Function of IRAK2 in macrophages and HECTD1 in B cells

Funktion von IRAK2 in makrophagen und HECTD1 in B zellen

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To

my

Mother Shrimati Sheeela Devi

Father Shri Ramesh Chand Joshi

Siblings Rekha, Rajesh and Manish

and nephew Udit

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List of Abbreviations

BM	Bone marrow
BSA	Bovine serum albumin
CCR	Chemokine receptor
CD	Cluster of differentiation
DC	Dendritic cell
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacidic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
g	Gram
h	Hours
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IP	Immunoprecipitation
KO	Knock-out
MHC	Major histocompatibility complex
mRNA	Messenger RNA
µg	Microgram
ml	Milliliter
mM	Millimolar
ng	Nanogram
PBS	Phosphate buffered saline
PE	Phycoerythrin
PMA	Phorbol 12-myristate 13-acetate
SD	Standard deviation
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WT	Wild type

Abstract

The Immune system exerts its response against invading pathogens via a cumulative, sequential cooperation of immune cells coordinated by their secreted products. Immune cells, such as macrophages and dendritic cells (DCs), express toll-like receptors (TLRs) to sense the presence of pathogens through pathogen-associated molecular patterns (PAMPs). The interaction of PAMPs with TLRs elicits a cytosolic signaling cascade that enhances the expression of genes to stimulate inflammation. Interleukin 1 receptor-associated kinase 2 (IRAK2) is a component of the TLR signaling pathway. IRAK2 transduces the TLR signal via a direct interaction with TNF receptor-associated factor 6 (TRAF6) and subsequent enhancement of its ubiquitination.

During my PhD thesis, I determined that a 55-amino acid long stretch at the C-terminal end of IRAK2 is important for TLR signaling. Overexpression of C-terminal truncated IRAK2 (IRAK2Δ55) in the murine macrophage cell line RAW 264.7 led to impaired CD40 expression after TLR4 stimulation by Lipopolysaccharide (LPS). I observed attenuated competency of IRAK2Δ55 in restoring a full TLR signaling response i.e. IL-6 secretion, NO production and CD40 expression in IRAK2-deficient RAW cells generated via CRISPR-Cas9 approach. Additionally, diminished TLR4 induced activation of nuclear factor κB (NF-κB) and extracellular signal related kinase (ERK) was observed with IRAK2Δ55 reconstituted RAW cells as compared to cell reconstituted with wildtype IRAK2.

IRAK2Δ55 reconstituted RAW cells also exhibited reduced TLR4-induced cell death and phosphorylation of receptor interacting protein kinase 3 (RIP3). Co-immunoprecipitation experiments in HEK 293T cells showed that IRAK2Δ55 was still able to bind to TRAF6 alike IRAK2 but failed to induce ubiquitination of TRAF6. In conclusion, the results suggest that the IRAK2 TRAF6 interaction is not sufficient to sustain full TLR signaling. An C-terminus-dependent unknown molecular mechanism is also involved.

Through my PhD work, I also analyzed a B cell lineage-specific HECTD1 knock-out mice. HECTD1 is an E3 ubiquitin ligase for various substrate proteins, such as heat shock protein 90 (HSP90),

adenomatous polyposis coli and phosphatidylinositol phosphate kinase type 1 γ. Hsp90 regulates a variety of signaling molecules in NF-κB activation pathways which are essential for an optimal B cell response.

HECTD1-deficient pro-B cells developed normally into mature B cells. However, TLR4 stimulated HECTD1-deficient B cells displayed reduced immunoglobulin (Ig) production in *in vitro* cultures. In addition, mice with HECTD1-deficient B cells showed a diminished Ig response after nitrophenylacetyl-keyhole limpet hemocyanin immunization. Thus, HECTD1 is necessary for efficient Ig secretion.

Zusammenfassung

Auf das Eindringen von Pathogenen in den Körper antwortet das Immunsystem mit einer kumulativen, sequenziellen und wechselseitigen Zusammenarbeit zwischen Immunzellen, ihren Oberflächenrezeptoren sowie den von ihnen sezernierten Mediatoren. Immunzellen, wie Makrophagen und dendritische Zellen (DZ), sind dabei in der Lage mittels Toll-like Rezeptoren (TLRs) das Vorhandensein von Pathogenen über Pathogen-assoziierte molekulare Muster (pathogen-associated molecular patterns, PAMPs) zu detektieren.

Die Bindung von PAMPs an TLRs führt über intrazelluläre Signalkaskaden zu einer verstärkten Expression pro-inflammatorischer Gene und damit zur Initiierung einer Immunreaktion. Die Interleukin 1 Rezeptor-assoziierte Kinase 2 (IRAK2) ist einer Komponente der TLR Signalkaskade. IRAK2 bindet direkt an den TNF-Rezeptor-assozierten Faktor 6 (TRAF6), welcher daraufhin verstärkt ubiquitiniert wird.

In meiner Promotionsarbeit habe ich einen 55 Aminosäure langen Abschnitt im C-Terminus von IRAK2 identifiziert, der für die Signalleitung von TLRs essentiell ist. Die Überexpression von mutierten IRAK2, dem dieser C-terminale Bereich fehlt (IRAK2Δ55), in der murinen Macrophagen Zelllinie RAW 264.7 führte zu einer verminderten Expression von CD40 nach Stimulation des TLR4 durch Lipopolysaccharid (LPS). Wurden IRAK2-defiziente RAW Zellen mit dem mutierten IRAK2Δ55 Gen rekonstituiert, zeigten diese Zellen verglichen mit Zellen, die mit dem wildtypischen Gen rekonstituiert wurden, eine verminderte Aktivierung des nuclear factor κB (NF-κB) und der extracellular signal related kinase (ERK) nach Stimulation des TLR4. Ebenso waren die Expression von CD40, die Sekretion von IL-6 und NO gestört. In IRAK2-defizienten und IRAK2Δ55 RAW Zellen war eine Reduktion des durch TLR4 induzierten Zelltodes sowie der TLR4-induzierten Phosphorylierung der Rezeptor-interagierenden Proteinkinase 3 (RIP3) zu beobachten. Ko-Immunpräzipitationsexperimente mit HEK 293T Zellen zeigten, dass IRAK2Δ55 genauso wie intaktes IRAK2 zwar in der Lage ist, TRAF6 zu binden, aber nicht dessen Ubiquitinylierung zu induzieren. Die

Ergebnisse dieser Arbeit zeigen, dass die Interaktion von IRAK2 mit TRAF6 für ein optimales TLR-Signal nicht ausreichend ist und deshalb ein bisher unbekannter Mechanismus an der Signalweiterleitung beteiligt sein muss. Dieser Mechanismus ist vom C-terminalen Ende von IRAK2 abhängig.

In einem zweiten Teil meiner Doktorarbeit analysierte ich B-Zellen von Mäusen, in denen HECTD1-spezifisch in der B-Zellentwicklungslinie deletiert wurde. HECTD1 ist eine E3 Ubiquitin-Ligase für zahlreiche Substratproteine, wie bspw. dem Hitzeschock-Protein (heat-shock-protein, HSP90), dem *adenomatösen Polyposis* coli Protein oder der Phosphatidylinositol Phosphatkinase Typ 1 γ. HSP90 reguliert eine Vielzahl an Signalmolekülen im NF-κB Signalweg, die für eine optimale B-Zell-Antwort wesentlich sind.

HECTD1-defiziente pro-B-Zellen entwickelten sich normal zu reifen B-Zellen. Die Stimulation des TLR4 auf HECTD1-defizienten B-Zellen führte *in vitro* zu einer im Vergleich zu wildtypischen B-Zellen reduzierten Immunglobulin-Sekretion. Eine reduzierte Immunglobulin-Antwort konnte auch in B-Zell-spezifischen hectd1$^{-/-}$ Mäusen beobachtet werden, wenn diese zuvor mit Schlitzschnecken-Hämocyanin (*Keyhole Limpet Hemocyanin*, NP-KLH) immunisiert wurden. Die reduzierte Produktion von Antikörpern durch HECTD1-defiziente B-Zellen zeigt, dass dieses Protein für diese zentrale Aufgabe von B-Zellen notwendig ist.

1. Introduction

1.1 Immune system

The word "immune" in "immune system" was derived from Latin word *immunitas*, a referral towards protection from legal prosecution to senators during the ancient Roman empire. Later, in historical medical terms, immunity meant protection from infectious diseases (Abbas et al., 2015; Janeway, 1989). The physiological function of the immune system is defense against pathogenic microbes (bacteria, viruses, protozoans, fungi etc.). Distinguished molecular determinants from pathogens are recognized by dedicated immune cells. Based on involved cells and the way of the response towards pathogens, the immune system is divided into innate and adaptive immunity (Figure 1.1).

Innate immunity makes the first line of defense against microbes. It stops pathogen infection via epithelial barrier, antimicrobial peptides, the complement system and cells. Innate immune cells *i.e.* neutrophils, macrophages, dendritic cells, natural killer (NK) cells and other innate lymphoid cells attack incoming microbes. Innate immune cells respond by phagocytosis or direct killing of pathogens. Innate cells express pattern recognition receptors (PRRs) which recognize pathogen associated microbial patterns (PAMPs) from invading microbes. Engagement of PRRs initiates an inflammatory cascade to facilitate recruitment of more immune cells at the site of infection. Innate cells *i.e.* macrophages and dendritic cell work as antigen presenting cells (APCs) which process the phagocytosed pathogen and present antigen peptides via major histocompatibility class II (MHC II) molecules. CD4 T cells recognize the MHC II bound peptide by T cell receptor and activate antigen specific adaptive immune response. Innate cells are critical for host defense and defects may cause chronic susceptibility to infection (Abbas et al., 2015).

The Immune system develops pathogen specific immunity with successive exposure to the same pathogen. This adaption for infection is called adaptive immunity or acquired immunity. The adaptive arm of the immune system consists of B and T cells, thereafter called B and T cells, which are equipped with unique antigen-specific receptors. B cell receptors (BCRs) and T cell receptors (TCRs) recognize specific antigenic determinants on pathogens with high specificity. The variable region

genes coding for the antigen-binding regions of BCR and TCR are randomly rearranged with cell maturation, which provides a set of unique receptors with the potential of recognizing a maximum of antigenic determinants from the same pathogen with high affinity. Activated B cells secrete BCRs in form of immunoglobulins (Ig), also known as antibodies, which bind the pathogens to neutralize or opsonize them.

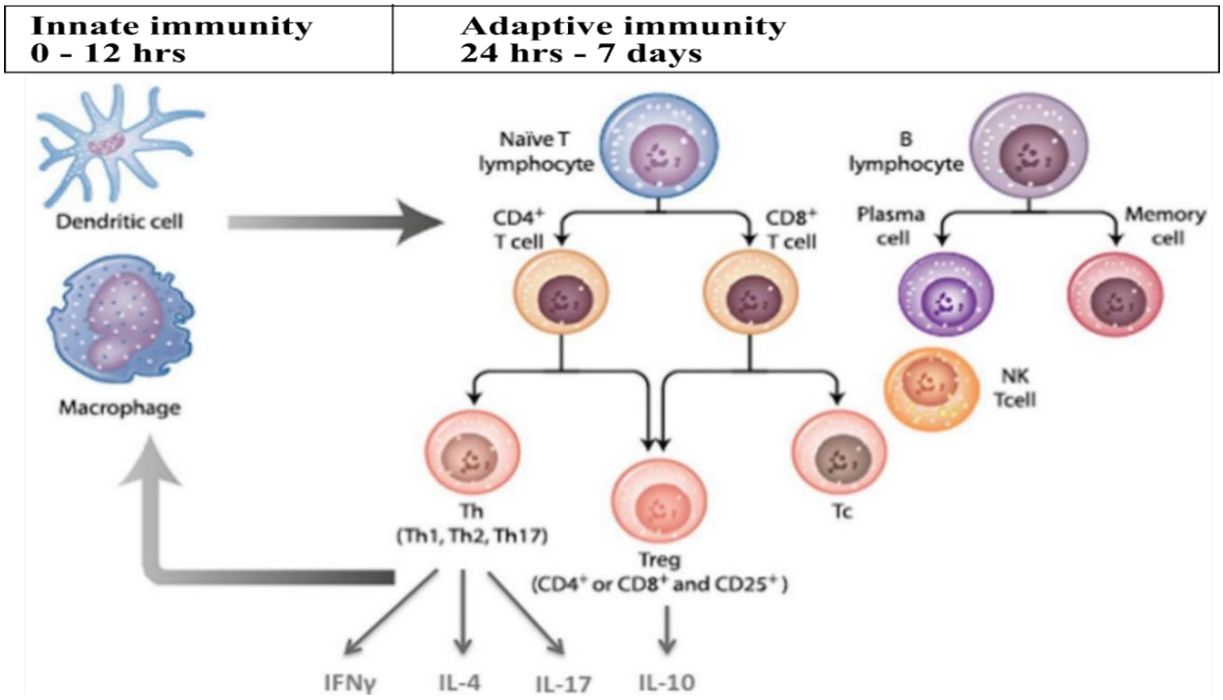

Figure 1.1: A schematic representation of immune system- Graphics describing sequential response from innate and adaptive arm after pathogen enters the host. Sketch is adapted from (Schiffrin, 2014).

T cells are divided in two types based on surface expression of CD4 or CD8 receptors. CD8+ T cells recognize foreign peptides presented by MHC I molecules on infected cells and perform direct killing. Antigen peptides presented via MHC II on APCs are identified by CD4+ T cells which lead to their differentiation and subsequent activation of other immune cells. Adaptive immune cells retain memory of the antigen by differentiating into memory cells.

The adaptive response is tightly regulated in a way that T and B cells do not undergo activation upon recognition of self-antigens to prevent damage to the host. Defects with the adaptive arm of the

immune system result in various immunopathologies *i.e.* autoimmunity or immunodeficiency leading to the destruction of certain tissues or increased infections, respectively (Abbas et al., 2015; Judith A Owen et al., 2013).

1.2 Pathogen recognition and immune response

Pathogens are microbes which could cause a disease and can elicit a specific immune response. However, pathogenicity of a microbe in host mostly depends on its ability to escape or hide from the immune system and subsequent colonization capacity. Different components of pathogen could hold a specific molecular pattern, such antigenic molecules are called PAMPs and recognized by specific receptors (PRRs) on immune cells (Judith A Owen et al., 2013).

As a consequence of recognition, phagocytic innate cells can internalize and kill the pathogens before they establish the infection to avoid any harm to the host (Abbas et al., 2015). PAMPs are components of a microbial cell or virus *i.e.* peptidoglycan, lipopolysaccharides (LPS), flagellin, single stranded DNA, unmethylated CpG DNA, G-rich oligonucleotides etc. The characteristic feature of PAMPs is their invariant nature because such molecules are essential for the survival. Thus, PAMPs are distinguishable from host molecules and easy to target by immune system (Figure 1.2).

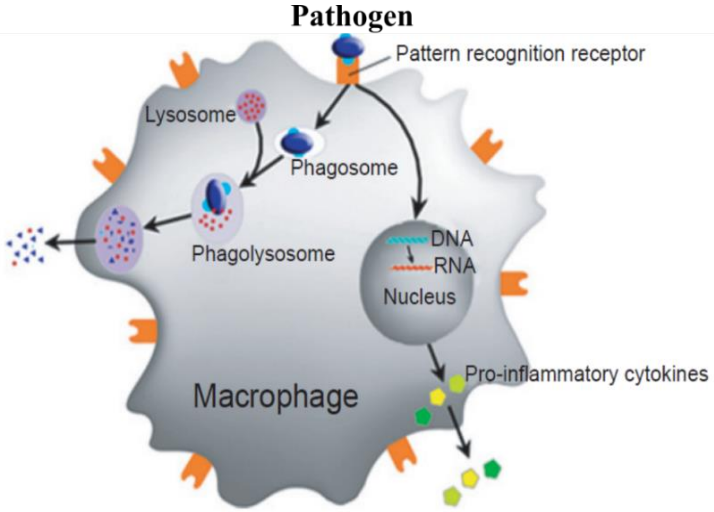

Figure 1.2: Response of macrophage towards pathogen- Pathogen recognized by macrophage killed via phagocytosis and initiate PRR pathways to induce inflammation. Diagram is adapted from (Ranson and Eri, 2013).

Janeway proposed the existence of a class of innate immune receptors capable of recognizing conserved microbial structures or "patterns" before the molecular identification of such receptors (Janeway, 1989). PRRs expression is an evolutionary conserved mechanism to sense infections. PAMPs interactions to PRRs initiate multiple intracellular signaling pathways to activate expression of genes encoding pro-inflammatory and antimicrobial mediators. Products of PRRs responsive genes from innate immune cells eliminate pathogens and activate adaptive arm of immune system to escalate required immune response to eliminate infection.

Innate immune cells and some other non-immune cells *i.e.* fibroblasts, epithelial, endothelial etc. express PRRs (e.g. TLRs, Nod like receptors and Rig-1 like receptors etc.). TLRs are extensively studied and crucial for microbial infection mediated immune responses (Judith A Owen et al., 2013).

1.2.1 Toll like receptors

Toll-like receptors (TLRs) are central and crucial for sensing microbes. Due to homology to the Toll protein from Drosophila melanogaster, which is important in dorso-ventral patterning during embryogenesis and in the antifungal response, the term TLR was derived. TLRs are membrane anchored glycoproteins consisting of a leucine-rich repeat (LRR) motif for an extracellular ligand and a cytoplasmic signaling Toll/interleukin-1 (IL-1) receptor homology (TIR) domain.

Ligand binding to the TLR induces its oligomerization, which subsequently initiates intracellular signal transduction to activate the transcription of inflammatory genes. The TLR family consists of 10 members (TLR1–TLR10) but in humans and mouse they slightly differ in their respective PAMPs recognition. TLRs expressed on the cell surface (TLR1, -2, -4, -5, -6 and -10) mainly recognize bacterial products whereas endosomal expressed TLRs (TLR3, -7, -8 and -9) are specific for nucleic acids (Figure 1.3).

APCs (macrophages, DCs and B cells) are the most important cell types expressing TLRs. TLR expression on these cells are further induced during the course of infection for better immune response (Cohen, 2014; Michael U. Martina and Wesche, 2002).

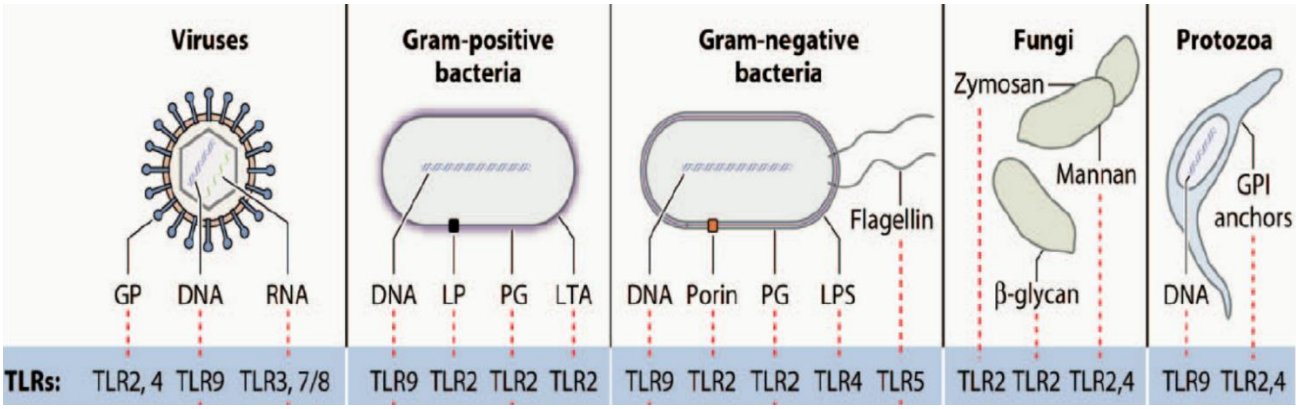

Figure 1.3: Types of PAMPs with their respective TLRs- PAMPs derived from various pathogens and specific TLRs on different cellular localizations in host cell. Adapted from (Mogensen, 2009).

PAMPs are either structural components or genetic material of microbes and recognized by unique PRRs to provide initial sensing. Structural PAMPs, for instance LPS from cell wall of gram-negative bacteria recognized by TLR4, whereas gram-positive bacteria containing lipoteichoic acid, lipoproteins and peptidoglycan are detected by TLR2.

Table 1.1: TLRs and recognized PAMPs from different microbial origin.

PRRs	**Cellular localization**	**PAMPs**	**Microbial origin**
TLR1/TLR2	Cell surface	Triacyl lipopeptides,	Bacteria
TLR2/TLR6	Cell surface	Diacyl lipopeptides Lipoteichoic acid,	*Mycoplasma,* Gram-positive bacteria
TLR2	Cell surface	Peptidoglycan Lipoproteins Envelope glycoproteins GPI-mucin Zymosan, β-Glycan	Gram-positive and -negative bacteria Various pathogens Viruses Protozoa, Fungi
TLR4	Cell surface	LPS	Gram-negative bacteria
TLR5	Cell surface	Flagellin	Flagellated bacteria
TLR7	Endosome	ssRNA	RNA viruses
TLR9	Endosome	CpG DNA	Viruses, bacteria and protozoa

Nucleic acids are recognized in intracellular compartments to exclude host DNA *i.e.* TLR3 recognizes dsRNA (viral replication), TLR7 and TLR8 are activated by single-stranded RNA (RNA viruses). Genomic DNA (unmethylated CpG DNA) from both viruses and bacteria is detected by TLR9 (Mogensen, 2009; Ranson and Eri, 2013) (Table 1.1).

A single pathogen can activate several different TLRs through different PAMPs and likewise, several unrelated pathogens can activate the same TLR. This facilitates a broad reach to innate immune cell for enhanced and simultaneous response against microbial infections.

1.3 TLR signaling

TLR activation is also considered as pro-inflammatory signaling pathway because it activates the innate immune cells and induces inflammation at the site of infection to eradicate pathogen (Boeglin et al., 2011). TLR signaling initiates after PAMP interaction to TLR which leads to conformational changes in cytoplasmic TIR domain to facilitate the binding of adaptor molecules. The adaptor protein MyD88 was first described as a common molecule in signaling pathways triggered by all TLRs (except TLR3) (Muzio et al., 1997). Studies with MyD88-deficient mice revealed the existence of MyD88-independent signal transduction through TLR activation (Takeda and Akira, 2004).

Later on, additional adaptor molecules *i.e.* TIR domain containing adaptor protein (TIRAP), TRIF-related adaptor molecule (TRAM) and sterile alpha armadillo motif containing protein (SARM) were also identified (Yamamoto et al., 2003). TLR signaling cascade can propagate dependent or independent of the MyD88 employment at TIR domain after TLR activation (Figure 1.4).

1.3.1 MyD88 dependent TLR signaling cascade

Upon TLR activation the TIR domain recruits MyD88 (in TLR4 with the help of TIRAP) to initiate the MYD88-dependent signaling cascade (Muzio et al., 1997). MyD88 then interacts with interleukin-associated receptor kinase 4 (IRAK4) and induces its oligomerization which further recruits IRAK1 and/or IRAK2 through N-terminal death domain (DD).

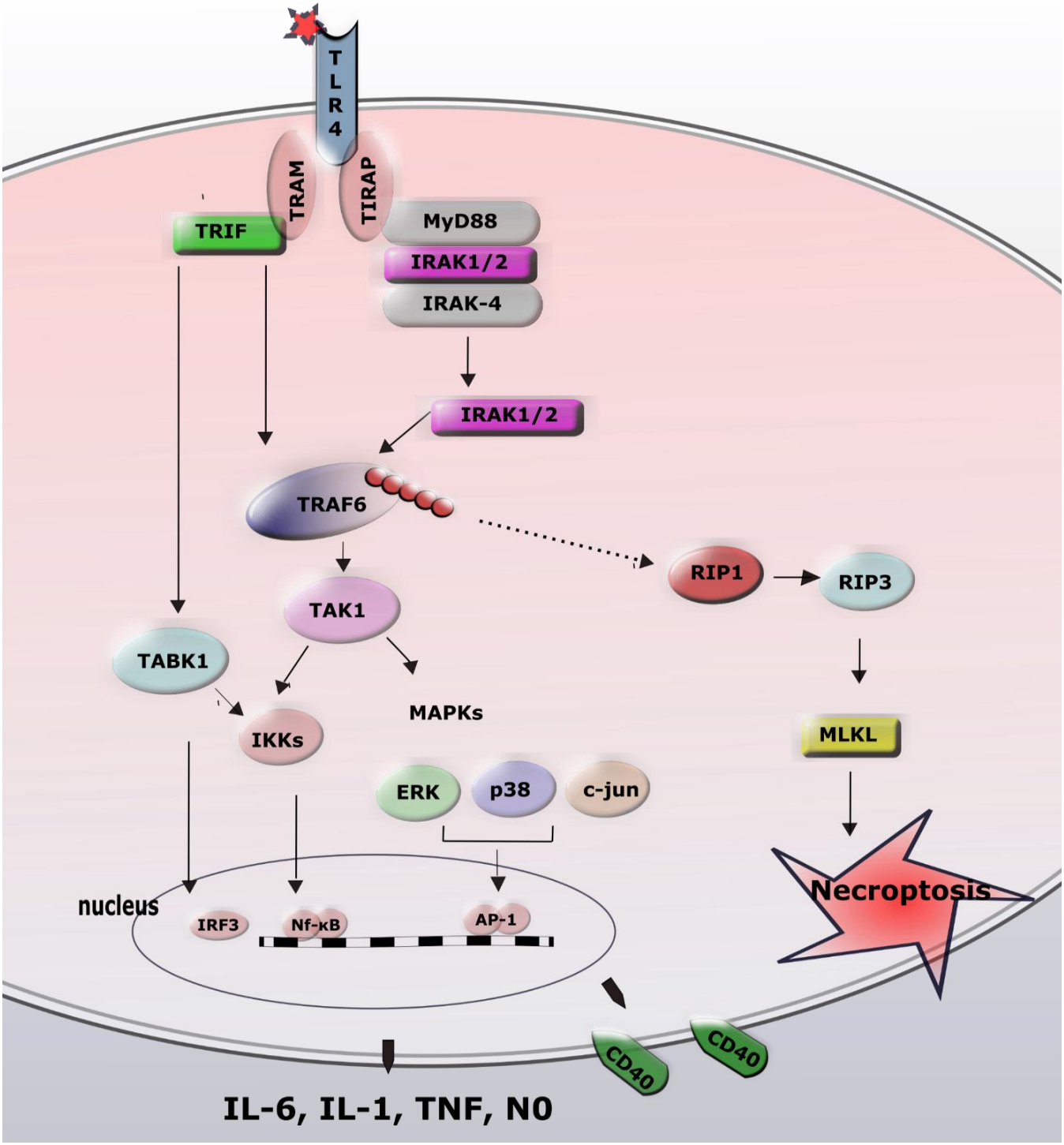

Figure 1.4: TLR4 activation pathway with MyD88 dependent and independent signaling cascade.

This high molecular weight complex is known as "myddosome" (Kawasaki and Kawai, 2014). Activated IRAK1/IRAK2 is released from the myddosome and binds TNF receptor associated factor 6

(TRAF6), an ubiquitin ligase (E3) and enhances its ligase activity (Meylan and Tschopp, 2008). TRAF6 catalyzes the synthesis of K63-linked polyubiquitin chains on TRAF6 itself and other molecules of the complex. Ubiquitinated TRAF6 is recognized by transforming growth factor-activated protein kinase 1 (TAK1) with the help of TAB1 and TAB3.

TRAF6 ubiquitination and subsequent TAK1 activation is crucial for IκB kinases (IKK) and MAPKs activation (Cohen, 2014). Recently, it was shown that TRAF6 can also directly modify IKKγ by K63 ubiquitination (Laplantine et al., 2009). Activated IKKγ interacts with IKKα and IKKβ, which results in phosphorylation of the inhibitory IκB protein. Phosphorylated IκB undergoes proteasomal degradation which results in release of NF-κB subunits (RelA, RelB). NF-κB activation involves phosphorylation and translocation to the nucleus. NF-κB binds to κB sites present in promoters and enhancer sequences of pro-inflammatory genes to enhance the transcription (Napetschnig and Wu, 2013) (Figure 1.4).

TAK1 mediates phosphorylation of several mitogen-activated protein kinase kinases (MKK3, -4, -6 and -7). MKK3/6 further phosphorylate and activate p38, while MKK4/7 activate c-Jun N-terminal kinase (JNK). While extracellular signal regulated kinase 2 (ERK2) is activated by MKK1/2. MAPKs activation collectively lead to activation of the transcription factor activator protein 1 (AP1) (Kawasaki and Kawai, 2014).

TLR pathway molecules mostly play exclusive roles while interleukin receptor associated kinase 1 (IRAK1) and IRAK2 used redundantly (Kawagoe et al., 2008). IRAK1 is essential for the early phase while IRAK2 is important to sustain the later phase of the TLR response (Pauls et al., 2013). Despite of a highly conserved protein sequence IRAK2 does not seem to be equally important in human and mouse TLR pathway (Sun et al., 2016). The variable role of IRAK2 protein makes it interesting molecule of TLR signaling cascade but reason for its specific behavior is not known.

1.3.2 MyD88 independent Signaling cascade

A MyD88-independent pathway was indicated by normal IFN-γ production in MyD88-deficient mice after TLR3 and TLR4 activation. TRIF was shown as an adaptor molecule responsible for signaling in the MyD88-independent pathway (Cohen, 2014). TRIF-mediated TLR pathway activates TBK1 and other IKKs, which further phosphorylate IRF3 and IRF7 to transcribe the *Ifng* gene (Napetschnig and Wu, 2013; Yamamoto et al., 2003).

NF-κB activation in TRIF-dependent pathway happens through binding of TRAF6 to TRIF and subsequent recruitment and activation of TAK1. Additionally, the receptor interacting protein 1 (RIP1) is recruited to TRIF (Youn et al., 2005). Ubiquitinated RIP1 forms a complex with TRAF6 and cooperates in facilitating TAK1 activation to activate NF-κB and MAPKs (Figure 1.4).

1.3.3 TLR signaling response

TLR signaling activates various transcription factors to enhance the expression of pro-inflammatory cytokines e.g. IL-1, IL-6, TNF and chemokines e.g. IL-8, RANTES (Weintz et al., 2010). Pro-inflammatory cytokines increase recruitment of immune cells to the site of infection and activate tissue-resident innate cells. Some non-immune cells *i.e.* epithelial, endothelial and hematopoietic cells also express TLRs to recognize PAMPs to help immune cells at sites of infection (Linthout et al., 2014). Macrophages and DCs also induce secretion of Type I IFN in response to TLR7/9 activation for antiviral defense. Furthermore, TLR signaling induces production of growth factors and anti-apoptotic proteins during inflammation to support host cells division and survival (Chen et al., 2010).

Infection clearance needs a quick, sustained and strong immune response from the innate and adaptive immune system. Initially, Janeway postulated a link between pathogen recognition and the induction of co-stimulation and hence activation of pathogen specific T cells (Janeway, 1989). Indeed, the TLR pathway activates macrophages and DCs to upregulate expression of costimulatory molecules (CD80, CD86 and CD40), antigen presenting MHC molecules and chemokine/cytokine receptors. These activated innate APCs further interact with T cells to induce their differentiation and

cytokine secretion (Schott et al., 2007). On the other hand, type II IFN produced by activated T cells further activates macrophages to escalate the inflammation process (Ranson and Eri, 2013). TLR signaling also activates B cells, induces their proliferation and Ig secretion (Pasare, 2005).

TLR induced innate immune cells exhibits upregulated anti-apoptotic proteins expression to sustain cell survival during acute inflammation (Jain et al., 2014). Under certain circumstances TLR signaling can induce a form of programmed cell death termed as necroptosis (Baker et al., 2014). Both MyD88 dependent and TRIF-mediated signaling cascades induce necroptosis (Lamothe et al., 2013). During inflammation, necroptosis is mediated by receptor interacting protein kinase-3 (RIP3) and its substrate mixed lineage kinase like (MLKL), without caspase activation (Pasparakis and Vandenabeele, 2015a).

1.4 IRAK2 is an ambiguous player of TLR signaling

IRAK2 belongs to the 4 member IRAK family proteins, among them IRAK1, IRAK2 and IRAK4 are ubiquitously expressed, while IRAK3 is restricted to macrophages and monocytes. All IRAKs share similar functional domains (Figure 1.5). They contain an N-terminal death domain (DD), a ProST domain (proline, serine and threonine rich domain), a conserved kinase domain (KD) and a C-terminal domain (except for IRAK4) (Rhyasen and Starczynowski, 2014).

The DD mediates interaction with MyD88 and IRAK4. The ProST region is rich in prolines, serines and threonines and is important for post-translation modifications. The KD of the IRAK family has an activation loop which is important for kinase activity. The C-terminal domain contains a TRAF6 binding motif (Jain et al., 2014).

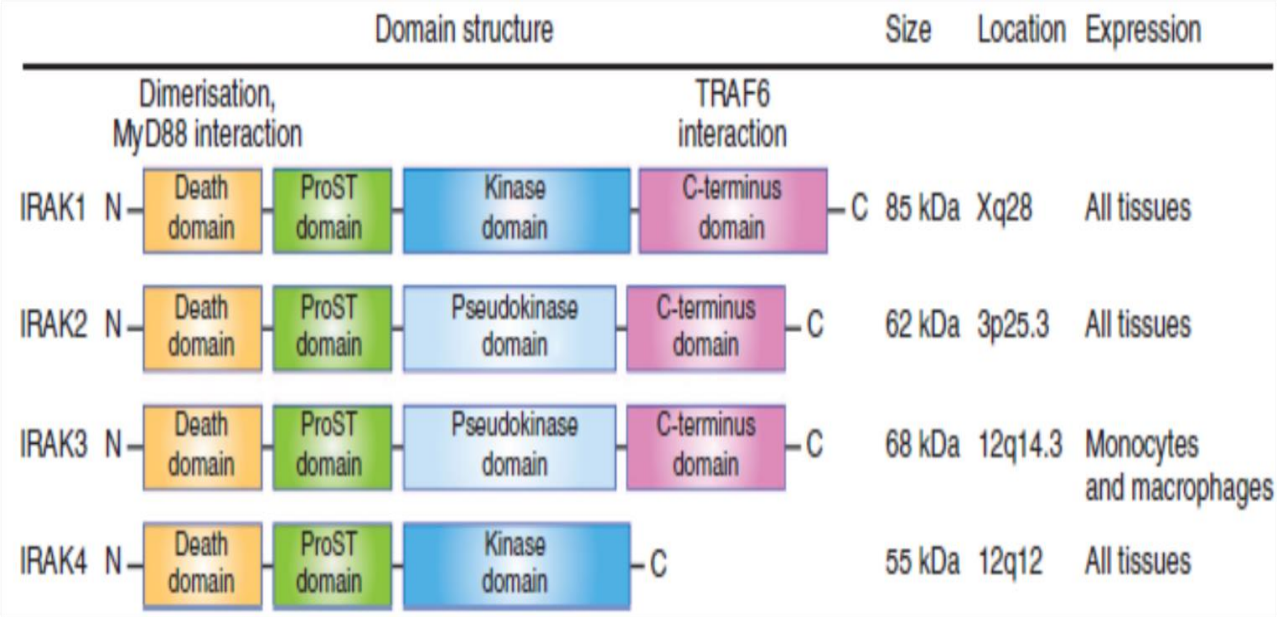

Figure 1.5: IRAK family member proteins and their functional domains- A comparison of IRAK family proteins with their respective differences and expression. Figure is adapted from (Rhyasen and Starczynowski, 2014).

The human IRAK2 protein was identified as homologue to IRAK1 and Pelle (IRAK2 orthologue in drosophila) (Muzio et al., 1997). It was characterized as a component of TLR/ IL-1 pathway. It was presumed to only have a redundant role with IRAK1 (Muzio et al., 1997; Olaf Rosati and Martin, 2002). However, the exclusive importance of IRAK-2 was described, when it was discovered that Vaccinia Virus protein A52 binds specifically to IRAK2 but not with IRAK1 and is capable of inhibiting TLR mediated NF-kB signaling (Harte et al., 2003; Maloney G et al., 2005). The murine *irak2* gene produces four splice variants of IRAK2 (a-d), but no variant is known in humans (Hardy and O'Neill, 2004).

Extended study with IRAK2 knock-out mice revealed the importance of IRAK2 in multiple TLR pathways (Meylan and Tschopp, 2008). IRAK2 mediates pathology by promoting Th17 cell development by enhancement of RAR-related orphan receptor gamma (RORγt) and Basic Leucine Zipper ATF-Like Transcription Factor (BATF) followed by IL-1β production (Smith et al., 2011). In humans, IRAK2 variants with mutations in non-TRAF6 binding site are associated with altered TLR

signaling and enhanced survival risk in colorectal cancer patients (Wang et al., 2015; Wang et al., 2014).

1.5 Regulation of TLR signaling response

TLR signaling initiates inflammatory responses which also harms host tissue. Unregulated TLR signaling causes chronic inflammatory disorders, autoimmunity and cancer progression. Hence, tight regulation of TLR pathway is essential to ensure the strong, albeit transient inflammation till pathogen elimination. TLR signaling is amplified during early infection but restricted by downregulation at later stages when infection is resolved. Regulatory molecules, *i.e.* suppressor of cytokine signaling 1 (SOCS1) and casitas B cell lymphoma protein b (Cbl-b) inhibit the MyD88 dependent pathway while the TRIF dependent pathway is inhibited by SARM (Cohen, 2014; Mogensen, 2009). Chaperones like heat shock protein 70 (hsp70) inhibits TRAF6 ubiquitination via direct interactions (Chen et al., 2006). IRAK1 is stabilized by direct interaction of HSP90 to sustain TLR induced signaling (Nardo et al., 2005). HSP90 also binds with IKKγ (NEMO) to maintain downstream NF-κB activation (Park et al., 2007). HSP90 regulates overall inflammation process through controlling various NF-κB activating molecules in the TLR pathway (Meike Broemer et al., 2004; Poulaki et al., 2007). This variety of molecules in the TLR signaling cascade indicate the complexity and necessity of this regulatory mechanism.

1.5.1 Post-transcriptional regulation

Pro-inflammatory genes induced by TLR signaling are also regulated at post-transcriptional level for balancing ongoing inflammation reaction. Most mRNAs of TLR responsive genes contain AU-rich element (ARE) at their 3' untranslated region. TLR signaling (mainly p38 MAPK pathway) induces ARE binding protein *i.e.* Tristetraprolin (TTP) to regulate mRNA degradation (Kawasaki and Kawai, 2014) (Barreau et al., 2005). TRAF6 ubiquitination (Yin et al., 2011) and IRAK2 are important in mRNA stabilization of various TLR induced pro-inflammatory cytokines (Wan et al., 2009; Yin et al., 2011).

Some microRNAs are also involved in the regulation of TLR pathway components *i.e.* TRAF6 and IRAK1 (Taganov et al., 2006). Various microRNAs *i.e.* miR-146a, miR-199a, miR-155, miR-126, miR-21, miR-29, miR-148/152 and miR-466 control the TLR pathway at different levels.

1.5.2 Ubiquitination mediated regulation

Many proteins in the TLR pathway are regulated by direct post-translational modifications *i.e.* phosphorylation, ubiquitination, sumoylation. Ubiquitination of TRAF6, TAK1, IKKγ (NEMO), IκB and IRAK1, IRAK2 are essential for TLR signaling (Chen, 2005 ; Wertz and Dixit, 2010). In addition to the direct ubiquitination of the signaling components, the activity of these proteins can also be regulated by ubiquitination of their regulatory proteins. For example, IRAK1 and IKKγ activity and stability are regulated by HSP90 ubiquitination (Nardo et al., 2005; Park et al., 2007).

The ubiquitin modification process is a complex mechanism and involves sequential action of 3 enzymes *i.e.* E1, E2 and E3 responsible for ubiquitin activation, transfer and ligation, respectively (Jadhav and Wooten, 2009). Ubiquitin is a small (8.5 kDa) protein added on a lysine residue of a substrate protein as monoubiquitin or as K48- or K63-linked polyubiquitin chains via E3 ubiquitin ligases. E3 ligases are divided in two categories based on their catalytic domain *i.e.* homologous to E6AP carboxy terminus (HECT) domain or really interesting new gene (RING) finger (Pickart, 2001).

There are ~30 HECT domain containing E3 ligases like UBE3A, ITCH, SMURF1 and HECTD1. The most E3 ligases belong to the RING finger family. Examples are: MDM2, PARKIN, TRAF2 and TRAF6. TRAF6 can ubiquitinate itself after activation induced by IRAK2 interaction (Wang et al., 2010). Other regulatory molecules *i.e.* HSP90 is a substrate of HECTD1 and regulates its intracellular localization by K63-polyubiquitination (Sarkar and E Zohn, 2012). Although exact involvement of E3 ligase activity in TLR signaling is unclear because specificity for more than one substrates bring more complexity in understanding.

Ubiquitinated proteins are regulated by ubiquitin editing enzymes and deubiquitinating enzymes (DUBs). DUBs like A20, CYLD etc. regulate TRAF6 and TAK1 ubiquitination (Kelly et al., 2011; Kim

et al., 2003; Vereecke et al., 2009). Ubiquitination mediated regulation is essential for TLR signaling and the subsequent inflammatory response (Tenekeci et al., 2016; Walczak et al., 2012). Certain E3 ligases can target multiple substrates and certain protein substrates can be modified by different E3 ligases (Taipale et al., 2012). Various immunopathologies are reported with defective ubiquitination regulation (Fan et al., 2010; Vereecke et al., 2009).

1.6 Abnormalities associated with TLR signaling molecules

TLR signaling is critical for innate immunity to eliminate pathogens as well as for wound healing. However, TLR signaling also supports tumor development. Several TLR signaling-dependent inflammatory molecules in the tumor microenvironment regulate the production of factors which in turn promote tumor growth, metastasis, immune suppression and chemotherapy resistance (Jain et al., 2014; Rhyasen and Starczynowski, 2014).

Thus, unregulated activation of TLRs, mutations in their signaling molecules, persistent inflammation and subsequent damage to host are key reasons for development of various diseases *i.e.* autoimmunity, chronic inflammation and allergies.

Table 1.2: Inherited defective TLR signaling and associated immune abnormalities (Mogensen, 2009).

TLR pathways	Associated disease complexities
TLR2	Susceptibility to leprosy and tuberculosis, staphylococcal disease, Increased severity of genital herpes, Protection from Lyme disease.
TLR4	Susceptibility to infection with gram-negative bacteria, malaria and RSV, meningococcal sepsis, Protection from Legionnaires' disease.
TLR7	Protection from hepatic fibrosis in chronic HCV infection.
TLR9	Susceptibility to SLE, Rapid progression of HIV infection, Increased risk of low birth in malaria during pregnancy.

Different studies have established a link between activation of TLRs and cancer (Rakoff-Nahoum S and R, 2009). Some types of diffused large B-cell lymphoma develop with inherited MyD88 mutations and subsequent aberrant activation of the transcription factors NF-κB, JAK and STAT (Carpenter and Lo, 2014; Ngo et al., 2011). Furthermore, certain mutations in A20 and TBK1 are also associated with B-cell lymphoma development and lung cancer, respectively (Compagno et al., 2009). Components of different TLRs pathways contribute to immune disorders, when they are altered, unavailable or in excess are listed in (Table 1.2). Different studies have reported inherited variants of IRAK2 proteins, which resulted in inefficient clearance of Hepatitis C virus (HCV) infection and enhanced survival risk in colon cancer patients (Rhyasen and Starczynowski, 2014; Wang et al., 2015; Wang et al., 2014). Increased levels of different IRAK proteins were also observed with a number of immune and non-immune cancers (Figure 1.6) (Rhyasen and Starczynowski, 2014).

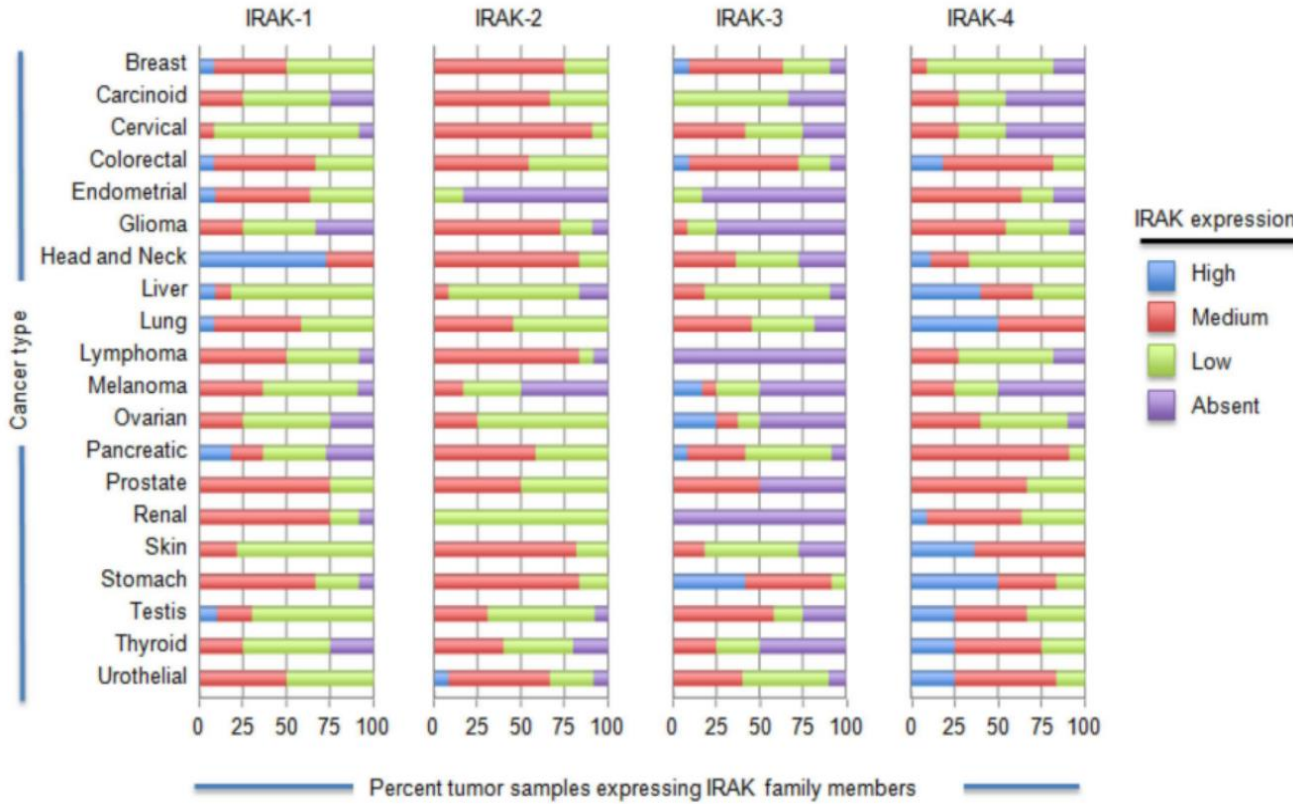

Figure 1.6: Expression level of IRAK proteins in different type of cancer- Schematic representation of the IRAK family members expression from cancer patient samples. Adapted from (Jain et al., 2014).

Other immunopathological complexities associated with malignancies were also found to be associated with IRAK proteins (Jain et al., 2014). Collectively, TLR signaling and the hereby induced inflammatory process play contradictory roles with the potential of promoting anti-tumor immune responses and paradoxically, supporting tumor growth at the same time (Kawasaki and Kawai, 2014; Maiorov et al., 2013). Defects in TLR pathway molecules enhance disease probabilities such as Inflammatory bowel diseases (IBD), Crohn's disease and ulcerative colitis which cause severe gastrointestinal dysfunction and reduced the quality of life (Ranson and Eri, 2013). Thus, detailed understanding of TLR signaling is mandatory to shift the balance from supporting to blocking cancer growth.

Specific aims and objectives of PhD thesis

TLRs induced signaling in innate immune cells is important for mounting an inflammation reaction and for activation of adaptive immunity. On B cells TLR pathways induce cell proliferation and Ig production via cross talk with BCR signaling. Defect in TLR signaling can lead to inefficient elimination of microbes or chronic inflammatory diseases as well as to autoimmunity and malignancies. Regulation of TLR pathway is essential for an optimal immune response and understanding of functional dynamics of its signaling components is required to treat related abnormalities.

IRAK2 is an essential part of TLR/IL-1 signaling cascade and its functional behavior is poorly understood. The aim of my PhD project is to investigate the role of the C-terminus of IRAK2 in TLR signaling by employing the mouse macrophage cell line RAW264.7. Further, I investigated the interaction of IRAK2 with the pro-survival protein A1.

Additionally, I analyzed B cell development and immunoglobulin response in mice with a B cell lineage-specific deletion of HECTD1.

2. Materials and Methods

2.1 Materials

2.1.1 Cells

RAW 264.7 macrophage cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin antibiotics. Human embryonic kidney (HEK) 293T cells were maintained in Modified Eagle's medium (MEM) and that supplemented with 1% penicillin/streptomycin antibiotics and 10% fetal bovine serum (FBS).

B cells: Splenic B cells enriched from Splenocytes via T cell depletion with Thy1 specific monoclonal antibody mediated complement system activation (Thy1, a protein expressed by all T cells, but not other leukocytes).

Obtained B cells cultured in Roswell Park Memorial Institute medium RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 0.1mM nonessential amino acids, 55 μM β-mercaptoethanol and 10mM HEPES.

2.1.2 Reagents

LPS from Escherichia coli (O111:B4) was purchased from Sigma-Aldrich.

CpG; Imiquoid; PamCSk4; IFN-γ was purchased from Sigma Alderich.

Antibodies for immunoblotting

Primary antibodies: anti-GFP (2956S), anti-Myc (2276S), anti-phosphorylated RelA (3033S), anti-RIP3 (15828S) were obtained from Cell signaling, anti-FLAG (F-3165) purchased from Sigma-Aldrich.

anti-ERK-2 (SC-154), anti-HA tag (Y-11), anti-GAPDH (FL-335), anti-phopsphorylated ERK1/2 (cell-signaling #9101), anti-RelA (622601) obtained from Biolegend.

Annexin-V (88-8007) from eBioscience.

Secondary antibodies: horseradish peroxidase -conjugated affinity pure Goat Anti- Rabbit IgG (Jackson immunoreaction laboratories, 111-035-144)

Antibodies for FACS staining:

Antibodies against surface markers, such as B220 **(**103206, 103210, 103210, 103226), CD8a (100706), CD21 (115506), CD25 (102006, 102016), CD27 (124216), IgD (405703), CD4 (100408) obtained from Biolegend; B220 (553087, 553088, 553100, 553089, 557683), CD4 (553651), CD8 (553033), CD19 (553785, 557398, 552854, 550992), CD21 (553818, 558658), CD23 (553138, 553139), CD25 (553866, 102016), CD27 (124216), CD117 (553354, 553355), IgD (553507, 553439), IgM (553409, 552867), CD11b (557397), CD40 (553791), CD138 (553714) from BD; CD43 (eBioR2/60), CD25 (61-0251-80) purchased from eBiosience.

Buffers

Table 2.1: Recipe for various buffers used during immunoblot assay.

Buffers	Components
RIPA	Tris-HCl pH 7.5 (50 mM), NaCl (150 mM), NaF (50 mM), EDTA (2 mM), Na-deoxycholate (0.5%), SDS (0.1%), Nonident-P 40 (1%)
Laemmlli buffer (5X)	Tris-HCl pH 6.8 (250 mM), SDS (10%), glycerol (20%), Bromophenol blue (0.25%), DTT (500 mM)
SDS Running buffer	Tris-HCl pH 8.3 (250 mM), glycine (1.92 M), SDS (1%)
SDS gel transfer buffer	Tris (25 mM), glycine (192 mM), methanol (10%)
TBST	Tris-HCl pH 7.5 (50mM), NaCl (150mM), Tween-20 (0.05% (v/v))
PBS	NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), KH_2PO_4 (0.24 g), di H_2O (800 ml) makeup in 1 Liter pH 7.4 adjusted with NaOH
FACS staining buffer	PBS with NaN_3 (0.1%)
PBST	PBS with Tween-20 (0.05% (v/v))
ELISA blocking buffer	PBS with Bovine serum albumin (1%)

2.1.3 Mice

B cell specific *hectd1*$^{-/-}$ mice on a C57BL/6 genetic background were generated by loxP-Cre strategy as described in (**Figure 2.3**). *hectd1*$^{flox/flox}$ mice provided by Dr. Marco Herold and Prof. Andreas Strasser from The Walter and Eliza Hall, Institute of Medical Research, Australia. Mice expressing Cre recombinase from the B cell-specific *Cd79a* locus (*Mb1*^{CRE}) were provided by Dr. Elias Hobeika,

University of Ulm and Prof. Michael Reth, Max Planck Institute of Immunobiology and Epigenetics, Freiburg. Colonies of Knock-out and control mice were maintained in our animal facility at Institute of Virology and immunobiology.

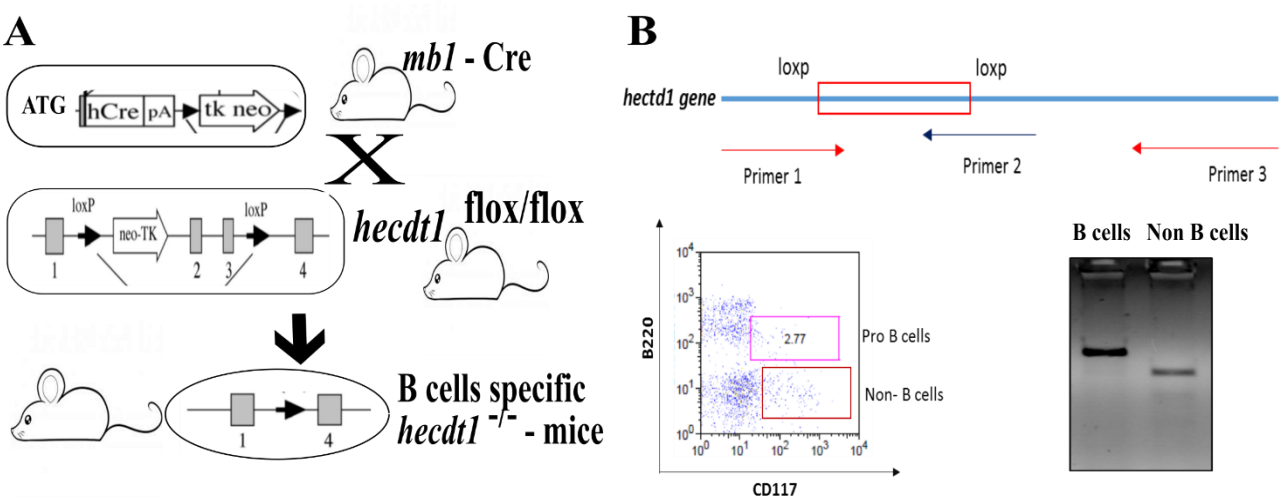

Figure 2.1: B cell specific *hectd1*$^{-/-}$ mice generation- Schematic representation of loxp and Cre strategy (A). Confirmation of *hectd1*$^{-/-}$ B cells in mouse by PCR using *hectd1* gene specific primer. FACS sorted B cells in and non-B cells were obtained from bone marrow (B).

All mice were kept under pathogen-free conditions. Use of mice in this study was according to a protocol approved by university of würzburg Animal Care and Use Committee.

2.2 Molecular Biological Methods

2.2.1 Polymerase chain reaction (PCR)

For site-directed mutagenesis and cloning gene constructs into plasmids, PCR was used. All PCR reactions were prepared with 4-100ng of DNA template, 0.2pmol of the forward and 0.2pmol of the reverse primers, 0.2mM deoxyribonucleotide triphosphates (dNTPs), 2mM MgSO4 and 2U of the thermostable *Pfu* polymerase in a reaction volume of 20µl containing the appropriate amount of the reaction buffer (Thermoscintific).

The PCR reactions were run on Biometra T3 thermocycler, which were programmed according to the parameters listed below (Table 2.2).

Table 2.2: PCR thermocycler program.

Program Steps	Cycles	Temperature (°C)	Time
Initial Denaturation	1	95	1min
Amplification	25-40	95	30s
		50-65	45s
		72	30s/1000bp
Extension	1	72	10min
Storage	1	4	hold

2.2.2 Quantitative real-time polymerase chain reaction

The Real-time Polymerase Chain Reaction is an updated version of standard PCR and provides real-time measurement of amplification with the progress of reaction, known as quantitative PCR (qPCR). This PCR was used to quantify the mRNA expression level of genes in RAW cells stimulated with TLR ligands.

RNA isolation

In order to assess the TLR signaling targeted genes expression level, total RNA was isolated from TLR activated RAW cells (1-1.5×105 cells/well from 24 well plate). RNA was extracted by Trizol reagent according to the protocol instructed by manufacturer. Isolated RNA was purified with 70% ethanol and measured for yield using Thermofischer nanodrop machine.

cDNA synthesis

Equal amount (2µg) of purified mRNA was transcribed to cDNA using first strand cDNA synthesis kit from Thermoscientific. RNA was diluted in distilled H2O including 1µl of oligo dT primers and heated at 65°C for 5 minutes and then reverse transcription master mix in provided buffer comprises of dNTPs, ribonuclease inhibitor and M-MuLV reverse transcriptase (RT) was added in tube. cDNA amplification was allowed on 37°C for an hour which was followed by a final inactivation period of 5

minutes at 95°C. For potential contamination of genomic DNA in RNA, control PCR reaction was performed in which the reverse transcriptase was replaced with distilled H2O.

qPCR

The quantification interested gene from total mRNA was done by adding specific primers in reaction mixture with SYBR Green qPCR master mix (Biotool). The qPCR reaction was done at Roche Light cycler 96 with amplification program suggested by Biotool. The relative expression of target mRNAs was calculated by normalization to β-actin mRNA with the ΔΔCt method.

Primers: (mouse genes)
β-actin - forward (5'-TCACCCACACTGTGCCCATCTACGA3') and
reverse (5'-GGATGCCACAGGATTCCATACCCA),
il-6 - forward (5'-TGGAGTCACAGAAGGAGTGGCTAAG-3') and
reverse (5'-TCTGACCACAGTGAGGAATGTCCAC),
tnf-α - forward (5'-ATAGCTCCCAGAAAAGCAAGC-3') and
reverse (5'-CACCCCGAAGTTCAGTAGACA-3')
il- β - forward (5'-GCCTTGGGCCTCAAAGGAAAGAATC-3') and
reverse (5'-GGAAGACACAGATTCCATGGTGAAG-3')

2.2.3 Plasmids and DNA purification

Plasmids bought and engineered to express desired protein were maintained in sufficient amount by sub-cloning in appropriate bacterial strain by transformation and subsequent plasmid DNA isolation. Bacterial transformation of DH5-α strain of *E. coli* with 100ng plasmid DNA was performed and allowed overnight growth on the agar plates containing suitable antibiotic (depends on antibiotic resistant gene plasmid contains).

Several distinct colonies were picked up and inoculated into 3-5ml LB medium (mini prep) or into 50ml-200ml LB medium (medi prep) supplemented with the required antibiotics. The cultures were

maintained for 16 hours at 37°C on continuous shaker incubator. Then the DNA was extracted from the bacteria using the Sigma-Aldrich kits for mini, medi and maxi prep.

2.2.4 Plasmid constructs and cloning strategies

Mammalian expressing plasmid constructs obtained from plasmid library developed by our research group. Mouse genes for required protein were amplified by RT-PCR from mRNA pool of murine cells. Mutant protein expressing constructs were obtained by site directed mutagenesis using PCR. All plasmids were cloned in competent *E.coli and* increased amount of plasmid DNA was purified via Medi preparation kit from Sigma-Alderich and stored at -20^{0}C. A representation of Myc-IRAK2 expressing plasmid is shown in below (Figure 2.1) and expression of other proteins were also achieved by similar plasmid constructs.

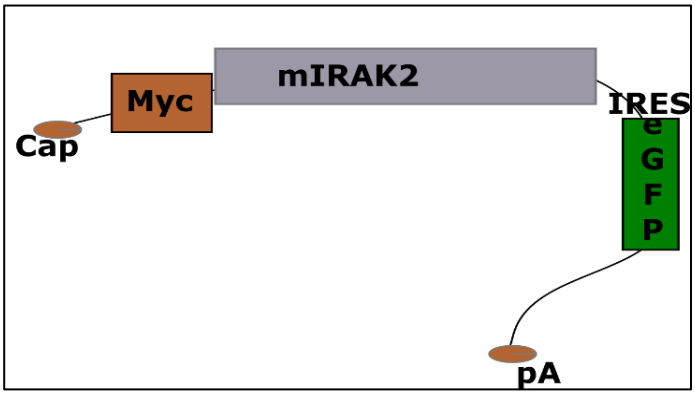

Figure 2.2. Graphic representation of mammalian plasmid construct- Plasmid expressed Myc- IRAK2 in mammalian cells. It contains GFP expressing gene under IRES control and used as marker for protein expression.

2.3 Genetic transformation in mammalian cells

2.3.1 Transient transfection

Polyethylimine (PEI) was taken as a transient transfection reagent for HEK293T cells and generally applied for gene expression assays or retroviral particle generation. 1.5 million HEK-293T cells were seeded in 3.5 mm cell culture plates 20 hrs before replacement of medium with fresh OptiMEM (life-technology). Transfection mixture contains 3X PEI of plasmid DNA used and mixed properly in a volume of 500 µl of OptMEM and incubated for 30 min at room temperature. This transfection mixture applied dropwise on seeded OptiMEM containing HEK-293T cells and placed in incubator.

Culture medium was replaced with fresh DMEM supplemented with 10% FBS after 6 to 8 hrs post transfection. Cells were harvested or supernatant collected after 20 to 40 hrs of transfection according to the requirement of experiments.

2.3.2 Stable transduction

A retroviral vector is an infectious virus used to introduce a non-viral gene into mitotic cells *in vivo* or *in vitro*. The advantage of this vector is that it has higher transduction efficiencies than transient transfection and allow the stable integration of the gene into recipient cell's genome. The gene of interest flanked at the 5' and 3' end by long term repeat (LTR). The vector was transfected in to a packaging cell line HEK 293T.

Since the vector contains the packaging sequence ψ, the RNA is recognized by capsid proteins encoded by the packaging cell line and packed into viral particles that are released into supernatant. The cultured supernatant is harvested after 48hours post-transfection and filter sterilized for further use.

Desired gene expressing vector has an internal ribosomal entry site (IRES) upstream to GFP expressing gene and antibiotic resistance protein expressing gene that allows the expression of GFP and antibiotic resistance gene, which makes stably transfected cell lines resistant to antibiotic selection.

Transduction and selection: Retroviral containing supernatant (3ml) from transfected HEK 293T cells applied with polybrene (10ug/ml) to RAW cells and centrifuged for 2 hrs at 2200 rpm after 30 minutes of gentle mixing. Cells that were successfully infected and integrated the genes of interest were selected by corresponding antibiotic (zeocin) or GFP associated sorting via FACS.

2.4 *irak2* gene deletion in RAW cells using CRISPR-Cas9 technique

Clustered regularly interspaced short palindromic repeats (CRISPR) are present in genomic DNA and transcribed CRISPR RNA (CrRNA) guides CRSIPR associated endonuclease (Cas9) to generate double stranded cut.

A short conserved sequence known as protospacer-associated motif (PAM) e.g. TGG, follows immediately 3´ of the crRNA and required for double-stranded endonuclease activity of Cas9.

This strategy is used by bacteria to defend from viruses and used in eukaryotic cells for altering specific genes to generate knock-out cells and organisms. We used this technique to obtain IRAK2 KO RAW cells.

Lentiviral particle generation

As described above for retroviral particle generation, HEK 293T cell plated at density of 1.5 million cells in 3.5mm cell culture plates. Transfection was done using PEI and CMV-mCherry-Cas9 or CRISPR gRNA expressing plasmid DNA with lentiviral packaging plasmids.

After 6 hrs culture media was replaced with fresh media. Following to that, after 24 hrs culture supernatant was collected and filter sterilized. Cas9 plasmid contained gene for mCherry and CRISPR gRNA plasmid had gene for eGFP constitutive expression which was used as marker for transduction.

Stable transduction of RAW cells:

RAW cells transduced with Cas9 containing lentiviral and mCherry expressing cells were sorted via FACS. mCherry expressing cells were sorted (using FACS Aria) and then further transduced in same way with plasmid containing CMV-BFP-IRAK-2 specific CRISPR guide RNA (gRNA) driven by the U6 promoter.

Critical exons of the IRAK2 genes were targeted with the following CRISPR gRNA constructs:

IRAK2, 5′- ACACGCTCAGTGAGTGGGAC -3′

Obtaining IRAK2 deficient RAW cells

CRISPR and cas9 co-expressing single cells were sorted by FACS. Each single clone was cultured and analyzed by FACS for LPS induced CD40 expression. Non-responding clones further

sequenced for IRAK2 genes gRNA targeted exons, disrupted genes given the non-responsive phenotype in LPS induced CD40 expression (Figure 2.2).

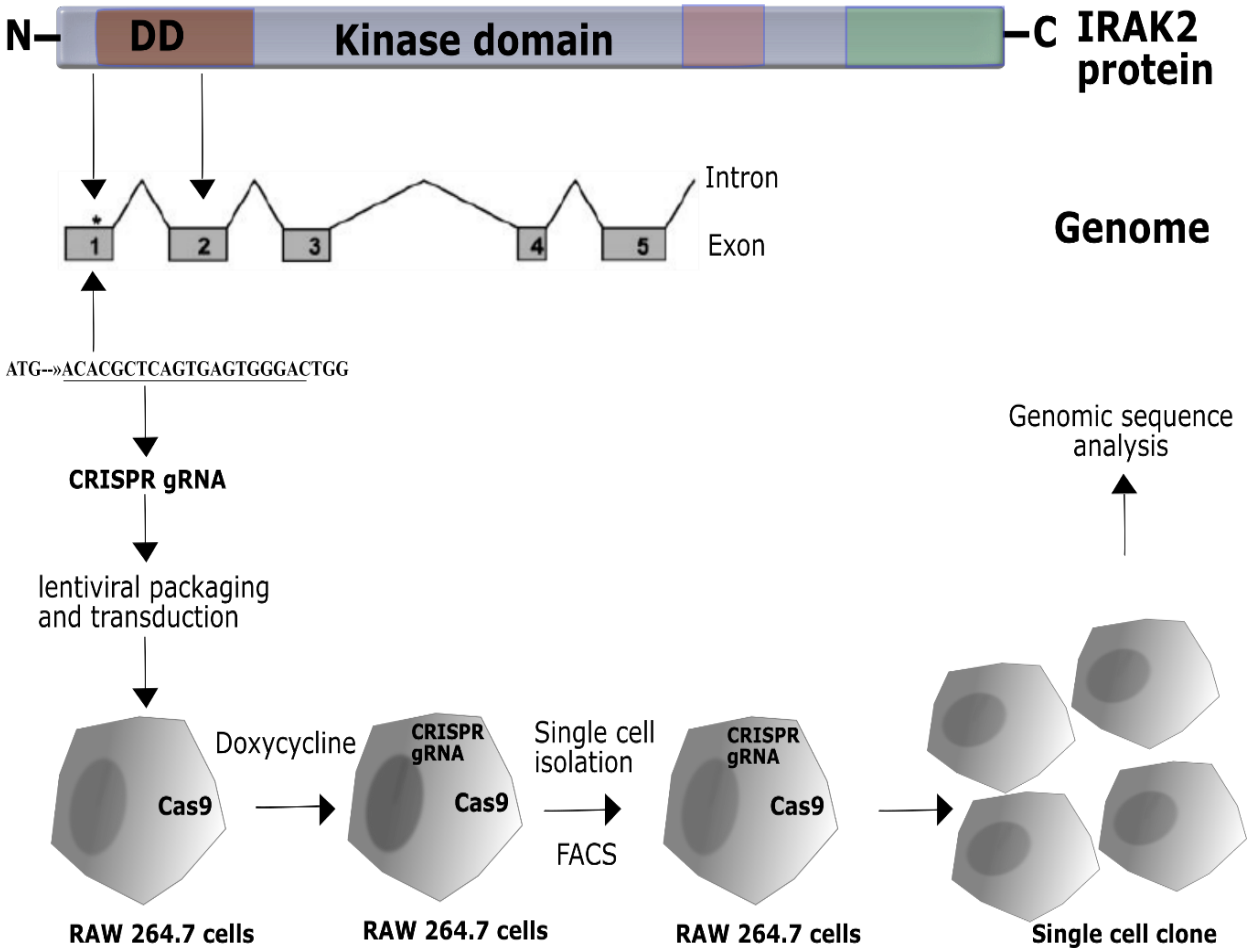

Figure 2.3: Schematic representation of CRISPR-Cas9 strategy for generation of IRAK2 deficient RAW cell.

Genomic sequence analysis of IRAK2 KO RAW cell

Isolated probable IRAK2 non-expressing single clone was analyzed for altered irak2 gene. For that, gene specific set of primers- Forward (5'- TGCTCGCTTACCCAGTGGGGC-3') and

Reverse (5'- GACAGATCCACGCCCCCCC-3')

were used and gene amplified by TOPO PCR cloning kit obtained from Thermo fischer scientific. Obtained genomic sequence from both alleles are shown in Figure 2.4.

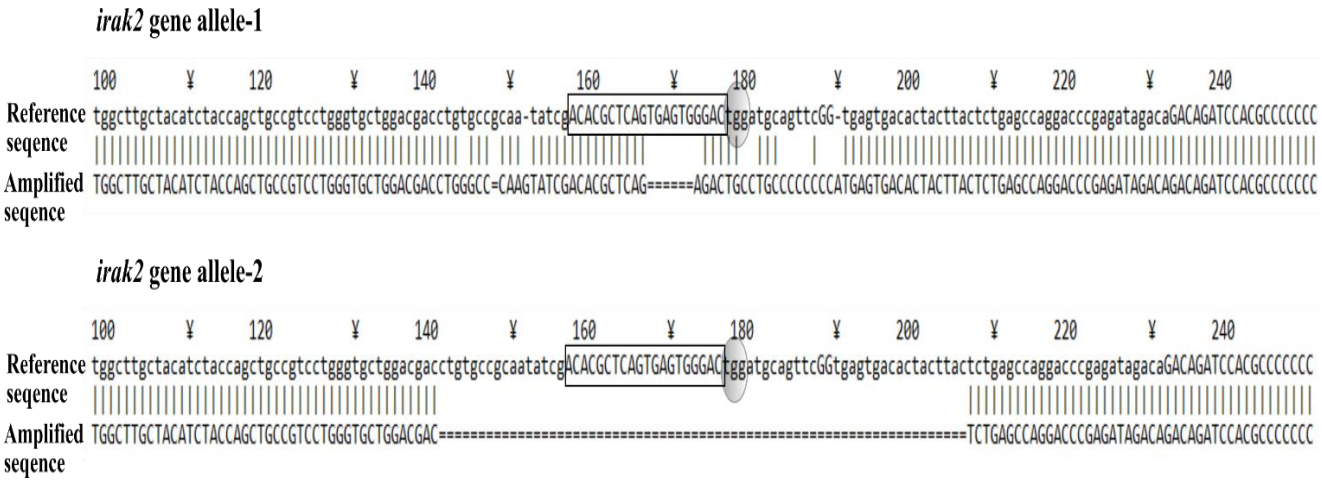

Figure 2.4: Genomic DNA sequence analysis of RAW cell single clone obtained by CRISPR-Cas9 technique- Both alleles of irak2 gene were amplified from single clone of RAW cell and aligned with reference sequence. Altered sequence with nucleotides insertion and deletion from RAW cell genome in irak2 gene is shown. CRISPR gRNA sequence is highlighted in boxed area and adjacent PAM site in circle.

2.5 Biochemical Methods

2.5.1 SDS-polyacrylamide electrophoresis

Cell lysate preparation: Cells were harvested and washed twice with ice-cold PBS and subjected to radio immunoprecipitation assay (RIPA) buffer containing a protease inhibitor cocktail (Sigma-Aldrich), Phosphatase inhibitor cocktail (Sigma-Aldrich) and 1mM DDT to lyse the cells for 15 min. This mixture centrifuged for 15 min at 4°C at full speed to settle down genomic DNA and major cell debris. Supernatant diluted with 5X lamellae buffer and boiled for 10 min and stored at -20°C.

Cell lysates were resolved with appropriate poly acrylamide gel electrophoresis (PAGE) in tris-glycine-SDS running buffer System (Bio-Rad assembly).

Resolution of cell lysate in SDS PAGE: The Sodium–Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed with the Bio-Rad Mini protean chamber system. Protein samples diluted with the 5X lamellae buffer and denatured at 90°C for 10min subjected to wells of polyacrylamide gel soaked in SDS-tris-glycine running buffer. Polyacrylamide gel loaded

with protein sample was run for 2 hrs at 120 Volts. Proteins with larger molecular weight were run upside down and pre-stained protein marker (PageRuler, Fermentas) was loaded on each gel and served as a reference for the molecular weight.

Table 2.3: SDS resolving gel recipe for 5ml or 1 mini gel.

Components	**8%**	**10%**	**12%**	**14%**
dd H2O	2.3 ml	1.9 ml	1.6 ml	1.3 ml
30% acrylamide (gel 30)	1.3 ml	1.7 ml	2.0 ml	2.3 ml
1.5 M Tris-HCl (pH 8.8)	1.3 ml			
10% APS	50 µl			
TEMED	10 µl			

Table 2.4: SDS stacking gel (4%).

Components	**2 ml**	**4 ml**
dd H2O	2.4 ml	4.8 ml
30% acrylamide (gel30)	0.5 ml	1 ml
1.5 M Tris-HCl (pH 6.8)	1 ml	2 ml
10% APS	100 µl	100 µl
TEMED	20 µl	20 µl

2.5.2 Immunoblot analysis

The immunoblotting is an antibody mediated detection of protein blotted on PVDF membrane from polyacrylamide gel.

Protein transfer on PVDF membrane from SDS-PAGE: It was performed with house made semi-dry transfer instrument using transfer buffer provided by soaked Whatman filter papers below PVDF membrane and above polyacrylamide gel. This arranged stack of gel with PVDF membrane (pre-incubated with transfer buffer following activation with methanol) was subjected to electricity of 12 volts for 90 minutes. The proteins were transferred to PVDF membrane with either 0.45µm pore size and transfer efficiency was confirmed by the transfer of the pre-stained protein marker. Then, the

PVDF membrane was blocked for 60 min with a 5% skimmed milk or BSA diluted in TBS with 0.5%Tween buffer.

Immunoblotting: PVDF membrane with transferred protein incubated with respective primary antibody (diluted desirably with blocking buffer) at 4°C overnight on rocker. After incubation, membranes were washed 3 times for 5min with washing buffer (TBS-0.5% Tween) on shaker. The membranes were incubated for 1.5-2 hrs at room temperature with horseradish peroxidase (HRP) conjugated secondary antibody diluted 1:10000 in blocking buffer.

Second wash was followed and enhanced chemical luminescence (ECL) solution obtained from advansta was applied on membranes. Chemi-luminescence excreted by HRP on interacted secondary antibody was detected in dark-box fitted with chemi-luminescence camera (Fujifilm). Re-probing for different antibody on same membrane was done by using primary antibody originated from different species.

2.5.3 Immunoprecipitation

To perform Co-IP, anti-FLAG antibody conjugated beads from Sigma-Aldrich were used to precipitate FLAG tagged protein and its interacting proteins. 293T cells were lysed with RIPA buffer after 20hr of transfection. 1/3rd of original volume of cell lysate was used as input and rest was incubated with anti-FLAG antibody conjugated beads for 2 hrs in cold room after 1:10 dilution with RIPA buffer (without SDS).

After incubation, beads were washed 3 times with RIPA buffer (without SDS) and 1 time with PBS. Precipitated protein was eluted in 2x lamellae dye by boiling for 3 minutes. Input and precipitation was resolved in SDS PAGE and analyzed by immunoblotting.

2.5.4 Measurement of NO release

Nitric Oxide (NO) release was measured by griess reagent assay according to manufacturer's instructions (Sigma-Alderich).

2.6 Immunological assays

Macrophages induce surface expression of CD40 molecule and release of cytokines (*i.e.* IL-6, TNF) after TLR activation. These proteins are detectable with specific antibodies conjugated with either fluorochrome or HRP which are measured by FACS or ELISA techniques.

2.6.1 FACS (Fluorescence associated cell sorting)

Proteins expressed on cell surface (also within cells) are detected by antibody specific to the proteins with the help of Flow cytometery. These antibodies for FACS analysis are conjugated with different fluorochromes and can be excited by suitable LASERs to detect presence of antibody bound protein.

For FACS analysis cells were stained with appropriate fluorochrome labeled antibodies in FACS buffer. Cells were incubated in dark cold room with agitation for 30 minutes. After incubation cells were washed once with FACS buffer and used for FACS acquisition in (BD LSR **II** machine) and analysis. FCS files from LSR machine analyzed by Flowjo software.

2.6.2 ELISA (enzyme linked immunosorbent assay)

ELISAs are used for the qualitative and quantitative assessment of cytokines, chemokines, growth factors, proteins, immunoglobulins and other immunological markers. Detection and quantification of protein analytes from various biological samples such as serum, plasma, cell culture supernatants and cell lysates indicate a multitude of biological and pathological events.

Supernatants from activated RAW cell culture after 16 hrs of stimulation were used to detect secreted IL-6 cytokine. ELISA was performed in flat bottom 96 well plates as described below and according to manufacturer's instructions.

Coating: wells were coated with anti-IL-6 antibody diluted in PBS (25 µl) for overnight at 4o C and washed 3X using PBST after incubation.

Blocking: wells were blocked with PBS containing 1% BSA (100 µ) for 1 hr at 37o C and washed 3X using PBST.

Sample addition: Blocked wells were used to incubate with supernatant collected from activated RAW cells (50 µl) and IL-6 protein (as standard) serially diluted (1:2) in 1% BSA containing PBST. Plate incubated at 37o C for 1hr and washed 3X with PBST after incubation time.

Detection: 50 µl of anti-IL-6 antibody conjugated with HRP (1:50000) was added in each well and incubated for 1 hr at 37oC and washed 6X with PBST. To detect bound antibody substrate for HRP was added (25 µl) and incubate till green blue color appeared in standard sample having wells and reaction was stopped with 0.5 M H2SO4. Blue color turned yellow in stopped reaction and intensity of color which is proportional to available IL-6 cytokine in well is measured in Spectrometer. OD values and calculated values from standard samples were used to plot IL-6 values from samples.

2.6.3 NP-KLH immunization in mice

For inducing T cell dependent antibody response, nitrophenylacetyl (NP) hapten conjugated to keyhole limpet hemocyanin (KLH**)** was used to immunize mice. NP-KLH (50µg) was diluted in saline, suspended in alum and injected intra-peritoneum into mice.

After 100 days mice were re-immunized with NP-KLH. Serum from immunized mice was collected regularly at different intervals and analyzed for NP-specific Ig titer by ELISA.

2.7 Software used for analysis

2.7.1 Statistical analysis

All cell percentages and FACS mean fluorescence intensities (MFI) were evaluated with 2 way anova test using Graphpad Prism software.

2.7.2 Online tool

National center for biotechnology information (NCBI) website and online library (https://www.ncbi.nlm.nih.gov/) was used to obtain gene and protein sequences and literature

search. For CRISPR gRNA design http://www.e-crisp.org/E-CRISP/designcrispr.html website was used and homology of CRISPR RNAs was predicted by RGEN tool from http://www.rgenome.net/mich-calculator/ website. Protein structure prediction was done with help of Robetta structure prediction server from www.bakerlab.org). DNA and protein sequence alignment was done using EMBL-EBI MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) software.

3. Results

3.1 Elucidation of IRAK2 C-terminal role in TLR signaling and molecular interaction of IRAK2 with anti-apoptotic protein A1/Bfl-1

TLR signaling is crucial for the activation of innate immune cells *i.e.* macrophages and DCs in the early phase of infection. Activation of TLR pathway also induces expression of various pro-survival proteins *i.e.* B cell lymphoma protein 2 (Bcl2), A1, Bcl-xL to sustain cell survival during acute inflammation (Orlofsky et al., 1999; Wong et al., 2004). Various malignancies are reported linked with TLR signaling components (Jain et al., 2014; Rhyasen and Starczynowski, 2014).

To better understand these processes, I investigated the TLR pathway molecule IRAK2, which is highly conserved in human and mice. In addition to TLR/IL-1 signaling, IRAK2 also involved in cell survival and important for ER stress signaling induced cell death (Benosman et al., 2013; Liu et al., 2015). In humans a hypo-functional IRAK2 variant was associated with increased risk of death in colorectal cancer patient (Wang et al., 2014). This potential dual contribution of IRAK2 in inflammation and cell death regulation, provides clue for immune abnormalities and malignancies. A detailed analysis of possible molecular interaction of IRAK2 with TLR signaling components and cell survival regulatory proteins is needed to answer the unsolved TLR associated immune response and cancer progression.

3.1.1 IRAK2 protein contains an unexplored evolutionary conserved C-terminal domain

Mouse IRAK2 with a change of glutamic acid to alanine at position 525 (E525A) causes abolished interaction with TRAF6 and subsequent TRAF6 ubiquitination, which was associated with reduced TLR signaling response (Flannery and Bowie, 2010; Pauls et al., 2013; Wang et al., 2014). TRAF6 protein sequence alignment displayed that crucial residues required for IRAK2 interaction are

conserved in human and mouse orthologues, thus explains the importance of this interaction (Figure 3.1A). But recent studies indicating functional inconsistency of IRAK2 in TLR signaling from human and mouse cells (Sun et al., 2016) suggested possibility of other factors or mechanisms involved in IRAK2 regulation.

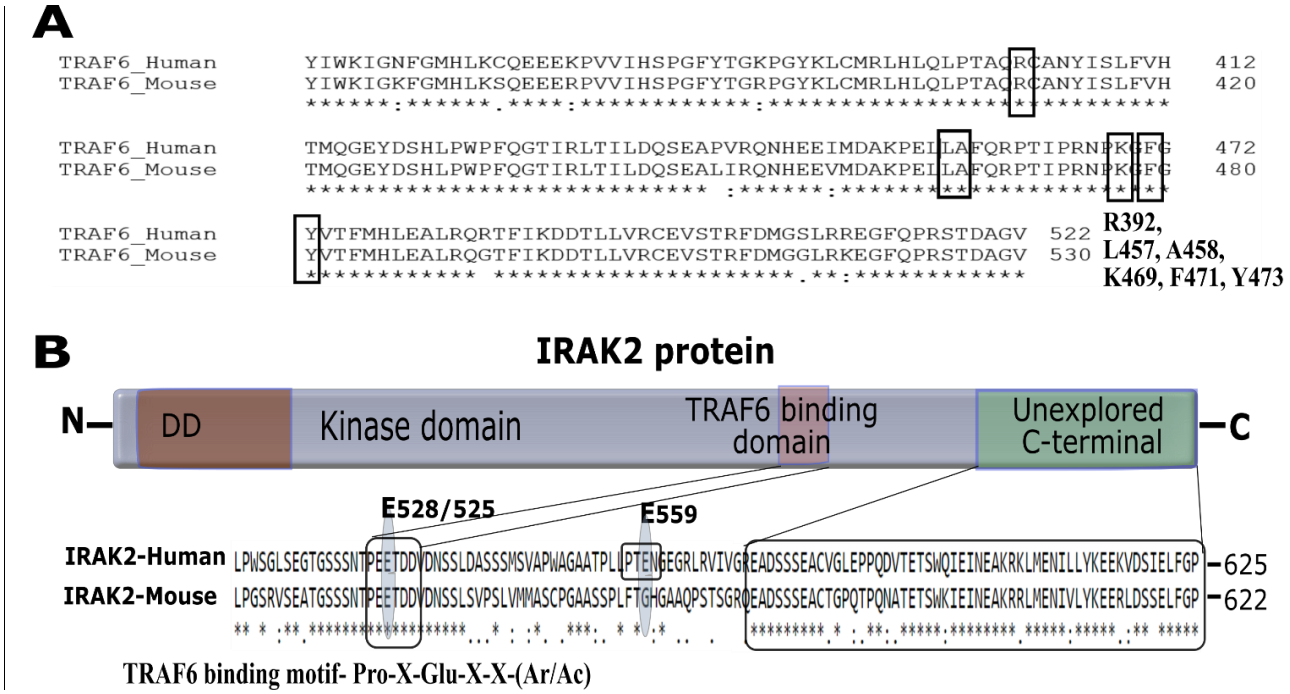

Figure 3.1: IRAK2 binding residues in TRAF6, domains architecture and sequence conservation of IRAK2- TRAF6 forms a IRAK2 interaction pocket which comprise of critical residues and conserved among human and mouse protein (A). Human and mouse IRAK2 protein sequence alignment shows conserved TRAF6 interaction domain and an C-terminal region with unknown function. In mouse IRAK2, E525 residue is crucial for TRAF6 interaction and in humans two possible TRAF6 interaction motifs are present at position 525 and 559 (B).

In order to explore additional functional relevance, I tested IRAK2 protein for its structural similarity between human and murine orthologues via sequence alignment using EMBL-EBI MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) software (Figure 3.1B). Protein sequence alignment revealed evolutionary conserved C-terminal regions in IRAK2 outside of the known TRAF6 binding site. This newly determined C-terminal region has not been studied for its functional importance yet.

Hence, investigation of this IRAK2 C-terminus might provide additional insight in TLR signaling in human and mouse cells.

3.1.2 C-terminal truncated IRAK2 protein is unable to enhance TLR4 signaling in RAW macrophage cells

To examine the possible contribution of newly determined IRAK2 C-terminal region in TLR signaling, I used the murine macrophage cell RAW 264.7 (RAW). These cells induce the costimulatory cell surface molecule CD40 and release pro-inflammatory cytokines after TLR engagement with the relevant PAMP (Benveniste et al., 2004; Björkbacka et al., 2004; Hambleton et al., 1996; Qin et al., 2005). Myc-tagged IRAK2 wildtype (WT) and C-terminal truncated (IRAK2Δ20, -Δ36 and -Δ55) versions of IRAK2 were overexpressed in RAW cells via recombinant retroviruses. The TRAF6 non-binding IRAK2 mutant E525A (IRAK2E525A) and IRAK2Δ100 were used as negative control (Figure 3.2A). These Myc-IRAK2 expressing retroviruses also code for green fluorescent protein (GFP) linked to IRAK2 expression via an internal ribosome binding entry site (IRES). GFP expression is used as control for IRAK2 protein expression (Figure 2.1).

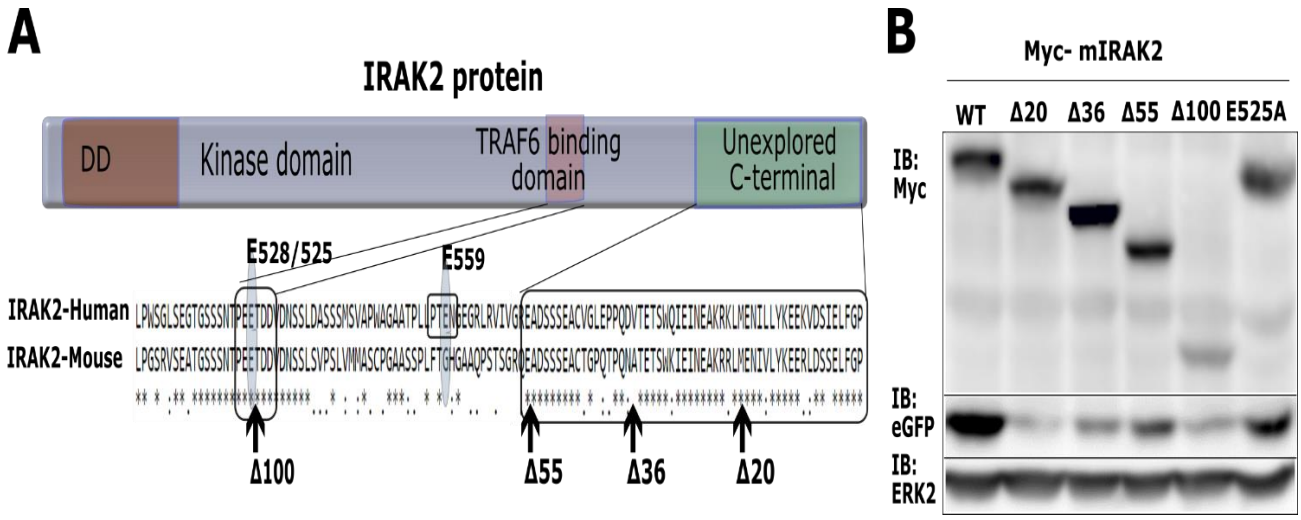

Figure 3.2: IRAK2 C-terminal truncation and overexpression in RAW cells- C-terminal of IRAK2 was sequentially deleted at indicated residues (A). RAW cells overexpressing IRAK2 proteins were sorted by flocytometry and cell lysates were resolved in SDS-PAGE to detect expressed proteins by indicated antibodies (B).

Initial experiments showed that these proteins were expressed at different levels in RAW cells (data not shown). RAW cell populations expressing comparable levels of the various IRAK2 proteins were obtained by cell sorting based on GFP expression. Finally, the sorted populations displayed comparable protein levels of the IRAK2 proteins was determined by immunoblot analysis of cell lysates (Figure 3.2B). Ectopic overexpression of IRAK2 protein enhances TLR signaling and subsequent expression of responsive genes e.g. CD40, IL-6 etc. (Meylan and Tschopp, 2008) and intact TRAF6 binding motif is critical for this contribution (Pauls et al., 2013).

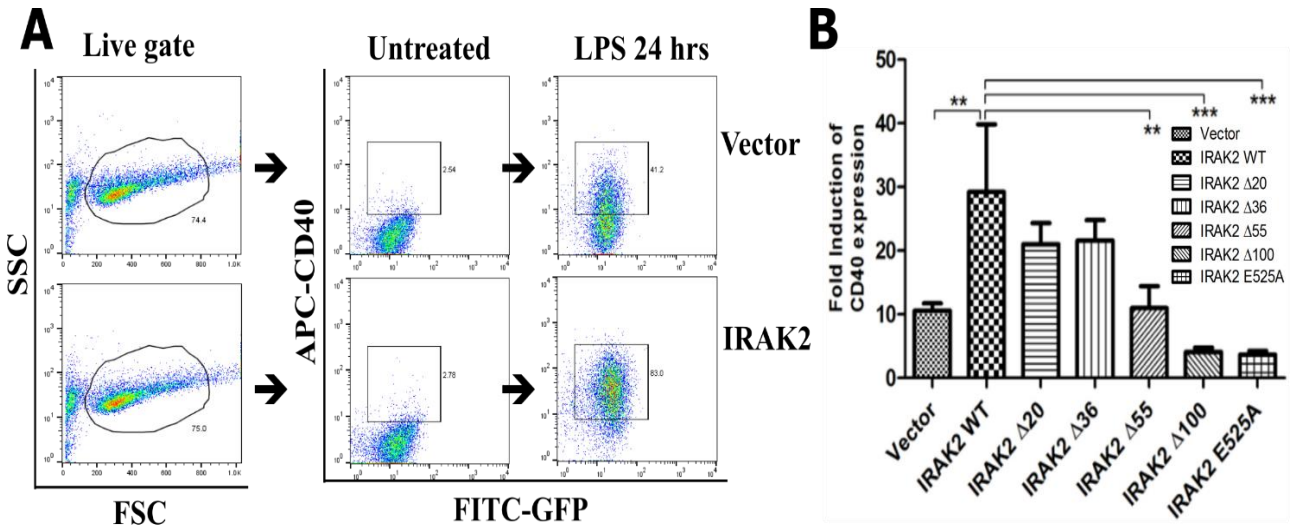

Figure 3.3: Ectopic expression of IRAK2 in RAW cells and analysis of TLR4 induced CD40 expression- Schematic representation of FACS analysis from IRAK2 overexpressing RAW cells shows that FSC/SSC gated live cells were used for CD40 expression induction analysis. APC-conjugated antibody used for surface stainigng of CD40 after 24 hrs of LPS (500 ng/ml) stimulation. Here FITC denotes GFP expression which corresponds to IRAK2 protein (A). Induced CD40 expression in IRAK2 overexpressiong RAW cells after LPS stimulation (24 hrs) presented as FACS calculated MFI mean of 3 independent experiments with SD error bars (B). Here, vector indicates recombinant retrovirus (rRV) expressing only GFP.

In order to investigate the ability of IRAK2 protein for its contribution in TLR4 signaling, IRAK2 overexpressing RAW cells were stimulated with LPS for 24 hrs. Induced CD40 expression was measured by surface staining with anti-CD40 APC conjugated antibody via FACS analysis. Ectopic expression of IRAK2WT enhanced CD40 expression (Figure 3.3A). FACS measured mean fluorescent intensity (MFI) analysis demonstrated reduced CD40 expression with -Δ55 truncation of

IRAK2 in comparison to WT protein. Inactive IRAK2 mutants -Δ100 and E525A imposed dominant negative impact while other shorter C-terminal deletions (Δ20 and Δ36) slightly influenced (Figure 3.3B).

This observation indicated towards a significant region (~55 residues) at IRAK2 C-terminal for TLR4 signaling.

Next, to examine whether this abrogated CD40 expression with IRAK2Δ55 was due to inefficient LPS stimulation. Overexpressing RAW cells were stimulated with increasing doses of LPS and for different time intervals. No further increment in CD40 expression was observed by enhancing the amount of LPS from 500ng/ml to 1500ng/ml for IRAK2Δ55 expressing cells in comparison to control cells (Figure 3.4A).

Furthermore, the reduced ability of IRAK2Δ55 to stimulate CD40 expression could be detected at 12 hours as well as at 24 hours after onset of stimulation (Figure 3.4B). Collectively, the data shows that 55 residues long C-terminal is critical and its deletion interferes with IRAK2 mediated contribution in TLR4 pathway.

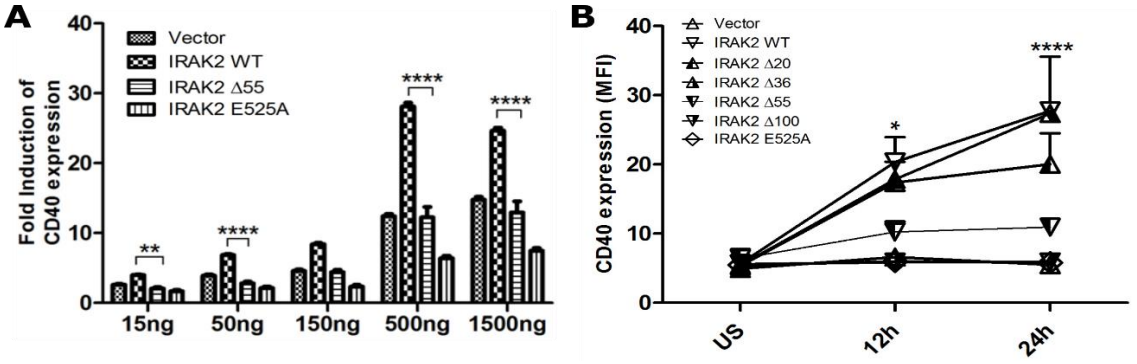

Figure 3.4: CD40 induction in IRAK2 overexpressing RAW cells with increased LPS stimulation- RAW cells were stimulated with LPS (10 – 1500 ng/ml) for 24 hrs (A) and with LPS (500 ng/ml) for 12 hrs and 24 hrs (B). CD40 expression was calculated as MFI via FACS analysis and presented as mean of 3 independent experiments with SD error bars.

Next, I asked if an increased protein level of IRAK2Δ55 could regain the CD40 expression induction. Therefore, overexpressing RAW cells were sorted for different IRAK2 protein levels by FACS.

Interestingly, LPS-induced CD40 analysis by flow cytometry even displayed a slight dominant negative impact on TLR4-induced CD40 expression with increased IRAK2Δ55 protein in comparison to the control population (Figure 3.5A).

Next, as control IFN-γ stimulation was used which doesn't share signaling cascade with TLR4 but induces CD40 expression (Lin and Levison, 2009). IFN-γ stimulated CD40 expression was comparable with all IRAK2 versions (Figure 3.5B). Whereas TLR4 and TLR4/IFN-γR co-stimulation demonstrated inhibited CD40 induction with IRAK2Δ55 in contrast to WT IRAK2 because of lesser TLR signaling contribution.

Collectively, the results from overexpressing RAW cells suggest a new important region in IRAK2 C-terminus and lacking IRAK2Δ55 show consistent and significant ineptness in TLR4 signaling induced CD40 expression.

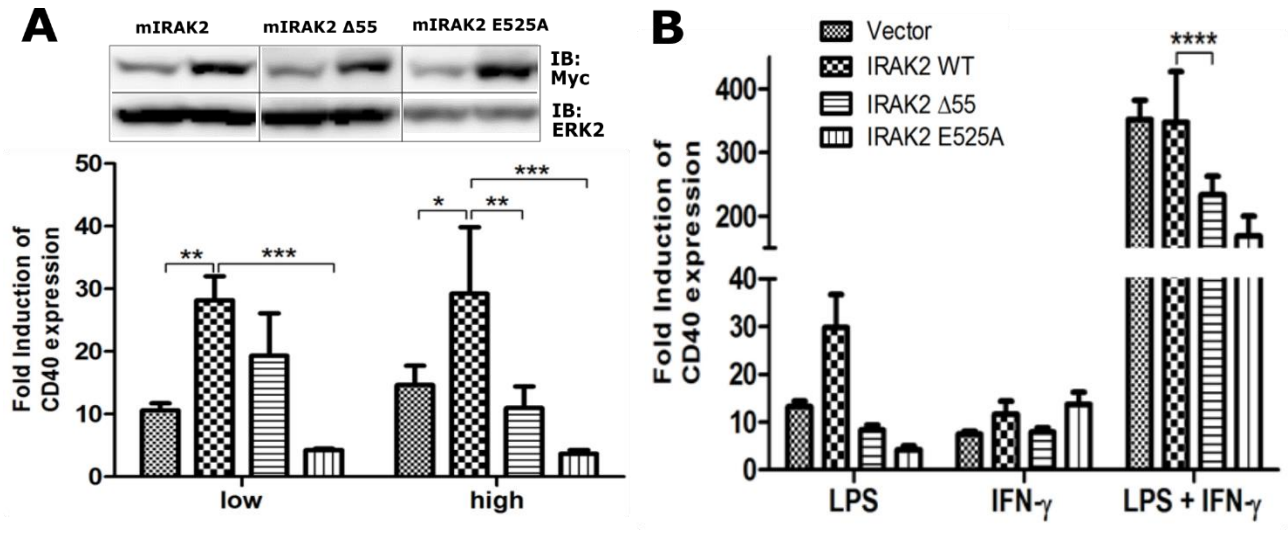

Figure 3.5: CD40 expression analysis with increased IRAK2 protein and IFN-γ stimulation- RAW cells were sorted for different protein levels of Myc- IRAK2 via FACS and subjected to immunoblot analysis. Here ERK2 serves as loading control. Obtained RAW cells with low and high IRAK2 protein expression were stimulated with LPS (500ng) for 24 hrs (A). Equal IRAK2 expressing RAW cells stimulated with LPS (500ng) or IFN-γ (500 U/ml) or IFN-γ together with LPS for 24 hrs. Fold change in CD40 expression was calculated in MFI from FACS analysis and presented as mean of 3 experiments with SD error bars (B).

3.1.3 TLR signaling is abolished in IRAK2 deficient RAW 264.7 cells

RAW cells also have endogenous IRAK2 protein which might interfere with overexpressed proteins. Therefore, IRAK2-deficient RAW cells would provide a more reliable model system to evaluate the potential of the mutant IRAK2 proteins. To obtain IRAK2 deficient RAW cells the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 approach was used. This technique uses endonuclease Cas9-mediated gene alteration which is guided by inducible *Irak2* gene specific CRISPR gRNAs (Figure 2.2).

Wild type RAW cells were infected with recombinant lentiviruses coding for Cas9 and gRNAs. TLR4-induced CD40 expression was decreased in RAW cells with an IRAK2-specific gRNA compared to the control population expressing only the relevant marker protein (Figure 3.6 left panel). Furthermore, selected single cell clones were analyzed for their CD40 induction after TLR4 activation and for *irak2* gene sequence. Altered *Irak2* gene (cas9-mediated insertion/deletions) was correlated with loss of TLR4 induced CD40 induction (Figure 3.6 right panel).

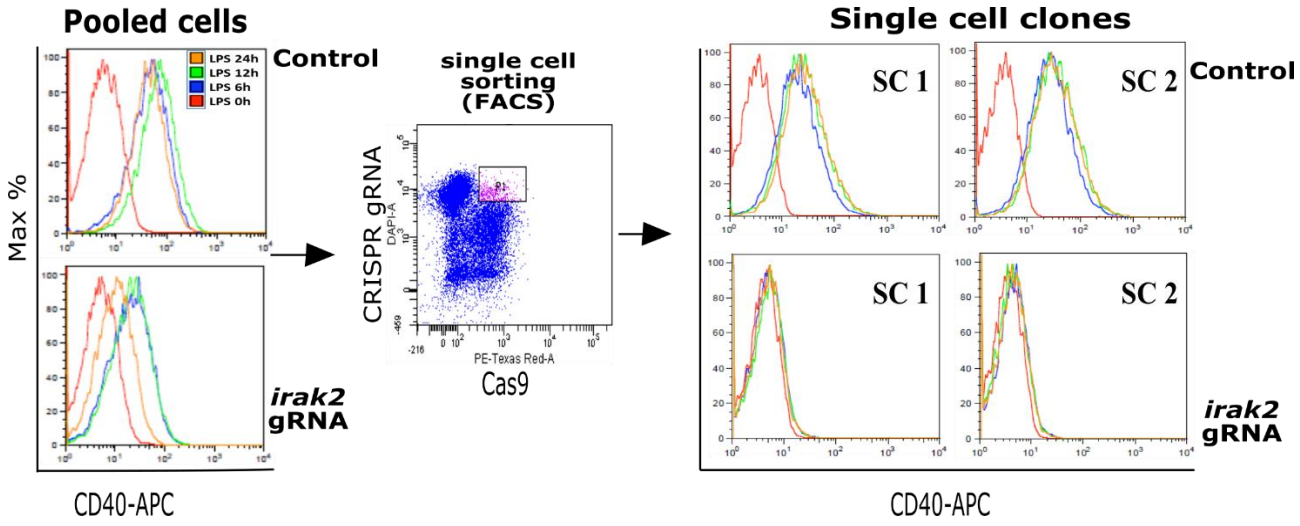

Figure 3.6: Schematic representation of obtaining IRAK2 knock-out RAW cells using CRISPR- Cas9 technique and FACS analysis- The pool of Cas9 and CRISPR gRNA co-expressing RAW cells were stimulated with LPS (500ng/ml) for 6, 12 and 24 hrs and analyzed for CD40 induction via flow cytometry. Single cell clone were sorted by FACS and stimulated by LPS as a above. Here control is cells containg non-CRSIPR gRNA expressing plasmid.

Further, detailed analysis was performed for one IRAK2 deficient single clone. In contrast to the control population, the IRAK2-lacking cells were unable to induce CD40 expression after TLR4 activation. Furthermore, with IFN-γR co-stimulation it was induced but lesser than WT cells (Figure 3.7A). In addition, increment in LPS dose and stimulation time could not rescue CD40 expression in IRAK2 knock-out (KO) cells (Figure 3.7B and C).

Consistently, lack of IRAK2 also interfered with TLR2-, TLR7- and TLR9-inducible CD40 expression (Figure 3.7D).

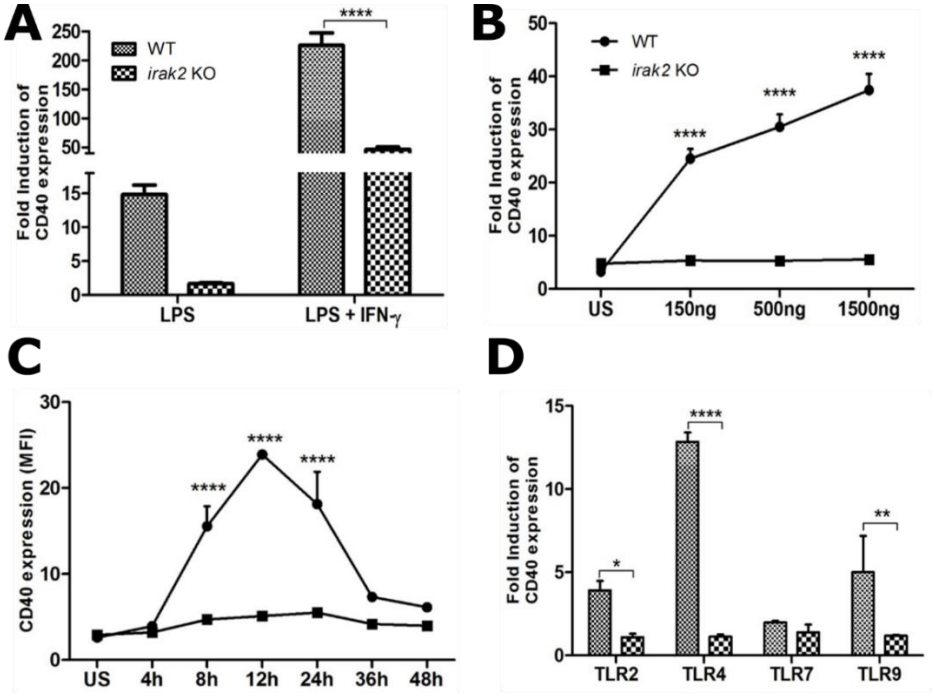

Figure 3.7: TLR induced CD40 expression in IRAK2 KO RAW cells derived from a single cell clone- WT and IRAK2 knock-out RAW cells were stimulated with LPS (500 ng) alone or together with IFN-γ (500U/ml) (A). with LPS (100 – 1500 ng) for 24 hrs (B) and with LPS (500 ng/ml) upto 48 hrs (C). With ligand for TLR2: pamcsk4 (500 ng/ml), TLR4: LPS (500 ng/ml), TLR7: Imiquoid (1µg/ml), TLR9: (5 µm) for 24 hrs (D). Induced CD40 expression was analyzed with FACS and calculated MFI displayed as mean of 3 independent experiments with SD error bars.

TLR pathways induce release of several effector mediators of inflammation e.g. nitric oxide (NO) and pro-inflammatory cytokines such as, IL-1, IL-6 and TNF in macrophages (Zhang et al., 2014). Our analysis of IRAK2-deficient RAW cells demonstrated impaired expression of *il-6* mRNA and IL-

6 cytokine secretion after TLR4 activation (Figure 3.8A and B). These results were consistent with *in vivo* studies with IRAK2 KO mouse macrophages (Kawagoe et al., 2008).

In addition, other TLR4 induced pro-inflammatory cytokines *il-1* and *tnf* expression was also abolished with IRAK2 deficiency (Figure 3.8C and D). Furthermore, NO was undetectable from supernatant of TLR4 stimulated IRAK2 KO RAW cells cultures (Figure 3.8E).

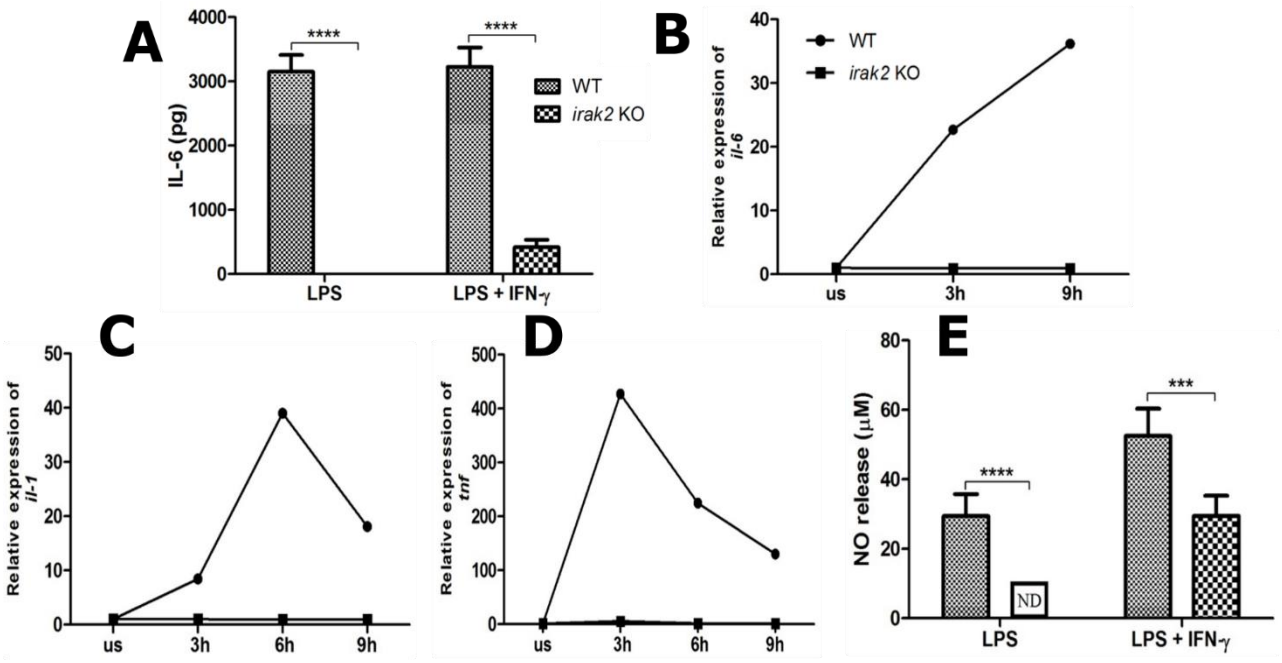

Figure 3.8: Pro-inflammatory mediators analysis in IRAK2 KO cells- WT and IRAK2 KO RAW cells stimulated with LPS (500 ng) alone or together with IFN-γ (500U/ml) for 20 hrs and IL-6 secretion was measured by ELISA from cuture supernant (A). Stimulated with LPS (500 ng) for indicated time and cell lysates were used to determine mRNA expression level of *il-6* (B) *tnf* (C) and *il-1* (D) with real-time PCR. NO release was estimated by griess reagent from supernatants of LPS (500ng) stimulated RAW cells after 20 hrs (E). Observations are presented in graphs as mean of 3 independent experiments with SD error bars.

Thus, IRAK2 deficiency displayed a detrimental effect on TLR pathway induced pro-inflammatory products indicating an impaired signaling cascade. To examine TLR4 induced MAPKs and NF-κB activation, lysates from LPS-induced RAW cells were subjected to immunoblot evaluation. IRAK2 KO cells presented impaired NF-κB (RelA/p65) and ERK2 phosphorylation, predominantly during later time points (1-8 hrs) as compared to WT cells (Figure 3.9A). NF-κB and ERK2 activation

measured by densitometry at later phase suggested less sustained TLR signaling with absence of IRAK2 (Figure 3.9B and C).

Basal activation of NF-κB and ERK2 at earlier time points (0-30 minutes) is consistent with the fact that TLR signaling uses rather IRAK1 in the early phase (Pauls et al., 2013; Wan et al., 2009).

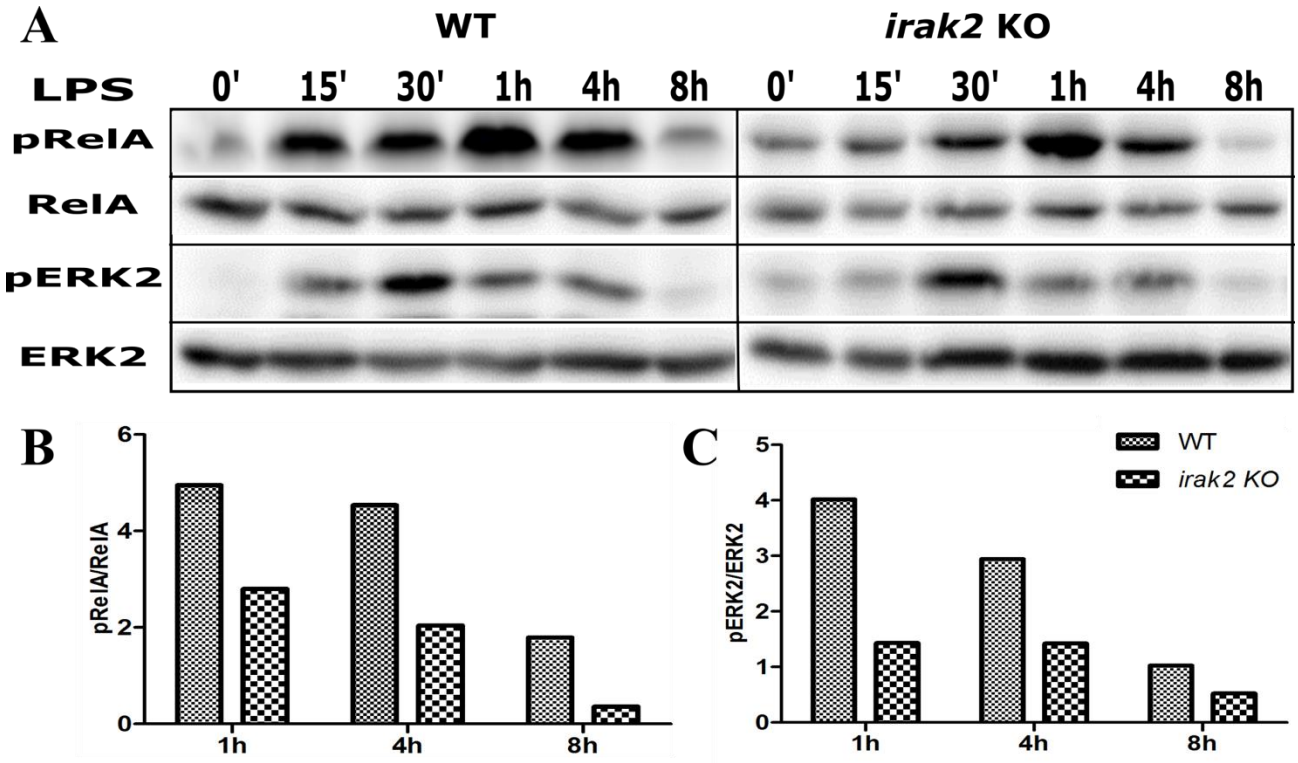

Figure 3.9: Immunoblot analysis for TLR4 induced MAPK and NF-κB activation in IRAK2 KO cells- IRAK2 KO and WT RAW cells were stimulated with LPS (1 µg/ml) for upto 8 hrs and cell lysates were resolved in SDS-PAGE and immunoblotted with NF-κB and ERK2 phosphorylation induction (A). Densitometery analysis is graphed from immunoblot for fold change in RelA (B) and ERK2 (C) phosphorylation with comparison to unstimulated cells.

Collectively, these observations from IRAK2 KO RAW cells demonstrated impaired TLR signaling. Hence, IRAK2 KO cells are an ideal system for further functional analysis of IRAK2 mutant proteins in TLR signaling.

3.1.4 IRAK2Δ55 protein is unable to restore TLR signaling

To further examine newly characterized C-terminal region, IRAK2-deficient RAW cells were reconstituted with WT and C-terminal truncated mutants of Myc-IRAK2 using retroviral mediated stable transduction. Reconstituted RAW cells were sorted for equal levels of IRAK2 proteins on the basis of GFP expression and evaluated by immunoblotting (Figure 3.10A).

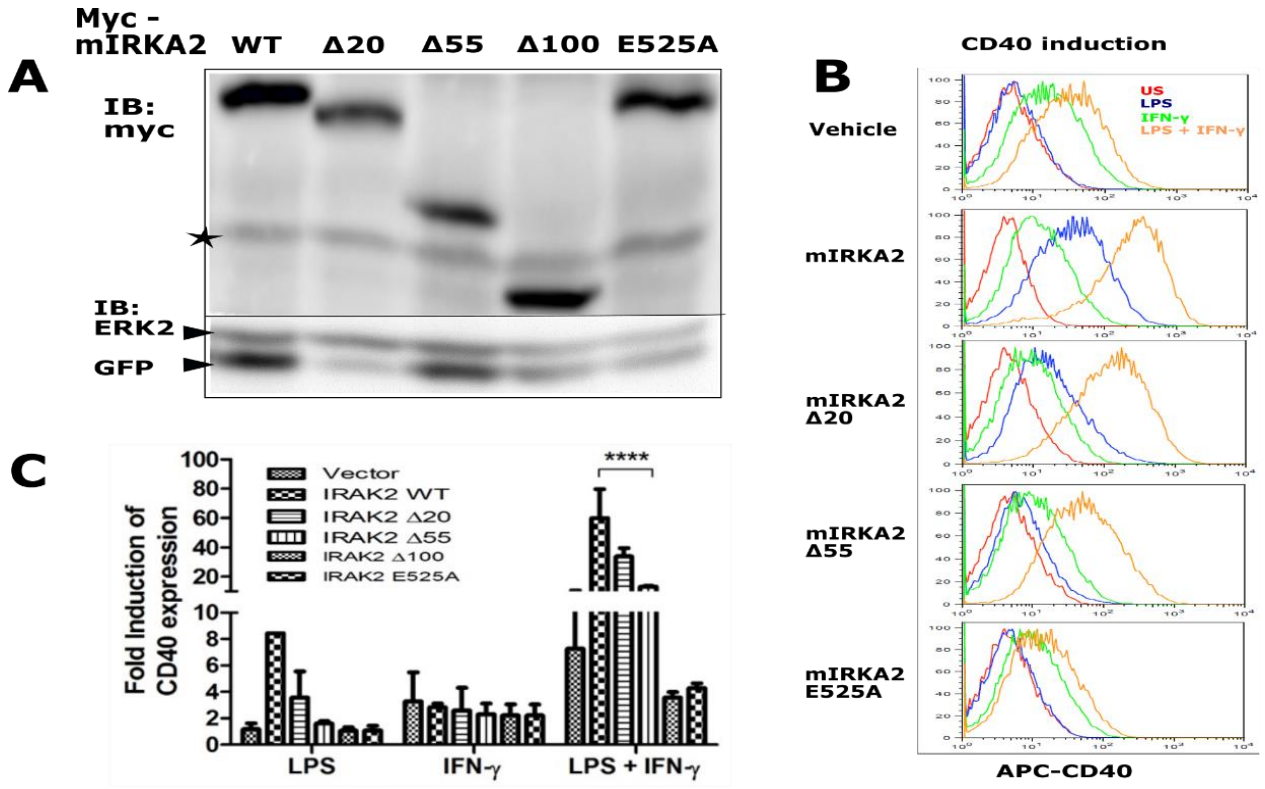

Figure 3.10: CD40 induction after reconstitution of IRAK2 deficient RAW cells- Myc- IRAK2 proteins reconstituted in *irak2* knock-out RAW cells, cell lysates were resolved in SDS-PAGE and immunoblotted for Myc, here GFP is control for plasmid expression and ERK2 for total protein (A). Reconstituted RAW cells activated with LPS (500 ng) and IFN-γ (500 U/ml). Induction in CD40 expression shown with FACS histograms (B) and fold CD40 change is presented as mean MFI of 4 independent experiments with SD error bars (C).

In order to determine functional efficiency of IRAK2 proteins with C-terminus truncation, CD40 induction was measured by FACS after TLR4 or IFN-γR stimulation. IRAK2Δ55 reconstituted cells exhibited minimal CD40 induction on TLR4 stimulation, in contrast to significant increase with WT IRAK2 and shorter deletion (IRAK2Δ20). Although, IFN-γ induced CD40 expression was equal

among all reconstituted RAW cells, IFN-γ plus LPS co-stimulation presented lesser induction with IRAK2Δ55 in contrast to a much stronger induction with IRAK2Δ20 and WT IRAK2 (Figure 3.10B and C).

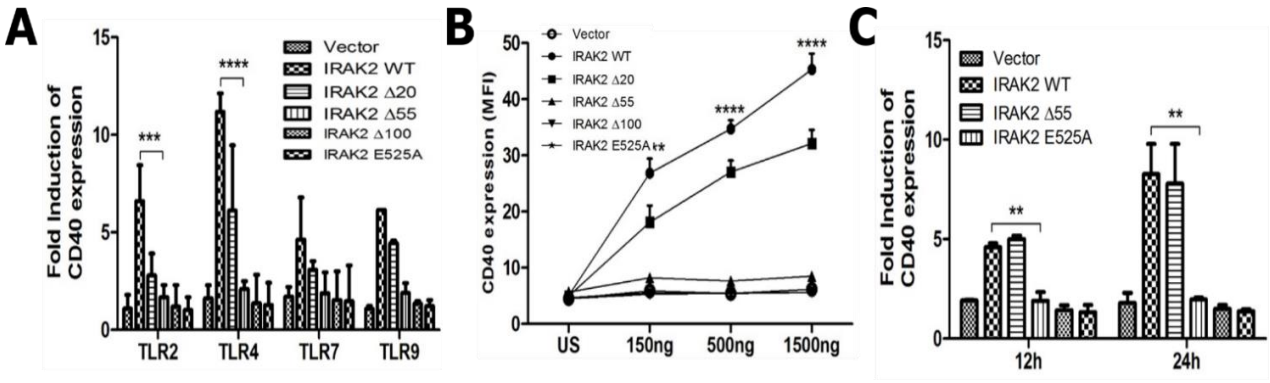

Figure 3.11: TLR induced CD40 induction after reconstitution of IRAK2 deficient RAW cells- Reconstituted RAW cells were stimulated with ligands for TLR2; pamcsk4 (500 ng/ml), TLR4; LPS (500 ng/ml), TLR7; Imiquoid (1µg/ml), TLR9; (5 µm) for 24 hrs (A). with LPS (100 – 1500 ng) for 24 hrs (B) and with LPS (500 ng/ml) for 12 hrs and 24 hrs (C). Induced CD40 expression analyzed with FACS and calculated MFI displayed as mean of 3 experiments with SD error bars.

Further analysis with other TLRs mediated CD40 induction confirmed the incompetency of IRAK2Δ55 (Figure 3.11A).

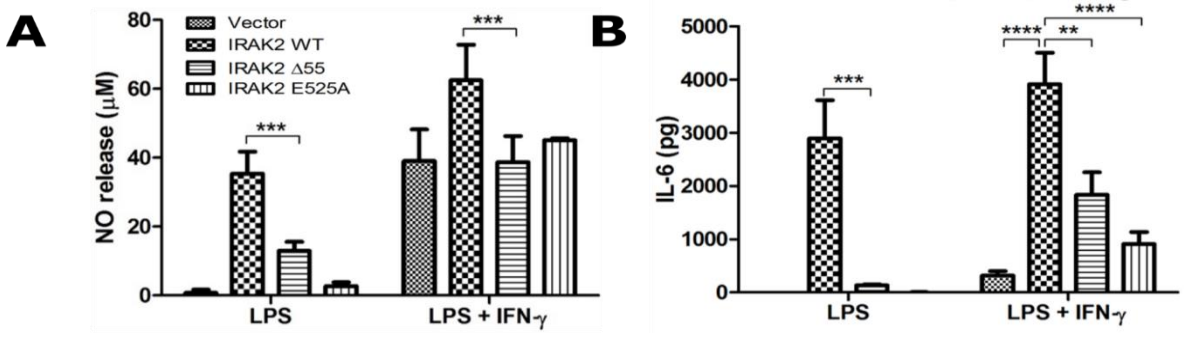

Figure 3.12: Pro-inflammatory mediators induction after IRAK2 reconstitution- NO release and (A) IL-6 secretion (B) measured by griess reagent and ELISA, respectively from supernatant of LPS (500 ng) alone or together with IFN-γ (500 U/ml) treated RAW cells after 20 hrs. Induced values subtracted with unstimulated values and mean of 4 experiments presented with SD error bars.

Next, reconstituted RAW cells stimulation with increasing doses of LPS or stimulation time revealed no further increment in CD40 expression with IRAK2Δ55, whereas, IRAK2Δ20 and WT IRAK2 could raise LPS stimulated CD40 expression in a dose and time dependent manner (Figure 3.11B and C). Furthermore, we determined the production of pro-inflammatory effector mediators *i.e.* NO release and IL-6 secretion after TLR4 and co-stimulation with IFN-γ in reconstituted cells. Supernatant analysis revealed significantly reduced NO and IL-6 secretion with IRAK2Δ55 in contrast to the WT IRAK2 expressing RAW cells. TLR4 co-stimulation with IFN-γ demonstrated induced NO and IL-6 detection but remained subordinate to WT counterpart due to the absence of TLR signaling (Figure 3.12A and B). These results are in consistency with our observations from overexpression experiments and suggested far lesser functional activity of IRAK2Δ55 as compared to IRAK2 WT in TLR signal restoration.

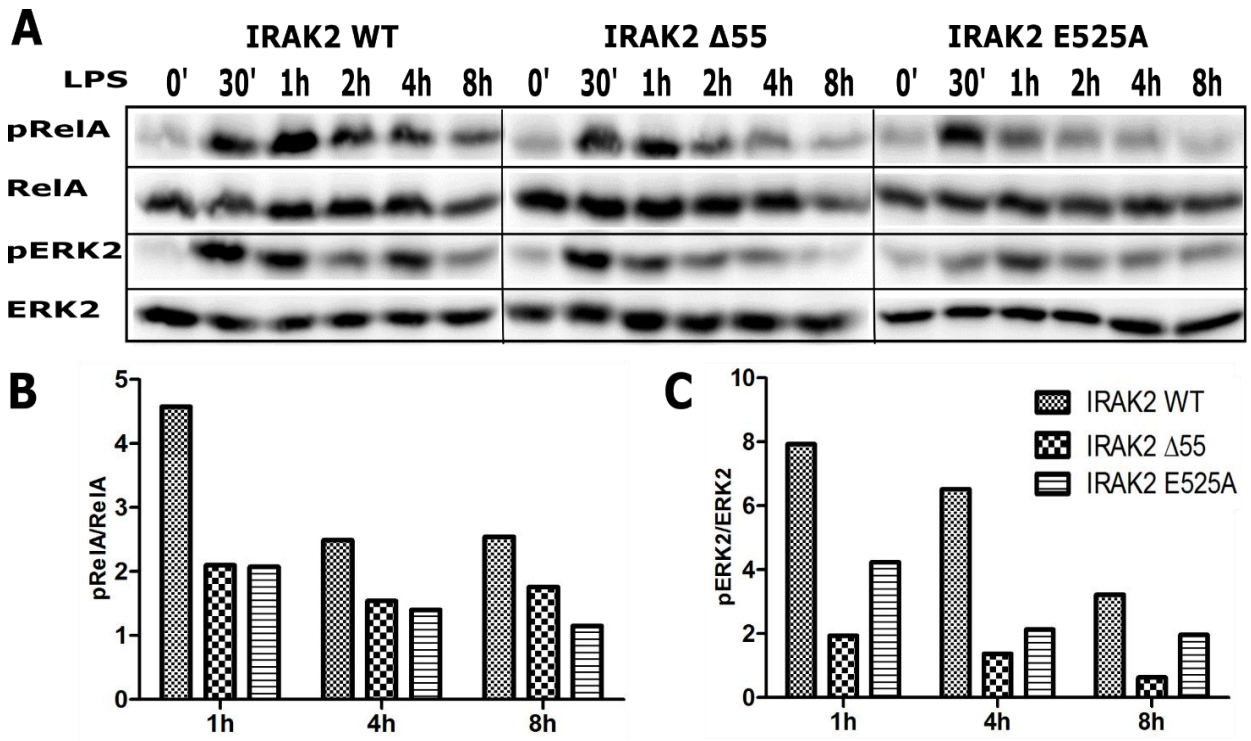

Figure 3.13: Immunoblot analysis for TLR4 induced MAPK and NF-κB activation in reconstituted RAW cells- RAW cells reconstituted with WT and mutants IRAK2 were stimulated with LPS (1 µg/ml) for upto 8 hrs and cell lysates subjected to immunoblotting for NF-κB and ERK2 phosphorylation induction (A). Densitometery analysis is presented from immunoblot for fold change in RelA (B) and ERK2 (C) phosphorylation with comparison to unstimulated cells.

In addition to TLR response, IRAK2Δ55 reconstituted RAW cells also showed weaker NF-κB (RelA) and ERK2 phosphorylation when compared to WT IRAK2 in immunoblots after LPS stimulation (Figure 3.13A). The difference was more prominent at later time points (1-8 hrs) and comparable to IRAK2E525A and IRAK2 KO cells (Figure 3.13B and C).

Again, this observation strengthens the observation from overexpression experiments that the ~55 residues long region of IRAK2 is important to sustain TLR signaling.

3.1.5 C-terminal truncation in IRAK2 does not influence its physically interaction with TRAF6 but ubiquitination induction is impaired

TLR signaling cascade involves IRAK2 interaction with TRAF6, which results in enhanced TRAF6 auto-ubiquitination (Keating et al., 2007; Wang et al., 2010; Ye et al., 2002). Our experiments with TLR signaling in RAW macrophages showed hypo-functional IRAK2Δ55 despite the presence of the known TRAF6 binding site.

In order to understand the potential mechanism responsible for the incompetence of IRAK2Δ55, physical interaction with TRAF6 was determined. HEK 293T cells were used to co-express FLAG-tagged TRAF6 with Myc-IRAK2 by transient transfection. Immunoprecipitation with an anti-FLAG antibody co-precipitated IRAK2Δ55 as well as WT and other shorter truncated (-Δ20, -Δ36) IRAK2. While TRAF6 non-interacting IRAK2Δ100 and IRAK2E525A were not detectable in immunoblot with anti-Myc antibody (Figure 3.14). This observation shows that the molecular interaction with TRAF6 was not lost in IRAK2Δ55.

Next, it was tested if TRAF6 ubiquitination induction was influenced with IRAK2Δ55. To do so, HA-tagged ubiquitin was co-expressed with FLAG-TRAF6 and Myc-IRAK2 in HEK 293T cells. Cell lysates were subjected to anti-FLAG immunoprecipitation.

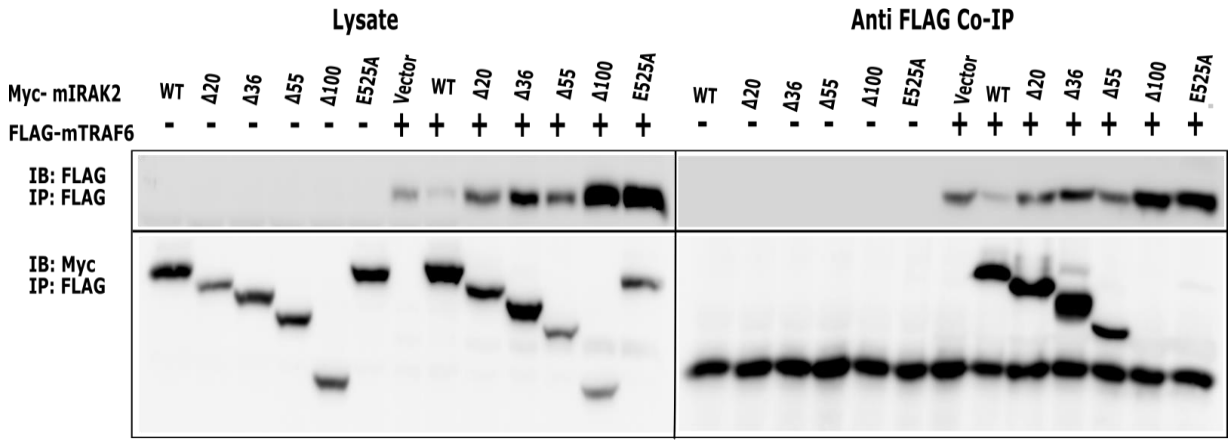

Figure 3.14: Co-immunoprecipitation of IRAK2 and TRAF6- Immunoprecipitation with anti-FLAG antibody was performed from cell lysates of HEK 293Tcells co-transfected with Myc- IRAK2 proteins and FLAG- TRAF6 after 24 hrs.

HA-tag specific immunoblot analysis displayed subordinate TRAF6 ubiquitination in the presence of IRAK2Δ55 in comparison to IRAK2 WT. As expected, in the presence of non-TRAF6 binding IRAK2E525A no TRAF6 ubiquitination was detectable (Figure 3.15A).

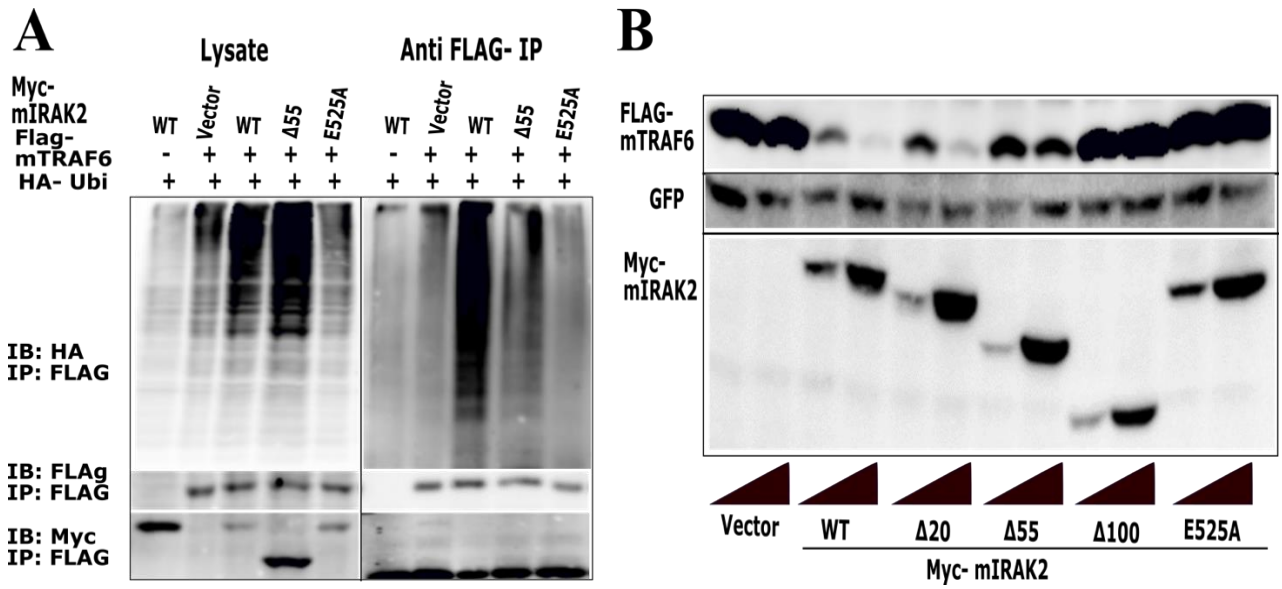

Figure 3.15: TRAF6 ubiquitination and protein availability with IRAK2 overexpression- HEK cells co-transfected with HA-tagged ubiquitin, Myc-IRAK2 and FLAG-TRAF6. After 24 hrs cell lysate was used for immunoprecipitation with anti-FLAG antibody. Polyubiquitinated form of TRAF6 was detected with anti HA immunoblot, here lysate is control for total protein availble before Co-IP (A). HEK cells co-expressin Myc-IRAK2 and FLAG-TRAF6. Cell lysate subjected for immunoblotting, GFP is control for TRAF6 expression (B).

Increased TRAF6 ubiquitination is associated with faster TRAF6 degradation (Keating et al., 2007; Pauls et al., 2013). Hence, I further analyzed the TRAF6 protein level in the presence of IRAK2 proteins. Immunoblotting analysis from FLAG-TRAF6 and Myc-IRAK2 co-expressing HEK 293T cells demonstrated almost non-detectable TRAF6 protein in the presence of WT IRAK2 protein as compare to vector control. While the protein level of TRAF6 is higher in the presence of IRAK2Δ55, but still lower as in the presence of non-TRAF6 binding IRAK2 proteins (IRAK2E525A and IRAK2Δ100) (Figure 3.15B).

These results show that IRAK2Δ55 co-operates less efficiently with TRAF6 to induce auto-ubiquitination of this protein. Collectively, these data suggest that the sole interaction of TRAF6 with IRAK2 is not sufficient for full TLR signaling. In addition, the C-terminus of IRAK2 characterized by our results is involved in unrecognized mechanism for TRAF6 ubiquitination induction.

3.1.6 TLR4 induced necroptosis is IRAK2 dependent

In addition to the induction of inflammatory mediators, the TLR pathway also promotes a certain form of programmed cell death, known as necroptosis, which is mediated by RIP3 phosphorylation (Baker et al., 2014; Lamothe et al., 2013; S.J. Kim and Li, 2013). Necroptosis was also described with autophagy inhibition induced ER stress signaling (Xu et al., 2003; Yue Xu et al., 2006).

In order to determine if LPS induces necroptosis in RAW macrophages, TLR4 signaling induced RIP3 phosphorylation was analyzed. Phosphorylated RIP3 was observed in WT RAW cells along the LPS stimulation (at 24 hrs) but was hard to detect in IRAK2 deficient cells, even after increased LPS dose. As control Bafilomycin A1, an autophagy inhibitor was used to induce RIP3 phosphorylation, which was independent of IRAK2 (Figure 3.16A and B). Reduced RIP3 phosphorylation was also observed with IRAK2Δ55 in comparison to WT IRAK2 reconstituted RAW cells. While IRAK2E525 displayed no RIP3 phosphorylation in response to TLR4 activation (Figure 3.16C).

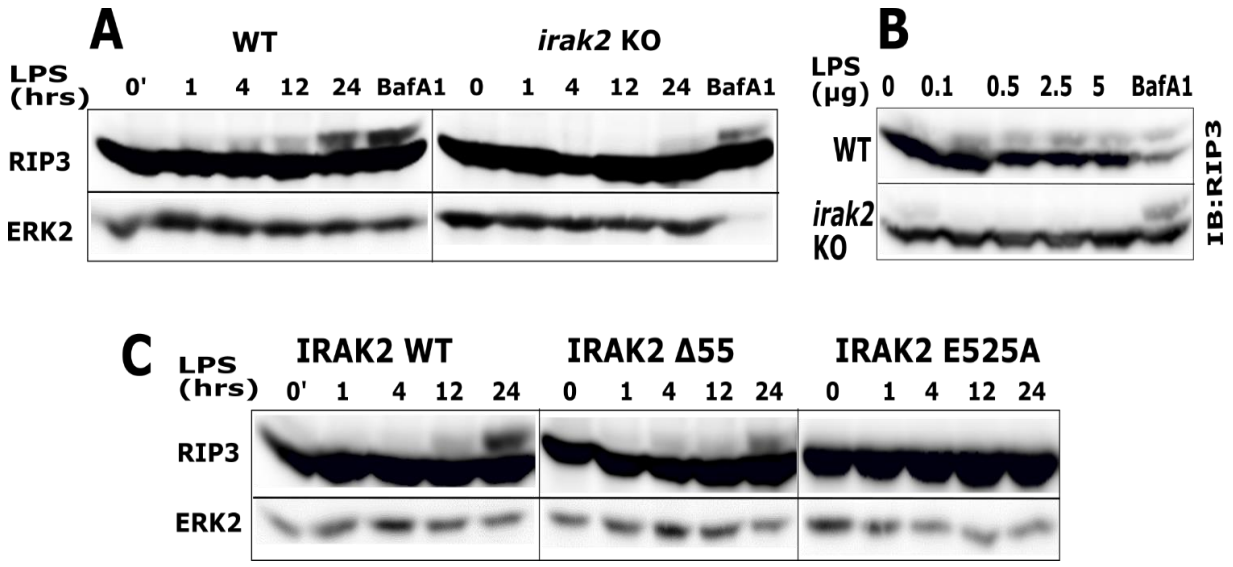

Figure 3.16: RIP3 phosphorylation with TLR4 activation- WT and IRAK2 deficient RAW cells were stimulated with LPS (1 µg) or treated with bafilomycin A1 (10 nm) (A). with LPS (100 ng to 5000 ng) and bafilomycin A1 for 24 hrs (B). RAW cells reconstituted for IRAK2 were activated with LPS (1 µg) upto 24 hrs (C). Cell lysates were subjected to SDS PAGE and immunoblotting for RIP3. The upper weaker band of RIP3 is phosphorylated form.

These results with reduced RIP3 phosphorylation suggest that IRAK2 mediated TLR signaling sustainability is critical for activation of downstream necroptosis inducing pathway.

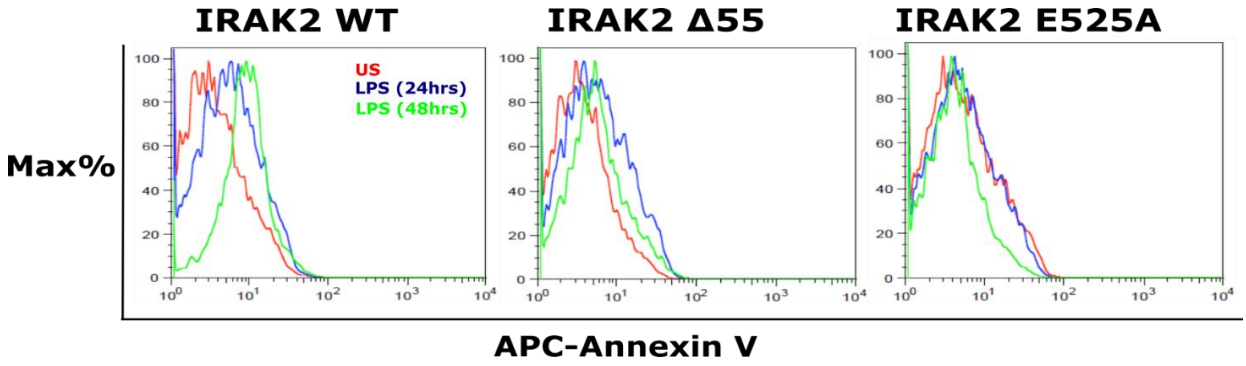

Figure 3.17: LPS induced cell death progression analysis- Reconstitute RAW cells were stimulated with LPS (1 µg) for 24 hrs and 48 hrs. Histograms presented for annexin V positive cells from FACS analysis.

Furthermore, I tested whether TLR4 induced RIP3 phosphorylation is in correlation with cell death progression. For this purpose, annexin-V staining was employed to determine dying cells after LPS stimulation and analyzed with flow cytometry. An increased frequency of annexin-V positive cells was observed from WT cells in comparison to IRAK2 lacking cells after LPS stimulation. Whereas

Bafilomycin A1 and Etoposide (an apoptosis inducer) induced annexin-V staining was not influenced with IRAK2 deficiency (data not shown).

In addition, IRAK2Δ55 reconstituted RAW cells also displayed significantly reduced annexin-V staining in contrast to WT IRAK2 after TLR4 activation (Figure 3.17). These observations indicate IRAK2 mediated TLR activation dependent progression of necroptosis upon LPS stimulation.

3.1.7 IRAK2 interacts with Bcl2-A1/Bfl-1

TLR pathway activation induces expression of pro-survival proteins to sustain cell survival during inflammation. Our results suggest that IRAK2 is critical in TLR signaling and subsequent necroptosis. On other hand IRAK2 also mediates ER stress signaling which induces cell death (Benosman et al., 2013). In addition, our research group observed IRAK2 interaction with anti-apoptotic protein, Bcl2-A1 (A1) through yeast 2 hybrid cDNA library screening (unpublished data).

A1 is a member of the Bcl-2 family of anti-apoptotic proteins. It contains Bcl-2 homology (BH) domains required for neutralizing pro-apoptotic proteins (Puma, Bid, Bax) and a shortened C-terminal transmembrane domain (Figure 3.18A). A1 is unique among Bcl-2 proteins as it has an extremely short half-life. It undergoes ubiquitination modification and subsequent degradation. It is a small ~21kDa protein and has a human orthologue Bfl-1 (Vogler, 2012).

A1 expression is induced in macrophages and neutrophils upon TLR activation during inflammation and helps in their survival during the inflammatory reaction (Orlofsky et al., 1999). First, I determined A1 protein expression in RAW macrophages with TLR4 activation if it is dependent on IRAK2. Immunoblot analysis displayed diminished expression of A1 protein in IRAK2 deficient cells as compared to WT cells (Figure 3.18B). This result again strengthens IRAK2 mediated TLR signaling also in anti-apoptotic proteins expression induction.

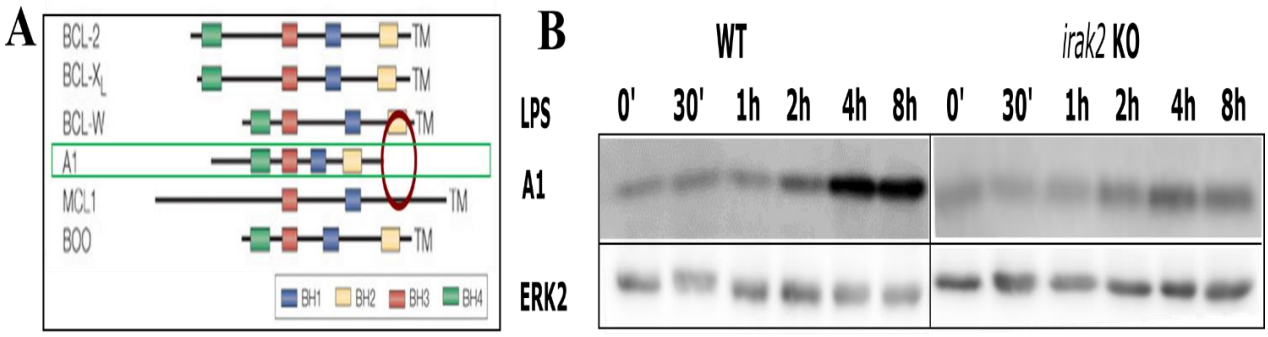

Figure 3.18: A1 protein comparison with Bcl-2 family members and its expression in IRAK2 KO RAW cells with TLR4 stimulation- A1 protein with Bcl-2 family member anti-apoptotic proteins showing all shared domain and relatively shorter C-terminal (A). Figure is adapted from (Strasser, 2005). WT and IRAK2 KO RAW cells were stimulated with 500 ng/ml LPS for indicated time and cell lysates immunoblotted for induced A1 protein. Here, ERK2 is a control for total protein.

Afterwards, a molecular interaction of A1 with IRAK2 was determined in mammalian cells. Anti-FLAG antibody mediated immunoprecipitation was performed from FLAG-A1 and IRAK2 co-expressing HEK 293T cells. And immunoblot analysis displayed significant detection of co-precipitated IRAK2 (Figure 3.19A).

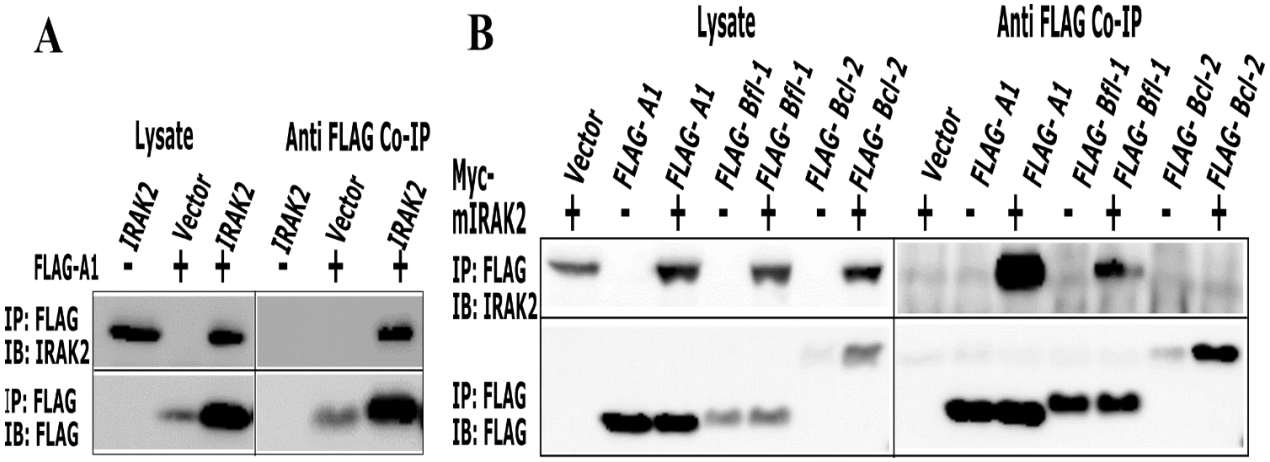

Figure 3.19: Interaction of IRAK2 with A1 and Bfl-1- HEK 293T cells co-transfected with IRAK2 and FLAG-A1 and cell lysate subjected for anti-FLAG immunoprecipitation. Precipitated IRAK2 with A1 is detected with IRAK2 specific immunoblot (A). IRAK2, FLAG- A1, Bfl-1 and Bcl-2 co expressing HEK 293T cell lysates precipitated with anti-FLAG antibody and co-precipitating IRAK2 is detected by immunoblot (B).

Furthermore, I tested IRAK2 interaction with Bcl-2 and the human orthologue Bfl-1. For this, FLAG-A1/Bfl-1/ Bcl-2 proteins were co-expressed with IRAK2 in HEK 293T cells and lysates were subjected to anti-FLAG immunoprecipitation. Co-precipitation of IRAK2 was observed with A1 and Bfl-1 but not with Bcl-2 protein (Figure 3.19B). These results suggest that IRAK2 interaction was specific to A1 and Bfl-1 in mammalian cells.

3.1.8 A1 exhibits more protein availability in presence of IRAK2 independent of its ubiquitination modification

Usually, physical interactions of A1 with various pro-apoptotic proteins e.g. p53 upregulated modulator of apoptosis (PUMA), Bid, Bax result in its increased protein level, probably via interference in its ubiquitination and subsequent proteasomal degradation (Herold et al., 2006; Vogler, 2012).

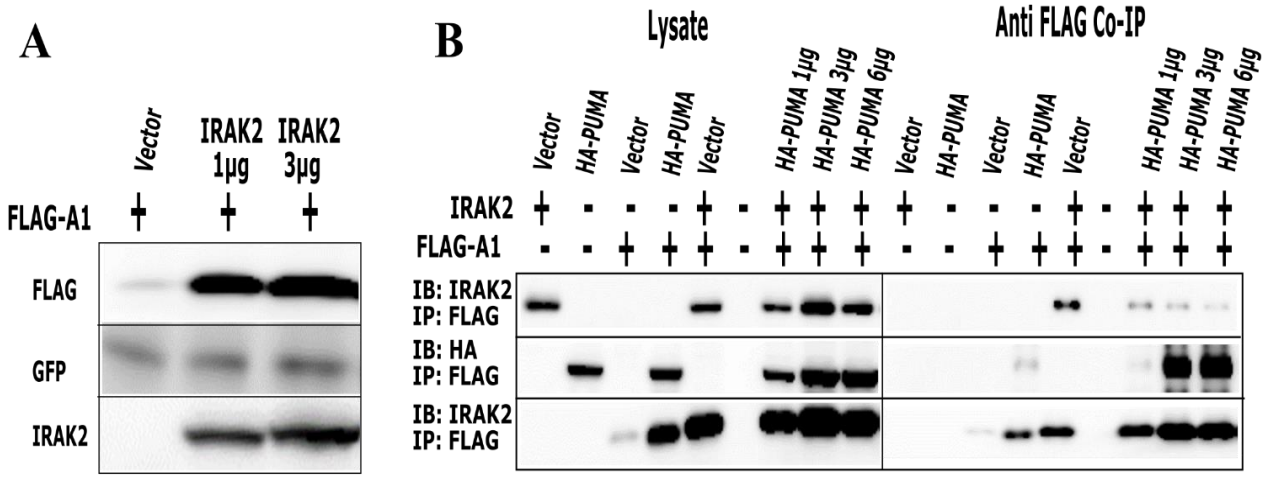

Figure 3.20: A1 protein availability with IRAK2 and PUMA co-expression- IRAK2 and FLAG-A1 co-expressing HEK 293T cell lysates immunoblotted for A1 by anti-FLAG antibody. Here GFP is expression control for A1 expressing plasmid (A). HEK 293T co-expressing IRAK2, FLAG-A1 and HA-Cherry-tagged PUMA protein precipitated with anti-FLAG antibody and co-precipitating IRAK2 and Puma was detected by immunoblot (B).

In order to investigate, whether the interaction of IRAK2 with A1/Bfl-1 also leads to increment in protein availability, FLAG-A1 was co-expressed with IRAK2 in 293T cells. Immunoblotting detection

presented enhanced availability of A1 protein with IRAK2 co-expression (Figure 3.20A). Increased A1 protein with IRAK2, indicates that this interaction might involve similar domain as pro-survival proteins. To examine this, IRAK2 was co-precipitated with FLAG-A1 in increased presence of HA-Cherry-tagged PUMA with anti-FLAG immunoprecipitation of co-expressing HEK 293T cell lysates. Simultaneous interaction of IRAK2 and PUMA with A1 was observed in immunoblot detection. Excess amount of PUMA protein efficiently displaced IRAK2 from A1 but not completely (Figure 3.20B, right panel).

This observation from PUMA and IRAK2 competition for A1 binding suggests either overlapping binding sites or differences in binding efficiency.

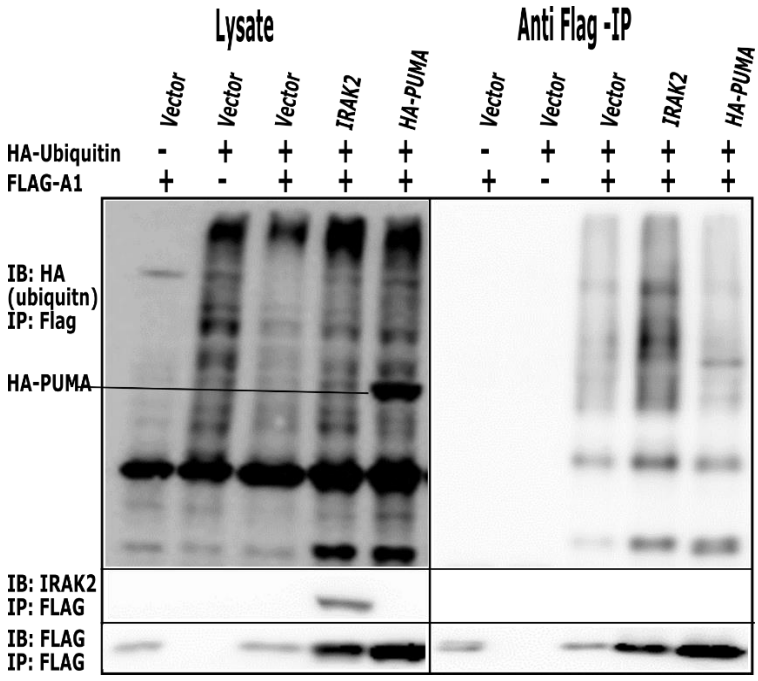

Figure 3.21: A1 ubiquitination with IRAK2 and PUMA- HEK cells co-transfected with HA-tagged ubiquitin, IRAK2, HA-tagged PUMA with FLAG-A1 expressing plasmids and after 24 h cell lysate used for immunoprecipitation with anti-FLAG antibody Cell lysate and ployubiquitnated A1 was detected with anti-HA immunoblot.

PUMA stabilizes A1 protein via blocking its ubiquitination. Therefore, I further investigated the A1 ubiquitination status in the presence of IRAK2. To analyze this, HEK 293T cells co-transfected with HA-ubiquitin, FLAG-A1, HA-Cherry-PUMA and IRAK2 than cell lysates were subjected to anti-FLAG immunoprecipitation. Despite of increased A1 precipitation, less ubiquitination was observed with PUMA in contrast to IRAK2 and vector control (Figure 3.21). Thus, IRAK2 does not seem to interfere with A1 ubiquitination.

3.1.9 A1 binding is not dependent on IRAK2 C-terminal

IRAK2 interacts with TRAF6 and also with A1. Next, I tested the possible incorporation of A1 during IRAK2 and TRAF6 interaction. For this, His-A1, FLAG-TRAF6 and Myc-IRAK2 were co-expressed in HEK 293T cells and TRAF6 was precipitated by anti-FLAG antibody. Immunoblot analysis exhibited that A1 protein was neither precipitated with TRAF6 alone nor with IRAK2-TRAF6 complex (Figure 3.22). This observation suggests that either A1 is not involved during IRAK2 and TRAF6 interaction or might be displaced by TRAF6 binding.

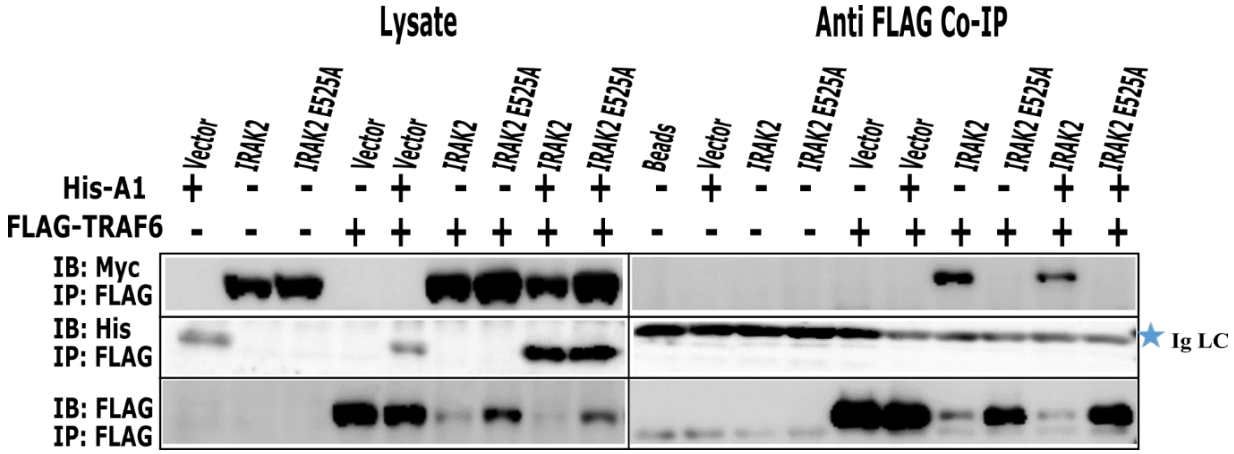

Figure 3.22: Presence of A1 interaction with TRAF6 and TRAF6-IRAK2 complex- HEK 293T cell lysate of co-expressing Myc-IRAK2, FLAG-TRAF6 and His-A1 protein subjected for anti-FLAG immunoprecipitation. Co-precipitated IRAK2 and A1 is detected with immunoblot.

To test if A1 interacts with the C-termius of IRAK2, HEK 293T cells co-expressing Myc-IRAK2 and FLAG-A1 were lysed and A1 was precipitated with FLAG specific antibody.

Immunoblot analysis revealed co-precipitation of all C-terminally truncated IRAK2 mutants (except IRAK2Δ100) as well as of IRAK2E525A and WT IRAK2 (Figure 3.23). Binding of A1 with IRAK2E525A suggested that TRAF6 interaction is not essential for this interaction. I conclude that neither the TRAF6 binding region nor the C-terminus of IRAK2 are involved in A1 binding to IRAK2.

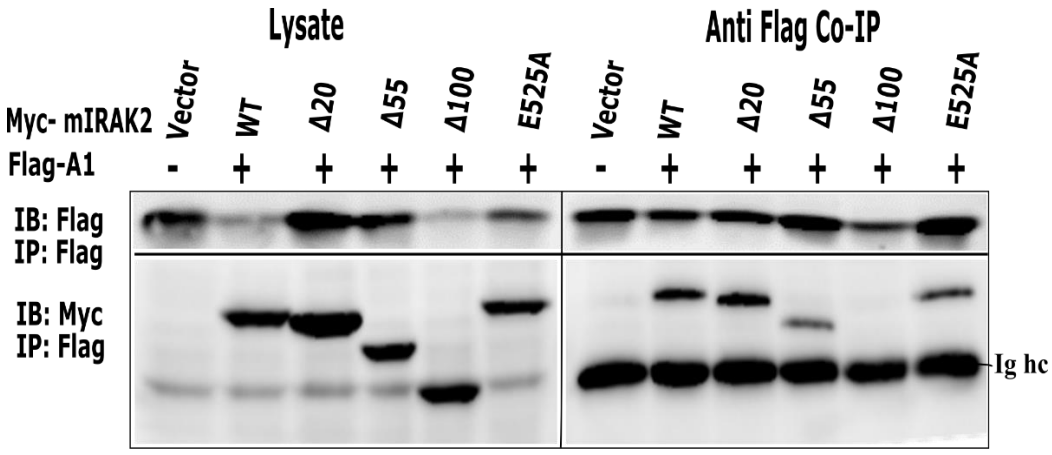

Figure 3.23: A1 interaction with IRAK2 protein with C-terminal truncation- Myc- IRAK2 and FLAG- A1 co-expressing HEK 293T cell lysates were precipitated with anti- FLAG antibody and co-precipitating IRAK2 is detected byanti myc immunoblot.

3.2 Importance of HECTD1 in B Cells

The humoral immune response is mediated by antibody producing B cells which are capable of recognizing an almost infinite number of foreign antigens and providing both short and long term protection from pathogen. Pathogen interaction induce B cells activation, proliferation and differentiation into immunoglobulin (Ig)-secreting plasma cells (Abbas et al., 2015; McHeyzer-Williams, 2003). The early response by pathogen activated B cells is characterized by the production of IgM which later switch to other isotypes and termed as Ig class switching. Various molecular modifications of signaling molecules *i.e.* ubiquitination, phosphorylation are involved in B cell activation and defects lead to improper Ig response or malignancies (Barbara A. Malynn and Ma, 2010; Chen, 2005 ; Wertz and Dixit, 2010).

In this study, we investigated if ubiquitin ligase HECTD1 has any role in B cells. HECTD1 is a large (~295 kDa) protein and contains HECT E3 ligase domain (Figure 3.24). It regulates different substrate proteins *i.e.* heat shock protein 90 (HSP90), adenomatous polyposis coli (APC) and phosphatidylinositol phosphate kinase type 1 γ (PIPKIγ) via conjugating poly-ubiquitination chain (Sarkar and E Zohn, 2012; Tran et al., 2013; Xiang Li et al., 2013).

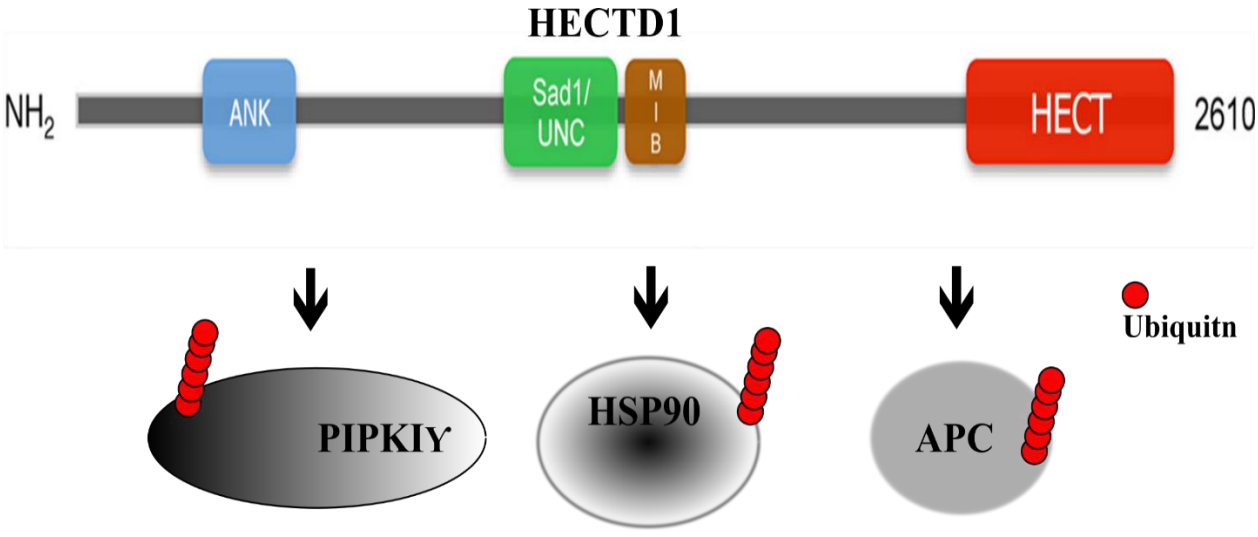

Figure 3.24: Domain architecture of HECTD1 ubiquitin ligase and its substrate protein- HECTD1 protein with its conserved motifs and various substrate proteins regulated by HECTD1 mediated polyubiquitination modification.

HECTD1 is an important protein as knock-out mice died during embryonic stage (Zohn et al., 2007). In order to investigate role of HECTD1 in B cells, our research group generated B cell specific HECTD1 knock-out C57BL/6 mice by Cre/loxP breeding. Mice expressing Cre recombinase from the B cell-specific *Cd79a* locus (*Mb1*^{CRE}) which encodes the Ig-α subunit of the BCR were crossed with loxP sites flanked *Hectd1* gene containing mice (Figure 2.1). B cell-specific HECTD1 KO mice (*Hectd1*$^{flox/flox}$) were analyzed for development, differentiation and Ig response from B cells.

3.2.1 HECTD1 is dispensable for B cell development in the bone marrow

B cells generation in mice initiates in liver from a hematopoietic stem cell and further development takes place in the bone marrow after birth (Abbas et al., 2015). Mature B cells develop in the bone marrow and every developmental stage is characterized by differential expression of immunoglobulin genes (Figure 3.25).

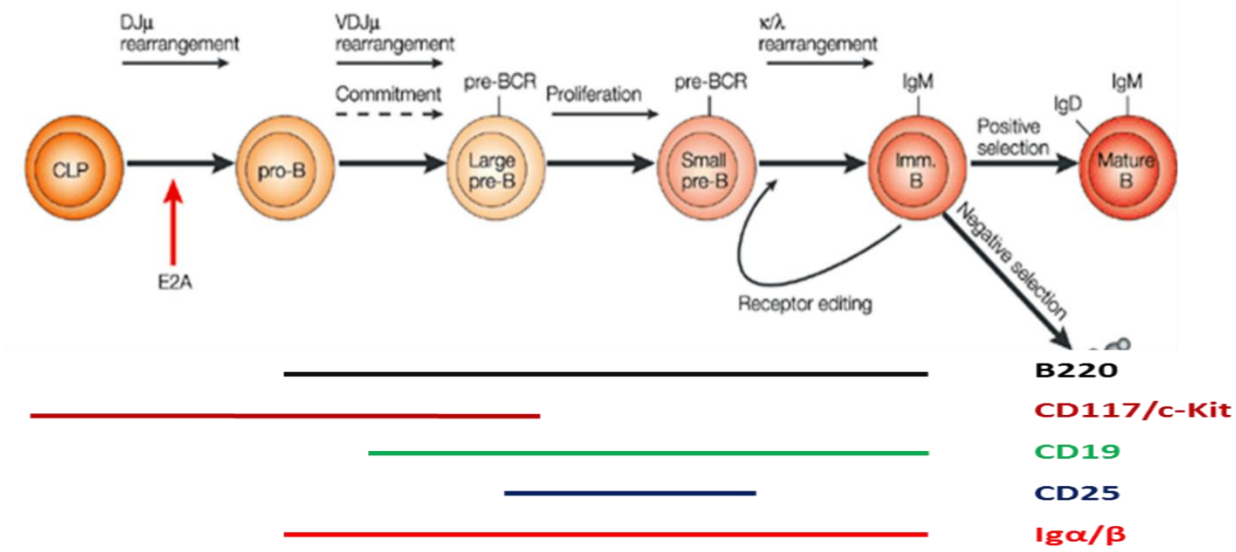

Figure 3.25: B cells development and BCR assembly in the bone marrow- B cell developmental stages from common proginator cell to mature B cell in bone marrow. It shows BCR assembly and surface marker expression different developmental stage. Graphics adapted from (Engel and Murre, 2001; Hardy and Hayakawa, 2001).

To investigate B cell development, bone marrow cells from WT and *Mb1*^{Cre} *X Hectd1*$^{flox/flox}$ were analyzed. Bone marrow cells were stained for different surface markers with fluorophore conjugated antibodies and subjected to FACS analysis (Figure 3.26A). B220 (CD45R) is a common surface marker for B cells and present throughout all developmental stages in the bone marrow (Hardy and Hayakawa, 2001).

Bone marrow cells were FACS stained for pro- (B220+, CD117+), pre- (B220+, CD25+), immature (B220+ IgM) and mature (B220+ IgD) B cells. FACS data showed that B cell frequencies from all developmental stages in the bone marrow were comparable in WT and *Mb1*^{Cre} *X Hectd1*$^{flox/flox}$ mice (Figure 3.26B and C).

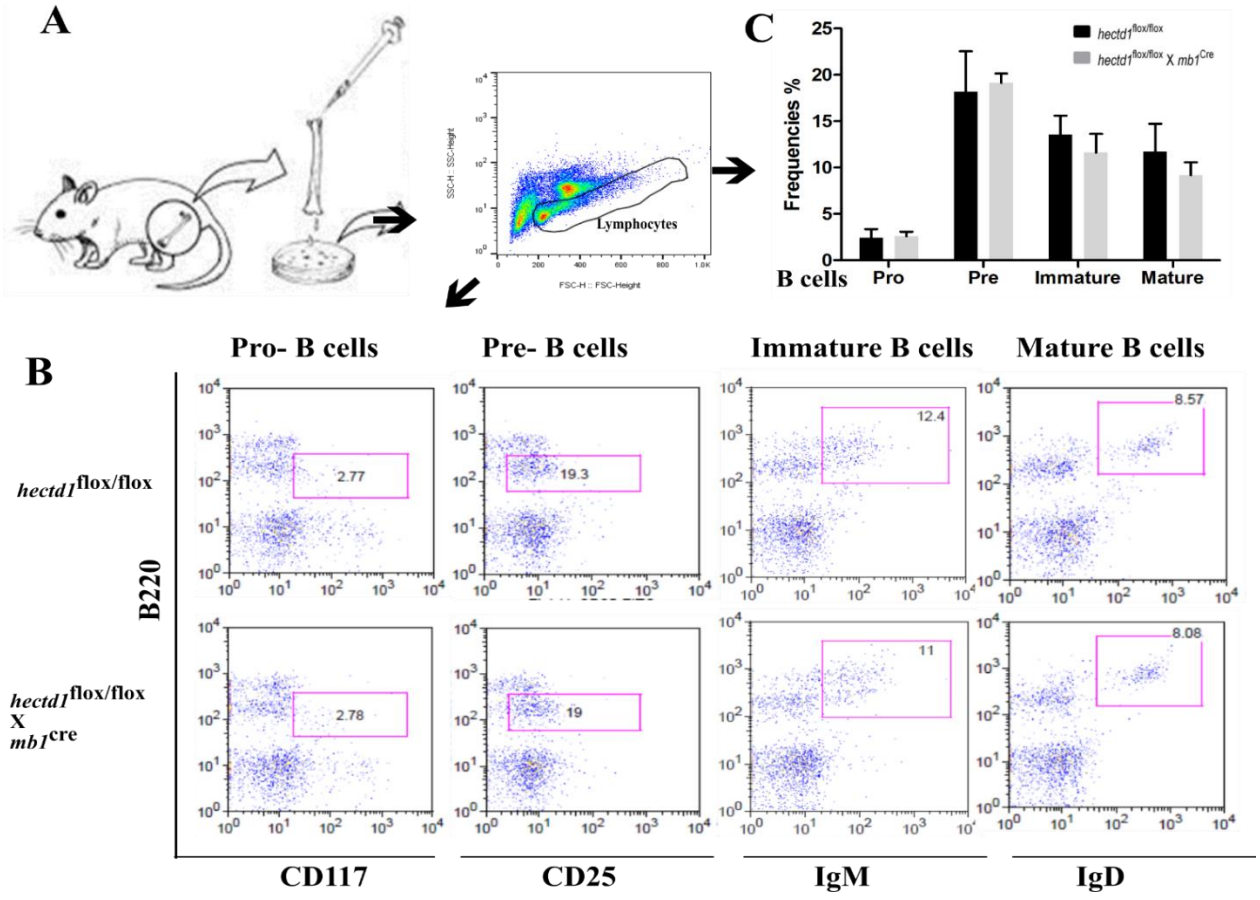

Figure 3.26: FACS analysis of bone marrow for B cells development with HECTD1 deficiency- Bone marrow cells after erythocyte lysis were analysed for different developmental stages of B cells (A). Representative gating scheme for analysis of B cells from bone marrow is shown (B). Frequency of B cells from different developmental satges shown as mean of 3 mice with SD error bars (C). Here *Mb1*^{Cre} *X Hectd1*$^{flox/flox}$ mice are carrying HECTD1 deficent B cell.

These results suggest that B cells develop normally in the absence of HECTD1. HECTD1 does not regulate the molecular events responsible for B cell development and the assembly of BCR.

3.2.2 HECTD1 is not necessary for development of follicular and marginal zone B cells

Immature B cells, generated from bone marrow migrate towards secondary lymphoid organs *i.e.* the spleen and lymph nodes. These cells rapidly pass through two transitional phases and develop into either marginal zone (MZ) or follicular (FO) B cells. Most mature B cells in the spleen are follicular B cells and are often called recirculating B cells as they tend to migrate between lymphoid organs. In secondary lymphoid organs, follicular B cells reside in specialized niches known as B cell follicles (Abbas et al., 2015; Hardy and Hayakawa, 2001; McHeyzer-Williams, 2003).

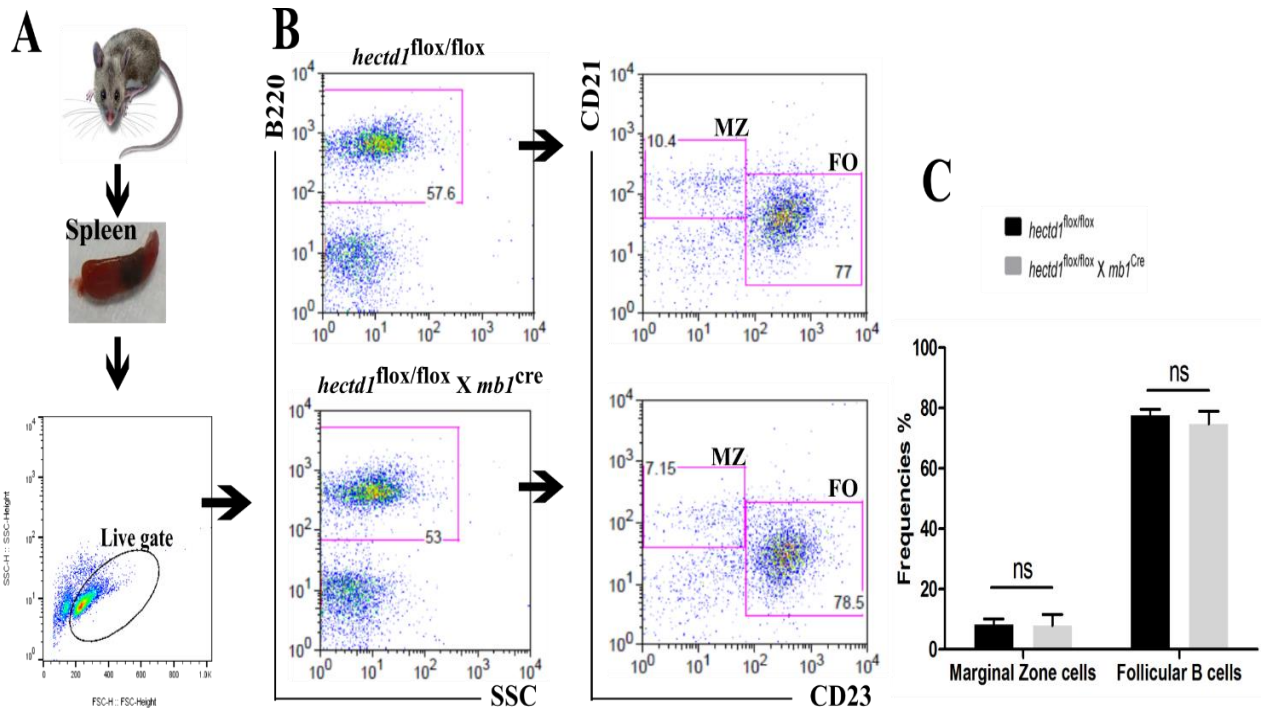

Figure 3.27: FACS analysis of B cells in spleen- Spleen cells from indicated mice were analysed for differentiated mature B cells (A). Distinguished fluorecent antibody staining was done for with indicated antigen and analysed from FSC, SSC denoted live cells freshly (B). Frequency of B cells are represented as mean of 3 mice experiments with SD error bar (C).

To investigate whether this late B cell development in the periphery is influence by HECTD1 deficiency, splenocytes were analyzed for MZ and FO B cells (Figure 3.27A). FACS analysis of B220+ B cells displayed equal frequency of $CD21^{hi}$ $CD23^{lo}$ marginal zone (MZ) and $CD21^{lo}$ $CD23^{hi}$ follicular B cells in both WT and B cell specific HECTD1 KO mice (Figure 3.27B and C).

These observations with splenic B cells indicate that HECTD1 is not involved in late B cell development.

3.2.3 Ig production is hampered in HECTD1 deficient splenic B cell cultures

Next, we asked if HECTD1 contributes to the effector response from B cells. Naïve and mature B cells residing in secondary lymphoid organs *i.e.* spleen, are capable of responding to pathogens after interaction with surface expressed BCR (Ig) and TLRs to produce antibodies (McHeyzer-Williams, 2003; Pasare, 2005) (Figure 3.28).

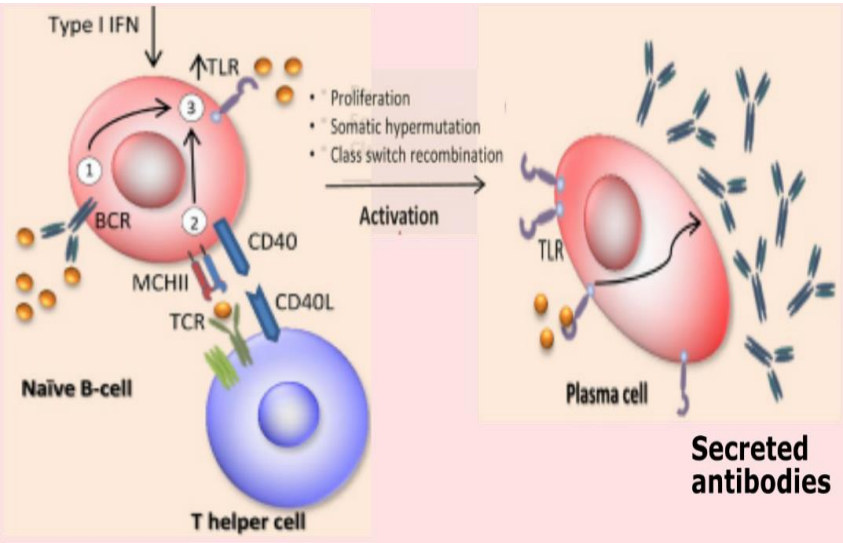

Figure 3.28: B cell activation and Ig secretion induction- Schematic representation of B cells activation with antigen engaged TLR together with BCR and CD40 co-stimulation. Adapted from (Isaza-Correa et al., 2014).

To compare Ig production efficiency of B cells in response to TLR4 activation, splenocytes obtained from WT and B cell specific *hectd1*$^{-/-}$ mice were cultured and stimulated with LPS or LPS plus CD40L (Figure 3.29A).

IgM secreted into culture medium was measured by ELISA at different days. Lower amount of IgM was observed in *hectd1*$^{-/-}$ B cells cultures in comparison to WT counterpart after LPS or LPS+CD40L

stimulation for 6 days (Figure 3.29B and C). Impaired Ig production in response to TLR and CD40 activation indicates HECTD1 might influence Ig expression inducing signaling cascade.

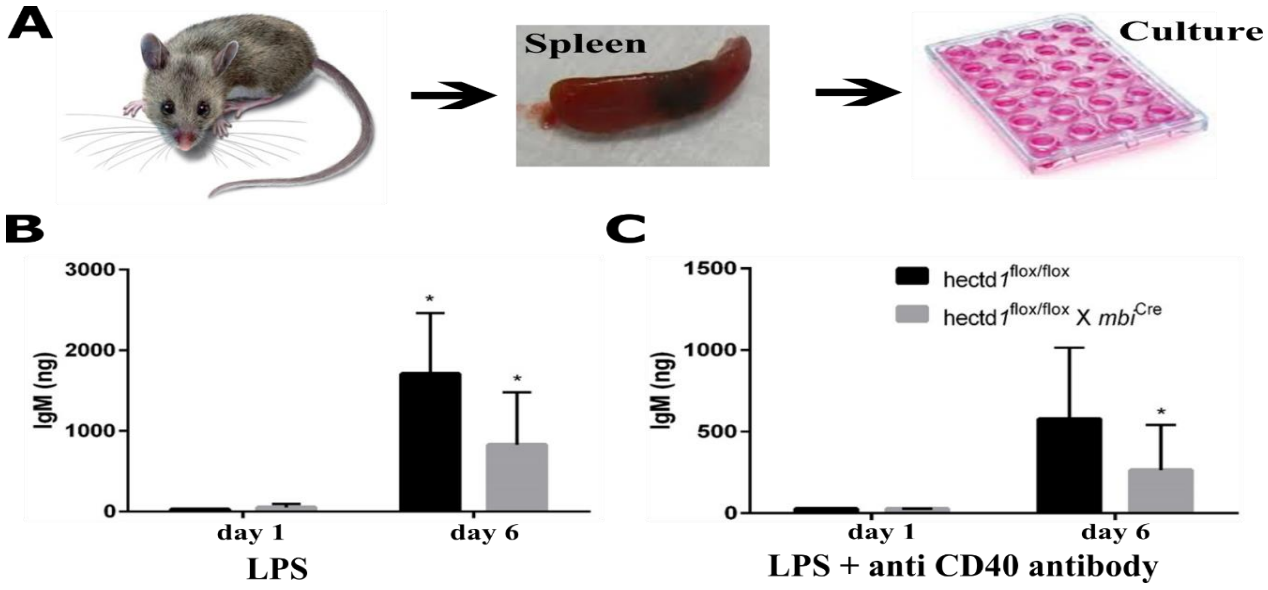

Figure 3.29: IgM production by cultured B cells with TLR4 and CD40 stimulation- Cells isolated from spleen and after erythrocyte lysis, cultured *in vitro* and stimulated with 10µg LPS/ml alone or with CD40L for 6 days. Culture supernatants were analyzed for IgM secretion by IgM specific ELISA from day 1 and day 6. Data shown are mean of 3 experiments with SD error bars.

3.2.4 NP-specific antibody response is significantly impaired in mice with HECTD1 deficient B cells after immunization

To test if the formation of natural antibodies is disturbed in the absence of HECTD1, serum Ig level was measured by Ig isotype specific ELISA kit. No significant difference was observed in serum level of antibodies (IgM and IgG3) between WT and B cell specific HECTD1 KO mice (Figure 3.30) suggesting no interference in Ig production machinery due to lack of HECTD1.

B cells exhibit a characteristic isotype specific antibody response after pathogen infection. During the first encounter B cells generate predominantly IgM antibodies but after successive or persistent infection other isotypes dominate the Ig response (Figure 3.31A).

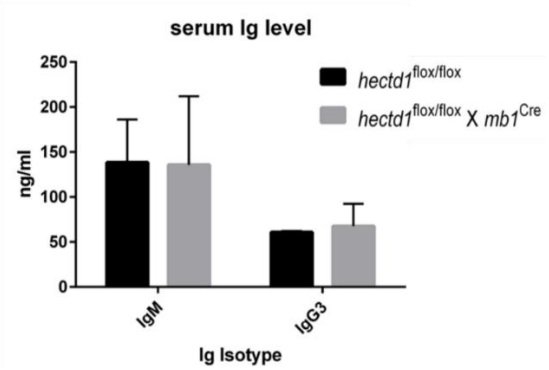

Figure 3.30: Immunoglobulins titer in mice serum- IgM and IgG3 level from serum of WT and B cell specific HECTD1 Knock-out mice was measured by isotype specific ELISA. Measured Ig amount is presented as mean of 5 mice with SD error bars.

To elucidate HECTD1 specific impact on this Ig response, mice were immunized with a T cell-dependent antigen, nitrophenylacetyl (NP) hapten conjugated to a carrier keyhole limpet hemocyanin (KLH). Mice were re-immunized on day 100th for secondary Ig response and serum was collected periodically as shown in (Figure 3.31B).

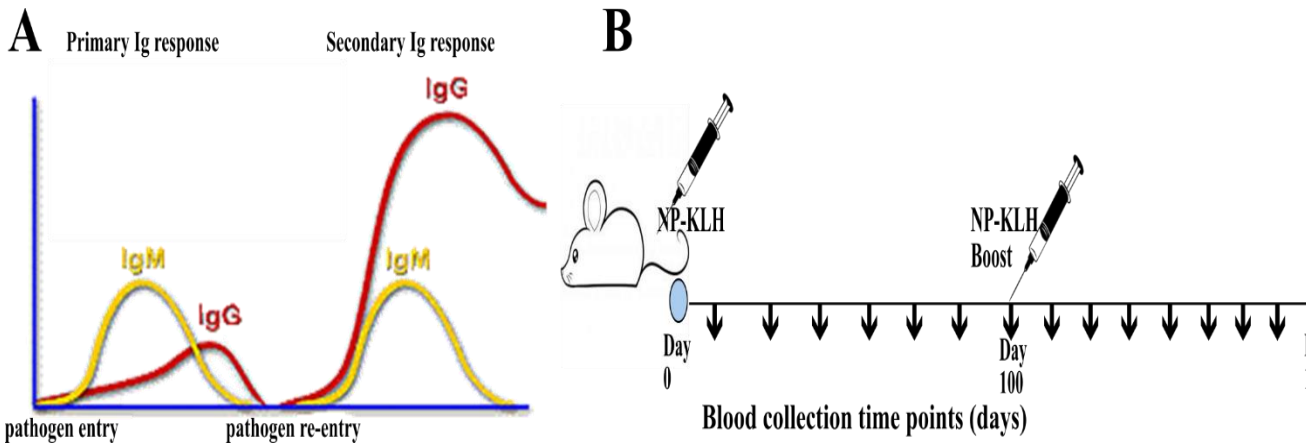

Figure 3.31: Mice NP-KLH immunization scheme- Isotype specific antibody response after infection and re-encounter of pathogen (A). Schematic representation NP-KLH immunization of mice for T cell dependent Ig response analysis (B).

NP-specific antibodies were measured from serum of immunized mice by ELISA. Analysis of two isotypes of NP-specific Ig in serum demonstrated a relatively weak primary and secondary IgM response in mice with HECTD1 KO B cells as compared to WT mice (Figure 3.32A). Another NP specific Ig isotype (IgG3) analysis revealed a lower primary response but severely abrogated secondary response in B cell specific *hectd1*$^{-/-}$ mice in contrast to WT counterpart (Figure 3.32B).

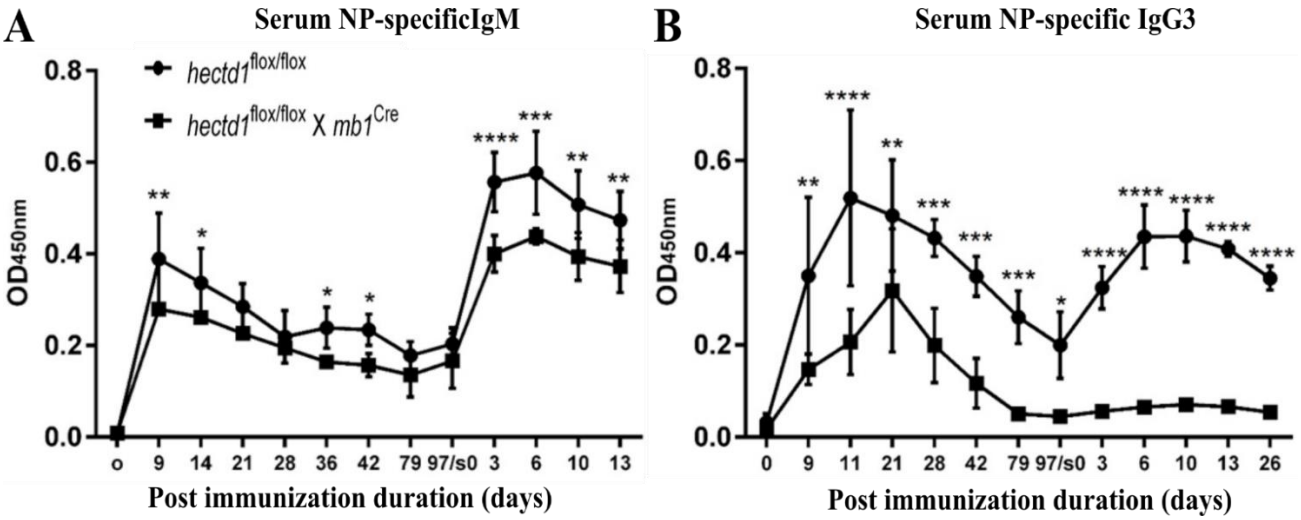

Figure 3.32: Antibody response after NP-KLH immunization- NP specific IgM (A) and IgG3 (B) measured from serum, collected on indicated time points after NP-KLH immunization. Ig specific ELISA extinction plotted as mean of 3 mice with SD error bars.

These results again indicate that HECTD1 regulates B lymphocyte activation and the subsequent Ig response. This reduced Ig response might be due to disturbance in germinal center reaction. Further investigations into germinal center reactions and T cell independent antibody responses might reveal more importance of HECTD1 in B cells.

4. Discussion

4.1 Newly characterized C-terminal region is important for IRAK2 in TLR signaling response

The IRAK2 protein sustains the TLR signaling cascade via transducing the signal by downstream interaction with TRAF6. The newly characterized IRAK2Δ55 was inefficient in restoring TLR response and imposed dominant negative impact on TLR-induced CD40 expression in RAW cells, despite of its interaction with TRAF6. Consistent with its inability to restore full TLR signaling, IRAK2Δ55 interaction was insufficient for the enhancement of TRAF6 ubiquitin ligase activity and resulted in reduced TRAF6 ubiquitination. Consequently, attenuated MAPK and NF-κB activation was exhibited by RAW cells after TLR activation in the presence of IRAK2Δ55. Our results suggest that the binding of TRAF6 to IRAK2 is not enough for optimal TLR signaling and an additional unknown mechanism in the signal transduction is required.

4.1.1 Abrogated TLR signaling with IRAK2Δ55 is associated with reduced TRAF6 ubiquitination

The IRAK2 protein is highly conserved in human and mouse counterparts. The essential role of IRAK2 in the TLR pathway in murine cells has previously been demonstrated (Kawagoe et al., 2008; Olaf Rosati and Martin, 2002) and these findings are supported by our results with diminished TLR signaling in IRAK2 KO and IRAK2Δ55-expressing RAW cells. Evolutionary conservation of this C-terminal region furthermore suggests a central importance in IRAK2-dependent TLR signaling.

Deletion of the C-terminal region might result in an altered IRAK2 protein conformation which might interfere with a required mechanism for the induction of TRAF6 ubiquitination and signal transduction. An online structure prediction tool (Robetta from www.bakerlab.org) revealed no stable structural domain in IRAK2Δ55 as it does not form any α-helices and β-sheets (data not shown).

Suboptimal TRAF6 ubiquitination induction in the presence of IRAK2Δ55 suggests the involvement of the IRAK2 C-terminus in this process. A phosphorylation site (S-X-X-D) within this region at S572-S-S-E for casein kinase II was predicated by an online server (http://scansite3.mit.edu/#proteinScan) as a possible active motif in the IRAK2 C-terminus. Phosphorylation at this site might be required for the optimal activation of IRAK2 and the subsequent induction of TRAF6 ligase activity. This SSS motif might provide a target in molecular therapies through the design of small molecular inhibitors to treat TLR-related diseases. Further studies focusing on this potential phosphorylation site in the IRAK2 C-terminal region should clarify if casein kinase II really interacts with IRAK2.

Additionally, the IRAK2-induced ubiquitination of TRAF6 stabilizes mRNAs for cytokines to enhance the inflammatory response following TLR activation. Consistently, *irak2*$^{-/-}$ mice displayed reduced cytokine mRNA stability (Tenekeci et al., 2016; Wan et al., 2009). TRAF6 mediated mRNA stabilization helps in sustaining pro-inflammatory response during infection. Our results with IRAK2Δ55 exhibited reduced TRAF6 ubiquitination and abstained expression of TLR induced CD40 or IL-6. If this reduced TLR response is also partially due to the lack of mRNA stabilization has to be analyzed in future studies.

In addition to self-ubiquitination and IRAK2-mediated induction, TRAF6 ubiquitination is also controlled by several other regulatory proteins (Wang et al., 2015; Zhang et al., 2014), such as Itch (Zhang et al., 2013a), Nur77 (Wu et al., 2016), A20 (Vereecke et al., 2009), TIFA (Ea et al., 2004), IRAK1(Muroi and Tanamoto, 2012), Hsp70 (Chen et al., 2006), UBE20 (Zhang et al., 2013b) and BAT3 (Lee et al., 2016). It is currently unknown if any of these TRAF6 regulatory molecules also interact with IRAK2 or its C-terminus and thereby possibly influence the activity of TRAF6.

4.1.2 Presence of inherited IRAK2 variants in humans emphasizes the importance of IRAK2 in TLR signaling

In contrast to murine cells, human cells seem to rely less on IRAK2 during TLR signaling (Mina et al., 2017; Sun et al., 2016). However, recent reports on single nucleotide polymorphisms (SNPs) in

IRAK2 in humans and linked abnormal TLR signaling suggest that IRAK2 also plays an important role in human cells. One non-synonymous IRAK2 variant rs35060588 (R214G) was associated with increased death risk in colorectal cancer patients (Wang et al., 2014). This variant was implicated with lesser TRAF6 ubiquitination and subsequent weaker NF-κB activation. Another inherited coding variant of IRAK2 rs3844283 (L329V) was linked to reduced hepatitis C virus (HCV) clearance and diminished IFN-α secretion (Wang et al., 2015). In contrast, IRAK2 variant rs708035 (D431E) exhibited enhanced TRAF6 ubiquitination and elevated NF-κB activation after influenza virus infection (Zhang et al., 2014). Interestingly, in all IRAK2 variants TRAF6 ubiquitination was influenced without disturbing the known TRAF6 interaction motif, which indicates an additional mechanism for TRAF6 activation aside from simple physical binding. These observations with human IRAK2 variants are in accordance with our findings on IRAK2Δ55. Above all, reports of coding variants and their association with pathologies emphasize the significance of IRAK2 in human TLR signaling and suggest the importance of our studies also for the human system. IRAK2 variants might serve as potential novel biomarker for TLR linked diseases or as a therapeutic intervention point.

4.1.3 IRAK2-associated malignancies might be a consequence of A1 interaction

Immune cell-related carcinogenesis is reported with overexpressed anti-apoptotic proteins of the Bcl-2 family, such as myeloid leukemia cell differentiation protein (Mcl-1) and Bfl-1 the human orthologue of A1 (Chen et al., 2010; Fan et al., 2010). Enhanced Bfl-1 protein is reported in myeloma, lymphoma and Hodgkin lymphoma (Fan et al., 2010; Loughran et al., 2011; Tarte et al., 2004). Various malignancies have been reported in humans with IRAK proteins overexpression, such as IRAK1 in non-squamous cell lung cancer (NSLC), melanoma and other cancers (Jain et al., 2014; Ngo et al., 2011; Rhyasen and Starczynowski, 2014). Inhibition of IRAK1 and IRAK4 signaling in melanoma, diffuse large B-cell lymphoma, myelodysplasia and acute myeloid leukemia exhibited prolonged mouse survival (Rhyasen et al., 2013; Srivastava et al., 2012). Innate immune cells such

as neutrophils, macrophages need anti-apoptotic proteins for survival to sustain intense inflammation (Metais et al., 2012; Ottina et al., 2012a; Ottina et al., 2012b; Vier et al., 2016).

In our experiments IRAK2 interacted with A1 and, interestingly, enhanced the availability of A1 when co-expressed in HEK 293T cells suggesting a possible interplay of IRAK2 and A1 in the progression of malignancies. As Bfl-1 is resistant to most anti-cancer drugs against Bcl-2 family member proteins, targeting IRAK2 to downregulate A1 might be an alternative to existing therapies to control the growth of cancer cells.

4.1.4 IRAK2 C-terminal can be targeted for molecular therapies in inflammatory diseases and associated cancers

Chronic inflammation induced by infections such as, *Helicobacter pylori*, *Chlamydophila psittaci*, *Borrelia burgdorferi* and Epstein–Barr virus (EBV) infections are associated with development of B cell lymphomas (Ferreri et al., 2009). Additionally, inherited mutations or deficiencies in TLR pathways molecules, such as MyD88 (L265P) is also a reason for various B cell lymphomas (Ngo et al., 2011; Vaqué et al., 2014). Abnormalities of the mediators of the TLR pathway such as IRAK4, A20 are linked with chronic inflammation, autoimmune disorders and leukemia (Chen et al., 2010; Kelly et al., 2011; Maiorov et al., 2013).

TLR signaling induced necroptosis pathway in macrophages required for tissue homeostasis (Baker et al., 2014). Over-expressed necroptosis mediators i.e. RIP3 and MLKL in inflammatory pathologies such as Crohn's disease, ulcerative colitis or allergic colitis (Pasparakis and Vandenabeele, 2015b). Our results demonstrate that IRAK2 deleted and IRAK2Δ55-expressing RAW cells exhibited reduced LPS-stimulated RIP3 phosphorylation. This observation suggests that IRAK2 is crucial for TLR signaling induced necroptosis and might serve as a target molecule for treatment in related diseases.

IRAK2 also mediates the unfolded protein response (UPR) and ER stress signaling which was shown to be responsible for cell death in human prostate cancer cell line (PPC1) (Benosman et al.,

2013). As part of IL-1 signaling pathway, IRAK2 is crucial for IL-1 induced ER stress signaling mediated myocytes apoptosis in cardiomyopathy (Liu et al., 2015). ER stress is also induced during excessive TLR activation and with extensive Ig production in B cells after bacterial infection (Liu and Ye, 2011; Vural and H., 2014). TLR activation induced persistent inflammatory microenvironment with increased risk of cancer and IRAK2 as important mediator of TLR signaling represent rational target for chronic inflammation and cancer.

Our results with the TLR-specific importance of the IRAK2 C-terminus suggests the C-terminal region as a target for small molecule based inhibitory therapies. Altogether, our findings with IRAK2 and previously reported TLR pathway linked pathologies, provide a scope for better insight into the involved mechanisms and offer an alternative target for molecular therapies. There are some inhibitors under development against TLR signaling molecules; an inhibitor with activity against IRAK2 has not been developed yet. Further elucidation of the IRAK2 C-terminal structure and mediated molecular mechanisms shall aid in resolving the complexities of inflammatory diseases and linked malignancies progression in humans and designing intervening therapeutics.

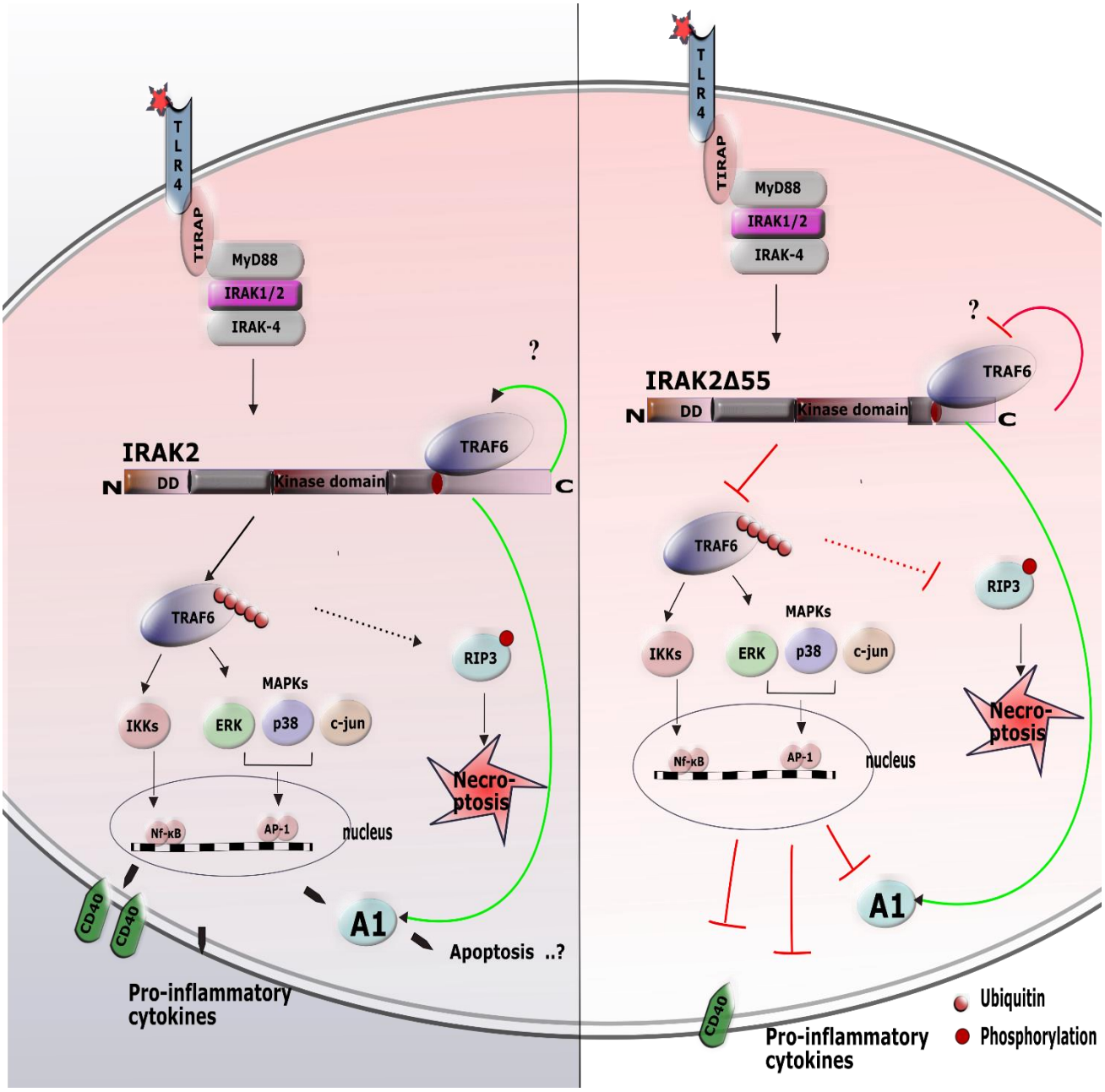

Figure 3.33: Schematic representation of comparison between IRAK2 and IRAK2Δ55 involvement in TLR signaling- IRAK2 interaction with TRAF6 induce its ubiquitnation and it is critical for downstream pathway to stimulate inflammation, antiapoptotic protein epression and necroptosis. Loss of newly determined ~55 long C-terminal in IRAK2 results in imapired TLR signaling and necroptosis because of reduced TRAF6 ubiquitnation.

4.2 Impaired Ig response from *hectd1*$^{-/-}$ B cells is probably because of dysregulated HECTD1 substrate proteins

The importance of HECTD1 was first observed when deficient mice died in the embryonic stage following incomplete neural tube closure (Zohn et al., 2007). Results from this thesis demonstrate an abrogated B cell response in the absence of HECTD1 which suggest the importance of this ligase in B cell physiology.

B lymphocyte activation involves TLR signaling which, together with BCR, leads to an increased Ig production and this process is regulated by various signaling molecules and transcription factors (Berberich and Schimpl, 1992; Pone et al., 2012; Rawlings et al., 2012). A chaperone protein HSP90 has been reported among various targets of HECTD1 to be involved in the regulation of NF-κB and MAPKs activation. In the NF-κB activation pathway, the central protein IKKγ is stabilized by HSP90 via direct interaction (Park et al., 2007). Additionally, HSP90 is also essential for stabilization of upstream component IRAK1 during TLR signaling (Nardo et al., 2005). Ig gene expression is NF-κB dependent (Nelsen and R, 1992), thus any disturbance in NF-κB activation results in a compromised Ig molecule production. Association of the HSP90 chaperone activity with a range of molecules shared by different signaling pathway indicate possible influence of HECTD1 deficiency in B cell response via unregulated HSP90 activity.

We observed an abandoned secondary IgG3 response from mice containing HECTD1 deficient B cells in immunization experiment. The loss of HECTD1 and subsequent influence on IgG3 response suggests a probable role of HECTD1 in the signaling events of the germinal center reaction. A defective Ig response towards T dependent antigen in various disease models is linked to faulty germinal center reactions (Niu et al., 2008). Disturbed PI3K activation is associated with an imbalanced Ig class switch in mice (Chen et al., 2015). The influence of HSP90 on the BCR signaling component Lyn and the subsequent activation of the PI3K pathway might explain how HECTD1 influences the germinal center reaction via HSP90 (Pauls et al., 2012; Woyach et al., 2012).

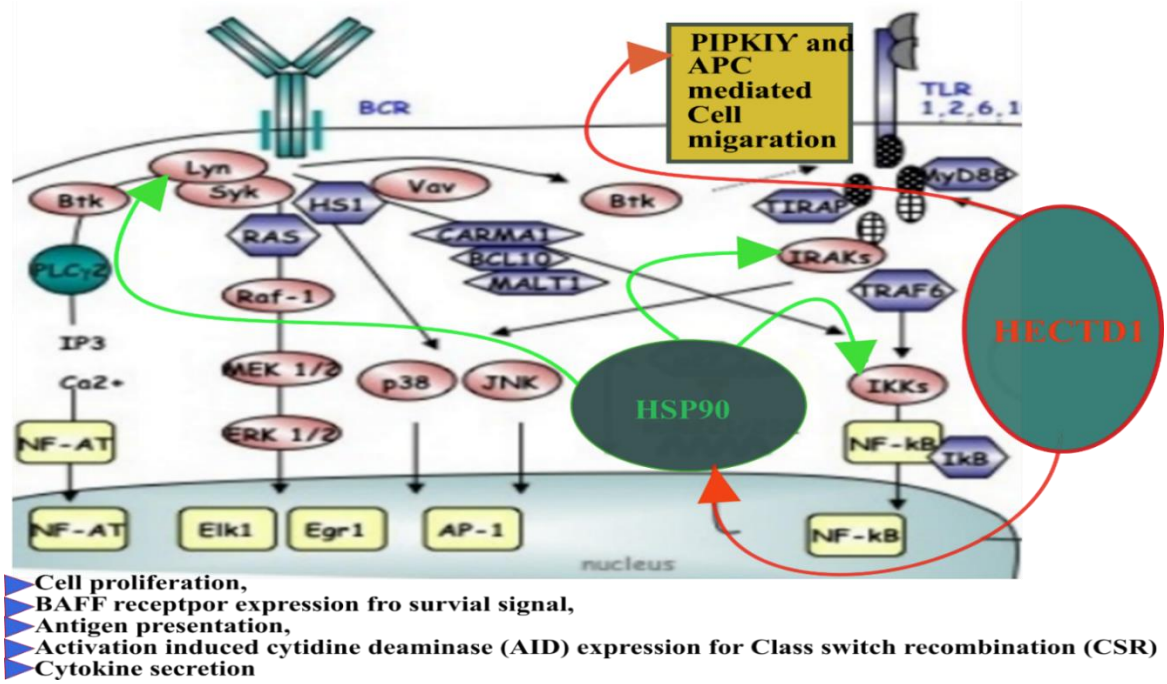

Figure 3.34: Graphical respresentation of hypothetical role of HECTD1 in B cells physiology. Modified from (Muzio, 2015).

I assume that adversary effects exerted by HECTD1 deficiency are probably due to the unregulated substrate protein HSP90 and result from disturbed BCR and TLR pathways (Figure 3.34). Loss of HECTD1 loci was linked with B cell leukemia (Hernandez et al., 2009). However, the direct association of HECTD1 with any signaling pathways has yet to be reported.

Additionally, HECTD1 regulates proteins such as Adenomatous Polyposis Coli (APC) which effects cell migration and homeostasis (Tran et al., 2013). Recently a unique DNA binding motif known as basic tilted helix bundle (BTHB) related similar sequence has been identified in HECTD1 (residue 1879-1966). This further suggests its possible role in various regulatory mechanisms (Helander et al., 2014). The exact mechanism of HECTD1's involvement in the immune system remains unclear and further investigations are needed to elucidate these mechanisms.

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Curriculum Vitae

Affidavit (Eidesstattliche Erklärung)

I hereby declare that my thesis entitled "Function of IRAK2 in macrophages and HECTD1 in B cells" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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