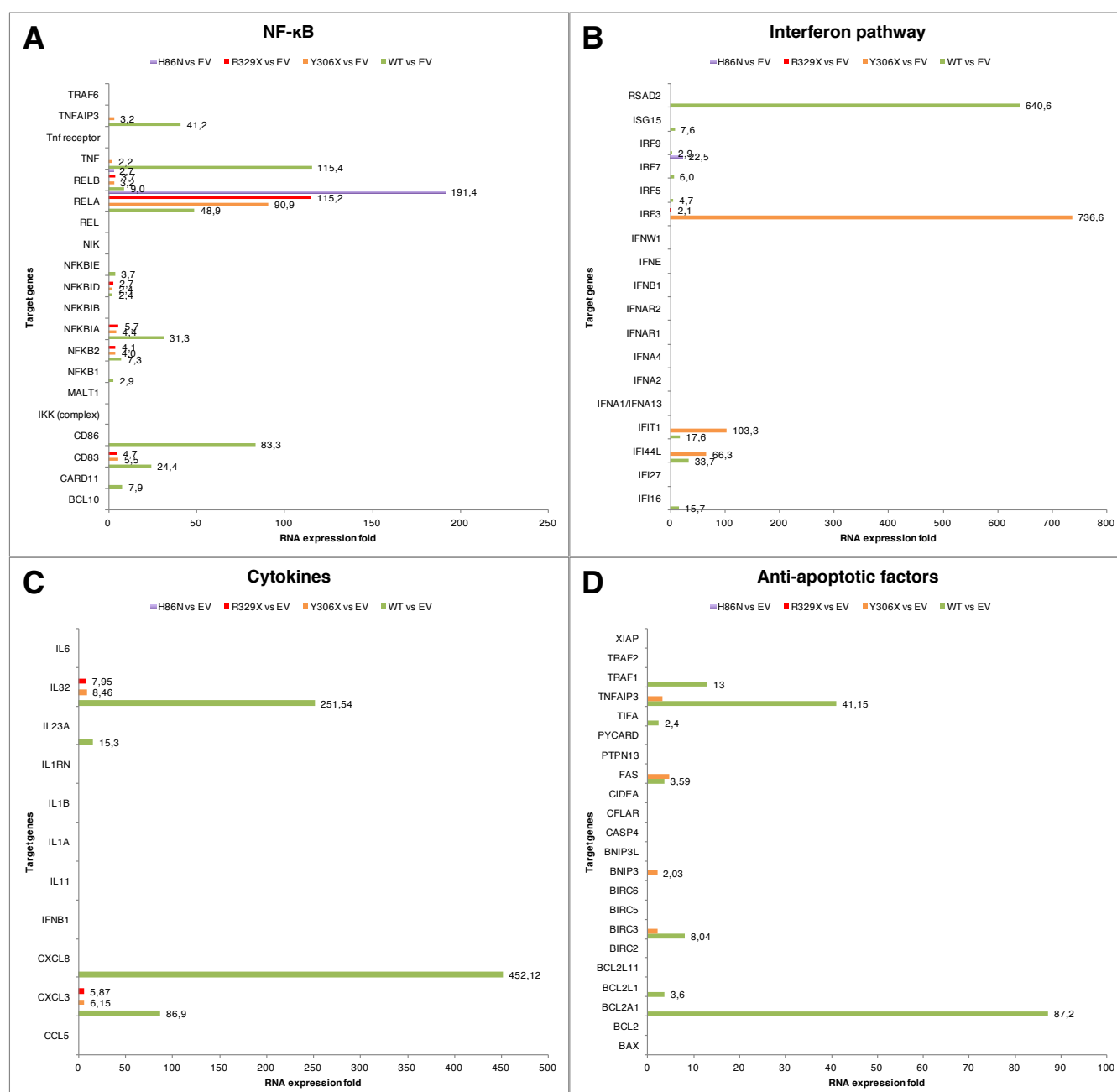
In blue  $RELA^{H86N}$  vs EV, in green  $RELA^{WT}$  vs EV, in red  $RELA^{R329X}$  vs EV in orange  $RELA^{Y306X}$  vs EV. EV= empty vector; U= up-regulated gene; D= down-regulated gene.

Then, we analyzed the list of genes generated by using ingenuity pathway analysis (IPA, from QIAGEN) (**Figure 33**). We analyzed differentially expressed genes in four different pathways, based on comparison of  $RELA$  transfected samples versus EV transfected samples. No down-regulated genes were found in the four

pathways. In the list NF- $\kappa$ B pathway (**Figure 33 A**), we found a significantly high expression fold of *RELA* for all conditions, which is consistent with the fact that they are transfected with *RELA* constructs. Interestingly in this ectopically and overexpressing system, the mutant *RELA* conditions had a higher expression fold of *RELA* (90.9 for *RELA*<sup>Y306X</sup>, 115.2 for *RELA*<sup>R329X</sup> and 191.4 for *RELA*<sup>H86N</sup>) compared to wild type *RELA* condition (48.9). Moreover, the samples transfected with *RELA*<sup>H86N</sup> had a higher expression fold than the samples expressing the truncated forms, whereas at the protein level we observed dramatically lower protein expressions for *RELA*<sup>H86N</sup> compared to *RELA*<sup>Y306X</sup> and *RELA*<sup>R329X</sup>. Also, we noticed that *TNFAIP3* (encoding A20) and *NFKBIA* (encoding I $\kappa$ B $\alpha$ ) were up regulated to 41.2 fold in *RELA*<sup>WT</sup> condition, consistent with negatively regulating an NF- $\kappa$ B activation. Also consistent with previous results of I $\kappa$ B- $\alpha$  protein expression, *RELA*<sup>Y306X</sup> and *RELA*<sup>R329X</sup> exhibited *NFKBIA* up regulation (respectively 4.4 and 5.7) but not in *RELA*<sup>H86N</sup> samples. Expressed target genes of an NF- $\kappa$ B activation were significantly up regulated such as *CD83* (24.3 fold) *CD86* (83.3 fold) *TNF* (115.4 fold) in *RELA*<sup>WT</sup> condition, but were only marginal with truncated *RELA* proteins. Because we observed ISG signatures in patients' cells, we investigated interferon targets in the RNA sequencing results (**Figure 33 B**). We did not find ISG differentially expressed genes in *RELA*<sup>R329X</sup> expressing cells, and only *IRF9* in *RELA*<sup>H86N</sup> expressing cells. *RELA*<sup>Y306X</sup> expressing cells showed high expression of *IRF3* (736.6), *IFIT1* (103.3) and *IFI44L* (66.3). As for *RELA*<sup>WT</sup> expressing cells, we found as up regulated genes *RSAD2* (640.6), *ISG15* (7.7), *IRF9* (2.9), *IRF7* (6.0), *IRF5* (4.7), *IFIT1* (17.6), *IFI44L* (33.7), and *IFI16* (15.7). Finally, we looked at pro-inflammatory cytokines and anti-apoptotic factors genes as described in the publication of *RELA* haploinsufficiency associated muco-cutaneous and inflammatory intestinal disease. For cytokines, genes differentially expressed in common *RELA*<sup>WT</sup>, *RELA*<sup>Y306X</sup> and *RELA*<sup>R329X</sup> expressing cells was IL-32 and CXCL3. As for anti-apoptotic factors, we found up regulated genes only in *RELA*<sup>WT</sup> *RELA*<sup>Y306X</sup> expressing cells with *BIRC3*, *FAS*, and *TNFAIP3*.

Overall, the RNA sequencing assay showed that *RELA*<sup>WT</sup> transfected HEK293T cells strongly up-regulates a vast number and broad variety of genes (in fold 2 filtered genes). Whereas HEK293T cells transfected with the truncated mutant *RELA*

constructs still featured a moderate number of significantly differentially expressed genes but not as strongly and numerous as  $RELA^{WT}$  expressing cells. Lastly,  $RELA^{H86N}$  expressing cells displayed few differentially expressed genes, which was comparable to EV transfected cells.



**Figure 33: Differential expression of indicated genes in HEK293T cells using RNA-seq data.**

Log2-fold change in the expression of indicated genes using RNA-seq data from two patients relative to EV controls

## 6 Discussion

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Recent studies have associated *RELA* haploinsufficiency to distinct clinical manifestations. First publication described human *RELA* haploinsufficiency as causative of TNF-dependent muco-cutaneous ulceration and inflammatory intestinal diseases with no apparent immune cell defect. Soon after, a following publication described an ALPS-like patient with autoimmune cytopenia, which they also have associated to *RELA* haploinsufficiency.

Herein for the first time, we describe the expression of three different mutated forms of *RELA* proteins in patients presenting CVID, pediatric SLE or familial form of SLE.

In the familial form of SLE (family 1), we detected a missense mutation c.256 C>A leading to the substitution of a p.H86N at an evolutionary highly conserved amino acid in the RHD of the *RELA* protein. In patients' T cells, the mutation results in the expression of a full length *RELA*<sup>H86N</sup> protein, as we do not observe a haploinsufficiency. Unfortunately, with the antibody at hand, we could not discriminate between the wild type *RELA* protein and the mutant *RELA* protein, thus we are not able to conclude on the nuclear translocation of the *RELA*<sup>H86N</sup> in patients' cells, based on our immunoblotting of nuclear protein extracts and cellular immunofluorescence staining. Ectopically over-expression of *RELA*<sup>H86N</sup> revealed its instability, although we overcame this apparent instability when we over-expressed concomitantly I $\kappa$ B $\alpha$ . We demonstrated that ectopically express *RELA*<sup>H86N</sup> interacts with protein partners such as I $\kappa$ B $\alpha$ , p50, *RELA*<sup>WT</sup> and with itself. In one hand, we could not conclude with the super-shift assay if *RELA*<sup>H86N</sup> binds to DNA in patients' cells due to lack of evidence for nuclear translocation. On another hand, super-shift assays of ectopically expressed *RELA*<sup>H86N</sup> showed very faint bands of shift and super-shift, thus making us unable to conclude on the mutant protein DNA binding. Still, these results could explain the impaired transcriptional activity of the ectopically expressed *RELA*<sup>H86N</sup> mutant protein when we perform luciferase reporter assays with

a  $\kappa$ B consensus target. Moreover, differential gene expression in HEK293T cells expressing  $RELA^{H86N}$  does cluster closely that of HEK293T cells transfected with an empty vector. Although, the observed differences we detect do suggest that  $RELA^{H86N}$  generates aberrant transcriptional activity. Interestingly, ectopically over-expression of  $RELA^{H86N}$  does not trigger expression of its stabilizer I $\kappa$ B $\alpha$  (seen in immunoblotting and RNA-seq).

Nevertheless,  $RELA$  protein expression levels appear to be similar between  $RELA^{H86N}$  patients' cells and healthy controls. Also,  $RELA^{H86N}$  can interact with other NF- $\kappa$ B proteins, thus competing with  $RELA^{WT}$  for dimer formations. This could result in aberrant NF- $\kappa$ B dimers and disturbed gene expression. Indeed, NF- $\kappa$ B heterodimers containing  $RELA^{H86N}$  could disturb nuclear localization and DNA binding. Although, RNA-seq experiment showed that cells ectopically over-expressing  $RELA^{H86N}$  significantly differed in genes expression from cells transfected with EV, suggesting that there is a residual activity mediated by  $RELA^{H86N}$  that has yet to be understood. Still, we suggest the hypothesis that  $RELA^{H86N}$  proteins generate abnormal NF- $\kappa$ B activity by a subtle dominant-negative like mechanism.

In patients' cells, there is no evidence of defect in immune cell development. Thus, how immune cells functions can be affected by  $RELA^{H86N}$  remains to be determined. Environmental triggers or genetic background could also explain the different onset and severity of the F1-I-2 (mother) and F1-II-2 (daughter).

In the pediatric SLE patient (family 2), we detected a *de novo* heterozygous nonsense mutation c.985 C>T leading to a premature stop codon p. R329X. The mutation resulted in the expression of a truncated form of  $RELA$ , corresponding to the RHD domain of  $RELA$  with an additional 23 amino acids. In unstimulated patient's T cells, we detected  $RELA^{R329X}$  in both cytoplasm and nucleus by cellular immunofluorescence and nuclear extracts immunoblotting, suggesting deregulation of its subcellular localization. Also, we observed slightly more  $RELA^{WT}$  in the nucleus, suggesting that the presence in the cytoplasm of  $RELA^{R329X}$  could impact cellular localization of  $RELA^{WT}$  without NF- $\kappa$ B activation. Indeed, immunoprecipitation assay revealed that the truncated  $RELA$  proteins interacted with p50, I $\kappa$ B $\alpha$ ,  $RELA^{WT}$  and also formed homodimers. We demonstrated by super-shift assay that  $RELA^{R329X}$

protein could bind to DNA. The ability of RELA<sup>R329X</sup> to bind to DNA and bind to protein partners show that its RHD domain is fully functional. Moreover, luciferase assays showed that RELA<sup>R329X</sup> protein is not transcriptionally active, as it lacks an activation domain and a 9AATAD. However, a 1:1 ratio of RELA<sup>WT</sup> RELA<sup>R329X</sup> shows an enhancement of the luciferase activity, and 1:3 ratio of RELA<sup>WT</sup> RELA<sup>R329X</sup> shows no dominant-negative effect. These results suggest that in a 1:1 ratio as in the patient, RELA<sup>R329X</sup> may behave as an enhancer of RELA<sup>WT</sup> when they form heterodimers by making it enter the nucleus with no stimulation required, and as seen in the immunofluorescence, by making it remain in the nucleus longer than normal. As for homodimers of RELA<sup>R329X</sup>, they act mainly as repressors of gene expression by binding to DNA targets without triggering transcription.

Nonetheless, RNA-sequencing assay of HEK293T cells over-expressing RELA<sup>R329X</sup> showed that NF-κB activation is detectable (up regulation of *CD83* negative feedback regulators *NFKBIA* and *NFKBIE*) but the amount of differentially expressed genes is drastically lower than cells over-expressing RELA<sup>WT</sup>. This important lower amount of differentially expressed genes could be explained by RELA<sup>R329X</sup> homodimers occupying most target promoters. Also, genes overexpressed could be due to heterodimers of RELA<sup>R329X</sup> with a transcriptionally potent NF-κB protein (c-REL, RELB, RELA) able to reach free target promoters. We suggest that RELA<sup>R329X</sup> affects NF-κB activity in a gain-of-function way when it forms heterodimers and in a dominant-negative way when it forms homodimers.

F2-II-1 has fully mature T and B cells, which indicate that the disruption of the homeostasis of NF-κB dimer formation does not affect immune development in early stages but rather can affect immune function and activation upon environmental trigger.

In the CVID patient (family 3), we detected a heterozygous nonsense mutation c.918 T>G leading to a premature stop codon p. Y306X. The mutation resulted in the expression of a truncated form of RELA, corresponding to exactly the RHD domain of RELA. Although we observed mediated decay of mRNA in patient T cells, we still detected protein expression of RELA<sup>Y306X</sup> at the same amount as RELA<sup>WT</sup>. Thus,



RELA<sup>Y306X</sup> could be very stable by being less prone to degradation. In patients T cells without NF-κB activation, RELA<sup>Y306X</sup> was detected mostly in the nucleus by immunoblotting. This abnormal subcellular localization could also explain the protein's putative stability, either because cytoplasmic RELA<sup>Y306X</sup> is rapidly degraded and nuclear RELA<sup>Y306X</sup> enters the nucleus without pathway trigger, or because RELA<sup>Y306X</sup> does not have a nuclear export signal. Also, immunoprecipitation of ectopically expressed RELA<sup>Y306X</sup> revealed that the truncated protein interacted with p50, IκBα, RELA<sup>WT</sup> and also formed homodimers. Moreover, super-shift assay showed that the truncated RELA proteins could bind to DNA. Furthermore, IgK-luciferase reporter assays showed that RELA<sup>Y306X</sup> is not transcriptionally active by itself, explained by the absence of the activation domain and a 9AATAD. Additionally, luciferase assays demonstrate that RELA<sup>Y306X</sup> interferes negatively with RELA<sup>WT</sup> when co-expressed. These observations of RELA<sup>Y306X</sup> on subcellular localization and transcriptional activity differ with what we have shown with RELA<sup>R329X</sup> protein.

Nevertheless, we observed in the RNA-sequencing assay of HEK293T cells over-expressing RELA<sup>Y306X</sup> activation of NF-κB-target genes (up regulation of *CD83* and negative feedback regulators *NFKBIA*, *NFKBID* and *TNFAIP3*). We observed this up regulation of *NFKBIA* previously in the immunoblotting of over-expressing RELA<sup>Y306X</sup> cells, where we noticed more IκBα protein expression than in the EV transfected cells. Though, the amount of differentially expressed genes was also importantly less abundant than in cells over-expressing RELA<sup>WT</sup>. In these settings, homodimers of RELA<sup>Y306X</sup> are most likely to be responsible of this reduced abundance of differentially expressed genes compared to cells over-expressing RELA<sup>WT</sup>. Similarly, to RELA<sup>R329X</sup> homodimers, RELA<sup>Y306X</sup> homodimers act as transcription repressors. Interestingly, p50 is structurally close to both truncated RELA proteins and p50 homodimers act also as a mainly transcription repressor. Regarding the genes differentially expressed that we still detect, heterodimers of RELA<sup>Y306X</sup> with a endogenous NF-κB protein (c-REL, RELB, RELA) could still activate some target genes. We suggest that RELA<sup>Y306X</sup> possibly interferes on NF-κB activity in dominant-negative way.

F3-II-2 had at the early stage of her life mature T cells and B cells, and she slowly developed lymphopenia with few T cells and very few B cells. This

immunological history showcases that the disruption of the homeostasis of NF-κB dimer formation does not affect immune development but rather can affect immune function and activation upon environmental trigger. Importantly, family 3 has a strong history of CVID, suggesting that there is other genetic modifying factors, or the mutation p.Y306X of RELA is the key modifying factor itself.

Because of a fair resemblance of the protein structures, we hypothesize that RELA<sup>Y306X</sup> and RELA<sup>R329X</sup> mutant proteins behave like a kind of p50 protein in some fashion, but with each truncated RELA having its own properties in the nuclear translocation capacities, strength of DNA binding, strength of IκBα binding during cytoplasmic sequestration or nuclear resolution of the activation.

Expressed stable truncated forms of RELA are able to form dimers with NF-κB proteins, and they disrupt the homeostasis of NF-κB dimer formation in response to stimuli. The mechanism in which they affect cell functions remains to be elucidated.

*RELA* mutations lead to different and heterogeneous clinical and immunological phenotypes (skin and intestinal inflammation, ALPS-like, CVID and SLE), with in common autoimmune or inflammatory traits. How defects in RELA can lead to disturbed immune responses needs to be understood. CVID are associated to other NF-κB protein partners such NFKB1 (185) (151) and NFKB2 (153, 186, 187). SLE and several autoimmune diseases are associated to type-1 interferon production (188). In mice, RelA enhances virus-induced expression of IFN-induced genes, as *Rela*<sup>-/-</sup> mouse embryonic fibroblasts (MEFs) express less IFN-α and IRF7 during viral replication (189) as well as in *nfkb1*<sup>-/-</sup> or *c-Rel*<sup>-/-</sup> MEFs (190). Also, increased levels of RELA induce interferon genes (191). Studies have shown that IRF5-RELA interaction and genomic binding was important in regulating the activity of human macrophages (192) and in human dendritic cells (193). Indeed, prolonged macrophage activity can cause skin lesions, and prolonged dendritic cell activity can lead to excessive antigen presenting activity and immune response. Surprisingly, *RelA*<sup>-/-</sup> *RIP1*<sup>K45A/K45A</sup> (abolished kinase activity of RIP1) mouse are viable and have normal lymphocyte development, which suggest that *RelA* is not essential for the development of the organism and for lymphopoiesis in mice (194). Interestingly,

those mice develop early skin inflammation and persistent upper respiratory tract infections, which are clinical manifestations that have been seen in humans with *RELA* defects.

Transcription factors allow complex regulation of gene transcription, thus changes in their expression level or dimerization kinetics can potentially lead to amplified changes in their associated network. Several publications described human defects in NF- $\kappa$ B activation, such as mutations in *IKBKG*, *NFKBIA*, and *IKBKA*, which are associated with primary immunodeficiency (132). Mucocutaneous ulceration was initially stated as not a universal finding of impaired NF- $\kappa$ B activation (*Badran et al., 2017*), as it was justified that it was not reported in patients with mutations in *NFKB1*. But here we show two different mutations of *RELA* associated to SLE with oral and skin ulceration. Although, *Badran* study compare human *RELA* haploinsufficiency with mucocutaneous ulceration with the *Rela*<sup>+/-</sup> mice that had a similar phenotype (195), *Comrie* study showed that *RELA* haploinsufficiency can also be linked to an ALPS-like phenotype with autoimmune cytopenia and arguing that variety of phenotypes is due to genetic/environmental modifying factors.

My thesis work brings evidence that CVID and SLE (pediatric and familial form) are additional possible manifestations of impaired *RELA* function. Furthermore, it highlights a critical role for impaired NF- $\kappa$ B activity in the event of autoimmune and inflammatory diseases in PID and PID associated with SLE as a larger clinical picture.

## 7 Perspectives

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Still many basic molecular aspects have to be clarified on how the mutations affect the RELA protein function. Indeed, studying the nuclear trafficking of both truncated forms RELA<sup>Y306X</sup> and RELA<sup>R329X</sup> will help understanding if the aberrant proteins enter the nucleus regardless of NF-κB activation or if they are unable to exit the nucleus (possible lack of protein nuclear export sequence). Actually, many phosphorylation and ubiquitination sites are absent due to the truncations and could affect subcellular localization regulation and proteasome degradation. To bypass the lack of antibody discriminating RELA<sup>H86N</sup> and RELA<sup>WT</sup>, we need to better study ectopically expressed RELA<sup>H86N</sup> localization in cells co-transfected with an IκB-α expression construct. Also, we should evaluate RELA<sup>H86N</sup> protein stability by using proteasome inhibitor MG132.

An important point that we realized by the end of this work, is the necessity to co-express with an IκB-α expressing construct when studying ectopically expressed RELA wild type and mutant proteins to improve the biochemical kinetics in NF-κB dimerization. In these settings, we will probably have more insight on transcriptional activity in luciferase assays and RNA-seq assays. Plus, we will be able to perform experiments with or without stimulation for NF-κB activation. An interesting control that has been missing in most experiments is an over-expression of p50, as the truncated forms and p50 are structurally close. In this regard, we should consider performing comparison between co-transfected cells with NFKBIA and 1:1 ratio of *RELA*<sup>WT</sup>/*RELA*<sup>MUTANT</sup> and co-transfected cells with NFKBIA and 1:1 ratio of *RELA*<sup>WT</sup>/NFKBI.

Nevertheless, overexpression data generated in the HEK293T cell line can be misleading when it comes to extrapolating to RELA functions in immune cells. Thus, we will perform RNA-seq on patients' cells. Likewise, we will perform extracts chromatin immunoprecipitation with massively parallel DNA sequencing (CHIP-seq) on patient's assays cells in order to determine how the RELA mutations affect the

binding to different NF- $\kappa$ B DNA consensus target sites in the genome. Also, performing assay for transposase-accessible chromatin with high throughput sequencing (ATAC-seq) will bring input on chromatin accessibility in patients' cells during NF- $\kappa$ B activation. Altogether, CHIP-seq, ATAC-seq and RNA-seq data will bring deep insight on the impact of the RELA mutant proteins on NF- $\kappa$ B physiological function.

Our collaborators are establishing murine models with patients *RELA* point mutations, as it could be a valuable tool to further investigate the pathomechanism of the diseases.

Finally, COVID patients with mutations in *RELA* have been lately identified, and could lead to a future collaboration with our research team.

## 8 Material and methods

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### Cell culture of SLE patients

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density-gradient centrifugation and washed twice with PBS. T cell blasts were obtained by stimulating  $1 \times 10^6$  cells per ml with staphylococcal enterotoxin (SEE) 0.1  $\mu\text{g/ml}$  (Toxin Technology) in Panserin 401 (PAN Biotech) supplemented with 10% human AB serum (Biowest), 1x penicillin - streptomycin (P/S) (Invitrogen), 1% glutamine,. After 3 days of activation, viable cells were separated by Ficoll centrifugation washed twice with Panserin and then cultured in Panserin supplemented with 10% human AB serum, 1% P/S and 100 U/ml interleukin 2 (IL-2). IL2 was refreshed every 2 to 3 days.

### Cell culture of CVID patients

Peripheral blood mononuclear cells were isolated by Ficoll-Paque density-gradient centrifugation and washed twice with RPMI 1640 GlutaMax medium (Invitrogen). T cell blasts were obtained by stimulating  $1 \times 10^6$  cells per ml in RPMI 1640 GlutaMax supplemented with 10% human AB serum, penicillin and streptomycin (Invitrogen), PMA (20 ng/mL; Sigma-Aldrich) and ionomycin (1  $\mu\text{mol/L}$ ). After 2 to 3 days of activation, viable cells were separated by Ficoll-Paque density-gradient centrifugation, washed twice with RPMI 1640 GlutaMax and then cultured in RPMI 1640 GlutaMax supplemented with 10% human AB serum and 100 U/mL IL-2.

### RNA and DNA preparation

DNA was isolated from the red blood cell pellet, obtained after Ficoll preparation of fresh blood samples, using the QiAamp DNA blood Mini kit. PCR was performed using Go Taq Flexi DNA polymerase (Promega) following manufacturer's instructions and with the following pairs of olinucleotides: forward Mut H86N 5'-

ggcgagaggagcacagatac -3', reverse Mut H86N 5'- cctgggtccagaaaggagta -3'; forward Mut R329X 5'- ggactgggaaagccagagag -3', reverse Mut R329X 5'- cccaggagtcttcatctcca -3'; forward Mut Y306X 5'- gcagattcagccaacagagg -3', reverse Mut Y306X 5'- tttcccagtcctcatctcac -3'. PCR products were purified on sephadex G-50 columns (GE healthcare). Purified PCR products were used as template for Sanger sequencing reaction using BigDye Terminator v3.3 cycle sequencing kit (Applied Biosystems) and 3500XL genetic analyser (Applied Biosystems). Forward primers were used for sequencing reaction (supplemental table). All sequences data were analyzed using ApE and 4peaks software.

Total RNA was isolated from PBMCs, T-blasts and B-EBV cell line using the RNeasy mini Kit following manufacturer's instructions (QIAGEN RNeasy mini kit) and cDNA was prepared using the Quantitect Reverse Transcription Kit according to manufacturer's instructions including DNase treatment to deplete genomic DNA, according to the manufactures instructions (RNase-Free Dnase Set from QUIAGEN). PCR was also performed on cDNA using Go Taq Flexi DNA polymerase (Promega) with the following pair of olinucleotides: forward Mut H86N 5'- gcgagaggagcacagatacc -3', reverse Mut H86N 5'- tggctccgtgaaatacacct -3'; forward Mut R329X 5'- caagtggccattgtgttccg -3', reverse Mut R329X 5'- ccccttaggagctgatctga-3'; forward Mut Y306X 5'- cgtgtctccatgcagctg -3', reverse Mut Y30X 5'- aggactcttctcatgatgctct -3'. Forward primers were used for sequencing reaction (supplemental table).

## Gene expression analysis

The expression of a set of 6 interferon-stimulated genes (ISGs) in PBMCs and T cell blasts was assessed by quantitative -RT-PCR using TaqMan Gene Expression Assays. Expression of ISGs was normalized to GAPDH. The following TaqMan Gene Expression Assays obtained from ThermoFisher Scientific were used: IFI27 - Hs01086370\_m1, IFI44L- Hs00199115\_m1, IFIT1 - Hs01675197\_m1, RSAD2 - Hs00369813\_m1, SIGLEC1- Hs00988063\_m1, ISG15 – Hs01921425\_s1, GAPDH- Hs03929097. Real Time quantitative PCR was performed in duplicate using the

LightCycler VIIA7 System (Roche). Relative quantification (RQ) represents the fold change compared to the calibrator (GAPDH). RQ equals  $2^{-\Delta\Delta C_t}$  where  $\Delta\Delta C_t$  is calculated by  $(C_{t\text{target}} - C_{t\text{GAPDH}}) \text{ test sample} - (C_{t\text{target}} - C_{t\text{GAPDH}}) \text{ calibrator sample}$ . Each value is derived from three technical replicates.

### **Confocal Microscopy**

T cell blasts ( $3 \times 10^6$ ) were stimulated in x ml of complete Panserin with either PMA (20 ng/mL; Sigma-Aldrich) and ionomycin (1  $\mu\text{mol/L}$ ) or 10ng/ml TNF- $\alpha$  (Invitrogen) for 15 minutes and 30 minutes at 37°C. Cells were coated on a poly-L-lysine matrix then fixed 20min with PFA 4% at room temperature (RT). Cells were permeabilized with PBS + 0.1% Triton X-100 10min at RT. To block unspecific staining samples were blocked for 1h with PBS + 5% BSA at RT before staining. Antibodies were diluted in PBS + 5% BSA. The primary antibodies used included rabbit monoclonal anti human NF- $\kappa\text{B/p65}$  (E379) C terminal (1/200; Abcam) and a mouse monoclonal anti human NF- $\kappa\text{B/p65}$  (F-6) N terminal (1/100; SantaCruz). Samples were incubated overnight at 4°C. For immunofluorescence, the following secondary antibodies were used: goat anti-rabbit IgG labelled with Alexa 488 and donkey anti-mouse labelled with Alexa 555 (1:1000; Invitrogen) 1h at RT in the dark. Slides were counterstained after 3 washes of PBS with 0.3  $\mu\text{g/ml}$  4,6-diamidino-2-phenylindole (Sigma-Aldrich). Images including Z-stacks were acquired on a Leica SP8 gSTED with an objective with x63 magnification. Nuclear localization of p65 was quantified by ImageJ software.

### **Immunoblotting of patient cells**

Protein samples were extracted in RIPA buffer (Bio-Rad- ThermoFisher ref 89900) supplemented with Complete Protease Inhibitor Cocktail (Roche). Equal amounts of extracted protein (10  $\mu\text{g}$ ) were loaded and run alongside Chameleon Duo Pre-stained Protein Ladder (LI-COR) on a NuPAGE 4–12% Bis-Tris Gel (Invitrogen) and transferred onto nitrocellulose membranes using iBlot2 (Invitrogen). The membranes were incubated with anti-beta actin (13E5 1:10,000 rabbit polyclonal; Cell Signaling) or anti-p65 (F-6 1:1000; mouse monoclonal Santa Cruz and 1: 2,000; E379 rabbit



monoclonal; Abcam) primary antibodies. Immuno-reactive bands detected following incubation with IRDye 680 RD anti-rabbit antibody (1:10000; LI-COR) and IRDye 680 RD anti-mouse antibody (1:10000; LI-COR) by using the CLx Odyssey Infrared Imaging System (LI-COR). Quantification of immunoblots was performed by using the Image Studio Lite software version 5.0 (LI-COR).

### **Expression plasmids**

Site directed mutagenesis using GeneART™ Site-Directed Mutagenesis System (Thermo Fischer Scientific) was performed following manufacture's instruction on the RELA<sup>WT</sup> expression constructs pEBB HA RelA (addgene #74892) and GFP-p65 (addgene #23255). The following oligonucleotide pairs were used for mutagenesis forward RELAH86N 5'-CCTCCTCACCGGCCTAACCCCCACGAGCTTG-3', reverse RELAH86N 5'-CAAGCTCGTGGGGGTTAGGCCGGTGAGGAGG-3', forward RELA329X 5'-CCCCGGCCTCCACCTTGACGCATTGCTGTGC-3', reverse RELA329X 5'-GCACAGCAATGCGTCAAGGTGGAGGCCGGGG-3', forward RELAY306X 5'-ACGTAAAAGGACATAGGAGACCTTCAAGAGC-3', reverse RELAY306X 5'- GCTCTTGAAGGTCTCCTATGTCCTTTTACGT-3'. The RELA sequence of all the plasmids were confirmed by DNA Sanger sequencing. IκB-α expression plasmid was a courtesy of James Di Santo laboratory (Pasteur Institute, Paris). IgK-luc (Firefly luciferase) and Renilla-Luciferase constructs were a courtesy of Elodie Bal (IMAGINE institute, Paris).

### **Ectopic expression of RELA mutant protein**

All transfection of HA-65 mutants or wild type expressing plasmids into HEK293T cells were carried out using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were lysed 48h after transfection for subsequent assays.

### **NF kappa B luciferase reporter assay**

HEK293T cells (4x10<sup>4</sup> per well) were seeded in 96-well plates and transfected with an NF kappa B reporter construct IgK-luc (Firefly luciferase), a Renilla-Luciferase construct and the different HA-p65 constructs. Luciferase assay was performed using

the Dual-Luciferase Reporter assay system (Promega) following manufacturer's instructions. Luminescence was analyzed with a VictorX4 plate-reader (PerkinElmer).

### **Immunoblotting of transfected cell lines**

For immunoblot analysis cell lysates of  $1 \times 10^6$  HEK293T cells were prepared with RIPA buffer 50 mM Tris pH7.4, 1% Triton X100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA with HALT protease inhibitor cocktail (Pierce). To ensure equal loading, protein concentrations were assessed via BCA<sup>TM</sup> protein assay kit (Thermo Fischer Scientific) before protein loading on SDS/PAGE 4-12% pre-casted polyacrylamide gels (Thermo Fischer Scientific). The antibodies NF- $\kappa$ B p65 (C20; recognizing a C-Terminal epitope of p65, sc-372), NF- $\kappa$ B p65 (F6; recognizing a N-Terminal epitope of p65; sc-8008), I $\kappa$ B $\alpha$  (E-1; sc-**376248**) and anti-I $\kappa$ B $\alpha$  (H-4, sc-1643) were obtained from Santa Cruz Biotechnology. Anti-HA rabbit (**H6908**; Sigma) and anti- $\alpha$ -tubulin (**T5168**; Sigma) were obtained from Sigma Aldrich. All antibodies were used according to the manufacturer's instructions. Secondary fluorescent antibodies were obtained from Li-Cor and were used in immunoblotting assays. Fluorescence analysis of immunoblots were performed on digitized images using ImageStudioLite version 5.2.5 software.

### **RNAseq gene expression profiling**

Total RNA were isolated using the RNeasy Kit (QIAGEN) including a DNase treatment step. RNA quality was assessed using RNA Screen Tape 6000 Pico LabChips with the Tape Station (Agilent Technologies) and the RNA concentration was measured by spectrophotometry using the Xpose (Trinean). RNAseq libraries were prepared starting from 2  $\mu$ g of total RNA using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) as recommended by the manufacturer. Half of the oriented cDNA produced from the poly-A<sup>+</sup> fraction was PCR amplified (11 cycles). The RNAseq libraries were sequenced on an Illumina HiSeq2500 (Paired-End sequencing 130x130 bases, High Throughput Mode). A mean of 18 millions of paired-end reads was produced per library sample (between 15 to 22 millions of passing filter reads).

### **Electrophoretic Mobility Super-shift assay**

Nuclear and cytoplasmic extracts for **electrophoretic mobility** super-shift assay were prepared using a NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific) according to manufacturer's instructions. Cells analyzed were treated with phorbol 12-myristate 13-acetate (PMA) (20 ng/mL; Sigma-Aldrich) and ionomycin (1  $\mu$ mol/L; Sigma-Aldrich) for 30 min or left untreated. DNA-protein interaction mixture was performed by using nuclear extracts and the Odyssey EMSA Buffer Kit with NFkB IRDye 700 Infrared labeled oligonucleotides 5'-AGTTGAGGGGGACTTTAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCC G-5' (underlined nucleotides are the kB consensus binding site), following manufacturer's instructions. Super-shift of complexes was produced by adding 1  $\mu$ g of anti-p65 (F6, sc-8008) antibody to the oligonucleotide-nuclear extract mixture 20 min prior to loading. Samples were subjected to PAGE (4 % gels, under non denaturation condition) and analyzed by Odyssey CLX Li-Cor. Fluorescence analysis of electrophoretic mobility super-shift assays were performed on digitized images using ImageStudioLite version 5.2.5 software.

### **Statistical analysis.**

Analyses were performed with PRISM software (version 7 for Macintosh, GraphPad Inc.). Statistical hypotheses were tested using two-way Anova tests or student's t-test, accordingly. A P value less than 0.05 was considered significant \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ .

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## 10 Acknowledgments

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**Jean-Paul Sartre** (*L’Être et le Néant*).

*“There is no real ending. It’s just the place where you stop the story.”*

**Frank Herbert**

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## 11 Publications

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*Mutations in the adaptor-binding domain and associated linker region of p110 $\delta$  cause Activated PI3K- $\delta$  Syndrome 1 (APDS1).*

Heurtier L, **Lamrini H**, Chentout L, Deau MC, Bouafia A, Rosain J, Plaza JM, Parisot M, Dumont B, Turpin D, Merlin E, Moshous D, Aladjidi N, Neven B, Picard C, Cavazzana M, Fischer A, Durandy A, Stephan JL, Kracker S.

Haematologica. 2017 Jul;102(7):e278-e281. doi: 10.3324/haematol.2017.167601. Epub 2017 Apr 20.

*Clinical and immunologic phenotype associated with activated phosphoinositide 3-kinase  $\delta$  syndrome 2: A cohort study.*

Elkaim E, Neven B, Bruneau J, Mitsui-Sekinaka K, Stanislas A, Heurtier L, Lucas CL, Matthews H, Deau MC, Sharapova S, Curtis J, Reichenbach J, Glastre C, Parry DA, Arumugakani G, McDermott E, Kilic SS, Yamashita M, Moshous D, **Lamrini H**, Otremba B, Gennery A, Coulter T, Quinti I, Stephan JL, Lougaris V, Brodzski N, Barlogis V, Asano T, Galicier L, Boutboul D, Nonoyama S, Cant A, Imai K, Picard C, Nejentsev S, Molina TJ, Lenardo M, Savic S, Cavazzana M, Fischer A, Durandy A, Kracker S.

J Allergy Clin Immunol. 2016 Jul;138(1):210-218.e9. doi: 10.1016/j.jaci.2016.03.022. Epub 2016 Apr 21.

### **Manuscript for submission:**

*RELA heterozygous dominant mutations are associated with pediatric and familial Systemic Lupus Erythematosus. (As 1<sup>st</sup> co-author)*

