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THE ROLE OF DUAL SPECIFICITY PHOSPHATASE -11 IN INNATE AND ADAPTIVE IMMUNE RESPONSES

Kalyan Chakravarthy Nallaparaju

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by

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A

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By

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DEDICATION

To my parents, thank you for your support and encouragement throughout my academic endeavors. Your words, thoughts and prayers kept me motivated and determined to do my best. To my sister, thank you for providing me with great support. To my grandparents, thank you for all your words of encouragement.

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Dual-specificity phosphatases (DUSPs) constitute a subfamily of protein tyrosine phosphatases characterized by their ability to dephosphorylate both phosphotyrosine and phosphoserine/phosphothreonine residues within a substrate, typically among members of the MAP kinase family. DUSPs have been shown to play a critical role in the regulation of various cellular processes including signal transduction, cell cycle regulation and cellular proliferation via modulation of MAP kinase activities. Also, many members of this family have been demonstrated to be potent immune regulators. Deregulated expression patterns of DUSPs have been associated with pathogenesis in a wide range of diseases. DUSPs are broadly classified into six subgroups, of which the atypical subgroup of DUSPs is the least studied for their physiological functions.

DUSP11 is an atypical dual specificity phosphatase. Several recent *in vitro* studies highlighted the importance of this molecule in cell proliferation, cancer suppression and chronic inflammation. However, the physiological function of DUSP11 has not been studied via genetic approaches *in vivo*. Further, the role of

DUSP11 in regulation of immune responses is not well understood. Our *in vitro* studies show that DUSP11 expression is induced after activation of innate and adaptive immune cells, suggesting that DUSP11 may play a role in the regulation of immune responses. To examine the function of DUSP11 in immune cells, we generated and analyzed *Dusp11-/-* mice. We hypothesized that DUSP11 is an essential regulator of innate and adaptive immune responses.

Interestingly, we found that *Dusp11-/-* mice were more susceptible to *Listeria monocytogenes* infection due to defective antigen-specific T cell responses when compared with wild-type mice. Further, *Dusp11-/-* mice were resistant to endotoxin-induced septic shock and Dusp11-/- dendritic cells had significantly decreased levels of pro-inflammatory cytokine production in response to TLR activation, associated with reduced NF-κB activation *in vitro*. Our mechanistic studies demonstrate that DUSP11 directly dephosphorylates the gamma binding domain (γbd) of IKK-β and thus positively regulates the activity of the IKK complex. Together, we demonstrate for the first time that DUSP11 is a critical regulator of immune responses and acts specifically by mediating IKK activation during innate immune responses. This phosphatase thus could serve as a novel target for therapeutic intervention for inflammatory and autoimmune diseases.

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ABBREVIATIONS

APC: Antigen Presenting Cell

CD: Cluster of Differentiation

CDC14s: Cell Division Cycle 14 phosphatases

DC: Dendritic Cell

DUSP: Dual-specificity phosphatases

ELISA: Enzyme-Linked Immuno Sorbent Assay

ERK: Extracellular signal-Regulated protein Kinases

ICS: Intracellular Cytokine Staining

IFN: Interferon

IL-2: Interleukin-2

IL-6: Interleukin-6

I.P.: Intraperitoneal

I.V.: Intravenous

JNK: c-Jun N-terminal kinase

MAPK: Mitogen Activated Protein Kinase

MAP2K: MAP Kinase Kinases

MFI: Mean Fluorescence Intensity

MHC: Major Histocompatibility Complex

MKPs: Mitogen activated protein Kinase Phosphatases

NK: Natural Killer

PAMP: Pathogen Associated Molecular Patterns

PRL: Phosphatases of Regenerating Liver

PRR: Pattern Recognition Receptor

PTENs: Phosphatase and Tensin homologues deleted on chromosome-10

PTK: Protein Tyrosine Kinase

PTP: Protein Tyrosine Phosphatase

TCR: T-Cell Receptor

TNF-α: Tumor Necrosis Factor

Treg cell: T regulatory cell

CHAPTER-1 INTRODUCTION

The immune system consists of specialized cells and molecules that defend the host against pathogens (Delves and Roitt, 2000a, b). Fundamentally, immune responses against microbes are classified either as innate or adaptive immune responses. Innate immune responses serve as a first line of defense against infections. However, they are not specific and are not usually sufficient to combat the infection. Adaptive immune responses come into play several days after the infection, but they are specific against the type of pathogen and also protect the host against reinfection from the same pathogen. Both innate and adaptive immune responses involve a wide range of cell types that functions as initiators, regulators and effectors (Dong et al., 2002). Innate immune cells include phagocytic cells such as macrophages, dendritic cells and neutrophils; granulocytes like basophils, mast cells and eosinophils; and lymphocytes like natural killer cells (NK cells) (Delves and Roitt, 2000a, b). Adaptive immune cells include B and T lymphocytes. All of these cells communicate and counter regulate one another by cell-cell contact or by secreting cytokines to generate optimum immune responses against pathogens (Dong et al., 2002). Any disruption in this regulation may result in chronic inflammation potentially leading to inflammatory and autoimmune diseases. The immune cells are tightly regulated at multiple levels during the activation as well as the termination of immune responses. This regulation involves both cell intrinsic mechanisms in the activated cells or cell extrinsic mechanisms mediated by other cell populations such as regulatory T cells (Tregs) (Peggs et al., 2008).

Innate immune cells recognize the evolutionarily conserved structures of pathogens known as pathogen associated molecular patterns (PAMPs). These PAMPs are recognized by pattern recognizing receptors (PRRs) expressed either on the surface or inside the innate immune cells (Lang et al., 2006; Mogensen, 2009). Several families of PRRs like Toll like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and the nucleotide-binding domain and leucine-rich repeat-containing receptors (NLRs) have been well characterized in last decade (Iwasaki and Medzhitov, 2010). Based on the nature of the infection one or more PRRs are activated by PAMPs, several signaling transduction pathways are rapidly activated to mediate gene expression and the synthesis of several immune molecules like cytokines, chemokines etc. Two major pathways that are involved in proinflammatory cytokine expression are the evolutionarily conserved MAP kinase signaling cascade and the NF-κB signaling cascade (Dong et al., 2002; Lang et al., 2006). The magnitude and duration of the innate immune responses are tightly regulated by several endogenous molecules like kinases and phosphatases that activate and deactivate these signaling pathways (Mogensen, 2009). In the case of adaptive immune cells, MAP Kinase and NF-κB signaling pathways are involved downstream of activating and inhibitory receptors to ensure optimum T cell and B cell response activation (Peggs et al., 2008). Therefore, these signaling pathways function as rheostats in both the innate and adaptive immune responses. Although their upstream activation signals vary, understanding the regulatory elements controlling this activation is essential. Regulation of cell signaling by protein tyrosine kinases and phosphatases will be discussed in detail in the next section.

1.1. Protein tyrosine kinases and protein tyrosine phosphatases are critical regulators of cell signaling

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs) regulate a wide variety of fundamental signaling and physiological pathways by reversible phosphorylation of proteins (Alonso et al., 2004; Hunter, 1995; Patterson et al., 2009; Tonks and Neel, 1996). Generally, the PTKs activate cell signaling by phosphorylating the protein substrates whereas the PTPs deactivate cell signaling by dephosphorylating the protein substrates (Patterson et al., 2009). However, in some exceptional cases phosphorylation of regulatory domains of proteins leads to deactivation of downstream signaling (Higashimoto et al., 2008). Though the role of PTKs in cell signaling is well established due to their early identification (Czernilofsky et al., 1980; Patterson et al., 2009), recent research is being focused on PTPs which has resulted in an increased understanding of the dynamics PTPs and PTKs play in fundamental physiological processes. This protein phosphorylation is reversible and tightly regulated and any aberrations in this process results in numerous diseases including cancer and immune deficiencies (Patterson et al., 2009; Tonks, 2006). Therefore, understanding this balance of signaling by PTKs and PTPs is critical for the development of new diagnostic and therapeutic strategies for the above mentioned diseases. Though PTPs and PTKs comprise of a large number of molecules and are classified into several sub families, in the interest of the present study the role of a sub family of PTPs, known as dual specificity phosphatases (DUSPs) in physiological processes and human disease will be further discussed.

1.2. Dual specificity phosphatases

Dual-specificity phosphatases (DUSPs) constitute a subfamily of protein tyrosine phosphatases that are characterized by their ability to dephosphorylate both phosphotyrosine and phosphoserine/ phosphothreonine residues within a substrate (Patterson et al., 2009; Pulido and Hooft van Huijsduijnen, 2008; Zhang and Dong, 2007; Zhang and Dong, 2005). DUSPs have been shown to play critical roles in the regulation of various cellular processes such as signal transduction (Zhang et al., 2004), cell cycle regulation (Karlsson-Rosenthal and Millar, 2006) and cellular proliferation (Zhang et al., 2009) via modulation of their substrate activities. Deregulated expression patterns of DUSPs have been associated with pathogenesis in a wide range of diseases such as colon cancer, multiple sclerosis etc. (Tonks, 2006). All the members of this subfamily have a highly conserved catalytic domain also known as the phosphatase domain which consists of a consensus sequence HCXXXXXR with conserved aspartic acid, cysteine and arginine residues forming the catalytic site (Huang and Tan, 2012). DUSPs share a common mechanism of catalysis in dephosphorylating their substrates by forming a stable phosphoryl-intermediate (Denu and Dixon, 1995; Niwa et al., 2002).

Currently, there are 61 members identified as DUSPs. Based on their domain homology and sequence similarity, these DUSPs are broadly classified into seven subgroups (Fig 1). They include slingshot phosphatases, phosphatases of regenerating liver (PRL), cell division cycle 14 phosphatases (CDC14), phosphatase

and tensin homologues deleted on chromosome-10 (PTEN), myotubularins, mitogen activated protein kinase phosphatases (MKP) and atypical DUSPs (Patterson et al., 2009). Among these, CDC14s are well studied for their role in cell cycle regulation and tumor suppression (Trautmann and McCollum, 2002) and PTENs (Wang and Jiang, 2008; Wishart and Dixon, 2002) and MKPs for their role in cell signaling (Zhang and Dong, 2007). The atypical subgroup of DUSPs is the least studied for their physiological functions (Patterson et al., 2009). MKPs (also known as typical DUSPs) and atypical DUSPs are both designated as DUSPs (Lang et al., 2006). Until recently the low molecular weight atypical DUSPs were grouped with MKPs as they also are thought to negatively regulate MAP Kinase activity.

Indeed, a few atypical DUSPs may function as MKPs as several members like DUSP14 (Marti et al., 2001), DUSP3 (Todd et al., 1999) and DUSP22 (Alonso et al., 2002; Chen et al., 2002) are shown to dephosphorylate MAPK substrates. But other members were shown to have other diverse substrates like STAT molecules for DUSP3 (Hoyt et al., 2007; Najarro et al., 2001) or Glucokinase for DUSP12 (Munoz-Alonso et al., 2000). Together, MKP and atypical sub group are comprised of 30 members out of which 11 are bona fide MKPs and 19 are atypical DUSPs(Alonso et al., 2004). The atypical DUSPs lack the N-terminal Cdc25 homology 2 (CH2) domains and a MAP Kinase-binding (MKB) motif or Kinase interacting motif (KIM) that is commonly present in MKPs (Huang and Tan, 2012; Lang et al., 2006). Recent studies indicate the potential of these atypical DUSPs as crucial signaling regulators (Patterson et al., 2009). Currently, the *in vivo*

physiological functions of most atypical DUSPs are still unknown. Studying the *in vivo* functions of atypical DUSPs by generating KO mouse models would provide a better understanding of their importance in various disease models. In the current study's interest, the MKPs and atypical DUSPs will be further described in the next section.

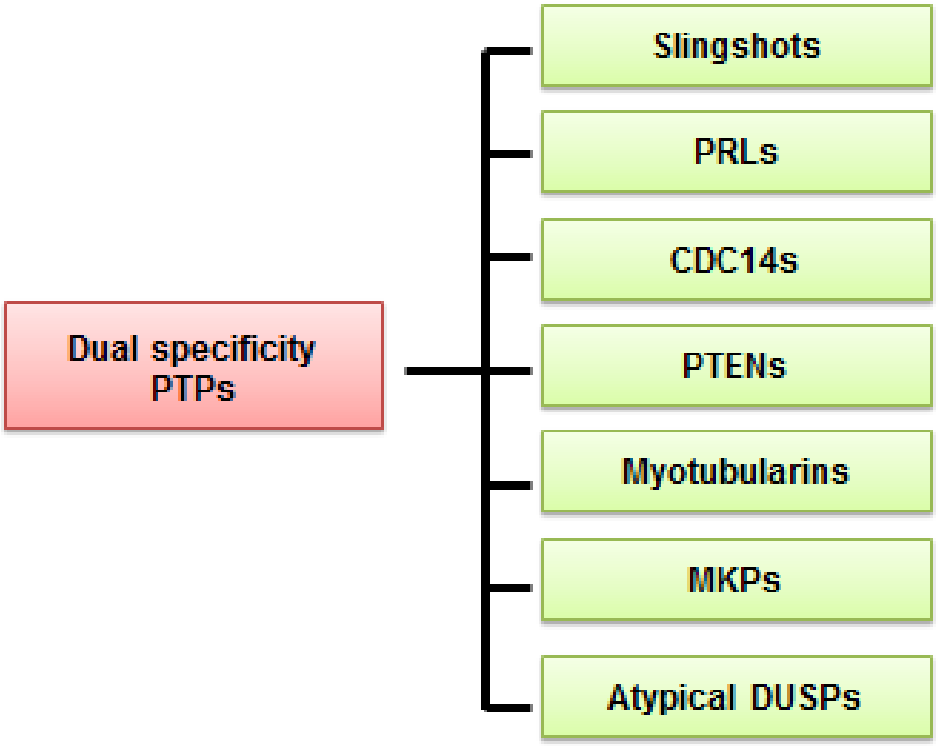

Figure 1. Classification of DUSPs. Based on their domain homology and sequence similarity, these DUSPs are broadly classified into seven subgroups (Patterson et al., 2009). They include slingshot phosphatases, phosphatases of regenerating liver (PRLs), cell division cycle 14 phosphatases (CDC14s), phosphatase and tensin homologues deleted on chromosome-10 (PTENs), myotubularins, mitogen activated protein kinase phosphatases (MKPs) and atypical DUSPs.

1.3. MAP Kinase signaling and their regulation by MKPs

Mitogen activated protein kinase (MAPK) signaling pathway is evolutionarily conserved, tightly regulated and is essential for many physiological processes (Dong et al., 2002). Mammalian species mainly have three families of MAPKs including extracellular signal-regulated protein kinases (ERKs), c-Jun NH2-terminal kinases (JNKs) and p38 MAPK. All these MAPK members have a Thr-X-Tyr motif in their activation loop and their phosphorylation by upstream MAP kinase kinases (MAP2Ks) is essential for their activation (Zhang and Dong, 2005). MAP kinases are activated by various extracellular and intracellular factors and they transduce the signals to alter gene regulation and ultimately cellular function. The magnitude and duration of MAPKs activation are critical for having optimum physiological effect. Therefore, tight regulation of these pathways is essential. The activities of these MAP Kinases are negatively regulated by MKPs (Zhang and Dong, 2007). As mentioned before, all the members of MKP subgroup and few members of atypical DUSP subgroups have MAPKs as their substrates. These members have substrate specificities for one or more MAPKs (Fig 2A). Unlike their substrate MAPKs which are ubiquitously expressed, the expression of MKP members varies between cell types and cellular localization (Zhang and Dong, 2005). Most of these members are early response genes i.e. their expression remains low in resting cells but increases rapidly upon appropriate stimulation, including cytokines or TLR ligand stimulations (Patterson et al., 2009). The degree of expression between the members varies

considerably for a given stimuli and is often dependent on their substrate MAPK activation (Ekerot et al., 2008; Keyse, 2000).

The substrate specificities of these DUSPs vary considerably between *in vitro* and *in vivo* studies (Zhang and Dong, 2005). For example, DUSP1/MKP-1 (Theodosiou et al., 1999) and DUSP6/MKP-5 (Tanoue et al., 1999) were previously demonstrated to dephosphorylate p38 and JNK in *in vitro* studies. However, using specific knock-out mouse models, our group and others have demonstrated that DUSP6 is JNK specific phosphatase *in vivo* (Zhang et al., 2004). Another example is DUSP2 where *in vitro* studies showed ERK & p38 are the substrates (Ward et al., 1994), but *in vivo* JNK is demonstrated to be the substrate (Jeffrey et al., 2006). This difference between *in vitro* and *in vivo* studies could be because of artificial settings *in vitro* or due to the compensatory mechanisms of other MKP family members or due to the involvement of scaffold proteins *in vivo* to facilitate the dephosphorylation of MAPKs. Therefore, it is essential to study the knock-out models of all the DUSP family members to identify the right substrate *in vivo* for these molecules (Zhang and Dong, 2005).

Several of these family members are also involved in development and this feature of MKPs is evolutionally conserved. In Drosophilla, puckered (*puc gene*) a JNK phosphatase is essential for embryogenesis (Martin-Blanco et al., 1998) and for *Caenohabditis elegans*, VHP-1 which is a JNK phosphatase is required for larval development (Mizuno et al., 2004). In mammals, DUSP9 is shown to be essential for

placental development (Christie et al., 2005). Further, our recent studies demonstrated that DUSP16/MKP7 is essential for embryonic development and absence or mutation of this gene results in arrest of embryonic development around E.13 to E.15 (Nallaparaju & Zhang et.al, unpublished data). Together, these studies highlight the essentiality of DUSPs in cell signaling regulation and development.

1.4. Atypical DUSPs have diverse substrates

Although several members of atypical DUSPs like DUSP3 (Todd et al., 1999) and DUSP14 (Marti et al., 2001) functions as MKPs these are phylogenetically distinct from the typical DUSPs and PTPs (Patterson et al., 2009). These members are closely related to VH1 (Vaccinia virus open reading from H1) phosphatase of Vaccinia virus (Liu et al., 1995). They are low in molecular weight and have wide range of substrate specificities (Fig 2B). For example, Glucokinase is demonstrated to interact with DUSP12 and is shown to be its substrate (Munoz-Alonso et al., 2000). In case DUSP22, STAT3 is demonstrated to be the substrate (Sekine et al., 2007; Sekine et al., 2006) in addition to JNK (Chen et al., 2002; Shen et al., 2001). Recent *in vitro* studies identified DUSP14 as a negative regulator of TNF and IL-1 induced NF-κB signaling by dephosphorylating TAK1 (Zheng et al., 2013). In case of DUSP22, a recent study identified focal adhesion kinase (FAK) as its interacting partner and is shown to involve in regulation of cell migration in lung cancer cells (Li et al., 2010). Together, these studies highlight the involvement of non MAPK proteins as substrates of atypical DUSPs (Huang and Tan, 2012). However, most of

the literature about atypical DUSPs is from *in vitro* studies and these findings are often controversial as in the case of DUSP3. One *in vitro* study using enzyme kinetics and cell transfections identified ERK1/2 as specific substrate (Todd et al., 1999) but other studies demonstrated that JNK could be the substrate but not the ERK (Zhou et al., 2002) (Todd et al., 2002). Therefore, it is essential to confirm these observations *in vivo* using knockout mouse models to understand the atypical DUSPs' roles in physiological processes and disease.

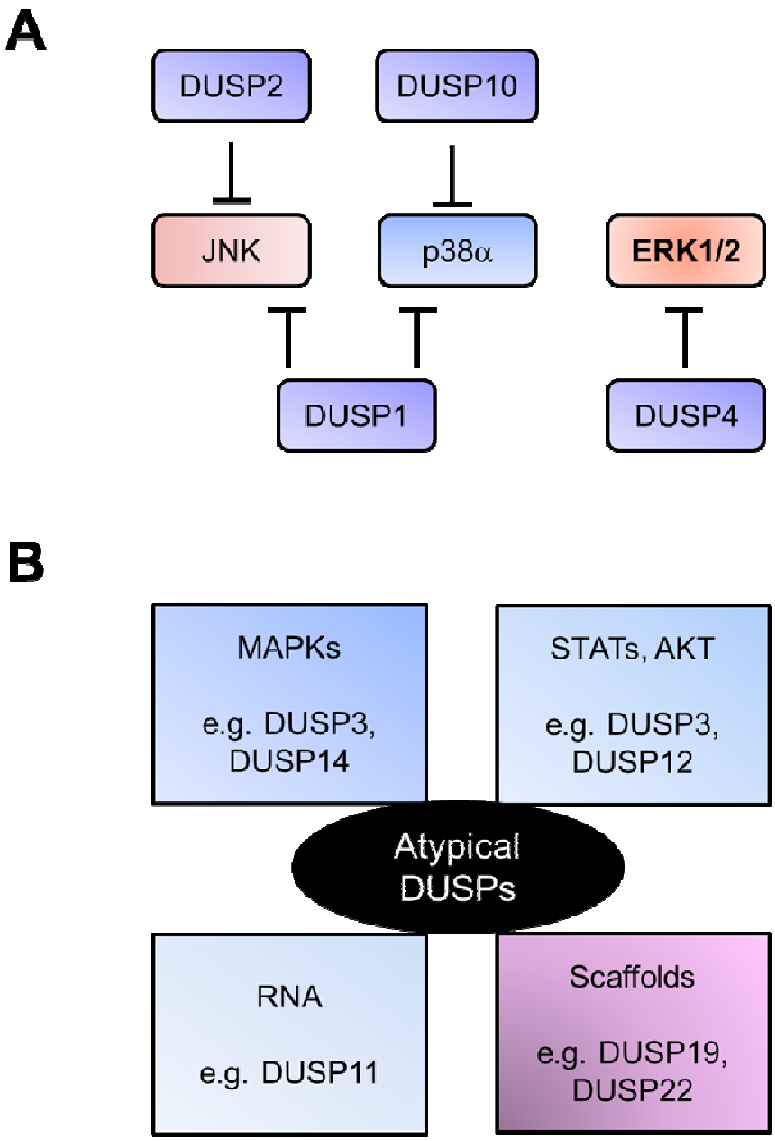

Figure 2. Substrate specificities of typical and atypical DUSPs. (A) Typical DUSPs mainly have one of the three MAP kinases as substrates as in case of DUSP2, DUSP4 and DUSP10 or two MAP Kinases JNK and p38α as in case of DUSP1. (B) In addition to MAP Kinases, atypical DUSPs have diverse range of substrates like STATs in case of DUSP3, AKT in case of DUSP12 or non-protein substrates like RNA in case of DUSP11.

1.5. DUSPs as regulators of innate and adaptive immune responses

Many members of typical and atypical DUSP families have been demonstrated to be potent immune regulators (Huang and Tan, 2012; Lang et al., 2006; Liu et al., 2007; Patterson et al., 2009). Our group and several other groups have highlighted the essentiality of DUSP family members in the regulation of both innate and adaptive immune responses (Huang et al., 2011; Zhang et al., 2004; Zhang et al., 2009). The first *in vivo* study showing the involvement of a DUSP family member in immune responses came from our group; using *Dusp10* knockout mice, Zhang et al. demonstrated that DUSP10 functions as a negative regulator of proinflammatory cytokine production by innate immune cells. Using *in vitro* TLR ligand stimulations and *in vivo* septic shock and *Listeria monocytogenes* infection experiments, this study demonstrated that *Dusp10*-deficient animals have enhanced proinflammatory cytokines like TNF-α and IL-6 compared to their WT counterparts. Further, this same study demonstrated that that DUSP10 acts intrinsically as positive regulator of T cell activation and proliferation but functions as a negative regulator of T cell effector function (Zhang et al., 2004). DUSP1 is one other such molecule whose function in innate and adaptive immune responses are well studied by several groups. *Dusp1-/-* macrophages and dendritic cells have enhanced proinflammatory cytokine TNF-α and IL-6 levels upon stimulation with various TLR ligands *in vitro* (Salojin et al., 2006; Zhao et al., 2005; Zhao et al., 2006). In the case of adaptive immune responses, our group has demonstrated that DUSP1 intrinsically is essential for T cell activation and proliferation by regulating JNK activity and

NFATc1 nuclear translocation. Further, this study demonstrated that *Dusp1-/-* mice have defective immune responses against influenza virus and are resistant to experimental autoimmune encephalomyelitis (EAE), a well-established mouse model for multiple sclerosis (Zhang et al., 2009). Another study from Chi and colleagues demonstrated that DUSP1 mediated signaling in dendritic cells is critical for the fate of adaptive T-cell immune responses to either become Th1 cells or Th17 cells. DUSP1 does this by regulating the polarizing cytokines and the respective cytokine receptors expression at the DC-T cell interface (Huang et al., 2011). One other DUSP family member DUSP2 is also well studied for its role in immune responses. *Dusp2-/-* mice were defective in proinflammatory cytokine production in macrophages *in vitro* and *in vivo* in rheumatoid arthritis model. This study highlighted the role of DUSP2 as positive regulator of in macrophages and mast cells (Jeffrey et al., 2006).

In a recent study from our lab, we showed that the expression of DUSP16, also known as MKP7, was induced in T cells upon TCR activation. To elucidate the physiological function of MKP7, we disrupted the *Mkp7* gene in mice and found that MKP7 is required for embryonic development. As *Mkp7* KO is embryonic lethal, to understand the function of MKP7 in immunity, we generated *Mkp7+/+* and *Mkp7-/-* fetal liver chimeras. *Mkp7-/-* CD4+ T cells exhibited enhanced ERK and JNK activation and produced increased amounts of IL-2 compared with Mkp7+/+ cells upon activation. *Mkp7-/-* CD4+ T cells, though their Th1 and Th2 differentiation was not affected, were defective in Th17 differentiation *in vitro*, which was rescued by

blocking IL-2 or inhibition of ERK activation. Furthermore, mice carrying *Mkp7-/-* T cells were deficient in their generation of Th17 and T follicular helper cells *in vivo* and were resistant to autoimmune experimental encephalomyelitis. Our results thus demonstrated an essential role of MKP7 in effector T cell function (Nallaparaju and Zhang et al.; Manuscript under review). Another recent study addressed the role of MKP7 as negative regulator of TLR induced proinflammatory cytokines in innate immune macrophages (Niedzielska et al., 2014). The roles of several other typical DUSP members like DUSP4 (Huang et al., 2012; Yu et al., 2012) and DUSP5 (Kovanen et al., 2008) in immune responses were also recently published by several other groups.

Several atypical DUSP subgroup members were shown to be potent immune regulators as well. DUSP14 was identified as interacting partner of T-cell co-stimulatory factor CD28 by yeast two-hybrid system and co-immunoprecipitation assays (Marti et al., 2001; Nakano, 2007). A recent *in vivo* study using *Dusp14-/-* mice identified this molecule as a negative regulator of T cell receptor (TCR) signaling by dephosphorylating TGF-β-activated kinase 1 (TAK1)-binding protein 1 (TAB1) (Yang et al., 2014). Another recent *in vitro* study identified DUSP14 as a negative regulator of TNF and IL-1 induced NF-κB signaling by dephosphorylating upstream TAK1 (Zheng et al., 2013). Another atypical DUSP, DUSP22 was recently identified as a regulator of TCR signaling by dephosphorylating Src-family tyrosine kinase member Lck. Further, this study reported that *Dusp22-/-* mice have enhanced T cell mediated immune responses and are more susceptible to EAE (Li et al.,

2014). Together, these studies demonstrate the indispensable role of typical and atypical DUSPs as regulators of immune responses.

1.6. DUSPs in inflammatory, metabolic disorders and cancer

Since DUPS members' role in immune responses is well established, it is reasonable to assume that they are critical in controlling various inflammatory diseases. Indeed, several mice and human studies have proved this point. As previously mentioned, DUSP10's involvement in EAE is studied by our group (Zhang et al., 2004). The role of DUSP2 in a mouse model of rheumatoid arthritis is published and is shown to play a proinflammatory role (Jeffrey et al., 2006). Contrastingly, DUSP1 is shown to play anti-inflammatory role in arthritis model (Salojin et al., 2006). Further, several members of this subgroup are shown to play regulatory roles in metabolic disorders like diet induced obesity and stress induced insulin resistance. DUSP1 deficient mice were shown to be resistant to diet-induced obesity due to an increase in fatty acid metabolism and enhanced energy expenditure (Wu et al., 2006). DUSP4 has a protective role in stress induced insulin resistance in mice by dephosphorylating ERK and JNK (Emanuelli et al., 2008).

In cancer, several DUSP members function as tumor suppressors (Patterson et al., 2009). One such member that is well characterized for its tumor suppressor role is DUSP6. In pancreatic cancer patients, DUSP6 is commonly under expressed in primary tumors and this is resulted by hypermethylation of the experimental

control region of DUSP6 (Xu et al., 2005). Further, this loss of expression of DUSP6 is correlated with tumor progression. DUSP6 is also shown to have a tumor suppressive role in ovarian cancers (Chan et al., 2008). However, as their *in vivo* target substrates greatly vary, not all members of DUSP family function as tumor suppressors. DUSP1 was shown to play a positive role in tumor progression of pancreatic cancer patients (Liao et al., 2003). However, in the case of other types of cancers like ovarian and testicular cancers, DUSP1 has a negative role in tumor progression (Murty et al., 1996). So, depending on the type and the stage of cancer, the DUSPs may have different roles. In summary DUSP family members provide a potential avenue for therapeutic approaches in autoimmune, inflammatory diseases and cancer. Further studying the role of uncharacterized members of these subgroups of PTPs is essential to design new therapies and diagnostic approaches. DUSP11 is one such member whose physiological role is not well established.

1.7. Dual-specificity phosphatase 11

DUSP11 is also known as PIR1 (phosphatase that interacts with RNA–ribonucleoprotein complex 1) due to its high affinity for RNA substrates *in vitro* (Deshpande et al., 1999; Yuan et al., 1998). Expression of DUSP11 is observed in several human cell lines derived from keratinocytes, epidermoid cells and myeloblastic cells, but its tissue distribution has not been studied in detail (Yuan et al., 1998). Using a yeast two-hybrid system, Yuan et al. demonstrated that DUSP11 associates with the splicing factors 9G8 and SRp30C. However, they could not

confirm this observation in mammalian cells (Yuan et al., 1998). Using affinity-purified GST (glutathione transferase)-tagged DUSP11 fusion protein, Deshpande et al. showed that DUSP11 has intrinsic phosphatase activity against phosphotyrosine residues *in vitro*, but no or weak activity against phosphoserine/ phosphothreonine residues (Deshpande et al., 1999). Also, they demonstrated that the activity of DUSP11 as an mRNA triphosphatase is several orders of magnitude greater than as a protein phosphatase. These studies suggest that DUSP11 may act in association with other mRNA phosphatases and facilitate mRNA maturation. Moreover, DUSP11 contains two arginine-rich and a proline-rich region that are commonly found in some RNA binding proteins (Deshpande et al., 1999). Schematic representation of DUSP11 in comparison to other well characterized DUSPs that are shown to have immunoregulatory function is depicted in Figure 3. Like other atypical DUSP members, DUSP11 lacks CH2 domain that is thought to be essential for interaction with MAP kinase substrates. Recent *in vitro* studies by Caprara et al (Caprara et al., 2009) and Hasler et al (Hasler et al.) highlight the importance of this molecule in cell proliferation, cancer suppression and chronic inflammation. Many DUSP genes have been demonstrated to be highly deregulated in the initial stages of cancer and have been shown to be tumor regulative. Recently, Helin and colleagues in an *in vitro* study using cell lines demonstrated that DUSP11 is up regulated in a p53 dependent manner after treatment with DNA damaging agents and suggested that DUSP11 contributes to p53-dependent inhibition of cell proliferation (Caprara et al., 2009). Using ShRNA knockdown experiments this study highlighted the role of DUSP11 as a tumor suppressor gene. Further, they demonstrated that DUSP11 interacts with a

splicing factor SAM68 (Src-associated protein in mitotic cells). Further, Dardousis et al. identified DUSP11 as one of the up regulated factors in HT-29 colon carcinoma cell lines (Dardousis et al., 2007). Moreover, studies in inflammatory bowels disease (IBD) patients demonstrate that expression of DUSP11 is down regulated in the mucosal tissue of colon compared to healthy individuals, highlighting its potentially suppressing role in chronic inflammation (Hasler et al., 2011). However, the physiological function of DUSP11 has not been studied via genetic approaches *in vivo*.

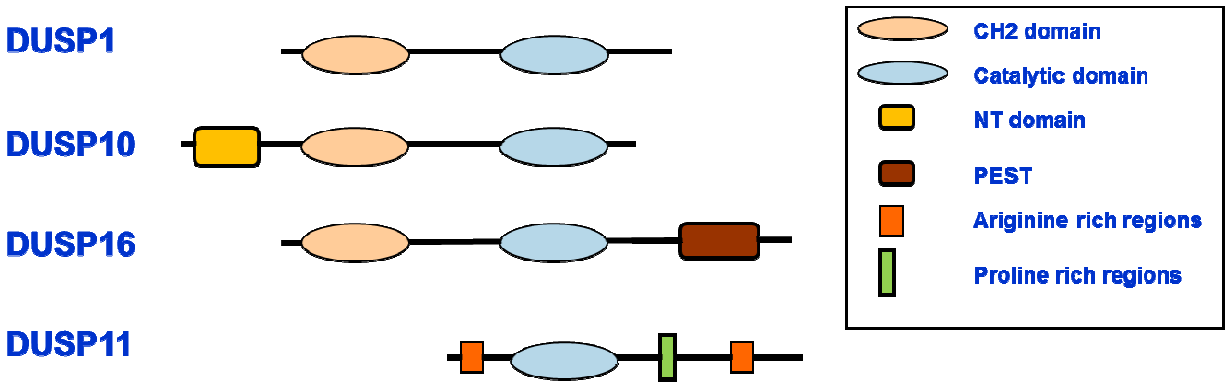

Figure 3. Schematic comparison of DUSP11 with other DUSPs that are shown to have immunoregulatory function. DUSP11 lacks CH2 domain that is thought to be essential for interaction of DUSP with MAPK substrates. Instead, it has proline and arginine rich regions that are usually present in mRNA interacting proteins.

1.8. Objective, hypothesis and specific aims of the present work

Though several studies evaluated the role DUSP11 in cell cycle regulation, tumor suppression and chronic inflammation *in vitro*, the role of DUSP11 in immune responses is unknown. A microarray screening performed by our laboratory to identify the potential regulators of innate and adaptive immune responses identified DUSP11 as one factor, along with other DUSP family members like DUSP1 (Zhang et al., 2009), DUSP10 (Zhang et al., 2004) and DUSP16 (Nallaparaju & Zhang et al., under review) that are highly induced in immune cells upon activation. The functions of three of these molecules DUSP1 (Zhang et al., 2009), DUSP10 (Zhang et al., 2004) and DUSP16 (Nallaparaju & Zhang et al., under review) as critical regulators of immune responses were confirmed *in vivo* by our group using gene knockout approaches. The phenotypes of these mice in innate and adaptive immune responses are summarized in Table 1. Since DUSP11 is identified in the same screening along with the other three members, we hypothesized that DUSP11 is a potential regulator of immune responses. Additionally, Arimura et al. identified DUSP11 as one of the seven highly expressed PTP family members among several immune cell lineages including dendritic cells, macrophages and T cells (Arimura and Yagi, 2010). Further, our *in vitro* studies showed that DUSP11 expression is induced after activation of innate immune cells by several TLR ligands and in adaptive immune cells by anti-CD3 and anti-CD28 agonistic antibodies (Fig 4), suggesting that DUSP11 may play a role in regulation of immune responses. Additional *in vivo* studies in animal models are required to elucidate the physiological

function of DUSP11. The main objective of my dissertation is to understand the role of DUSP11 in innate and adaptive immune responses against pathogens by employing the DUSP11 KO mice we generated in our laboratory. In particular, our aim is to understand the molecular mechanisms and the signaling events regulated by DUSP11 in immune cells. To our knowledge, this is the first study to investigate the essentiality of DUSP11 in regulating immune responses. Once the role of this protein is elucidated, modulating its expression may be a potential avenue for future therapy in inflammatory and autoimmune diseases.

Based on our *in vitro* studies and published literature about DUSP11 we hypothesized that DUSP11 regulates innate and adaptive immune responses by regulating TLR and TCR signaling pathways respectively. To address our hypothesis, in chapter 2 we have determined whether DUSP11 is critical to induce optimal immune responses against pathogens. Further, in Chapter 3 we evaluated the signaling pathways regulated by DUSP11 to identify substrate of DUSP11 in immune cells.

Table 1. Role of DUSP molecules in generation of innate and adaptive immune responses. The innate and adaptive immune responses phenotype observed in the mice deficient in each of the DUSP members identified in our microarray screen is summarized in the following table.

Knockout mouse	Change in MAPK signaling	Phenotypic changes	
		Innate immunity	Adaptive immunity
Dusp10-/-	• ↑ JNK in macrophages & T cells	• ↑ TNF-α, IL-6, TGFβ by macrophages	• ↓ CD4+ T cell proliferation • ↓ Th1 & Th2 cytokine production
Dusp1-/-	• ↑ p38 in Macrophages & DCs • ↑ JNK In T cells	• ↑ IL-6, TNF-α, IL-1b by macrophages	• ↓ CD4+ T cell proliferation • ↓ Th1 & Th2 cytokine production
Dusp16-/- (Embryonic lethal)	• ↑ JNK In Macrophages & DCs • ↑ JNK & ERK in T cells	• ↑ IL-12 by macrophages	• ↑ CD4+ T cells proliferation & activation • ↓ Th17 responses

CHAPTER 2 – REGULATION OF IMMUNE RESPONSES BY DUSP11

BACKGROUND

DUSPs have been shown to play critical roles in regulation of various cellular processes such as signal transduction, cell cycle regulation and cellular proliferation via modulating MAP kinase activities (Patterson et al., 2009). Also, many members of this family have been demonstrated to be potent immune regulators (Lang et al., 2006; Zhang and Dong, 2007; Zhang and Dong, 2005). Deregulated expression patterns of DUSPs have been associated with pathogenesis of a wide range of diseases including inflammatory, autoimmune diseases and several types of cancer (Patterson et al., 2009; Pulido and Hooft van Huijsduijnen, 2008). DUSPs are broadly classified into six subgroups, among which the atypical subgroup of DUSPs is the least studied for their physiological functions (Patterson et al., 2009).

DUSP11 (also known as PIR1 (Deshpande et al., 1999; Yuan et al., 1998)) is an atypical dual specificity phosphatase (Patterson et al., 2009). An *in vitro* study in cell lines by Caprara et al. showed that DUSP11 is up-regulated in a p53 dependent manner after treatment with DNA damaging agents and suggested that DUSP11 contributes to p53-dependent inhibition of cell proliferation (Caprara et al., 2009). This study highlighted the potential role of DUSP11 as a tumor suppressor gene. Two other *in vitro* studies by Yuan et al. and Deshpande et al. demonstrated

DUSP11s' ability to phosphotyrosine and phosphoserine/ phosphothreonine substrates *in vitro* (Deshpande et al., 1999; Yuan et al., 1998). Further, they demonstrated that the activity of DUSP11 as an mRNA triphosphatase is several orders of magnitude greater than as a protein phosphatase. These studies together suggest that DUSP11 may act in association with other mRNA phosphatases and facilitate mRNA maturation. Though these studies together suggested the plausible physiological role of DUSP11, it is essential to the study the physiological function of this phosphatase *in vivo* using genetic knockout mouse strain specifically lacking this protein to better understand its function in physiology and disease so that it can be potentially targeted for therapeutic or diagnostic purposes.

The role of DUSP11 in immune responses remains largely unknown. A study in inflammatory bowel disease patients demonstrate that expression of DUSP11 is down regulated in the mucosal tissue of colon compared to healthy individuals, highlighting its potential role in suppressing chronic inflammation (Hasler et al., 2011). Additionally, Arimura et al. identified DUSP11 as one of the seven highly expressed PTP family members among several immune cell lineages including dendritic cells, macrophages and T cells (Arimura and Yagi, 2010). Moreover, a microarray screening performed by our laboratory to identify the potential regulators of innate and adaptive immune responses identified DUSP11 as one factor, along with other DUSP family members like DUSP1 (Zhang et al., 2009), DUSP10 (Zhang et al., 2004) and DUSP16 (Nallaparaju & Zhang et al., under review), whose functions as critical immune regulators is confirmed *in vivo* by gene knockout

approaches. Further, our *in vitro* studies showed that DUSP11 expression is induced after activation of innate immune cells by several TLR ligands and in adaptive immune cells by anti-CD3 and anti-CD28 agonistic antibodies, suggesting that DUSP11 may play a role in regulation of immune responses. Based on these studies, we hypothesized that DUSP11 is a critical regulator of innate and adaptive immune responses against pathogens.

To examine the function of DUSP11 in regulating immune responses, we generated and analyzed *Dusp11*-/- mice. *Dusp11*-deficient mice exhibit increased susceptibility to *Listeria monocytogenes* infection than wild-type animals and are protected from endotoxin-induced septic shock. *In vitro*, DUSP11 KO dendritic cells produce decreased levels of pro-inflammatory cytokines in response to TLR activation. Together, we demonstrate for the first time that DUSP11 is a critical regulator of immune responses and acts specifically by modulating innate immune responses. This phosphatase thus could serve as a novel target for therapeutic intervention for innate cell-mediated inflammatory and autoimmune diseases.

RESULTS

2.1. DUSP11 expression is enhanced in dendritic cells upon TLR activation and in T cells upon TCR activation

As a first step to understand the role of atypical DUSP11 in immune responses, we analyzed the expression of DUSP11 in cells of the immune system. To address the role of DUSP11 in innate immune cells, bone marrow derived dendritic cells (BMDCs) were cultured from WT B6 mice in presence of GM-CSF for 7 days. These BMDCs were then stimulated with different TLR ligands *in vitro* to study the expression DUSP11 using RT-PCR. Our studies showed that DUSP11 mRNA expression was highly induced after activation of BMDCs by with various TLR ligands like peptidoglycan (PGN) that activates TLR2, Poly (I:C) that activates TLR3 and LPS that activates TLR4 but not by CpG which activates TLR9 (**Fig. 4a**). Further, we studied the kinetics of DUSP11 induction in BMDCs upon stimulation with LPS (**Fig. 4b**) and identified that DUSP11 is induced as early as 30 min after stimulation suggesting that it is an early response gene. Together, these data suggest a potential regulatory role of DUSP11 in innate immune responses.

To address the possible role of DUSP11 in T cells, we examined the expression of DUSP11 in naïve CD4+ T cells after stimulation with anti-CD3 antibody for various time points and in effector Th cells, including Th1, Th2 and Th17 cells, using quantitative RT-PCR. DUSP11 mRNA expression was greatly increased

at 1h after anti-CD3 stimulation and the expression level was maintained at 1.5 h after stimulation (**Fig. 4C**). DUSP11 was also expressed in Th1, Th2, Th17 effector cells at a comparable level (**Fig. 4D**). These data suggest a possible regulatory role of DUSP11 in naïve CD4+ T cell activation and CD4+ effector cell function.

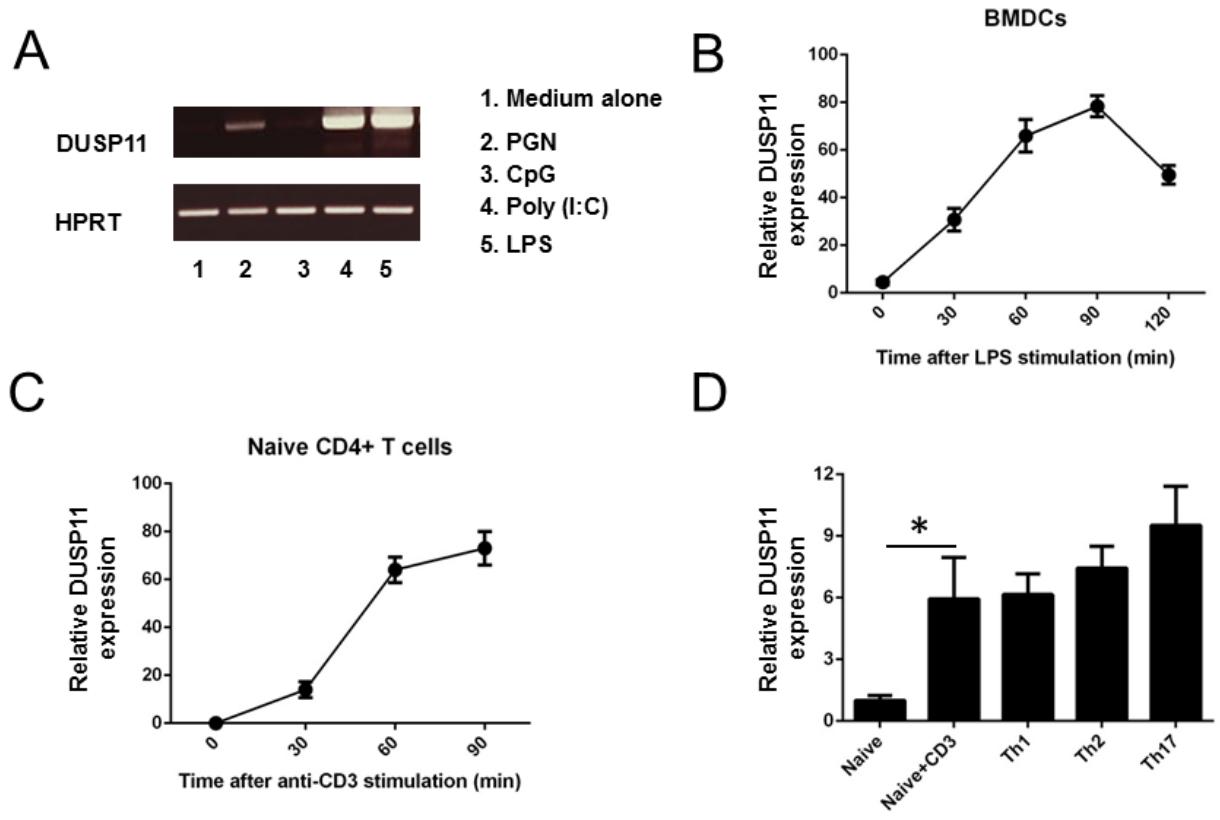

Figure 4. DUSP11 expression is induced in DCs in response to TLR activation and in T cells upon TCR activation. (A) BMDCs were stimulated with different TLR ligands for 4h and DUSP11 expression was analyzed by quantitative real-time PCR (QRT). (B) BMDCs were stimulated with LPS (10 ng/ml) for different time points and DUSP11 induction kinetics were analyzed by QRT. (C) FACS-sorted naïve CD4+ T cells were activated with anti-CD3 (1 μg/ml) antibody for different time points and DUSP 11 induction kinetics was analyzed by QRT. (D) *In vitro* differentiated Th1, Th2 or Th17 cells were activated with anti-CD3 antibody for 4h. DUSP11 expression was determined by QRT. * $P < 0.01$ (t-test). The data are a representative of three independent experiments.

2.2. Generation of *Dusp11-/-* mice

To determine the function of DUSP11 in immune responses, mice deficient in DUSP11 were generated using an ES cell (BB0918) containing an insertion mutation, or gene trap, in the 1st intron of the *DUSP11* gene. The gene trap vector generates a spliced fusion transcript containing DUSP11, the β-geo cassette encoding β-Gal and the neomycin resistance gene (**Fig. 5A**). Genotypes of the embryos were determined by PCR using primers flanking the insertion in the wild-type allele **(P1 and P2, Fig. 5A)** and the same forward primer **(P1)** plus a reverse primer in the vector **(P3)** (**Fig. 5B**). Further, western blotting analysis on bone marrow-derived dendritic cells (BMDCs) was used to confirm the disruption of the DUSP11 protein expression (**Fig. 5C**). Homozygous *Dusp11-/-* mice were fertile and indistinguishable in appearance from wild-type (WT) littermates. Further, the lymphoid immune cells developed normally in the absence of the DUSP11 gene (**Fig 6**). Therefore, DUSP11 is dispensable for the development of the immune system.

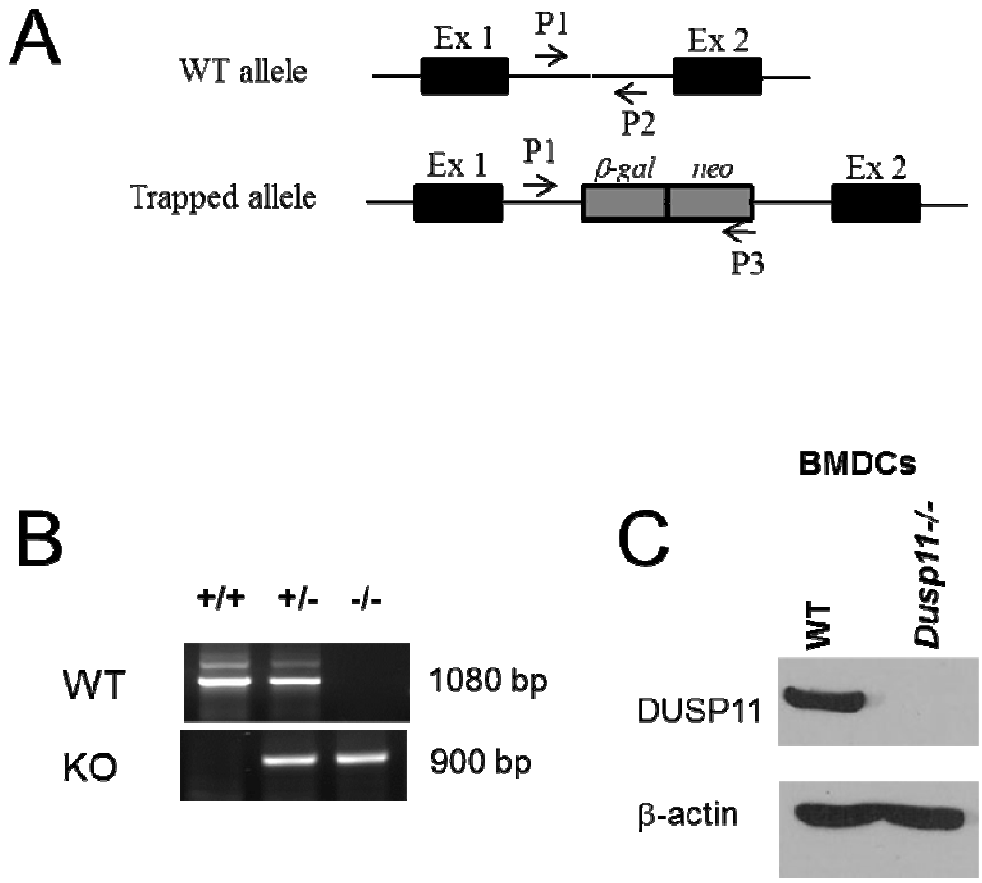

Figure 5. *Dusp11 -/-* (gene trap) mouse generation. (A) Schematic representation of WT and DUSP11 trapped alleles respectively. The gene trap vector containing β-galactosidase (β-geo) and neomycin transferase (neo) genes was inserted into the 1st intron of the DUSP11 gene.(B) Genomic DNA was isolated from embryonic tissues from *Dusp11+/-* x *Dusp11+/-* breeders for genotyping using P1/P2 and P1/P3 primer pairs shown in fig A to identify WT and mutated alleles, respectively. (C) DUSP11 deficiency in *Dusp11-/-* bone marrow derived dendritic cells was confirmed by western blotting. The data are representative of three independent experiments.

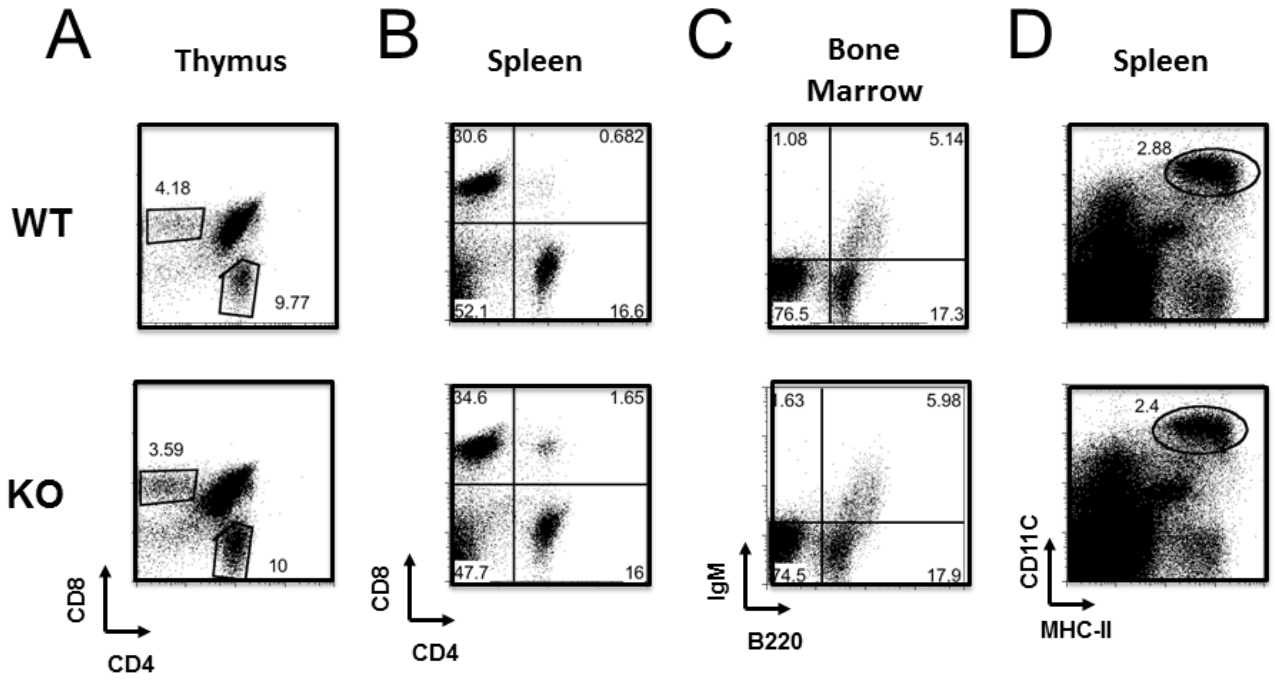

Figure 6. Development of T, B, and dendritic cells is normal in *Dusp11-/-* mice. (A) Thymocytes and (B) splenocytes from WT and DUSP11 KO mice were stained with antibodies against TCR-β, CD4 and CD8 to analyze various T cell subpopulations. (C) Bone marrow cells are stained for B220 expressing IgM+ cells. (D) Splenocytes were stained with CD45R, CD11C and MHC-II antibodies and CD45R- populations were analyzed. The data are representative of three independent experiments that yielded similar results.

2.3. *Dusp11-/-* mice are defective in immune responses against *Listeria monocytogenes* infection

Innate and adaptive immunity plays principal protective roles in response to intracellular pathogen infection such as *Listeria monocytogenes* infection (Zenewicz and Shen, 2007). To study the role of DUSP11 in regulating pathogen-specific immune responses, we infected WT and DUSP11 knockout (KO) mice with 10^4 CFU of *Listeria monocytogenes* expressing ovalbumin (LM-Ova) on day 0. Serum IL-6 and TNF-α were measured at different time points post infection (**Fig 7A**). DUSP11 KO mice showed substantially reduced levels of pro-inflammatory cytokines compared to WT mice at 24h and 48h post infection. To examine if DUSP11 KO mice had any defect in bacterial clearance, on Day 3 or Day 7 post infection, livers were homogenized and plated to determine *Listeria* burden (**Fig 7B**). We found that the bacterial burden in KO mice was significantly higher than in WT mice, indicating that DUSP11 KO mice are impaired in *Listeria* clearance.

On day 3, splenocytes were isolated and the IFN-γ expressing cells were measured by intracellular staining after the surface staining of NK1.1 and TCR-β (**Fig 8A**). DUSP11 KO mice exhibited defective innate immune responses both in NK cells and NK T cells compared to the corresponding WT controls. Both $CD4^+$ and $CD8^+$ T cells are important in anti-*Listeria* immunity and bacterial clearance (Pamer, 2004). To examine the regulation of anti-bacterial CD4+ and CD8+ T cell responses by DUSP11, 7 days post infection; splenocytes were stimulated with LLO peptide

and SIINFEKL peptides, respectively. Cells expressing IFN-γ, IL-17 or granzyme-B were measured by intracellular staining after surface staining with antibodies to CD4 and CD8 (**Fig 8B**). We found that both $CD4^+$ and $CD8^+$ T cell responses against *Listeria* antigens were highly compromised in DUSP11-deficient mice. Together, these results demonstrated that DUSP11 is essential for anti-*Listeria* immunity.

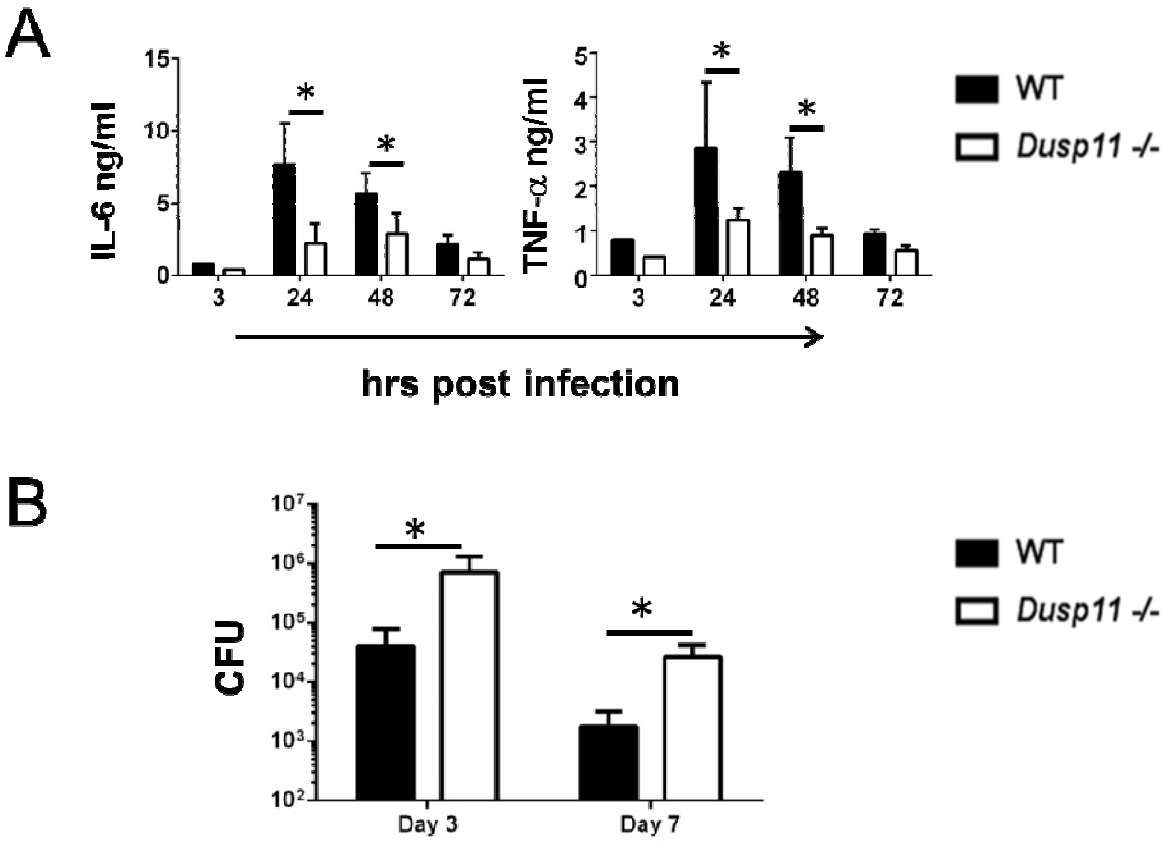

Figure 7. *Dusp11-/-* mice have defective proinflammatory cytokines secretion and defective bacterial clearance upon *Listeria monocytogenes* infection. (A) WT *or Dusp11-/-* mice were intravenously infected with 104 LM-Ova (n=5 per group). Serum TNF-α and IL-6 were measured by ELISA at different time points post infection. (B) On Day 3 or Day 7 post infection, livers were homogenized and plated to determine Listeria burden. * $P < 0.05$ (t-test). The data are representative of three independent experiments with similar results.

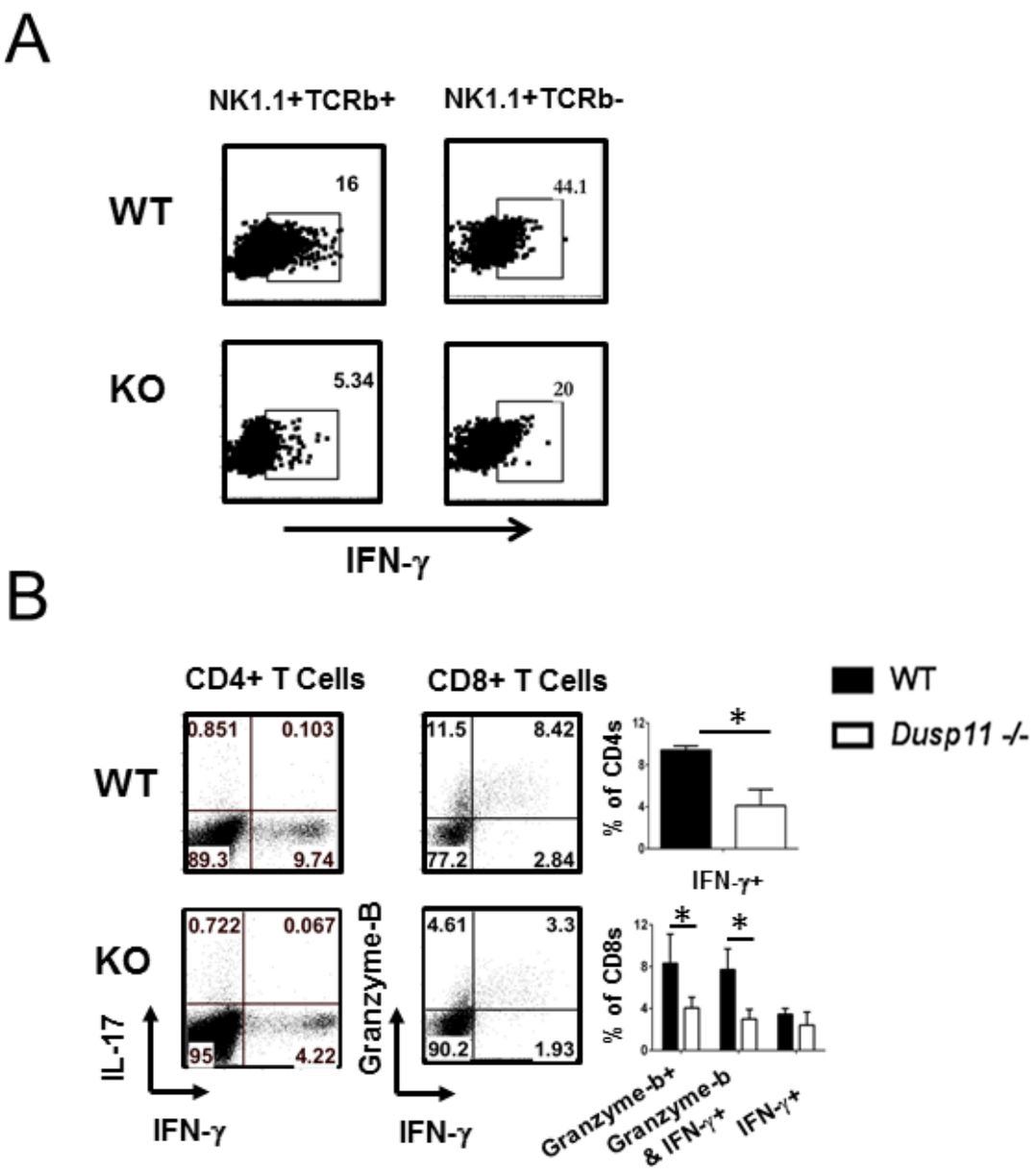

Figure 8. *Dusp11-/-* mice have defective innate and adaptive immune responses against *Listeria monocytogenes* infection *in vivo.* (A) Three days post infection, splenocytes of the listeria infected mice were obtained, and the IFN-γ expressing cells were measured by intracellular staining after the surface staining of

NK1.1 and TCR-β. (B) Seven days post infection; splenocytes were stimulated with LLO peptide or SIINFEKL peptide overnight including 4 hour incubation with Golgi-Stop and Golgi-Plug. Cells expressing IFN-γ, IL-17 or granzyme-B were measured by intracellular staining after the surface staining of CD4 and CD8. * $P < 0.05$ (t-test). Data shown are representative of three independent experiments.

2.4. *Dusp11-/-* mice have defective antigen specific immune responses *in vivo*

To further confirm if *Dusp11-/-* mice are defective in antigen-specific T-cell responses, we employed a KLH immunization model. WT and DUSP11 KO mice were immunized with KLH in Complete Freunds Adjuvant (CFA). On day 7, splenocytes were isolated and were stimulated with varying concentrations of KLH protein. Cytokine production was determined by intracellular staining after overnight stimulation with KLH (**Fig 9A**) or by ELISA (**Fig 9B**) after 72h KLH stimulation. DUSP11 KO mice expressed decreased levels of both Th1 and Th17 cytokines (**Fig 9A and Fig 9B**), suggesting that DUSP11 is required antigen-specific T cell responses *in vivo*. Further, to identify if humoral immune responses are compromised in *Dusp11-/-* mice, we also studied T follicular helper (Tfh) cells and germinal center B cell population in these mice. We found reduced percentage of GL7+Fas+ germinal center B cells which is correlated with decreased CXCR5+Bcl6+ Tfh cells in the lymph nodes of *Dusp11-/-* mice compared to their WT counterparts (**Fig 9C**).

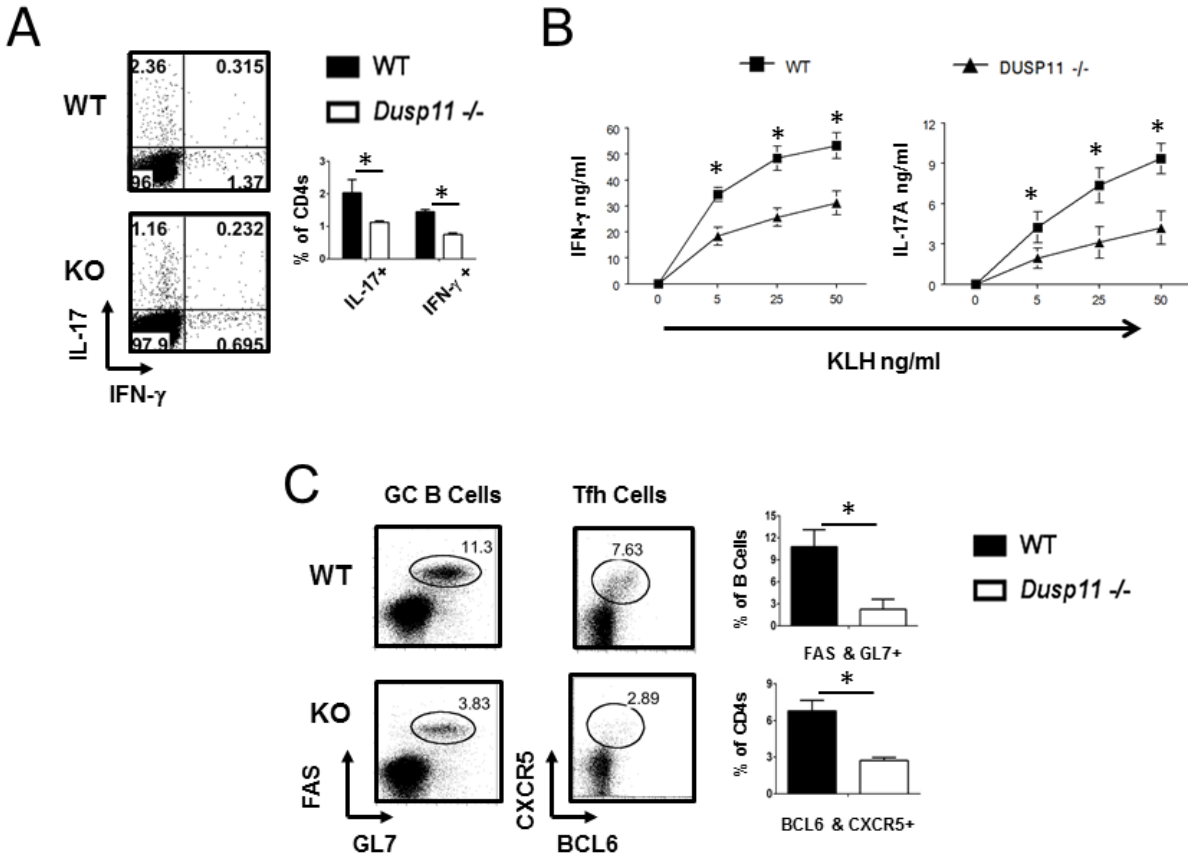

Figure 9. *Dusp11-/-* mice have defective KLH specific immune responses *in vivo*. (A) WT and *Dusp11-/-* mice were immunized with KLH in CFA. On day 7, splenocytes were isolated and stimulated with KLH peptide. Cytokine production was determined by intracellular staining after KLH re-stimulation. (B) 7 days post KLH immunization splenocytes were stimulated with different doses of KLH for 72h and IFN-γ and IL-17 were analyzed by ELISA. (C) Germinal center B cells were determined by staining with GL-7, FAS and B220 antibodies and Tfh cells by CD4, CXCR5 and BTLA antibodies. * $P < 0.05$ (t-test). Data shown are a representative of three independent experiments.

2.5. *Dusp11-/-* mice have defective innate immune responses *in vivo* and *in vitro*

Considering that innate immune responses are compromised in *Dusp11-/-* mice in the early phase during *Listeria* infection (**Fig 7 A&B**), we directly studied their response to intraperitoneal injection of LPS. Following injection with 15 μg of LPS per g of body weight, WT and KO mice were monitored for signs of sepsis for 48 h and serum IL-6 and TNF-α levels were measured at several time points. Similar to innate responses against Listeria, pro-inflammatory cytokine production in KO mice was attenuated post LPS injection (**Fig 10A**). Further, the KO mice were resistant to septic shock compared to their WT counterparts (**Fig 10B**). Together, these results highlight the importance of DUSP11 in innate immune responses *in vivo*.

To investigate how DUSP11 regulates innate immune responses *in vitro*, DCs were generated from bone marrow cells of WT and DUSP11 KO and stimulated with various concentrations of LPS. IL-6 and TNF-α protein (**Fig 11A**) and mRNA (**Fig 11B**) levels were measured. We found that upon LPS stimulation, the production of pro-inflammatory cytokines IL-6 and TNF-α was decreased in KO DCs compared to WT cells at both mRNA and protein levels (**Fig 11**). Further, we tested if macrophages have similar phenotype and identified that the bone derived macrophages from *Dusp11-/-* mice have phenotype similar to that of DCs but the defect is less compared to DCs.

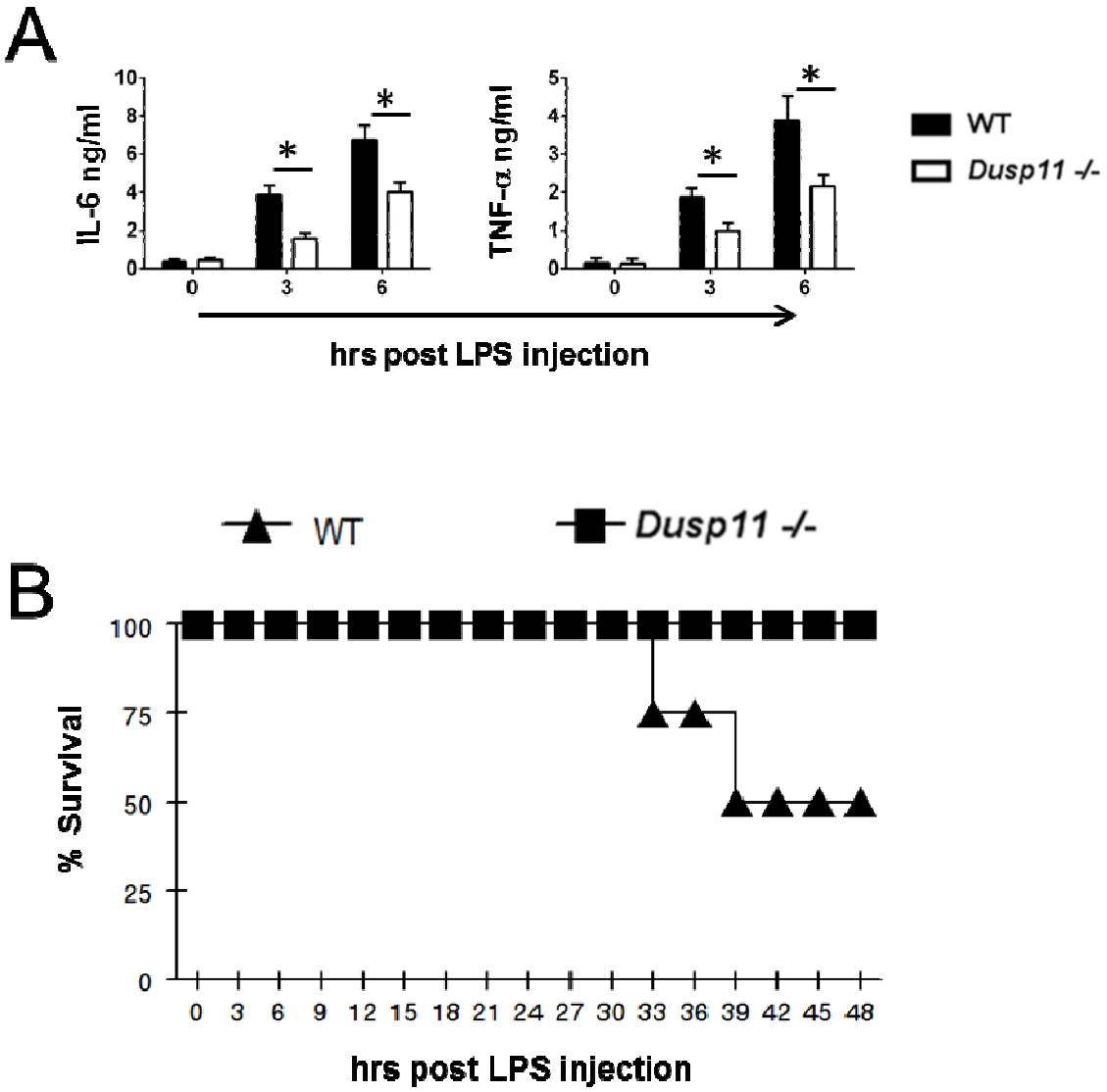

Figure 10. *Dusp11-/-* mice have defective innate immune and inflammatory responses *in vivo*. (A) WT or *Dusp11-/-* mice were intraperitoneally injected with LPS. Serum TNF-α and IL-6 were measured by ELISA at different time points post infection. * $P < 0.05$ (t-test). (B) Post LPS injection, survival of the mice was monitored over the next 48 hours. Data are combined from two independent experiments and are from a total of 10 mice of each genotype.

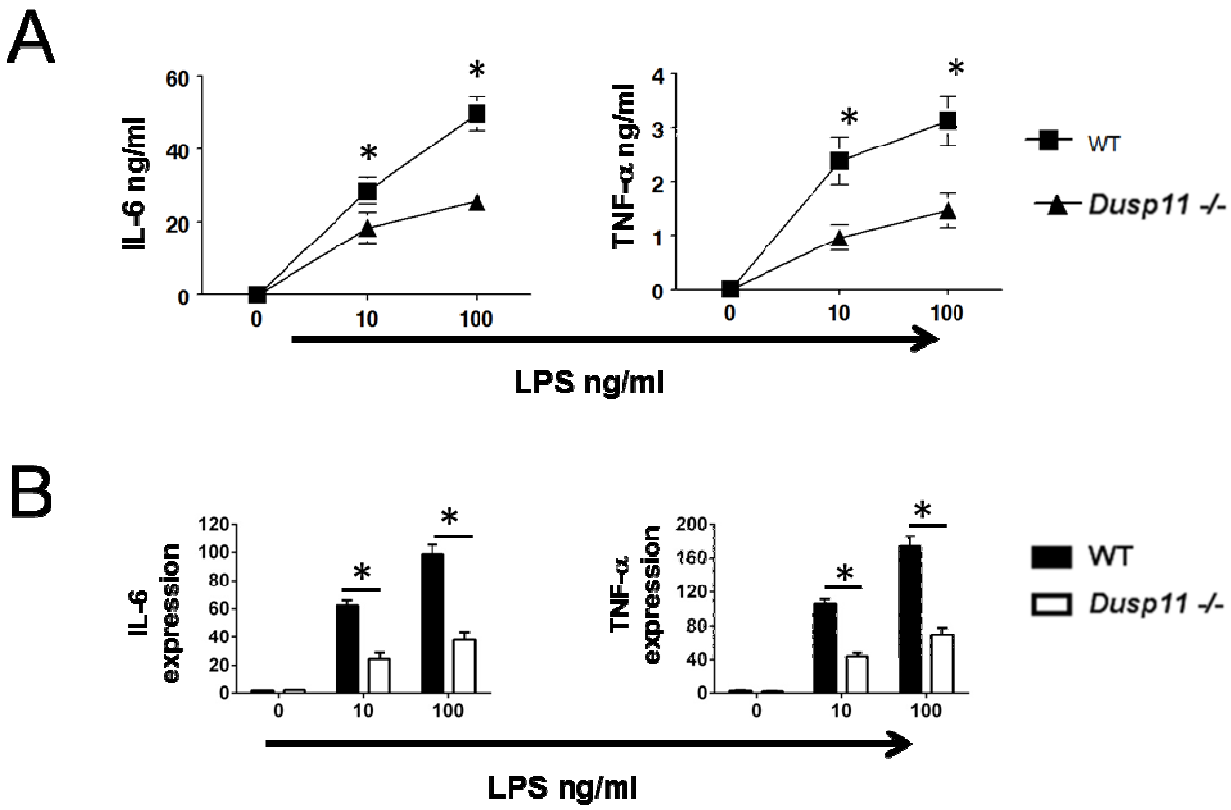

Figure 11. *Dusp11-/-* mice have defective proinflammatory cytokines secretion *in vitro* in dendritic cells upon stimulation with LPS. CD11c+ bone marrow derived dendritic cells from WT and *Dusp11-/-* were stimulated with different doses of LPS and mRNA and protein levels of IL-6 and TNF-α were measured using ELISA (A) and quantitative real-time PCR (B) respectively. * $P < 0.05$ (t-test). Data shown are representative of three independent experiments.

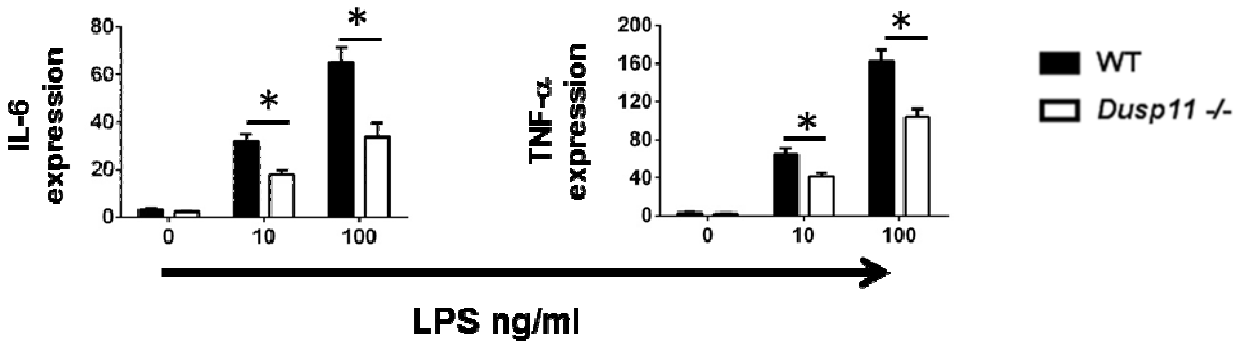

Figure 12. *Dusp11-/-* mice have defective proinflammatory cytokines secretion *in vitro* in macrophages upon stimulation with LPS. CD11b+ bone marrow derived dendritic cells from WT and *Dusp11 -/-* bone marrow macrophage cells were stimulated with different doses of LPS and mRNA levels of IL-6 and TNF-α were measured using quantitative real-time PCR. * $P < 0.05$ (t-test). Data shown are representative of three independent experiments.

2.6. DUSP11 is required for CD4 T cell activation and proliferation but dispensable for CD4 T cells differentiation *in vitro*

To address the regulation of $CD4^+$ T cell activation and proliferation by DUSP11, naïve CD4+ T cells from WT and DUSP11 KO mice were isolated and stimulated with plate-bound anti-CD3 antibody or anti-CD3 plus anti-CD28 antibodies. IL-2 production in culture supernatants was determined by ELISA (**Fig 13A**). We found that upon stimulation with anti-CD3 alone or anti-CD3 plus anti-CD28 antibody, the production of IL-2, a hallmark of naïve T cell activation, was higher in DUSP11 KO $CD4^+$ T cells than in WT cells. Furthermore, DUSP11 KO $CD4^+$ T cells exhibited enhanced proliferation compared with WT cells in response to anti-CD3 antibody stimulation (**Fig 13A**). To examine the regulation of DUSP11 in T helper cell differentiation and effector function, WT and DUSP11 KO naïve CD4+ T cells were cultured under Th1, Th2 or Th17 differentiation conditions *in vitro*. After 4 days of differentiation, effector Th cells were analyzed by intracellular cytokine staining. As shown in **Fig 13B**, *Dusp11-/-* Th1, Th2 and Th17 cells produced similar amounts of effector cytokines compared to WT cells. Together, these data demonstrate that DUSP11 is dispensable for Th1, Th2 and Th17 differentiation and function *in vitro*.

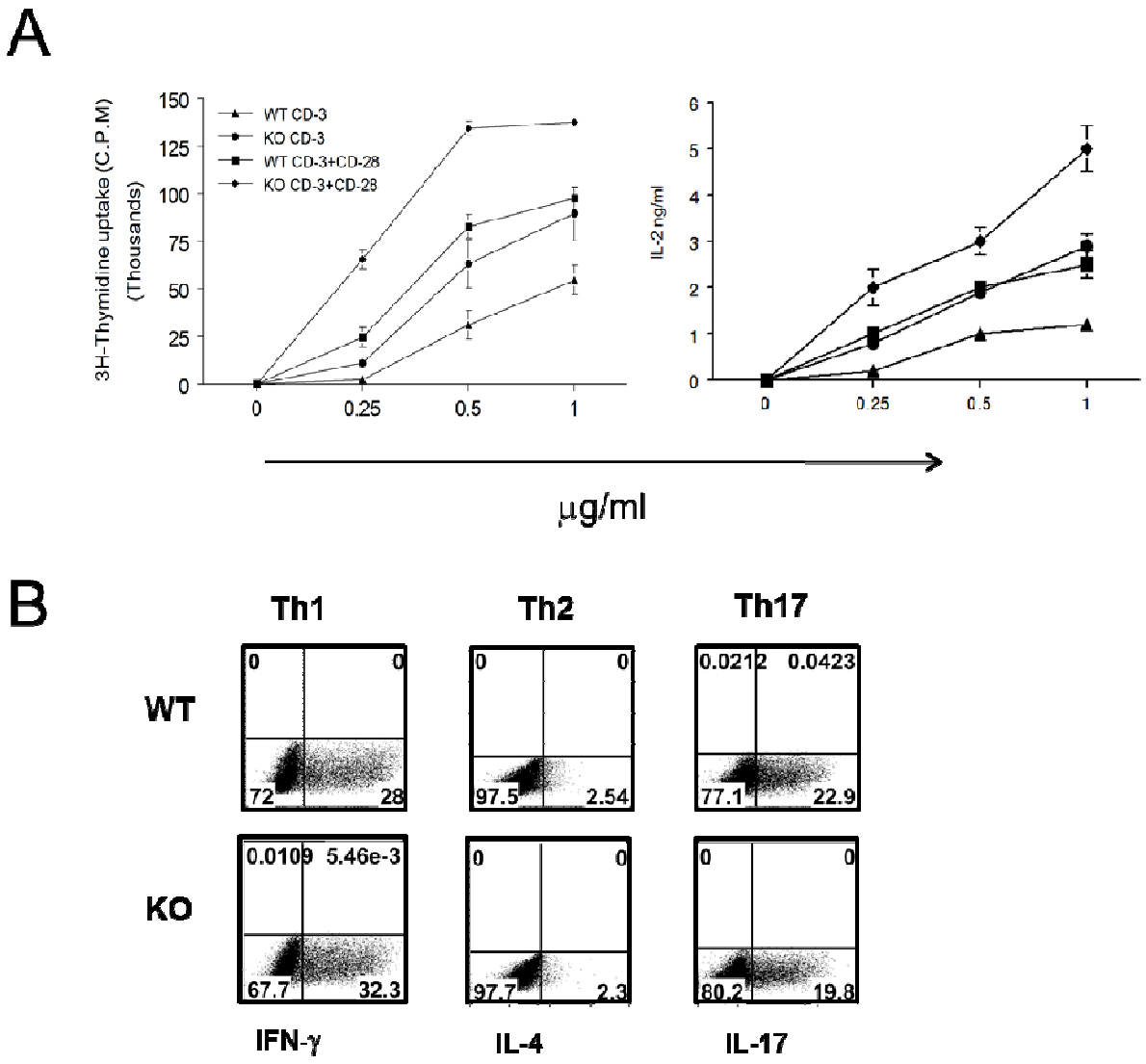

Figure 13. DUSP11 is required for CD4 T cell activation and proliferation but dispensable for CD4 T cell differentiation *in vitro*. (A) Naïve CD4+ T cells were activated with different concentrations of anti-CD3 in the presence or absence of anti-CD28 and their IL-2 production and [3H]-thymidine uptake was measured. (B) Naïve CD4+ T cells from WT and *Dusp11-/-* mice were differentiated into Th1, Th2, and Th17 cells, followed by anti-CD3 stimulation before intracellular cytokine staining. Data shown are representative of three independent experiments.

2.7. DUSP11 expression in dendritic cells is required to regulate the T helper cell responses *in vitro* and *in vivo*

Though we did not observe any defect in Th differentiation by DUSP11 KO T cells *in vitro* (**Fig 13B**), we observed defective adaptive immune responses by DUSP11 KO mice *in vivo* (**Fig 8B and Fig 9 A & B**), which may possibly results from the defective innate immunity in these mice. To assess this idea, we employed a previously described DC-CD4 T cell co-culture system (Chung et al., 2009). CD4+ T cells from WT or KO mice were co-cultured with BM-derived DCs (WT or KO) in Th1 or Th17 skewing conditions. IL-17- or IFN-γ–expressing T cells were measured by intracellular staining. We found that WT and KO T cells produced similar amounts IFN-γ or IL-17 when cultured with WT DCs, whereas KO DCs always exhibited reduced ability to induce cytokine expression by WT or KO T cells (**Fig 14**).

To assess the *in vivo* relevance of these observations we performed an OT-II cells adoptive transfer experiment where Ly5.1+ positive OT-II CD4 T cells were adoptively transferred into Ly 5.2+ positive WT or KO recipient mice. Following the transfer, mice were immunized subcutaneously with OVA protein in CFA. Seven days post immunization; antigen specific responses produced by the transferred cells were analyzed by flow cytometry. We found that the transferred cells produce more IFN-γ in case of WT mice compared to the KO mice (**Fig 15A**).

To investigate whether CD4 T cells lacking DUSP11 have an intrinsic defect *in vivo*, we employed CD4 transfer EAE model (Zhang et al., 2009). We isolated CD4+ T cells from WT and DUSP11 KO mice and transferred 5×10^6 WT or KO cells into *Rag1–/–* mice, followed by immunization with MOG in CFA to induce EAE disease (Chung et al., 2009). *Rag1-/-* mice that received WT and *Dusp11-/-* CD4 T cells developed disease around day 6 after second immunization and all the mice had disease scores between 2 and 3 by day 12 (Please refer to Chapter 5 to find the scoring scheme we followed). We did not observe any significant difference in disease kinetics between *Rag1-/-* group that received *Dusp11-/-* CD4 T cells compared to the group that received WT CD4 T cells demonstrating that DUSP11 deficient T cells do not have intrinsic defect in EAE model (**Fig 15B**). Together, these results suggest a more important role of DUSP11 in innate immune cells, i.e. DCs, which ultimately regulates the adaptive T cell responses.

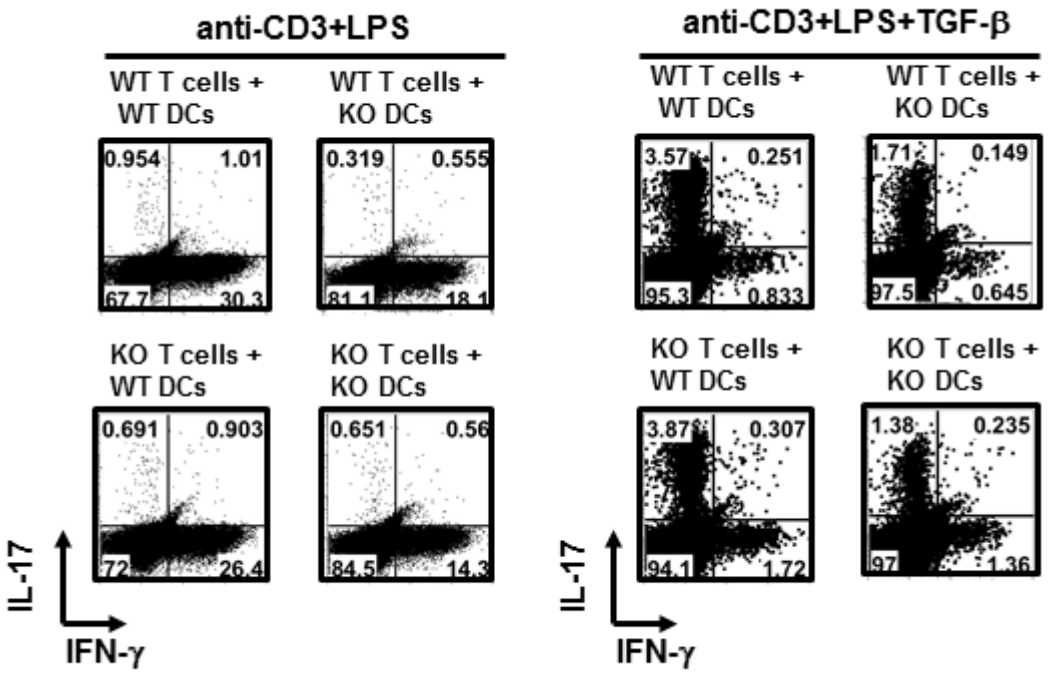

Figure 14. DUSP11 expression in dendritic cells modulates Th1 and Th17 responses *in vitro.* FACS-sorted naive CD4+ T cells from WT or KO mice were co-cultured with BM-derived DCs (WT or KO) in the presence of soluble anti-CD3 Ab and LPS to skew the cells towards Th1 condition (A) or soluble anti-CD3 Ab and LPS plus TGF-β to skew the cells towards Th17 condition (B). IL-17- and IFN-γ expressing cells were measured by intracellular staining. Data shown are a representative of at least two independent experiments.

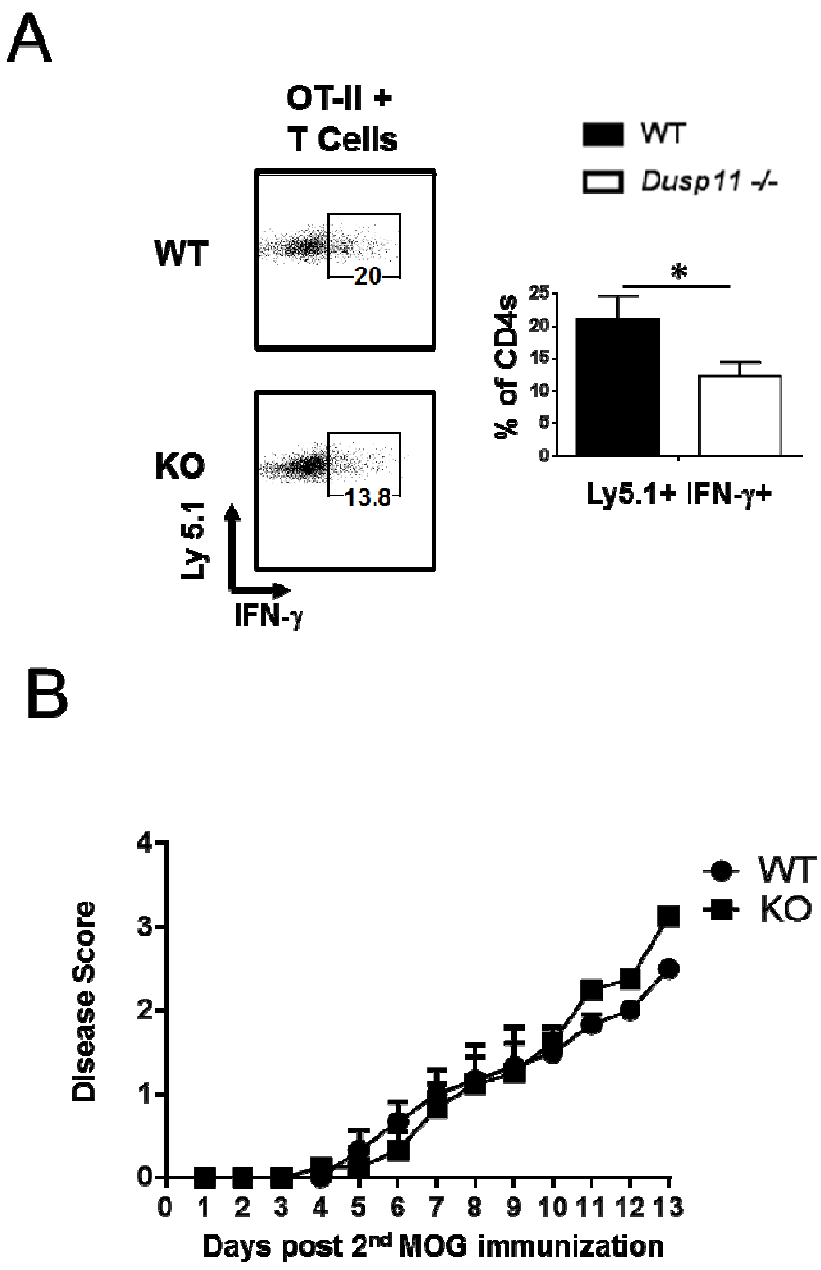

Figure 15. DUSP11 expression in dendritic cells modulates Th1 and Th17 responses *in vivo*. (A) Naïve CD4+ T cells ($5X10^6$) from Ly-5.1+ OT-II mice were adoptively transferred into Ly-5.2+ WT or KO recipient mice and then the recipient mice were immunized subcutaneously with OVA. Seven days post immunization, LN cells of the recipient mice were harvested and stimulated in vitro with OT-II peptide

and stained for Ly-5.1, Ly 5.2, CD4 and IFN-γ. IFN-γ producing adoptively transferred cells was analyzed. Data shown are a representative of at least two independent experiments. (B) Naïve CD4+ T cells ($5X10^6$) from WT or *Dusp11-/-* mice were transferred into *Rag1-/-* recipient mice. One day post transfer the recipient mice (5 mice per group) were immunized with MOG35–55 peptide to elicit EAE and disease was scored. Data shown are a representative of at least two independent experiments.

DISCUSSION

DUSPs comprise a group of protein phosphatases that are characterized by their ability to dephosphorylate both phosphotyrosine and phosphoserine/Phospho-threonine residues (Patterson et al., 2009). DUSPs role as critical immune regulators is only being appreciated recently. Several recent studies by ours and several other groups highlighted the essentiality of these molecules in regulating innate and adaptive immune responses by modulating MAP kinase activities (Huang et al., 2011; Zhang et al., 2004; Zhang et al., 2009). We showed in this chapter that DUSP11 is critical for innate immune responses mediated by dendritic cells.

DUSP11 was previously demonstrated to bind to ribonucleic acid-ribonucleoprotein (RNA-RNP) complexes and RNA-splicing factors(Deshpande et al., 1999; Yuan et al., 1998) and is a p53 target gene (Caprara et al., 2009). Recent *in vitro* studies by Caprara et al (Caprara et al., 2009), Dardousis et al (Dardousis et al., 2007) and Hasler et al (Hasler et al., 2011) highlighted the possible function of this molecule in cell proliferation, cancer suppression and chronic inflammation. However, the physiological function of DUSP11 has not been studied via genetic approaches *in vivo*. In this study, we identified DUSP11 as an essential regulator of innate immune responses. Our findings demonstrate that although DUSP11 expression is induced *in vitro* in both innate and adaptive immune cells upon stimulation by TLR ligands and TCR activation respectively, DUSP11 is dispensable for adaptive responses intrinsically *in vitro* and *in vivo*.

Detailed analysis of innate and adaptive responses *in vitro* and *in vivo* by employing novel DUSP11-deficient mice that we generated highlighted the essential function of DUSP11 in proper innate responses, which eventually regulate the generation of adequate adaptive responses. Our *in vivo* experiments using *Listeria monocytogenes* infection showed that *Dusp11-/-* mice had compromised serum proinflammatory cytokine levels and increased liver bacterial burdens on day 3 after infection (**Fig 7 A and B**). Correlating with these observations, we found that compared with WT mice, *Dusp11-/-* mice had defective pro-inflammatory cytokine production by DCs in response to LPS stimulation *in vitro* (**Fig 11**) and were resistant to endotoxin induced septic shock *in vivo* (**Fig 10**). Further, studying the adaptive immune responses against *L. monocytogenes* infection on day 7 (**Fig 8B**) and using KLH immunization model (**Fig 9**) we demonstrated that antigen specific CD4+ T cell responses and B cell responses were compromised in the absence of DUSP11.

Though we observed proliferation defect in *Dusp11-/-* CD4 T cells, we did not observe any defect in *in vitro* differentiation (**Fig 13**). This result suggested that DUSP11 is dispensable for adaptive immune responses. However, our in vivo KLH immunization (**Fig 9**) and *Listeria* infection (**Fig 8**) studies showed that the *Dusp11-/-* mice had compromised adaptive immune responses. To address this discrepancy between *in vitro* and *in vivo* data, using CD4 T cell-DC co-culture experiments we clearly demonstrated that DUSP11 is critical for innate immune responses and that the defects in innate responses ultimately affect the adaptive immune responses. In

conclusion, our findings establish DUSP11 as a critical regulator of innate immune responses which eventually impacts the induction of adaptive immune responses *in vivo*. To our knowledge, this is the first report to demonstrate the regulation of immune responses by DUSP11. This is exciting to us because DUSP11 provides a potential target to treat inflammatory and infectious diseases. However, to pursue this avenue it is essential to understand the molecular mechanisms regulated by DUSP11 to affect the immune responses. In chapter 3 we attempted to understand this mechanism and identify the target molecule of DUSP11.

CHAPTER 3 – MOLECULAR MECHANISMS REGULTED BY DUSP11 IN DENDRITIC CELLS

BACKGROUND

In Chapter 2 we demonstrated that DUSP11 is critical for proinflammatory secretion by dendritic cells. In case of adaptive immune CD4+ T cells we have identified that DUSP11 is dispensable for intrinsic regulation. In this chapter we attempted to understand the molecular mechanisms regulated by DUSP11 to mediate the proinflammatory cytokines secretion in dendritic cells upon stimulation by LPS, a TLR4 ligand. We hypothesized that DUSP11 regulates signaling pathways downstream of TLR receptor to mediate the proinflammatory cytokines secretion. The two main signaling pathways involved in proinflammatory cytokines secretion upon TLR stimulation are MAP Kinase and NF-κB signaling pathway (Newton and Dixit, 2012). So, we tested these two pathways in *Dusp11-/-* DCs in comparison to their WT counterparts upon LPS stimulation. Additionally, several studies to date have suggested that DUSP11 may function as RNA phosphatase. Therefore, we have tested whether DUSP11 effects the stability of proinflammatory cytokine-encoding mRNA.

We have demonstrated that the decreased levels of pro-inflammatory cytokines produced by *Dusp11-/-* dendritic cells in response to TLR activation is

associated with reduced NF-κB activation *in vitro*. Furthermore, DUSP11 directly dephosphorylated the gamma binding domain (γbd) of IKK-β and thus positively regulated the activity of the IKK complex. Together, we demonstrate for the first time that DUSP11 is a critical regulator of immune responses and acts specifically by mediating IKK activation during innate immune responses. This phosphatase thus could serve as a novel target for therapeutic intervention for inflammatory and autoimmune diseases.

RESULTS

3.1 DUSP11 regulates MAP Kinase signaling in dendritic cells

To understand the molecular mechanisms regulated by DUSP11, we analyzed the MAP kinase signaling in WT and *Dusp11-/-* DCs following LPS stimulation by western blotting. *Dusp11-/-* DCs showed enhanced ERK and P38 phosphorylation, but no difference in JNK phosphorylation in comparison to WT DCs (**Fig 16A**). To test if DUSP11 can directly regulate ERK and P38 activation, we overexpressed DUSP11 in 293 T cells by transfecting 293 T cells with increasing doses of DUSP11 plasmid with either MEK1 plasmid to activate ERK or MKK6 to activate P38. Only ERK but not P38 activity is decreased by DUSP11 in a dose-dependent manner (**Fig 16B**). These data suggest that DUSP11 directly regulates ERK activity. To determine whether enhanced ERK activity is responsible for defect in proinflammatory cytokines secretion by *Dusp11-/-* DCs, we treated WT and *Dusp11-/-* DCs with ERK inhibitor and measured the proinflammatory cytokines secretion upon LPS stimulation by ELISA (**Fig 17**). ERK inhibitor treatment did not rescue proinflammatory cytokines defect in *Dusp11-/-* DCs suggesting that enhanced ERK activity is not responsible for the defect.

DUSP11 is previously suggested to function as mRNA phosphatase (Deshpande et al., 1999; Yuan et al., 1998). To test whether DUSP11 regulates stability of proinflammatory cytokine - encoding mRNA, we stimulated WT or

Dusp11-/- MEF cells with LPS followed by treatment with actinomycin-D (**Fig 18**). The half-lives of TNF-α and IL-6 mRNAs' are similar between WT and *Dusp11-/-* MEF cells. These results demonstrate that DUSP11 does not regulate stability of proinflammatory cytokine- encoding mRNA.

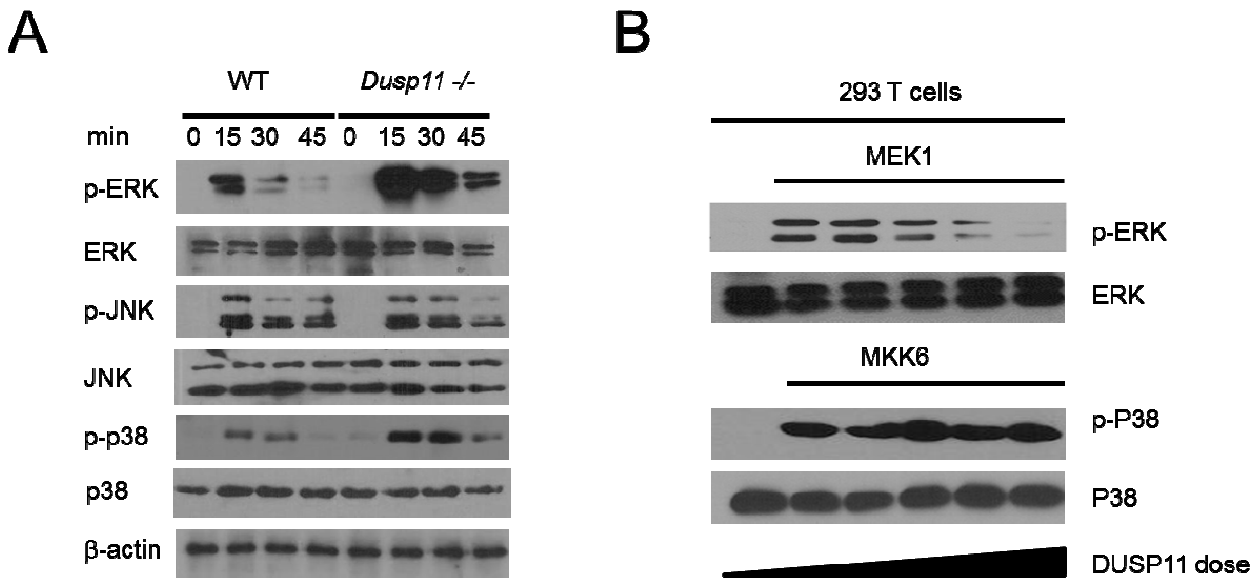

Figure 16. DUSP11 regulates MAP Kinase signaling. (A) Dendritic cells from WT or *Dusp11-/-* mice were stimulated with LPS (100 ng/ml) for the indicated times and western blots were performed for the respective proteins. (B) DUSP11 is expressed in 293 T cells by transfection in increasing dose of plasmid in presence of MEK1 to activate ERK or MKK6 to activate P38. Western blots were performed for the respective proteins. Data shown are a representative of three independent experiments.

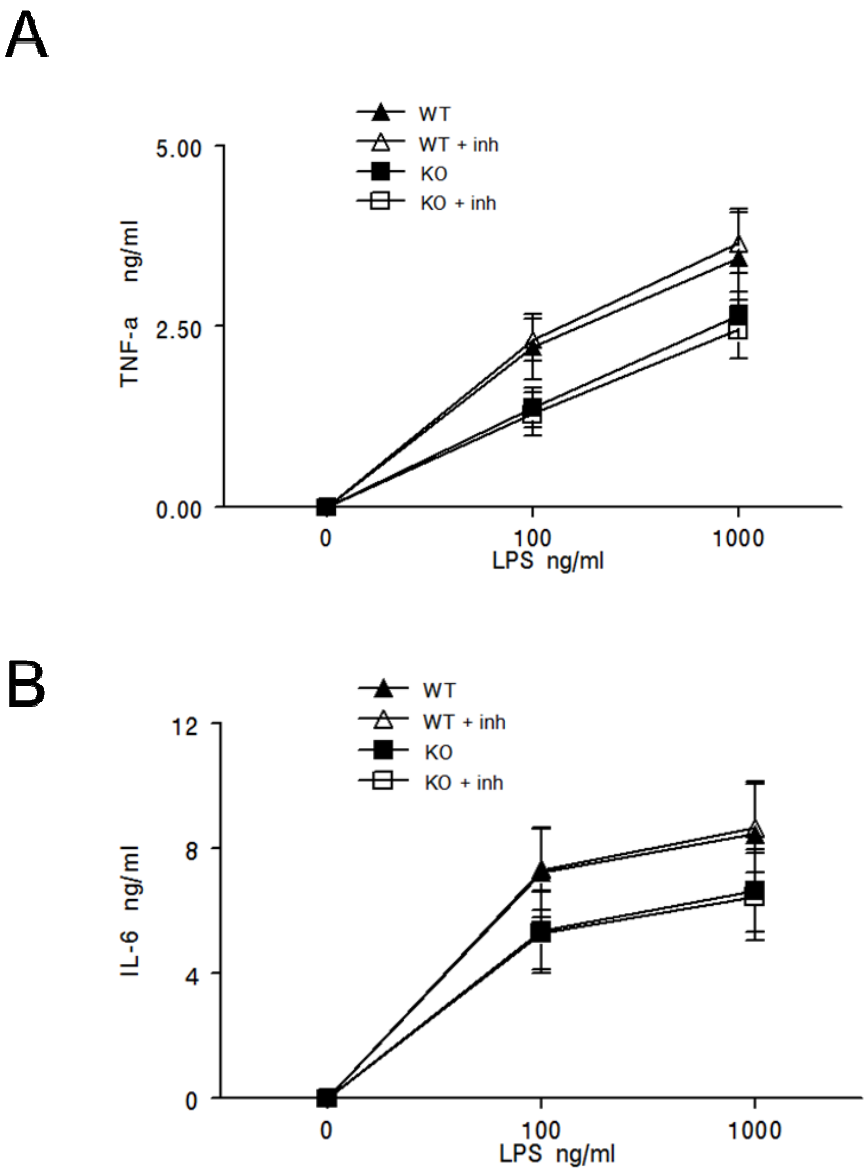

Figure 17. ERK inhibitor treatment cannot rescue defective proinflammatory cytokines secretion in *Dusp11-/-* DCs. Dendritic cells from WT or KO mice were cultured in presence or absence of ERK inhibitor (5 ng/ml) for 2h followed by stimulation with LPS (100 ng/ml) for 24h and ELISAs were performed on the supernatants for the respective cytokines. Data shown are a representative of at least two independent experiments.

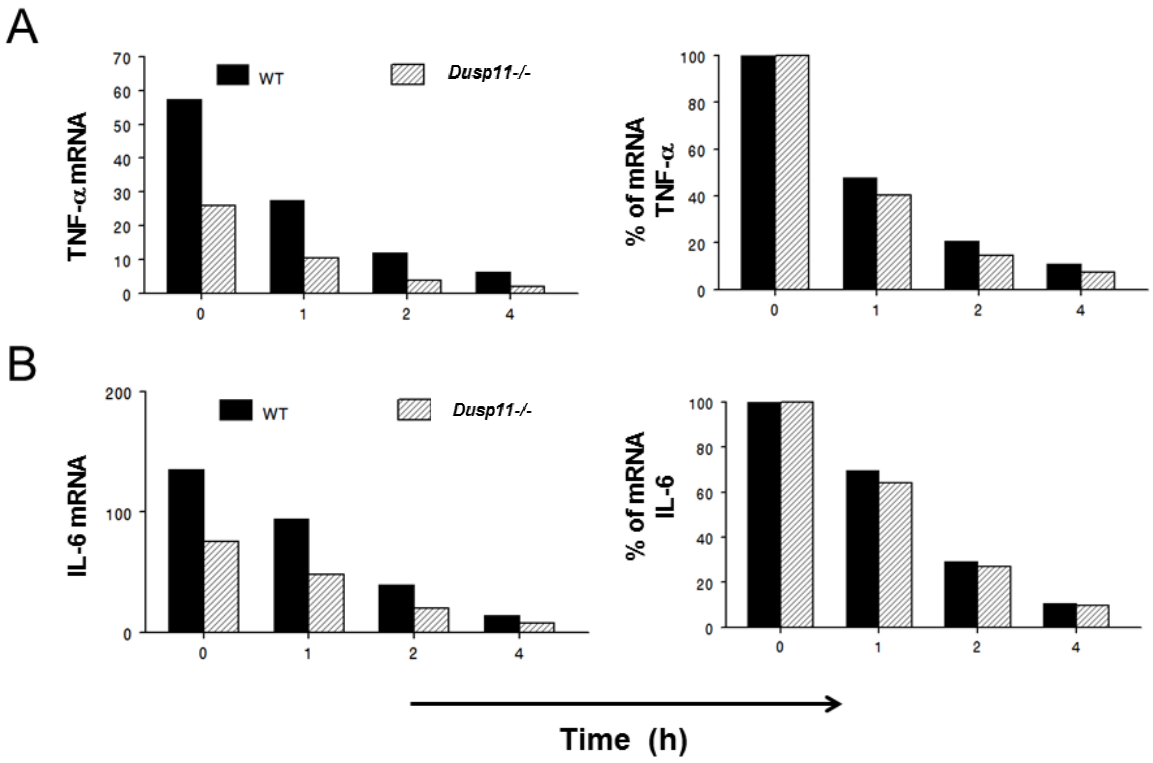

Figure 18. DUSP11 does not regulate stability of proinflammatory cytokine-encoding mRNA. Real-time PCR analysis of TNF-α (A) and IL-6 (B) mRNA in WT and *Dusp11–/–* MEFs pretreated for 1 h with LPS (100 ng/ml), followed by treatment for 0–4 h (horizontal axes) with actinomycin D (5 μg/ml). Data shown are a representative of at least two independent experiments.

3.2 DUSP11 regulates NF-κB signaling in dendritic cells

The increased ERK and p38 phosphorylation cannot possibly account for the defective pro-inflammatory cytokine production in *Dusp11-/-* DCs. We thus analyzed other signaling pathways involved in the production of pro-inflammatory cytokines. NF-κB signaling pathway is essential in the activation of pro-inflammatory cytokine production in addition to MAP kinase pathway (Ghosh and Hayden, 2012). So, we analyzed the NF-κB signaling pathway by studying the phosphorylation of IκB-α and found it was defective in the *Dusp11-/-* DCs compared to the WT controls (**Fig 19A**). An electrophoretic mobility shift assay (EMSA) using nuclear extracts from WT and *Dusp11-/-* DCs after stimulation with LPS further supported that the NF-κB binding was dramatically diminished in *Dusp11-/-* cells compared to WT cells (**Fig 19B**). Also, *in vitro* kinase assay using WT and KO DC lysates post LPS stimulation revealed that IKK function was defective in KO DCs (**Fig 20A**). Further, luciferase assay using WT and phosphatase inactive DUSP11 constructs demonstrated that DUSP11 directly regulates NF-κB activity in a phosphatase-dependent manner (**Fig 20B**).

Also, RT-PCR analysis demonstrated that induction of several NF-κB target genes was defective in KO DCs compared to WT DCs (**Fig 21**). Together, our experiments indicate that DUSP11 deficiency impairs NF-κB signaling. Since ERK signaling was enhanced in *Dusp11-/-* cells, we tested if there is a crosstalk between

ERK and NF-κB pathways. We used an ERK inhibitor and found that inhibiting ERK signaling did not restore NF-κB activation in *Dusp11-/-* DCs (**Fig 22**). DUSP11 thus may have an unexpected direct effect on the NF-κB signaling pathway.

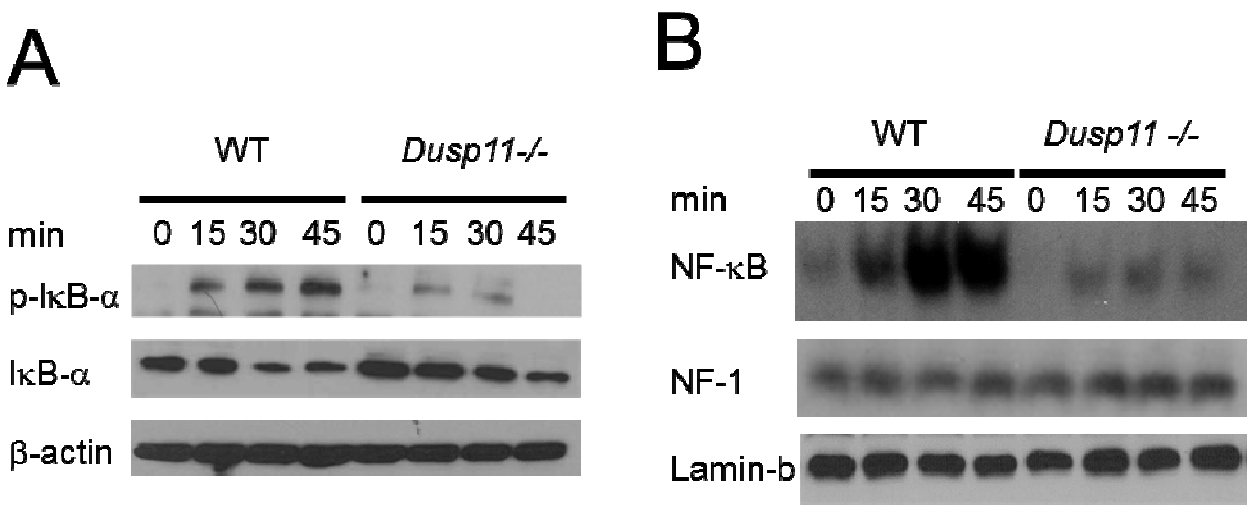

Figure 19. DUSP11 regulates NF-κB signaling. (A) Dendritic cells from WT or *Dusp11-/-* mice were stimulated with LPS (100 ng/ml) for the indicated times and western blots were performed for the respective proteins. (B) Electrophoretic mobility-shift assay of NF-κB and control DNA binding factors (NF-Y) was performed using nuclear extracts from WT and KO DCs stimulated with LPS (100 ng/ml). Data shown are a representative of at least two independent experiments.

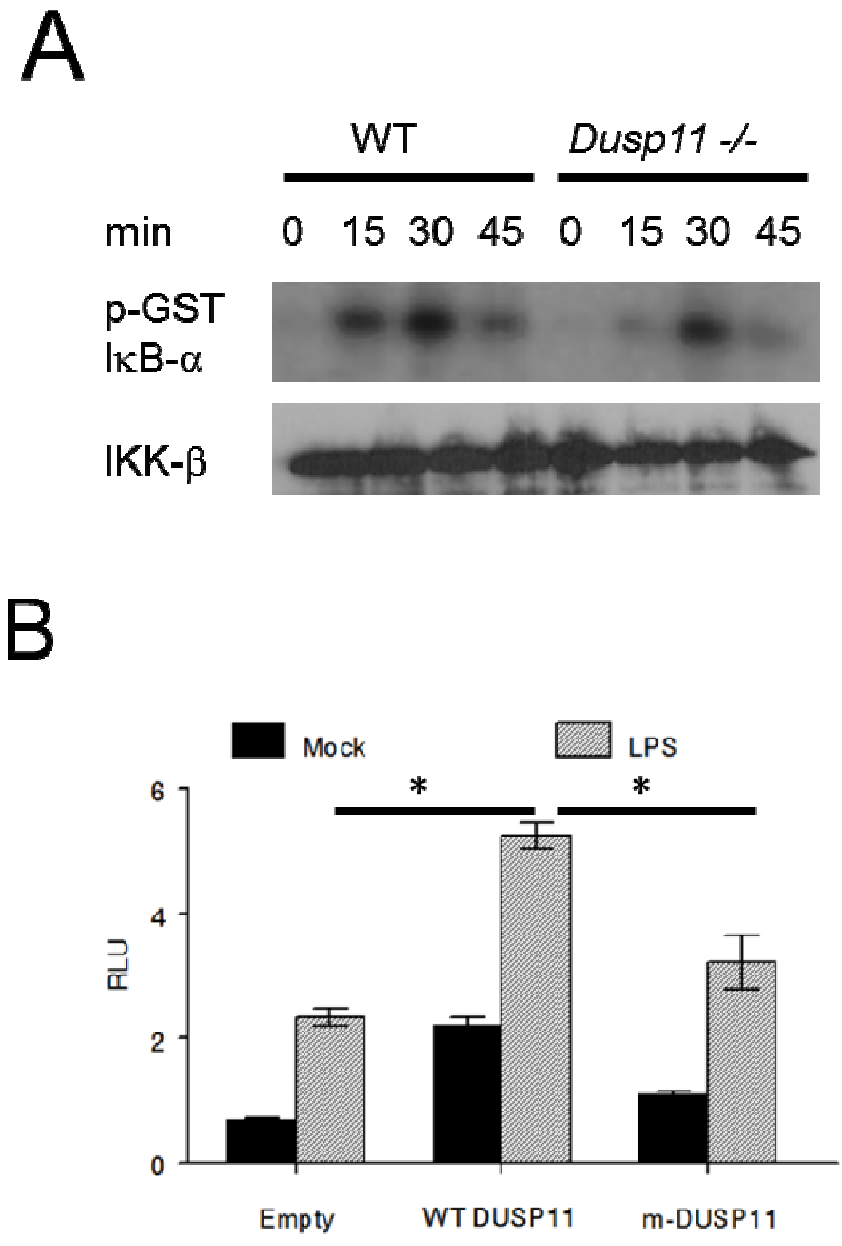

Figure 20. DUSP11 regulates NF-κB signaling. (A) IKK complex is immunoprecipitated from WT and KO DCs by using anti-IKKβ after stimulation with LPS (100 ng/ml) and subjected to kinase assays using GST-IκBα as substrate. (B) Luciferase activity in HeLa cells transfected with NF-κB firefly luciferase reporter

plasmid, renilla luciferase plasmid and empty vector (empty) or vector encoding Flag-tagged DUSP11 (WT DUSP11) or vector encoding Flag-tagged phosphatase inactive DUSP11 (m-DUSP11), followed 20 h later by no stimulation (mock) or stimulation for 8 h with LPS (100 ng/ml) is measured; results are presented relative to the activity of renilla luciferase. Data shown are representative of at least two independent experiments.

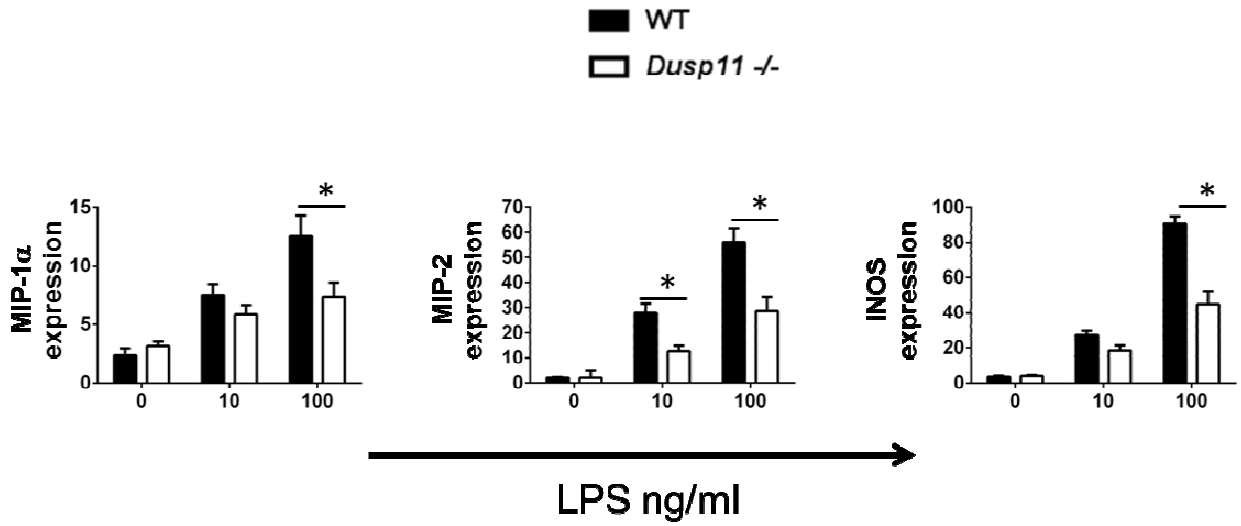

Figure 21. NF-κB target genes are down regulated in the absence of DUSP11. Several known NF-κB target genes expression are tested by Real-time PCR in WT and *Dusp11-/-* DCs upon stimulation with LPS (100 ng/ml)**.** Data shown are a representative of at least two independent experiments.

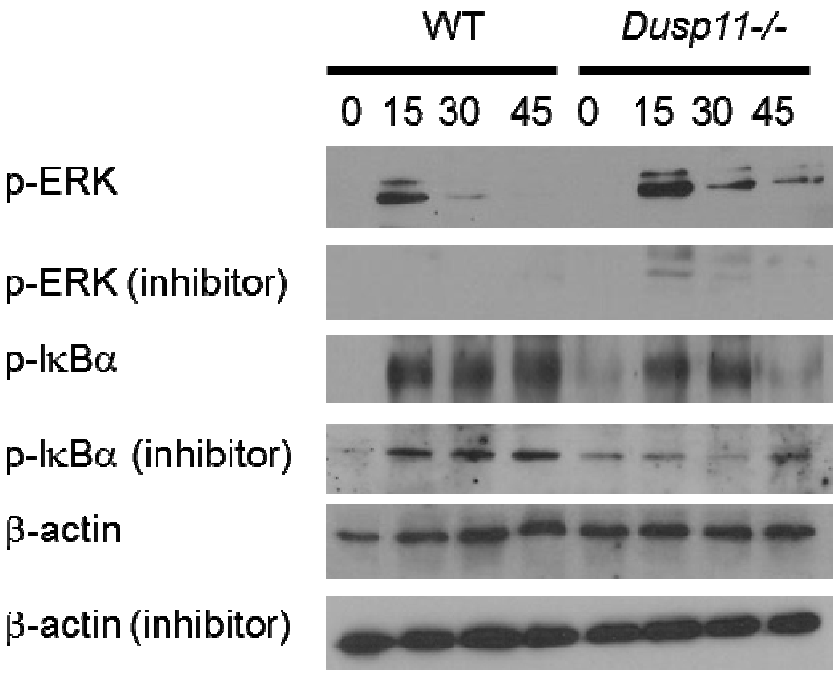

Figure 22. ERK inhibitor treatment cannot rescue defective NF-κB signaling in *Dusp11-/-* DCs. Dendritic cells from WT or *Dusp11-/-* mice were cultured in presence or absence of ERK inhibitor (5 ng/ml) for 2h followed by stimulation with LPS (100 ng/ml) for the indicated times and western blots were performed for the respective proteins. Data shown are a representative of at least two independent experiments.

3.3 DUSP11 dephosphorylates the gamma binding domain (γbd) of IKK-β

We further studied the upstream signaling components in NF-κB signaling pathway and found that TAK1 activation and IKK-α and IKK-β phosphorylation (Ser176/180 phosphorylation) was not defective in *Dusp11-/-* DCs upon LPS stimulation (**Fig 23**). However, our *in vitro* kinase assay showed that IKK activity is defective in the absence of DUSP11 (**Fig 20A**). Previous studies have demonstrated that IKK-α and IKK-β phosphorylation (Ser176/180 phosphorylation) is not sufficient for activation of IKK complex (Grabiner et al., 2007; Shambharkar et al., 2007). Several other modifications like ubiquitination and phosphorylation of other sites are also required (Grabiner et al., 2007; Higashimoto et al., 2008; Shambharkar et al., 2007). Of particular interests, the gamma binding domain (γBD) at the C-terminus of IKKβ has been shown to be phosphorylated, which negatively regulates IKK complex activity (Higashimoto et al., 2008). Polo like kinase 1 (PLK1) was described as the kinase that phosphorylates γBD (Higashimoto et al., 2008) but the phosphatase that dephosphorylates this domain has not yet been identified. To examine if DUSP11 dephosphorylates γBD, we employed the HeLa cell line for *in vitro* studies as anti-p-γBD antibody is only available in human. Overexpression of WT but not phosphatase-inactive mutant of DUSP11 induced dephosphorylation of γBD (**Fig 24A**). Conversely, knocking down of DUSP11 expression using a DUSP11-specific shRNA resulted in inhibition in LPS-induced de-phosphorylation of γBD (**Fig 24B**). Using co-immunoprecipitation (Co-IP), we found that DUSP11 directly

interacted with IKK-β (**Fig 24C**), which was enhanced following LPS stimulation. Thus, DUSP11 appears to regulate the dephosphorylation of γBD. To test whether DUSP11 can directly de-phosphorylate γBD, an *in vitro* phosphatase assay was conducted using immunoprecipitated DUSP11 and IKK from 293T cells. Indeed, DUSP11 directly dephosphorylated γBD of IKK-β *in vitro* in a dose dependent manner (**Fig 25A**). To verify if DUSP11 regulates the dephosphorylation of γBD in mice, we employed *in vitro* phosphatase assay where WT and KO DC lysates were incubated with IKK-β purified from HeLa cells and γBD phosphorylation was tested. As shown in Fig **25B**, WT DC lysates dephosphorylated γBD more efficiently than KO DCs.

To examine the γBD phosphorylation in WT and KO DCs directly, we generated mouse specific phosphoantibody against γBD (See under "Materials and Methods"). By performing western blots on DC lysates from WT and KO we confirmed the above results that γBD dephosphorylation is defective in the absence of DUSP11 (**Fig 24D**). Due to sensitivity issues of mouse phospho γBD antibody, these western blots were performed after immunoprecipitation of IKK complex using anti IKK-β antibody. It was previously proposed that γBD de-phosphorylation is essential for interaction of IKK-β and IKK-γ (Higashimoto et al., 2008). We tested if DUSP11 KO DCs had any defect in this interaction by performing co-IP of IKK-β and IKK-γ but failed to find any difference between WT and KO DCs (**Fig 26**). Together, we demonstrate that the γBD of IKK-β is a target of DUSP11 in innate immunity.

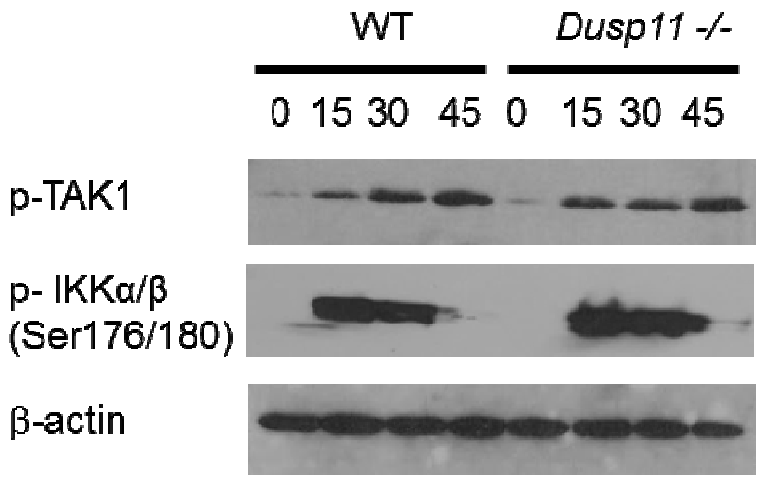

Figure 23. TAK1 and IKK-α/β activation site phosphorylation is not defective in *Dusp11-/-* dendritic cells. Phospho TAK1 and phospho IKK-α/β levels are measured in WT and DUSP11 KO DCs after stimulation with LPS (100 ng/ml). Data shown are a representative of at least two independent experiments.

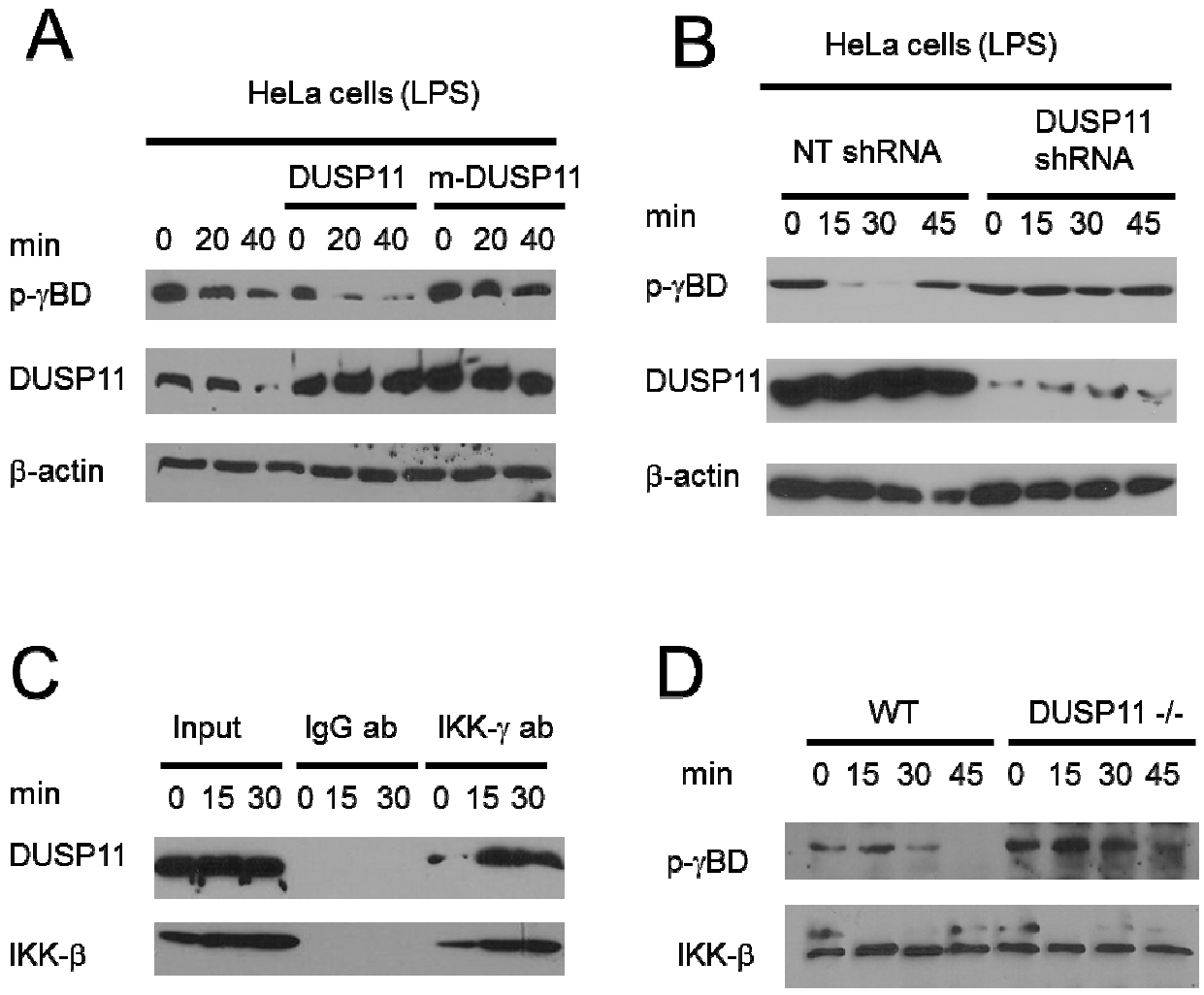

Figure 24. DUSP11 dephosphorylates γ-binding domain of IKK-β. (A) WT (DUSP11) or phosphatase inactive DUSP11 (m-DUSP11) is overexpressed in HeLa cells and γBD phosphorylation was analyzed by western blotting upon stimulation with LPS (100 ng/ml). (B) HeLa cells were transfected with lentivirus expressing non target control shRNA (NT shRNA) or DUSP11 specific shRNA (DUSP11 shRNA)

and γBD phosphorylation was analyzed by western blotting stimulation with LPS (100 ng/ml). (C) WT dendritic cell lysates were subjected to immunoprecipitation upon stimulation with LPS (100 ng/ml) with control immunoglobulin (Ig) or with antibody against IKK-γ.The immunoprecipitates were analyzed by western blotting with antibodies against DUSP11. IKK-β serves as a loading control. (D) Dendritic cells from WT or KO mice were stimulated with LPS (100 ng/ml) for the indicated times and IKK-β is immunoprecipitated from these cell lysates. Further, phosphorylation of γBD of IKK-β is analyzed in the immunoprecipitated samples using the mouse specific p-γBD antibody. Data shown are a representative of at least two independent experiments.

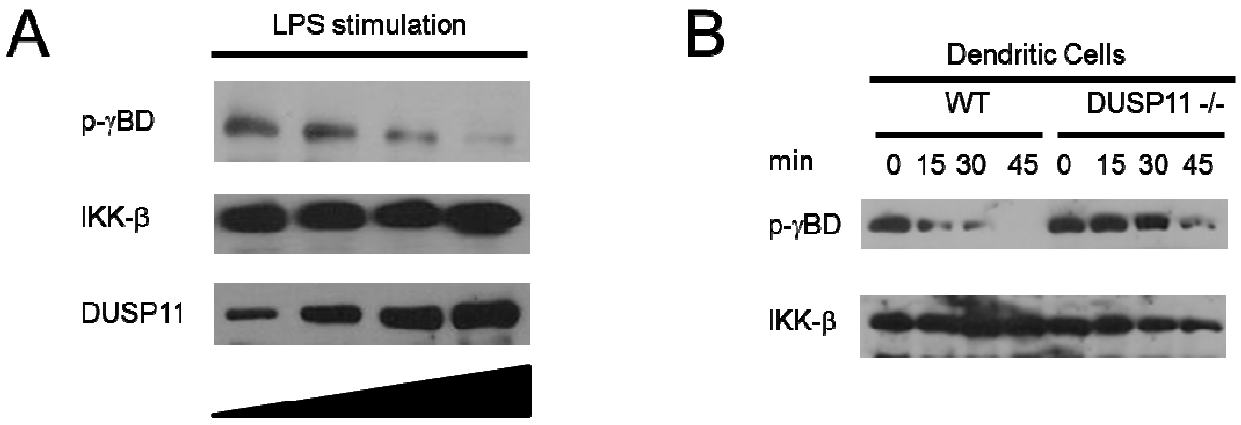

Figure 25. DUSP11 dephosphorylates γBD of IKK-β *in vitro*. (A) Immunoprecipitated IKK complex from HeLa cells were incubated with increasing doses of immunoprecipitated DUSP11 and γBD phosphorylation was analyzed by western blotting. IKK-β is used as loading control. (B) WT and DUSP11 KO DC lysates were incubated with immunoprecipitated IKK complex from HeLa cells and γBD phosphorylation was analyzed by western blotting upon stimulation with LPS (100 ng/ml). Data shown are a representative of at least two independent experiments.

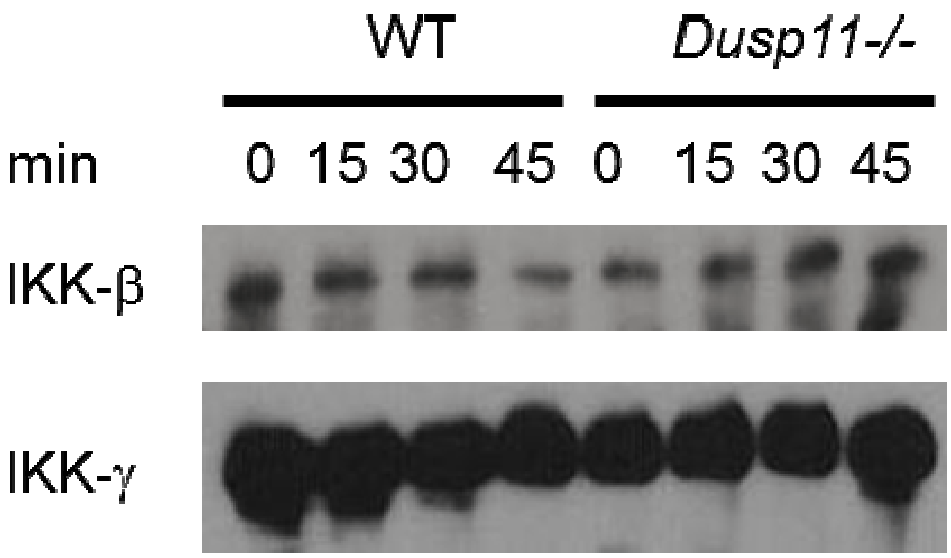

Figure 26. IKK-β and IKK-γ interaction is not defective in *Dusp11-/-* dendritic cells. WT and *Dusp11-/-* dendritic cell lysates were subjected to immunoprecipitation upon stimulation with LPS (100 ng/ml) with antibody against IKK-γ. The immunoprecipitates were analyzed by western blotting with antibodies against IKK-β. IKK-γ serves as a loading control. Data shown are a representative of at least two independent experiments.

DISCUSSION

In previously published *in vitro* studies, DUSP11 has been proposed to have a role in RNA metabolism (Deshpande et al., 1999; Yuan et al., 1998). However, these observations had not been confirmed *in vivo*. To understand the mechanisms by which DUSP11 regulates pro-inflammatory cytokine levels, we performed RNA stability experiments on IL-6 and TNF-α pro-inflammatory cytokine RNA from WT and *Dusp11-/-* DCs. However, we did not find any difference in the stability of these two RNAs (**Fig 18**), suggesting that DUSP11 may not regulate RNA metabolism *in vivo*. Several other members of the DUSP family, such as DUSP1(Zhang et al., 2009), DUSP10(Zhang et al., 2004), and DUSP16(Masuda et al., 2001), have been shown to specifically target the MAP kinase signaling pathway and dephosphorylate the MAP kinases ERK, JNK and P38. We observed that, similar to other family members, DUSP11 also targeted MAP kinase signaling by dephosphorylating ERK and P38 (**Fig 16A**). Furthermore, we observed that DUSP11 positively regulates the NF-κB signaling pathway (**Figs 19 & 20**). These observations suggested that DUSP11 can be involved in cross talk between the MAP kinase and NF-κB signaling pathways. However, we found that inhibiting ERK signaling did not restore NF-κB activation in *Dusp11-/-* DCs (**Fig 22**). DUSP11 thus may have an unexpected direct effect on the NF-κB signaling pathway. Our in vitro kinase assay (**Fig 20A**) confirmed that NF-κB signaling is compromised at IKK complex in the absence of DUSP11. Further, our detailed mechanistic studies (**Figs 24 & 25**) confirmed that DUSP11 specifically targets and dephosphorylates the gamma binding domain of

IKK-β. Thus far, we have shown that DUSP11 specifically targets and dephosphorylates the gamma binding domain of IKK-β. It was previously proposed that γBD de-phosphorylation is essential for interaction of IKK-β and IKK-γ (Higashimoto et al., 2008). We tested if *Dusp11-/-* DCs had any defect in this interaction by performing co-IP of IKK-β and IKK-γ but failed to find any difference between WT and KO DCs when the cells are activated (**Fig 26**). However, we identified that in the absence of DUSP11 the basal level phosphorylation of γBD is higher (**Fig 24D**) and accordingly the IKK-β and IKK-γ interaction is lower in the basal condition (**Fig 26**). These finding suggest us that in addition to dephosphorylating γBD, DUSP11 or its binding partners may additionally be required effective interaction of IKK-β with IKK-γ. So, the role of γBD of IKK-β in regulation of IKK activation and the requirement of DUSP11 in this process needs to be further studied.

In summary, although its deficiency enhances ERK and P38 activation in DCs, DUSP11 is unique among DUSPs in that it is the only known protein that positively regulates the activation of NF-κB pathway by serving as a phosphatase of the γBD of IKKβ. In resting cells the γBD remains phosphorylated by polo like kinase 1 (PLK1) due to which the IKK complex remains inactive so that the signaling remains in the basal state. Upon stimulation with LPS, the γBD will be dephosphorylated by DUSP11 to activate the IKK complex there by activating the downstream signaling. How DUSP11 is activated upon TLR4 stimulation by LPS

needs further understanding (**Fig 27**). Considering this novel regulation by DUSP11 is critical for the NF-κB activation in innate immunity, it may be considered as an attractive target to treat immune and septic diseases.

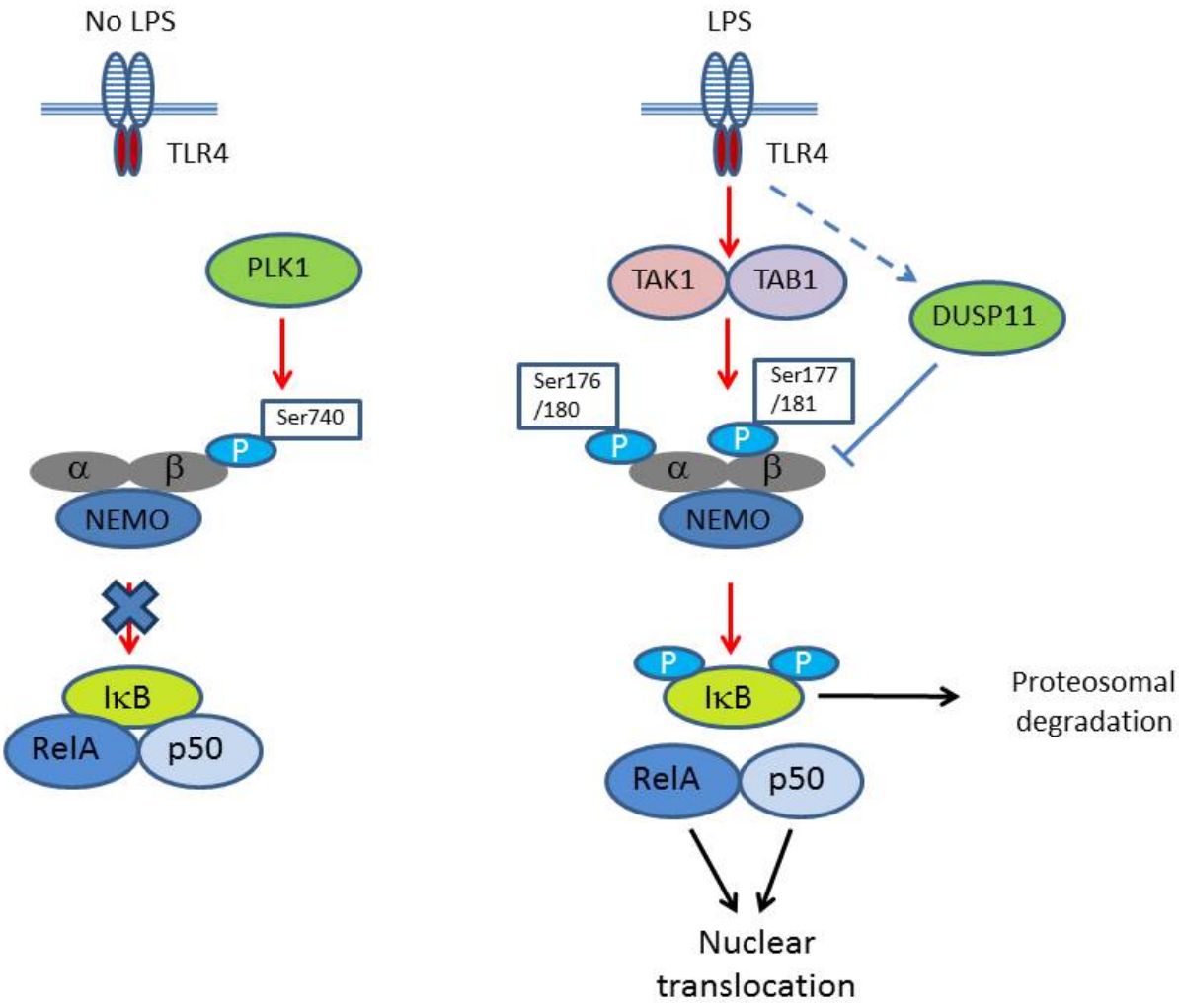

Figure 27. Proposed model for DUSP11 mediated regulation of NF-κB signaling. In resting cells the γBD remains phosphorylated by PLK1 due to which the IKK complex is inactive so that the signaling remains in the basal state. Upon stimulation with LPS, the γBD will be dephosphorylated by DUSP11 in addition to IKK-α and IKK-β phosphorylation at Ser 176/180 and Ser 177/181 respectively so that the IKK complex activity is enhanced there by activating the downstream signaling. How DUSP11 is activated upon TLR4 stimulation by LPS needs further understanding.

CHAPTER 4 – GENERAL DISCUSSION AND FUTURE DIRECTIONS

DUSPs role as critical regulators of several physiological functions is discussed in Chapter 1. Herein we have focused on the role of DUSP11, an atypical DUSP, as regulator of immune responses and the molecular basis of this regulation. We analyzed the role of DUSP11 in innate and adaptive immune responses by generating the *Dusp11-/-* mice and studying them using several *in vitro* and *in vivo* experimental models. We found that DUSP11 regulates proinflammatory cytokine secretion by dendritic cells by dephosphorylating a regulatory domain of IKK-β known as gamma binding domain (γBD). In Chapter 2, our findings demonstrate that although DUSP11 expression is induced *in vitro* in both innate and adaptive immune cells upon stimulation, DUSP11 is dispensable for adaptive responses *in vitro*. Further, our *in vivo* studies highlighted the requirement of DUSP11 in developing optimal innate responses, which regulates the generation of the adaptive responses *in vivo*. Together, we demonstrate for the first time that DUSP11 is a critical regulator of immune responses and acts specifically by modulating innate immune responses. This phosphatase thus could serve as a novel target for therapeutic intervention for innate cell-mediated inflammatory and autoimmune diseases.

Additionally, in Chapter 3 we investigated the molecular mechanisms regulated by DUSP11 in immune responses. Since, we identified in Chapter 2 that DUSP11 is induced in dendritic cells upon TLR stimulation and that DUSP11 is critical in mediating innate immune responses, we focused our mechanistic studies

in dendritic cells upon TLR4 stimulation with LPS. Several other members of the DUSP family, such as DUSP1 (Zhang et al., 2009), DUSP10 (Zhang et al., 2004), and DUSP16 (Nallaparaju & Zhang et al. under review), were shown to specifically target the MAP kinase signaling pathways by dephosphorylating the MAP kinases ERK, JNK and P38. We observed that, similar to other family members, DUSP11 also targeted MAP kinase signaling by dephosphorylating ERK. Furthermore, we observed that DUSP11 positively regulates the NF-κB signaling pathway. These observations suggested to us that DUSP11 may be involved in cross talk between MAP kinase and NF-κB signaling pathways. IKK2 is the critical molecule of the NF-κB signaling pathway shown to negatively regulate MAP kinase signaling (Waterfield et al., 2004). Our observations in *Dusp11-/-* mice with increased ERK activity and decreased NF-κB activity suggested that IKK2 could be a potential target of DUSP11. Of particular interest, the gamma binding domain (γBD) at the C-terminus of IKKβ has been shown to be phosphorylated, which negatively regulates IKK complex activity (Higashimoto et al., 2008). In this study Zandi and colleagues demonstrated that Polo like kinase 1 (PLK1) phosphorylates serines 733, 740, and 750 of γBD in IKK-β during resting state and that this phosphorylation regulates the interaction of IKK-β with IKK-γ and thereby effects the IKK complex activity (Higashimoto et al., 2008). However, the phosphatase that dephosphorylates this domain has not yet been identified. Based on our phenotype in dendritic cells we hypothesized that DUSP11 is the phosphatase that dephosphorylates γBD. Our detailed mechanistic studies confirmed DUSP11 indeed regulates NF-κB activity by dephosphorylating the gamma-binding domain of IKK2. These mechanistic studies

highlight the role of DUSP11 as a positive regulator of NF-κB signaling, which ultimately affects the secretion of proinflammatory cytokines upon TLR stimulation in dendritic cells.

In this chapter, we will relate our results to recent reports on DUSP11 and other family members' role as regulators of immune responses and how this molecule can be targeted in future studies to treat inflammatory diseases and cancer.

4.1. Role of DUSP11 in NF-κB signaling mediated proinflammatory cytokines secretion

DUSP members regulate immune responses mainly by regulating MAP Kinase signaling. Being a member of DUSP sub group we hypothesized that DUSP11 regulates MAP kinase activity by dephosphorylating one or more of their members. Though we identified that *Dusp11-/-* DCs have enhanced ERK and P38 phosphorylation, this enhanced MAP kinase activity could not explain the defect in proinflammatory cytokines secretion by *Dusp11-/-* dendritic cells as enhanced MAP kinase signaling would result in enhanced cytokines secretion. MAP Kinase pathway and NF-κB signaling pathways are the two critical pathways involved in the secretion of proinflammatory cytokines (Newton and Dixit, 2012). To explain the proinflammatory cytokines secretion phenotype in *Dusp11-/-* DCs we analyzed the NF-κB signaling pathway and identified that NF-κB signaling is defective in the

absence of DUSP11 at the IKK complex level. We identified that upstream of IKK complex the NF-κB signaling pathway is intact in *Dusp11-/-* DCs. Further, we identified that IKK-α and IKK-β activation site phosphorylation (Ser176/180 phosphorylation) is also comparable between WT and *Dusp11-/-* DCs. However, several recent studies have demonstrated that this phosphorylation by itself is not sufficient for activation of the IKK complex (Grabiner et al., 2007; Shambharkar et al., 2007). Several other modifications like ubiquitination and phosphorylation of other sites are also additionally required (Grabiner et al., 2007; Higashimoto et al., 2008; Shambharkar et al., 2007). We went on to analyze additional phosphorylation sites of IKK-β due to its importance in regulating both NF-κB signaling and the ERK signaling and identified that DUSP11 regulates the activity of IKK complex by dephosphorylating γBD of IKK-β. Therefore, DUSP11 positively regulates the activation of the NF-κB pathway and promotes the secretion of proinflammatory cytokines.

4.2. Role of DUSP11 in IKK complex activity

The transcription factor NF-κB is widely studied for its gene regulatory function as it activates large groups of genes in response to wide range of extracellular signals (May and Ghosh, 1999). Understanding the molecular mechanism that regulates NF-κB signaling is essential as this pathway is deregulated in wide range of human diseases including cancer, autoimmune diseases and chronic inflammation (Sun and Liu, 2011). In case of unstimulated

cells NF-κB is sequestered in the cytoplasm by inhibitory proteins IκBs. Upon stimulation by proinflammatory signals or other external stimuli like PAMPS, these IκBs are phosphorylated and degraded to release the NF-κB dimers so that they can translocate into nucleus and activate the transcription of their target genes. The upstream protein complex that phosphorylates the IκBs includes two catalytic subunits IKK-α and IKK-β and a regulatory subunit IKK-γ (also known as NEMO) (Ghosh et al., 1998). Together, this complex is known as IKK complex and the activity of this complex is tightly regulated by several modifications like phosphorylation at multiple sites and ubiquitination (Grabiner et al., 2007; Shambharkar et al., 2007). Previous studies have demonstrated that a domain in C-termini of IKK-α and IKK-β known as Gamma binding domain (γBD) or nemo binding domain (NBD) is critical for their association with NEMO (May et al., 2000; May et al., 2002). However, this domain of IKK-β but not that of IKK-α is essential for TNF-α and IL-1β mediated NF-κB activation (Solt et al., 2009). This domain in IKK-β is previously demonstrated to be auto-phosphorylated by helix-loop-helix domain of IKK-β and is responsible for IKK-β activity (Delhase et al., 1999). Further, the same group has shown that mutating the serines in this domain to alanines resulted in higher basal activity as well as prolonged activation in response to the proinflammatory stimuli (Delhase et al., 1999). Another recent study by Zandi and colleagues demonstrated that PLK1 is the kinase that phosphorylates the γBD of IKK-β (Higashimoto et al., 2008). But the phosphatase that dephosphorylates this domain has not yet been identified. Our detailed mechanistic studies demonstrated

that DUSP11 directly dephosphorylates γBD of IKK-β to regulate IKK complex activity in canonical NF-κB pathway.

Further, Zandi and colleagues showed that phosphorylation of γBD regulates the interaction of IKK-β with IKK-γ and therefore plays a critical role for IKK activation (Higashimoto et al., 2008). We tested if *Dusp11-/-* DCs had any defect in this interaction by performing co-IP of IKK-β and IKK-γ but failed to find any difference between WT and KO DCs when the cells are activated. However, we identified that in the absence of DUSP11 the basal level phosphorylation of γBD is higher and accordingly the IKK-β and IKK-γ interaction is lower in the basal condition. These finding suggest us that in addition to dephosphorylating γBD, DUSP11 or its binding partners may additionally be required effective interaction of IKK-β with IKK-γ. So, the role of γBD of IKK-β in regulation of IKK activation and the requirement of DUSP11 in this process needs to be further studied. Cell permeable γBD peptide is a selective non- catalytic IKK inhibitor that disrupts the interaction of the catalytic subunits with NEMO, effectively blocking kinase activity that is triggered by many different inflammatory stimuli. This peptide is currently being evaluated in clinical studies with promising results for B-cell lymphoma and other forms of cancer (Habineza Ndikuyeze et al., 2014; Gamble et al., 2012) and inflammatory diseases (Edwards et al., 2009). Since we demonstrated that DUSP11 directly regulates the activity of this domain, targeting DUSP11 may be a promising strategy in these diseases.

4.3. Role of DUSP11 in adaptive immune responses

Although DUSP11 expression is induced *in vitro* in both innate and adaptive immune cells upon activation, we have identified that DUSP11 is intrinsically dispensable for adaptive responses *in vitro.* Our results demonstrate that *Dusp11*-/- CD4 T cells have enhanced proliferation upon activation with anti-CD3 and anti-CD28 antibody. This is in correlation with previous observations by Caprara et al that DUSP11 regulates cellular proliferation in a p53 dependent manner. However, when these CD4 T cells were differentiated into Th1, Th2 & Th17 subtypes *in vitro.* There was no defect or enhancement when compared to their wild type counterparts. But in a *L. monocytogenes* infection model and KLH immunization model our studies demonstrated that *Dusp11-/-* mice have defective adaptive immune responses. To address this discrepancy between *in vitro* and *in vivo* data, we used *in vitro* co-culture experiments and OT-II transfer experiments and demonstrated that DUSP11 has an intrinsic defect in dendritic cells but in case of CD4 T cells DUSP11 is dispensable. However, the defect in innate immune cell production of proinflammatory cytokines eventually leads to defective adaptive immune responses *in vivo*. These results support the growing body of evidence that intrinsic pathways in innate immune cells can modulate T cell lineage differentiation (Huang et al., 2011).

4.4. DUSP11's role as RNA phosphatase

Several *in vitro* studies suggested that DUSP11 may function as an RNA – 5' – phosphatase and has been proposed to have a role in RNA metabolism (Deshpande et al., 1999; Yuan et al., 1998). Further, these studies identified that DUSP 11 interacts with splicing factors like 9G8, SRp30C (Deshpande et al., 1999) and SAM68 (Caprara et al., 2009). However, these observations were not confirmed *in vivo*. To understand the mechanisms by which DUSP11 regulates proinflammatory cytokine levels, we performed RNA stability experiments on IL-6 and TNF-α proinflammatory cytokine RNA from WT and *Dusp11-/-* DCs. However, we did not find any difference in the stability of these two RNAs, suggesting that DUSP11 may not regulate RNA metabolism of these cytokines *in vivo*. However, these studies are preliminary and further detailed mechanistic studies into RNA metabolism may provide better understanding into the role of DUSP11 as an RNA phosphatase.

In case of *C. elegans*, a homologue of DUSP11 known as PIR-1 is shown to interact with Dicer DCR1 and was suggested to have a role in RNA silencing. Moreover, PIR-1 is essential for development, as the PIR-1 embryos fail to mature into adult worms (Duchaine et al., 2006). However, in the case of mice we have identified that DUSP11 is dispensable for development and the analyzed immune cell components are fully intact. So, although due the homological similarity of these proteins, it may be interesting to study the role of DUSP11 in RNA silencing, there

may be difference in function for DUSP11 in mammals as observed in the case of development.

Bioinformatics analysis using Human Protein-Protein Interaction Mining Tool from Wei Li's laboratory (He et al., 2009) helped us identify AUF-1 as a strong interacting partner of DUSP11 (Data not shown). AUF1, like DUSP11 is identified as an RNA binding protein and is suggested to have a role in RNA metabolism. A recent study identified AUF1 as a critical regulator of NF-κB signaling by maintaining proper levels of TAK1 an upstream kinase that phosphorylates IKK-β and activates NF-κB signaling (Sarkar et al., 2011). These findings suggest that AUF1 interacts with DUSP11 and that these two proteins along with several other interacting partners may work as a complex that regulates NF-κB signaling at different levels.

4.5. DUSP inhibitors in preclinical metabolic, autoimmune diseases and cancer therapy trials

Many members of DUSP family are overexpressed or hyper reactive in several human diseases including cancer and autoimmune diseases making them potential candidates for targeted therapy approach. The involvement of DUSPs in cancer and autoimmune diseases is well discussed in chapter-1. Here we will discuss the current status of their inhibitors in preclinical and clinical trials of autoimmune diseases and cancer. DUSP1 is a DUSP member whose role and molecular mechanisms are well documented in autoimmune diseases like

rheumatoid arthritis and in several varieties of cancer (Jeffrey et al., 2007; Rios et al., 2014; Wancket et al., 2012). Depending on the type and the stage of disease its expression patterns and the regulatory mechanisms varies. Since this molecule is well studied in various disease models now it is being actively pursued as a therapeutic target (Pulido and Hooft van Huijsduijnen, 2008; Wancket et al., 2012). Targeted therapy against DUSP1 has been evaluated both at protein level and at mRNA level. In case of patients with major depressive disorder (MDD), DUSP1 mRNA is overexpressed in the hippocampus resulting in diminished ERK activity. This condition is reverted by anti-depressant drug fluoxetine (Prozac). Additionally, DUSP1-/- mice were shown to be resistant to stress induced depressive like symptoms making the inhibition of DUSP1 a promising approach to treat depressive disorders (Duric et al., 2010). This approach is currently being considered for clinical trials in MDD patients (Rios et al., 2014).

In cancer, DUSP1 expression patterns vary between tumor types and the stages of tumors and DUSP1 is shown to have both oncogenic and tumor suppressive functions depending on the type of cancer (Chan et al., 2008; Murty et al., 1996). These studies are discussed earlier in Chapter 1. It is essential to diagnose the stage of cancer and DUSP1 expression patterns in the patients before they are treated with DUSP1 inhibitors. Several plant based derivatives like sangunarine and quinone derivatives or synthetic small molecular inhibitors like tricyclic pyrolle-2-carboxamide were shown to have inhibitory effects on DUSP1 in micro molar ranges (Nunes-Xavier et al., 2011). One such inhibitor, a plant based

triptolide-derivative Minnelide is currently evaluated in phase-II clinical trial in patients with advanced stage GI tumors where DUSP1 is overexpressed (ClinicalTrials.gov id: NCT01927965). Minnelide diminishes DUSP1 expression at the transcriptional level (Liu et al., 2011). Further, in mouse models of spontaneous pancreatic cancer, Minnelide treatment is shown to promote survival of mice and is effective in reducing tumor growth (Chugh et al., 2012). In cancer types where DUSP1 functions as a tumor suppressive factor like in case of ovarian cancer (Murty et al., 1996) or breast cancer, Bowman-birk inhibitor (BBI) could be used for therapy as this inhibitor significantly enhances DUSP1 expression upon addition to the cell lines (Chen et al., 2005).

In case of atypical DUSP sub group members, DUSP3 inhibitors are currently being actively investigated in several disease models. Tertronic acid and phenylpyrazole derivatives have been demonstrated to have inhibitory effects on DUSP3 by inhibiting its enzyme activity (Nunes-Xavier et al., 2011). Currently these molecules are studied in disease models (Rios et al., 2014). Some of the inhibitors like AS07234-4 are shown to have cross-inhibitory effects against both DUSP2 and DUSP 10 (Gobert et al., 2009). Together, these studies highlight the potentiality of DUSP inhibitors as therapeutics against human diseases. Having identified the mechanisms by which DUSP11 regulates proinflammatory cytokines secretion by antigen presenting cells, DUSP11 targeting as a therapeutic strategy can now be tested in septic shock mouse models or patients or additionally in cancer mouse models or patients where NF-κB signaling is hyper activated (Staudt, 2010).

Recently, the high resolution structure of DUSP11 catalytic core has been reported. (Sankhala et al., 2014). Now, small molecule inhibitors from natural sources and chemical libraries should be screened to identify molecules that can specifically inhibit DUSP11 activity or expression. Such inhibitors can have potential advantages over NF-κB inhibitors because unlike NF-κB inhibitors which block proinflammatory cytokines secretion completely, based on our KO data DUSP11 inhibitors only blocks them partially. As these cytokines have many immunological and physiological functions, partial blockade will be more advantageous than complete blockade. Thus, therapeutic inhibition of DUSP11 in cancers and septic shock patients deserves further exploration. Moreover, DUSP11 is overexpressed in colon carcinoma and glioblastoma cell lines (Dardousis et al., 2007) suggesting a causative or biomarker role for DUSP11 in their pathology which can be further explored.

4.6. Significance of the study and future directions

In summary we have identified DUSP11 as a critical regulator of immune responses against intracellular pathogens. In LPS induced septic shock mouse model that mimics human sepsis, we have shown that DUSP11 functions as positive regulator of proinflammatory cytokines secretion and the resultant multiple organ failure. Further, we have demonstrated that DUSP11 positively regulates proinflammatory cytokines secretion by innate immune cells. Mechanistically, we found that DUSP11 directly dephosphorylates the gamma binding domain (γbd) of

IKK-β and thus positively regulates the activity of the IKK complex. DUSP11 is unique that it is the only known DUSP member that positively regulates the activation of NF-κB pathway. Considering this novel regulation by DUSP11 is critical for the NF-κB activation in innate immunity, it serves as an attractive target to treat immune and septic diseases.

Although we have demonstrated that DUSP11 regulates NF-κB signaling by dephosphorylating γBD of IKK-β, the upstream signaling pathway that activates DUSP11 is not known. Further studies need to be carried out to identify the signaling pathway. Since NF-κB signaling is involved in regulation of several other signaling pathways like activation of inflammasome pathways or necrosis, the role of DUSP11 as regulator of NF-κB in these pathways should be further studied. Also, we did not investigate the modifications required on DUSP11 like phosphorylation or ubiquitination to activate it. Further understanding the regulation of DUSP11 activity would provide a potential target molecule to moderately regulate NF-κB signaling pathway. Moreover, like discussed before, the role of DUSP11 as RNA phosphatase and its involvement in RNA silencing deserves further exploration. We also identified that *Dusp11-/-* DCs have enhanced ERK and p38 activation. Though our studies using the respective inhibitors have demonstrated that the enhanced MAP kinase activity is not responsible for defective proinflammatory cytokines secretion, this phenotype may be responsible for other physiological functions like cellular proliferation or cell cycle regulation. The role of DUSP11 in these physiological processes needs to be further studied. Together, this study demonstrates that

DUSP11 is critical for innate immune responses and, as the first MAP kinase phosphatase that positively regulates NF-κB pathway; it may be targeted to treat immune diseases.

CHAPTER V – EXPERIMENTAL PROCEDURES

Generation of *Dusp11-/-* mice: A targeted embryonic stem cell line with disruption of mouse DUSP11 by means of a gene-trapping vector inserted into intron 1 (BB0918; Bay genomics) was injected into mouse blastocysts and subsequently transferred into pseudo pregnant foster mothers on a mixed C57BL/6:129 background for the generation of chimeras, as described previously(Dong et al., 1998). Heterozygous (+/-) mice were intercrossed to generate wild-type (WT) and mutant mice. To get the mice in B6 background we backcrossed the mixed background mice for six generations. All the experiments were performed first in mixed background mice and then were confirmed in B6 background mice with appropriate WT controls. OT-II mice with Ly5.1 congenic marker were generated by crossing OT-II mice with Ly5.1+ mice. C57BL/6 and RAG1-deficient mice were purchased from Jackson laboratories. Mice were maintained in the animal care facility at The University of Texas MD Anderson Cancer Center, and the animal studies were approved by the Institutional Animal Care and Use Committee.

***In vitro* DC and macrophage assays:** Dendritic cells were cultured from bone marrow of WT and DUSP11 KO mice in presence of GM-CSF for 7 days as described previously(Chung et al., 2009). Macrophages were cultured from bone marrow of WT and DUSP11 KO mice in presence of M-CSF (L929 supernatant) for 7 days as described previously (Zhong et al., 2013). CD11C+ or CD11B+ cells were sorted out from these cultured cells by staining with CD11C or CD11B magnetic

beads for DCs and macrophages respectively (Miltenyi Biotec). These cells were stimulated with varying concentrations of LPS for 4 h or 24h and proinflammatory cytokine levels were determined by real-time PCR and standard ELISA (BD Pharmingen, San Diego, CA) respectively.

***In vitro* T cell assays:** Naïve CD4+ T cells were purified from lymph nodes and spleens of mice by FACS based on the CD4+CD62LhiCD44lo and CD8+CD62LhiCD44lo surface phenotypes. To analyze the effects of DUSP11 on T cell activation and proliferation, T cells were incubated with different concentrations of plate-bound anti-CD3 antibody or anti-CD3 plus anti-CD28 antibodies. IL-2 production by T cells was measured by ELISA (BD Pharmingen, San Diego, CA) 24 h after T cell activation. Cell proliferation was determined at 72 h, after incubation with [3H] thymidine in the last 8 h. To examine the role of DUSP11 in effector function, naïve CD4+ T cells were cultured under Th1-, Th2-, or Th17-skewing conditions as described previously(Angkasekwinai et al., 2007). After 4 days of differentiation, cells were washed and treated with 2 μg/ml of plate-bound anti-CD3 for cytokine measurement or treated with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of GolgiPlug (BD Biosciences) for intracellular cytokine staining, and samples were analyzed by flow cytometry.

Real-time PCR analysis for mRNA expression: Total RNA was prepared from cells with TRIzol reagent (Invitrogen). Complementary DNA (cDNA) was synthesized with SuperScript reverse transcriptase and oligo(dT) primers (Invitrogen), and gene

expression was examined using a Bio-Rad iCycler iQ real-time PCR kit with iQ SYBR Green reagent. The data were normalized to the reference gene β-actin or GAPDH. The list of primers used in this study are shown in Table 1.

Table 2. List of Primers.

a) Genotyping primers for genomic DNA (5' to 3')		
WT Forward	GGCAGAAACCACATCCCCGAAAG	
WT Reverse	TTACATCTATGAGCTGTCTC	
KO Forward	GGCAGAAACCACATCCCCGAAAG	
KO Reverse	TACAGTCCTCTTCACATCCATGCT	
b) Real time PCR primers		
Gene name	**Primer sequence forward (5' to 3')**	**Primer sequence reverse (5' to 3')**
DUSP11	GGCAGAAACCACATCCCCGAAA	CTGGGAGATAGTCTTTCC
IL-6	CACTTCACAAGTCGGAGGCTTA	GCAAGTGCATCATCGTTGTTC
TNF-α	AATGGCCTCCCTCTCATCAGT	GCTACAGGCTTGTCACTCGAATT
iNOS	CGAAACGCTTCACTTCCAA	TGAGCCTATATTGCTGTGGCT
MIP-1α	CTGCCCTTGCTGTTCTTCTCTGT	GGCTGCTGGTTTCAAAATAGTCA
MIP-2	CCACTCTCAAGGGCGGTCAA	CCCCTTATCCCCAGTCTCTTTCA
GAPDH	GAGAACTTTGGCATTGTGG	ATGCAGGGATGATGTTCTG
β-Actin	TCCTTCGTTGCCGGTCCAC	ACCAGCGCAGCGATATCGTC

mRNA stability experiments: Mouse embryonic fibroblasts (MEF) cells from WT or *Dusp11-/-* mice were stimulated for 1 h with LPS (100 ng/ml), followed by treatment (for mRNA decay) or no treatment (for steady state mRNA level) with 5 μM Actinomycin-D. Up to 4h hours post treatment, cells were harvested and mRNA was extracted as mentioned above (Zhong et al., 2012).

KLH immunization: WT and DUSP11 KO mice were immunized were subcutaneously immunized at the base of tail (200 ul/ mouse) with keyhole limpet hemocyanin (KLH) protein emulsified in complete Freund's adjuvant (CFA). Seven days post immunization the mice were sacrificed and analyzed individually for germinal center formation and antigen specific antibody and cytokine responses. Briefly, total spleen cells or LN cells (Tfh and GC B cell staining shown in figure are from LN cells) from these mice were stained for T follicular helper cells (Tfh) using PerCP labeled anti-CD4, biotinylated anti-CXCR5 mAb followed by APC labeled streptavidin and PElabelled anti-BCL6 mAbs (BD Biosciences). Germinal center B cells were detected by staining with FITC-labeled anti-GL7 and PE-labeled anti-Fas mAbs, and PerCP labeled anti-B220 mAb (BD Biosciences). To measure effector cytokine levels, ELISAs and intracellular staining techniques were used. On day 7, splenocytes or lymphocytes were isolated and stimulated with varying concentrations of KLH peptide. Cytokine production was determined by ELISA after 3 days of stimulation or by intracellular staining after overnight stimulation. For intracellular staining, cells were first stained with PerCPCy5.5-labeled anti-CD4 and then permeabilized with permeabilization buffer (BD Biosciences). They are further

stained with APC-labeled anti-IFN-γ and PE-labeled anti-IL17 (BD Biosciences). To measure KLH specific antibody responses, sera from immunized mice were collected and IgM, IgG, IgG1, and IgG2a antibodies were measured by ELISA. Briefly, plates were coated with 10 μg/ml of KLH protein followed by addition of sera from immunized mice in 3-fold serial dilution. Antigen specific antibodies are then detected using HRP conjugated anti mouse IgM, IgG, IgG1 and IgG2A antibodies (Southern Biotechnology Associates) respectively (Nurieva et al., 2008).

Listeria monocytogenes **infection:** An erythromycin-resistant strain of Ova-expressing Listeria monocytogenes (LM-Ova) was kindly provided by Dr. Michael Bevan (University of Washington). The bacteria were grown in brain-heart infusion media supplemented with 5 μg/ml erythromycin (Chung et al., 2013). The bacteria were harvested at mid-log growth phase and were intravenously (i.v.) injected into animals ($1x10^4$ colony forming units (CFU)/mouse). Three or 7 days after infection, the spleens and livers of the infected mice were harvested. The Listeria burden in the liver was determined by plating serial dilutions of liver cells, suspended in PBS with 0.1% NP40, on brain-heart infusion plates overnight. Splenocytes were stimulated with SIINFEKL peptide or LLO peptide overnight for intracellular cytokine staining or for 3 days for ELISA analysis (BD Pharmingen, San Diego, CA).

Adoptive transfer and EAE induction: CD4+ T cells from spleens and LNs of WT and *Dusp11-/-* mice were purified by automacs sorting (Mitenyi Biotec, Auburn, CA) and $5X10^6$ cells were adoptively transferred i.v. into *Rag1*$^{-/-}$ recipient mice. After 24h,

EAE was induced in these recipient mice by immunization with MOG35-55 peptide as described by our group previously (Dong et al., 2001). Briefly, mice were immunized with the peptide in CFA twice and two subsequent treatment of pertussis toxin, 1 day after each immunization. The mice were observed daily for clinical signs and scored on a scale of 0-5 with gradations of 0.5 for intermediate scores: 0, no clinical signs; 1, loss of tail tone; 2, wobbly gait; 3, hind limb paralysis; 4, hind- and forelimb paralysis; 5, death. Preparation and stimulation of mononuclear cells from brain and spinal tissues were done as described.

DC/T cell co-culture experiments: FACS-sorted naive CD4+ T cells from WT or KO mice were co-cultured with bone marrow-derived DCs (WT or KO) in the presence of soluble anti-CD3 antibody (0.2 mg/ml) and LPS or LPS (100 ng/ml) plus TGF-β (1 ng/ml). IL-17- or IFN-γ-expressing cells were measured by intracellular staining (Chung et al., 2009).

Adoptive transfer experiments: FACS-sorted naïve CD4+ T cells ($5X10^6$) from **spleens** and lymph nodes of Ly-5.1+ OT-II mice were injected i.v. into congenic WT or KO recipient mice. One day later the recipient mice were subcutaneously immunized with OVA protein. Seven days post immunization the recipient mice were sacrificed and lymph nodes cells were stimulated overnight with OT-II peptide followed by intracellular staining (Soroosh et al., 2006).

Western blot analysis: For analysis of ERK, JNK, p38, and IκBα activation, WT and KO dendritic cells were activated with LPS for different times. In case of ERK and p38 inhibitor treatment experiments, ERK inhibitor (U0126 (Cell Signaling Technology, Beverly, MA)) or p38 inhibitor (SB203580 (Cell Signaling Technology, Beverly, MA)) is included in the cultures for 2 hrs before stimulation with LPS. Cell lysates prepared as described previously(Zhang et al., 2009) were subjected to Western blot analysis with anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, anti-phospho-IkBα, and anti-total IkBα antibodies (Cell Signaling Technology, Danvers, MA). Anti-total IKK-β and IKK-γ antibodies are purchased from Santacruz biotech. DUSP11 antibody is purchased from ProteinTech group. Anti p-γBD antibody is obtained from Antagene Inc. The signal was detected with ECL reagent (Pierce Protein Biology Products).

shRNA knock down: The short hairpin RNA (shRNA) lentiviral plasmid pLKO.1-shDUSP11 (Oligo IDs: V3LHS_381810 & V3LHS_381808) or pLKO.1-shNS was purchased from MD Anderson shRNA and ORFeome Core. HEK 293T cells were co-transfected with the appropriate lentiviral plasmid together with the packaging vectors pRRE, VSV-G, and RSV-Rev to obtain lentiviruses. Packaged viruses were then used to infect HELA cells, which were treated with polybrene (8 mg/ml) and then selected in the presence of puromycin (1 mg/ml) for 7 days. The puromycin-resistant cells were used for experiments.

EMSA: Nuclear extracts were prepared from WT and KO DCs after stimulation with LPS for indicated times and subjected to EMSA using the following ^{32}P-radiolabelled oligonucleotide probes:NF- κB (5-CAACGGCAGGGGAATTCCCCTCTCCTT3) and NFY (5AAGAGATTAACCAATCACGTACGGTCT3) (Hu et al., 2013)

Luciferase reporter assay: To identify if DUSP11 modulate NF-κB signaling, we constructed DUSP11 and phosphatase in active mutant DUSP11 expression plasmids and transfected those together with pGL3–NF-κB luciferase reporter (0.1 μg) and a control pRL-TK renilla luciferase reporter (0.02 μg) into HeLa cells through the use of Lipofectamine 2000. 20 h after transfection, cells were stimulated with LPS for 8 h before luciferase assays (Zhong et al., 2012).

Constructs: Mammalian expression plasmids for Flag- or Myc-tagged DUSP11 were constructed in the pcDNA6.0 vector. Plasmids encoding phosphatase in active DUSP11 mutant was made with a site-directed mutagenesis kit (200519; Stratagene).

LPS induced septic shock: Age- and sex-matched WT and DUSP11–/– mice were injected intraperitoneally with LPS (15 mg/kg). Survival was monitored every three hour for 48 hours. Blood was obtained at 3h and 6h after challenge, and the concentrations of TNF-α and IL-6, in the sera were measured by ELISA (Zhong et al., 2013).

***In vitro* kinase assay:** Whole-cell lysates from WT and KO DCs were prepared in a kinase cell lysis buffer supplemented with phosphatase inhibitors after stimulation with LPS for indicated times and subjected to coimmunoprecipitation using IKK-γ antibody. The immunoprecipitates are then subjected to *in vitro* kinase assays as described previously (Zhang et al., 2008).

***In vitro* phosphatase assay:** *In vitro* phosphatase assay was performed using immunoprecipitated DUSP11 from 293T cells overexpressing DUSP11 and immunoprecipitated IKK complex from HELA cells. IKK complex was incubated with increasing doses of DUSP11 in phosphatase assay buffer(Lorenz, 2011) and phosphorylation of γBD of IKK-β was analyzed by western blotting. To test if DUSP11from dendritic cells can dephosphorylate γBD, DC lysates from WT and KO were incubated with immunoprecipitated IKK complex from HELA cells and phosphorylation of γBD of IKK-β was analyzed by western blotting.

Co-immunoprecipitation: Co-IPs were performed as described previously(Zhong et al., 2013). Briefly, standard immunoprecipitations were performed under normal conditions in lysis buffer [20 mM tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40], and the immunoprecipitates were subjected to standard Western blotting analysis.

Generation of mouse specific phosphoantibody against γ-binding domain: Mouse specific phosphoantibody against γ-binding domain was generated in rabbits

against phosphorylated FTTLDW(pS)WLQME peptide using outside vendor (Pierce Biotechnology, Inc.). Phosphoantibodies are enriched in the serum by affinity purification process; first by passing through non-phospho-peptide column to remove non phospho antibodies followed by phospho-peptide column.

Antibodies used for flow cytometric analysis: The following antibodies were used for cell surface and intracellular staining: PerCP/Cy5.5 or FITC-labeled anti-TCR-β(clone H57-597), PerCPCy5-5-labeled anti-CD4 (clone GK1.5), Alexa 488-labeled anti-CD8 (clone 5H10-1), and APC-labeled anti-CD11b (clone M1/70), all from Bio legend; and PE- or Alexa 488-labeled anti-IFN-γ (clone XMG1.2), PE-labeled anti-IL-17 (clone TC11-18H10), Alexa 647-labeled anti-granzyme B (clone GB11), FITC- or PerCPCy5.5-labeled anti-NK1.1 (clone PK136), and PerCPCy5-5-labeled anti-Ly6C (clone AL21), all from BD Biosciences. For intracellular staining, cells were permeabilized with permeabilization buffer (BD Biosciences) and then further stained with the intracellular staining antibodies described above. These cells were analyzed by using an LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Inc.).

Statistical analysis: The Student t test was used to assess the statistical values. P values were determined, along with standard error of the mean (SEM) or standard deviation (SD) using STATISTICA software (StatSoft, Inc., Tulsa, OK). P values < 0.05 were considered significant.

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VITA

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