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LRRFIP1 negatively regulates IL-17 signalling by binding to CIKS

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LIST OF PUBLICATIONS

This dissertation is based upon the following manuscript in preparation:

Paola Mastrovito, Domenico Somma, Vincenzo Volpe, Marianeve Grieco, AlessioLepore, Antonio Leonardi. *LRRFIP1 negatively regulates IL-17 signalling by binding to CIKS*

Moreover I also contributed to the following publications:

- Volpe V., Raia Z., Sanguigno L., Somma D., Mastrovito P., Moscato F., Mellone S., Pacifico F., Leonardi A. **"**NGAL controls the metastatic potential of anaplastic thyroid carcinoma cells." *J Clin Endocrinol Metab*. 2013 ;98(1):228-35
- Domenico Somma, Paola Mastrovito, Marianeve Grieco, Alfonso Lavorgna, Anna Maria Salzano, Andrea Scaloni, Antonio Leonardi *CIKS/DDX3X interaction controls the stability of the Zc3h12a mRNA induced by IL-17* **Submitted**

ABSTRACT

Interleukin-17 (IL-17), the signature cytokine produced by T helper 17 (Th17) cells, plays crucial roles in host defense against microbial organisms and in the development of inflammatory diseases. IL-17 promotes the expression of cytokines and proteins involved in inflammatory responses, via the induction of gene transcription and post-transcription stabilization of mRNA. These functions are mediated by CIKS, an adaptor protein that is recruited to the receptor after IL-17 stimulation.

We shows here that LRRFIP1 isoform 3 is a new CIKS interactor. Over-expression of LRRFIP1 isoform 3 blocks the IL-17 induced gene expression, via down-regulation of NF-κB and AP-1. Accordingly, down-regulation of LRRFIP1 enhanced the expression of *cxcl1* and *il-6* after IL-17 stimulation. LRRFIP 1 isoform 3 exerts this function by interfering with the recruitment of CIKS to the cytoplasmic domain of the IL-17 receptor.

Collectively our finding defines a new mechanism regulating the inflammatory response.

BACKGROUND

T_H17 linfocites

T helper 17 cells (Th17) are a subset of T helper cells producing interleukin 17 (IL-17) developmentally distinct from Th1 and Th2 cells (Harrington et al., 2005; Park et al., 2005).

Originally IL-17 was thought to be produced exclusively by T cells, but it is now known to be secreted by a variety of innate cells including macrophages, mast cells, dendritic cells, and natural killer (Korn et al., 2009).

Whereas it was known for decades that IL-12 induces Th1 cells (IFN-γ producers) and IL-4 induces Th2 cells (IL-4, IL-5 and IL-13 producers), it was only recently demonstrated that Th17 cells differentiate upon exposure to combinations of IL-6 and transforming growth factor-β (TGF-β).

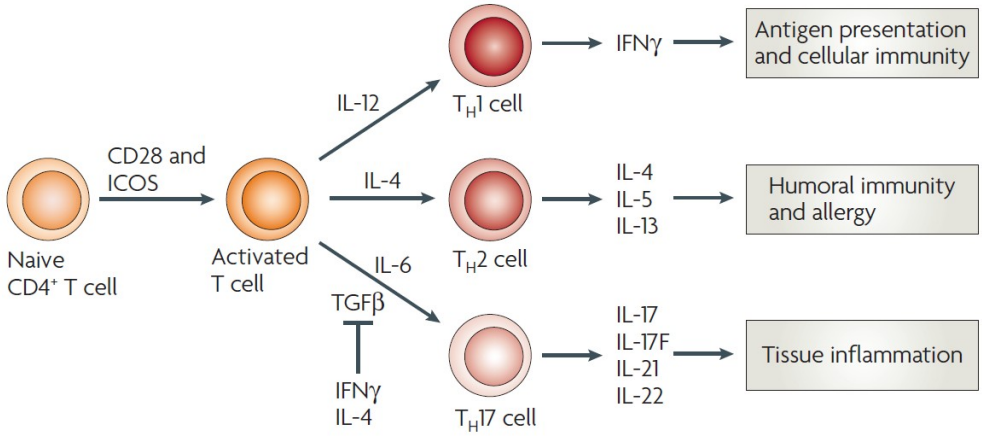

Figure 1: ***General scheme of T-helper-cell differentiation.*** *Nat Rev Immunol*. 2008 May; **8**(5):337-48.

Initial differentiation fase of mouse TH17 cells requires the presence of transforming growth factor-β (TGFβ) and IL-6 as well as STAT3 signalling (Bettelli et al., 2007). Analogous to the differentiation of TH1 and TH2 cell lineages, TH17 cells require additional lineage-specific transcription factors. Both retinoid-related orphan receptor-α (RORα) and RORγt are required for TH17 cell differentiation (Dong, 2008a), and these transcription factors can be induced by the combined presence of TGFβ and IL-6 in naive T cells (Chung et al., 2009; Martin et al., 2009; Sutton et al., 2009).

It has been argued that differentiation of human TH17 cells could

potentially occur independently of TGFβ, as a combination of IL-23 and IL-1β has been shown to be sufficient to induce TH17 cell differentiation. However, murine and human TH17 cells are very similar; both cell types express IL-17A, IL-17F, IL-21, IL-22, IL-23R and IL-1R1, and require RORα and RORγt as crucial transcription factors for their differentiation (Ghoreschi et al., 2010).

IL-17 is essential for host defence against many microbes, particularly extracellular bacteria and fungi, in particular for mucosal epithelia (O'Quinn et al., 2008). Interleukin-17 and other Th17 cytokines are linked to the pathogenesis of diverse autoimmune and inflammatory diseases. The IL-17 receptor is expressed ubiquitously, and hence most cells can potentially respond to this cytokine (Yao et al., 1997).

It has been noted that IL-17A expression can be induced within hours after infection or immunization with an adjuvant and before the differentiation of TH17 cells from native progenitors, which takes several days. This suggests that cells other than TH17 cells can produce IL-17.

Mast cells also produce several members of the IL-17 family. Following Fcε receptor I-mediated activation, mast cells produce substantial amounts of IL-17E, which can influence TH2-type responses (Ikeda et al., 2003). Mast cells also produce IL-17A in response to their activation by Toll-like receptor 2 (Mrabet-Dahbi et al., 2009); this has been shown in several diseases, including asthma, psoriasis (Lin et al., 2011), rheumatoid arthritis (Hueber et al., 2010; Moran et al., 2011), and amyotrophic lateral sclerosis (Fiala et al., 2010).

The respective contribution of IL-17 produced by these different cell types to disease pathology is still unknown.

Interleukin-17 mediates adverse effects in many autoimmune diseases. Before the discovery of the Th17 it was considered that Th1, Th2 and B cells were the main mediators of pathology in autoimmunity.

Based on these observations, inhibitors — mostly monoclonal antibodies — have been designed, and the results of these clinical trials are now being released.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disorder that may affect many tissues and organs, but principally attacks flexible (synovial) joints. It can be a disabling and painful condition, which can lead to substantial loss of functioning and mobility.

Inflammatory infiltrate of the joint synovium membrane leads to bone and cartilage destruction. The presence of IL-17-positive cells in the affected synovium and the production of functionally active IL-17 by this tissue was

first demonstrated in this disorder (Lubberts et al., 2002).

Interleukin-17 induces pro-inflammatory cytokines such as TNFα, IL-1β and IL-6 from cartilage, synoviocytes, macrophages and bone cells (Shen and Gaffen, 2008). Collectively, these pro-inflammatory cytokines contribute to RA increase and also establish a chronic inflammatory state by a positive feedback loop wherein IL-17-induced IL-6 maintains the Th17 T-cell population (Ogura et al., 2008). The IL-17 also stimulates the production of multiple chemokines, including IL-8/CXCL8, CXCL1, CXCL2, CCL20, CCL2 and CCL7 (Fossiez et al., 1996; Park et al., 2005; Shen and Gaffen, 2008; Shen et al., 2005a). These serve to recruit neutrophils, macrophages and lymphocytes to the synovium, thereby enhancing inflammation.

Inhibition of IL-17 with antibodies against the ligand IL-17A or its receptor IL-17RA protected against the development and consequences of arthritis (Lubberts et al., 2004). Furthermore, mice lacking IL-17RA develop a very mild form of experimental arthritis.

Psoriasis

Psoriasis is a chronic inflammatory skin disorder characterized by dermal hyperplasia. The key histological features of psoriatic skin are epidermal keratinocyte hyperproliferation, vascular proliferation and infiltration of Dentritic Cells, macrophages, neutrophils and T cells (Nestle et al., 2009).

Psoriasis has been successfully treated using TNFα inhibitors in some but not all patients. Skin biopsy samples taken from patients with psoriasis showed high expression of IL-17 together with high expression of IL-23, IL-22 and IL-6 (Zheng et al., 2007). Furthermore, increased numbers of TH1 and TH17 cells were found in blood and skin lesions of patients, showing a positive correlation with disease activity (Lowes et al., 2008). Local production of TH17 cytokines within the plaques appears to contribute to the increased production of chemokine CC motif ligand 20 (CCL20), a key chemokine that is necessary for the migration of TH17 cells (Harper et al., 2009). Mast cells and neutrophils represent additional sources of IL-17 in skin that is affected by psoriasis (Lin et al., 2011).

Multiple sclerosis

Multiple sclerosis is a chronic inflammatory disease that leads to brain inflammation in which the myelin around the axons of the brain and spinal cord are damaged, leading to demyelination leading to a broad spectrum of signs and symptoms

Experimental autoimmune encephalomyelitis (EAE) is a model of multiple sclerosis, a T-cell-mediated autoimmune disease of the central nervous system. It is elicited by immunization of neuroantigens, such as myelin basic protein and proteolipid protein. As with many other autoimmune conditions, Th1 cells were long thought to be responsible for EAE pathology, despite the fact that IFN-/-, IFNcR-/- and IL-12p35-/- mice were susceptible (Becher et al., 2002; Ferber et al., 1996; Zhang et al., 2003). Landmark studies comparing the IL-12p35-/- and IL-23p19-/- mice showed clearly that the Th17 path-way was responsible for pathology (Langrish et al., 2005).

Further evidence for the role of Th17 cells in driving EAE was shown in STAT6-/- / T-bet-/- doubly deficient mice, lacking Th1 and Th2 cells (Das et al., 2009). IL-17 drive the tight junctions distruption that form the blood–brain barrier, enabling Th17 cells to migrate into the central nervous system and cause neuronal damage (Kebir et al., 2007). Furthermore, EAE in the mice could be ameliorated by treatment with anti-IL-17 antibodies.

Asthma

Asthma is a common airway disorder that is characterized by chronic airway inflammation, mucus production, and airway hyperresponsiveness with airway remodeling.

Mouse models of asthma have yielded significant new insights recently. In particular, the cytokine IL-17E (IL-25) has been shown to have a role (Tamachi et al., 2006).

IL-17E was proposed to initiate and maintain Th2 responses. Furthermore, by directly and/or indirectly inducing the expression of, in particular, IL-13 and IL-5, IL-17E may be responsible for the observed mucus production and lung infiltration of eosinophils during allergic reactions (Claudio et al., 2009).

In asthmatic patients, IL-17 expression has been shown to increase in sputum, lung cells, bronchoalveolar lavage fluids, and peripheral blood (Barczyk et al., 2003).

Interleukin 17 Family

The IL-17 gene and IL-17 protein were first discovered as a product of T cells in rodents, but it was not immediately recognized as a cytokine, owing to its unusual amino acid sequence (Rouvier et al., 1993).

It is well established that IL-17 activity contributes to various aspects of acute inflammation. The IL-17-mediated release of IL-6 and IL-8 from mesenchymal cells leads to fever, an acute phase response (caused by IL-6) and the accumulation of neutrophils in blood and tissue (caused by IL-8)(Fossiez et al., 1996). IL-17 activity also contributes to chronic inflammation (Miossec, 2003), which is often — but not always — associated with matrix destruction.

The IL-17 molecule is composed of two monomers that are linked by intramolecular disulphide bonds on cysteine residues to form a homo or eterodimer. IL-17 is now formally referred to as IL-17A in the literature and is the founding member of the IL-17 family, which is composed of six members — from IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25), and IL-17F (Aggarwal and Gurney, 2002).

IL-17A and IL-17F are the best characterized cytokines of the IL-17 cytokine family. Both are covalent homodimers, and recent findings show that they also form IL-17A–IL-17F heterodimers (Chang and Dong, 2007).

IL-17A, IL-17F and the IL-17A–IL-17F heterodimer signal through the same receptor subunits: IL-17RA and IL-17RC (Kuestner et al., 2007; Wright et al., 2008). Results from mouse models, comparing IL-17A-/- and IL-17F-/- mice, indicate that some of the pro-inflammatory functions are not identical: IL-17A has a more important role in driving autoimmunity than IL-17F (Yang et al., 2008).

This effect is probably due to more activity of IL-17A in inducing inflammation: IL-17F-induced responses are 10–30 fold weaker in terms of downstream gene activation than those of IL-17A, with IL-17A–IL-17F heterodimers acting at an intermediate level (McAllister et al., 2005).

In contrast to the other members, IL-17E (IL-25) induces allergic responses and activation of the TH2 pathway and it is produced by mucosal epithelial cells and many immune cell types (Wang et al., 2007). Moreover, IL-17E inhibits Th17 cell development by inducing the expression of IL-13 by dendritic cells (DCs) or by inhibiting IL-23 production (Dong, 2008b; Kleinschek et al., 2007).

IL-17B, IL-17C and IL-17D functions are poorly defined .

IL-17 Receptors

The first receptor to be identified for IL-17 was initially referred as IL-17 receptor (IL-17R) but is now known as IL-17RA (Yao et al., 1995). Afterwards additional receptor components, required to form a functional receptor complex for IL-17 signaling, were identify (Yao et al., 1997).

Four additional receptors have been identified in the IL-17R family, based on sequence homology to IL-17RA: IL-17RB, IL-17RC, IL-17RD and IL-17RE, thus IL-17RA appears to be a common receptor chain for the IL-17 family of ligands.

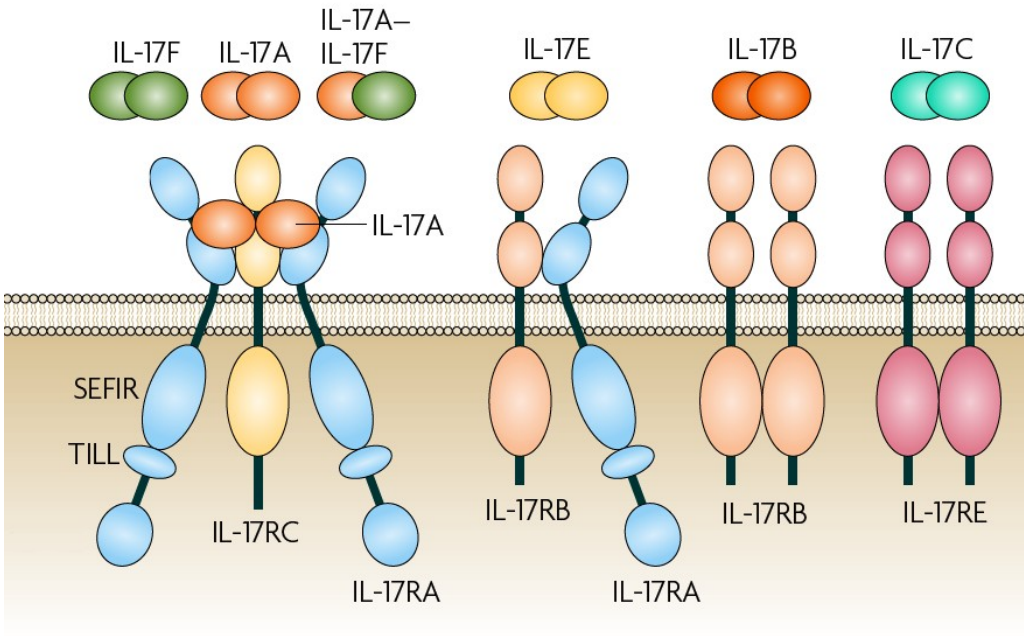

Figure 2: ***IL-17R family ligand–receptor relationships and main structural features.*** *Nat Rev Immunol.* 2009 Aug;**9**(8):556-67.

All receptor subunits are single transmembrane domain-containing proteins, these receptor subunits contain certain conserved structural motifs, including an extracellular fibronectin III-like domain and a cytoplasmic domain named SEFIR, a member of STIR-domain superfamily.

STIR-domain superfamily comprising TIR (Toll/Il-1 Receptor) domain, and SEFIR (SEF/IL-17R) domain.

TIR domain has three 'boxes' of conserved residues and a important binding loop named BB-loop, SEFIR domain keep the same structure of TIR

domain, but lack the TIR box 3 subdomain and the BB-loop (Novatchkova et al., 2003). However, a region at the carboxy-terminal side of the SEFIR domain in IL-17RA has marked sequence homology to BB-loops. Deletion or point mutations in this region render IL-17RA non-functional, so this motif is referred to as a TIR-like loop (TILL)(Maitra et al., 2007; Shen et al., 2009).

Surprisingly, although all IL-17Rs have a SEFIR motif, the TILL domain seems to be unique to IL-17RA, perhaps explaining why IL-17RA functions as a common subunit shared by several receptors in the family.

As mentioned IL-17RA is necessary for signal transduction mediated by IL-17A, IL-17A–IL-17F and IL-17F (Hymowitz et al., 2001). Indeed, IL-17RA pairs with IL-17RC to induce responses to IL-17A and IL-17F (Toy et al., 2006). Similarly, IL-17RA–/– mice are refractory to the effects of IL-17E, suggesting that IL-17RA is also a component of this receptor complex (Rickel et al., 2008).

IL-17RA is expressed ubiquitously, with particularly high levels in haematopoietic tissues (Ishigame et al., 2009; Yao et al., 1995), but only a limited number of IL-17A-induced genes has been documented in lymphocytes, and these genes are distinct from those induced by IL-17A in other cell types (Hsu et al., 2008; Ishigame et al., 2009), like endothelial cells and fibroblasts.

Another function of IL-17RA seems to limit signalling by receptor-mediated internalization of the ligand. Indeed, surface expression of IL-17RA rapidly decreases after IL-17A binding (Lindemann et al., 2008).

The precise stoichiometry of the IL-17A-binding receptor complex has not been determined, but some data suggest the existence of a trimeric complex containing two IL-17RA subunits and one IL-17RC subunit (You et al., 2006).

In contrast to IL-17RA, IL-17RC expression is low in haematopoietic tissues and high in non-immune cells of the prostate, liver, kidney, thyroid and joints (Haudenschild et al., 2002; Kuestner et al., 2007). This differential expression of IL-17R subunits is coherent with a mechanism for tissue-specific signalling by IL-17A and/or IL-17F.

IL-17RB instead binds both IL-17B and IL-17E. This subunit is expressed by various endocrine tissues as well as kidney, liver and Th2 cells (Lee et al., 2001). Recent evidence indicates that IL-17RB pairs with IL-17RA to form a functional receptor complex for IL-17E (Rickel et al., 2008). IL-17RB is the one IL-17R that has a TRAF6-binding motif in its cytoplasmic tail (Maezawa et al., 2006).

IL-17RD and IL-17RE has no known ligand and their function is unknown.

IL-17: molecual pathway

IL-17 stimulation leads to two major biological responses: 1) de novo transcription and, 2) stabilization of mRNA. Both biological responses are medieted by CIKS (Sun et al., 2011) (also known as ACT1 or TRAF3IP2) that is recruited to the receptor after IL-17 stimulation. Indeed genetic ablation of CIKS gene in mice, leads to unresponsiveness to IL-17 stimulation (Claudio et al., 2009).

CIKS contains a SEFIR domain, an E3 ubiquitin ligase domain (Liu et al., 2009), and a binding site to TRAF6 (Sønder et al., 2012). The binding between CIKS and IL-17RA is mediated by a SEFIR-SEFIR domains interaction. In particular CIKS binds to the CC′ loop region of the cytoplasmic tail of IL-17RA.

After receptor binding CIKS recruits and ubiquitinates TRAF6 through K63 in a U-box – dependent manner (Liu et al., 2009).

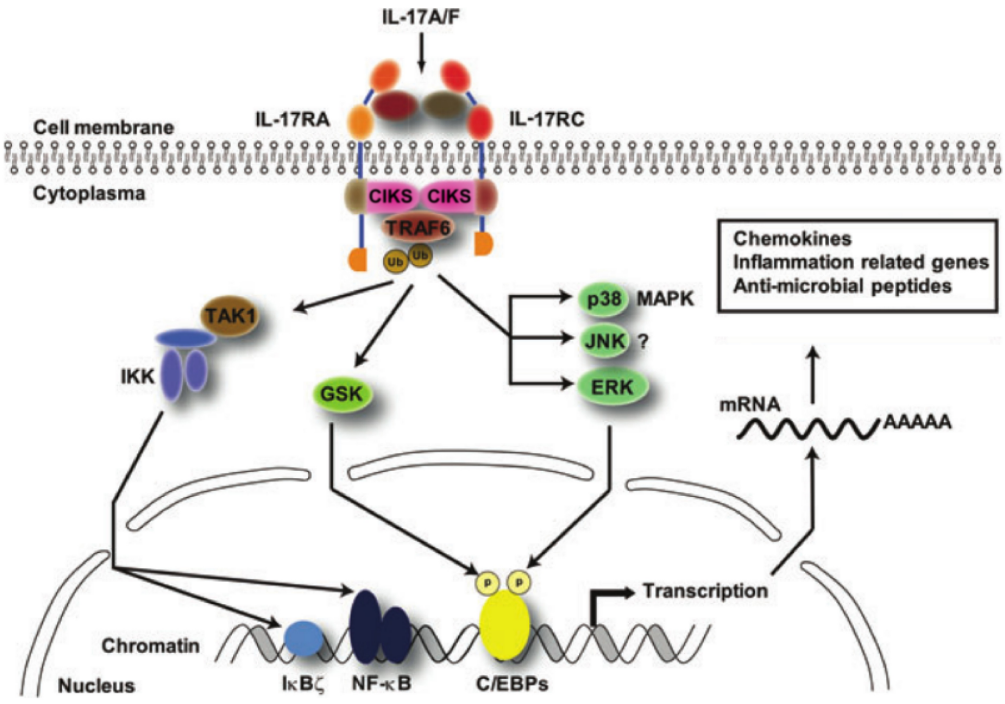

Figure 3: ***IL-17 signaling pathway****. Modified by Ann N Y Acad Sci.* 2011 Jan;1217:60-76

This signal drives TRAF6 to the activation of NF-κB via TAK-IKK

complex (Deng et al., 2000). Like the activation of NF-κB by TLR, IKK complex induces the phosphorylation, degradation of IκBα and subsequent activation of NF-κB (Liu et al., 2009; Schwandner et al., 2000). Some data demonstrate that NF-κB p65 and p50 subunits quickly associate with NF-κB response element upon IL-17 stimulation (Ruddy et al., 2004; Shen et al., 2006). There is no evidence of activation of the noncanonical NF-κB subunits. Interestingly, IL-17 also induces expression of IκBζ (Shen et al., 2005a), a positive regulator of NF-κB activation (Yamamoto et al., 2004). Recently, additional evidence suggests that IκBζ plays an important role in IL-17–induced expression of human β-defensin 2 and neutrophil gelatinase-associated lipocalin (Karlsen et al., 2010).

IL-17 signaling also activates C/EBPs. Among all C/EBP family members, only C/EBP- β and C/EBP-δ are transcriptionally up-regulated by IL-17 (Shen et al., 2005b). The cooperation of NF-κB and C/EBPs can only partially explain the synergistic effect of IL-17 and TNFα.

IL-17A also activates the MAPK pathway. Within 30 minutes of IL-17A stimulation, ERK is rapidly phosphorylated and then regulates various downstream signaling components, including NF-κB, C/EBP, and AP1 (Maitra et al., 2007).

Expression of the IL-17 target genes dependent

IL-17 target genes can be categorized into five major groups:

1) chemokines;
2) proinflammatory cytokines and inflammatory mediator related genes;
3) antimicrobial peptides;
4) tissue remodeling genes;
5) signaling components such as C/EBPβ , C/EBPδ , and IκBζ

CXCL1, CXCL2, and CXCL5 are the major mediators of the IL-17–induced neutrophil response in host defense and various inflammatory disease models. IL-17F alone or in combination with IFNγ can stimulate CXCL10 production in bronchial epithelial cells (Kawaguchi et al., 2007). Other CXC chemokines such as CXCL6, CXCL8, and CXCL12 are also regulated by IL-17. CCL2 and CCL20 are among the few CC chemokines up-regulated by IL-17 (Kao et al., 2005).

IL-17A induces production of other proinflammatory cytokines in various cell types. For example, IL-6 is one of the earliest target genes reported in IL-17A–stimulated fibroblasts (Yao et al., 1995). G-CSF is another important cytokine that is induced by the IL-17 pathway.

IL-17 (like IL-1 and TLR) also induce a anti-inflammatory RNase: Zc3h12a (also know as MCPIP or MCPIP1) that selectively degrades specific target mRNA, like IL-1, IL-6 or IL-12, to modulates the immune response and inflammation (Huang et al., 2012). Zc3h12a recognizes the 3'-untranslated region (UTR) of the mRNA, it is able to cleaved the mRNA and it is required for normal decay of mRNA. It triggers apoptosis and promotes angiogenesis in response to the binding of CCL2 to CCR2 (Matsushita et al., 2009). Zc3h12 is also a Interferon Stimulated Genes (IGSs) induced by interferon-γ, and it has been hypothesized to suppress the antiviral state (Abe et al., 2011).

Antimicrobial peptides are important mediators of innate immunity; and IL-17A is a potent inducer of various antimicrobial peptides. Lipocalin 2 (24p3) plays an important role in host defense against E. coli and Klebsiella pneumoniae by limiting bacterial siderophores to bind free iron (Goetz et al., 2002). In fibroblast cells, IL-17A alone or with TNFα results in quick induction of 24p3 protein (Shen et al., 2006). As another example, human β-defensin 2 (hBD2) is an antimicrobial peptide and a chemo-attractant to CCR6+ cells. hBD2 is markedly up-regulated by IL-17A in human airway epithelium and during virus infection (Kao et al., 2008).

IL-17A also induces expression of MMPs, including MMP1, MMP2, MMP9, and MMP13, which are the major players in extracellular matrix degradation and bone resorption (Sylvester et al., 2004). Mice lacking IL-17RA fail to express MMPs in streptococcal cell wall induced-arthritis and show amelioration of cartilage destruction (Koenders et al., 2005).

Leucine-Rich Repeat Interacting Protein-1 – LRRFIP1

Leucine-rich repeat in Flightless-1 interaction protein 1/GC-binding factor 2 (LRRFIP1/GCF2) belongs to a small family of proteins whose function and regulation have been poorly studied. It was originally identified either as repressor of the epidermal growth factor receptor gene (Reed et al., 1998) or as interactor of the mammalian homolog of *Drosophila* Flightless I (Fliih-I), a member of gelsolin family (Liu and Yin, 1998). LRRFIP1 was found in both nuclear and cytoplasmatic compartments in cells (Ariake et al., 2012; Ohtsuka et al., 2011).

The study of LRRFIP1 gene and cDNA, showed that the gene encodes

different splice isoforms in human and mouse species, which may be differentially expressed and regulated (Fong and de Couet, 1999; Liu and Yin, 1998; Reed et al., 1998; Rikiyama et al., 2003; Suriano et al., 2005). Five human LRRFIP1 isoforms have been reported and three of them, namely, GCF2, LRRFIP1 and TAR RNA interacting protein (TRIP), have been described as proteins functionally different. Indeed, GCF2 is a trascriptional repressor, LRRFIP1 is a modulator of the innate immune response translation, and TRIP is an RNA-binding protein and possible regulator of mRNA translation (Gubern et al.). Northern blot analysis showed that LRRFIP1 mRNA was differentially expressed in many human tissues and cell lines. LRRFIP1 mRNA was expressed in most human tissues with the highest expression level in peripheral blood leukocytes and lowest expression in brain and testis. Therefore, LRRFIP1 protein is present at high levels in Burkitts' lymphoma and several other cancer cell lines (Rikiyama et al., 2003). LRRFIP1 has been indentified as one of the cancer-associated genes (Sjöblom et al., 2006) which promotes cell invasion and metastasis (Arakawa et al., 2010). Finally, LRRFIP1 plays also a role in the formation of thrombosis. Indeed, its shRNA might represent a promising prevention strategy for deep vein thrombosis(Yin et al., 2013).

LRRFIP1 isoform1 contains an N-terminal domain of unknown function, a conserved 87-amino acid domain predicted to be a coiled coil, and a nucleic acid binding domain. The coiled coil domain, which is found in all LRRFIP1 genes (isoform 1, isoform 2 and isoform 3), is highly conserved across mammalian species and is required for interaction with other proteins such as the leucine-rich repeat (LRR) domain of Fliih-I (Fong and de Couet, 1999).

With regard to the mouse isoforms, three different proteins have been reported, two of which, Fli-I leucine-rich repeat associated protein 1 (Flap-1) and Lrrfip1, have been studied. Dai *et al.* found that Flap-1 compete with Fliih-I at the same binding site at MyD88, suggesting a functional role in TLR-mediated NF-κB activation and cytokine production(Dai et al., 2009). On the other hand, it is able to directly bind dsRNA as well as GC-rich ds-DNA. Indeed, LRRFIP1 detects exogenous dsDNA through its nucleic acid-binding domain and increases the production of type I interferon, possibly by promoting β-catenin activation (Yang et al., 2010). Both Flap-1 and Lrrfip1 directly interacts with β-catenin and activates β-catenin-dependent trascription activity(Lee and Stallcup, 2006).

The three isoforms appear to be different from each other, as shown in fig. 4A. Isoform 1 and isoform 2 have the same exon 1 which is different from exon 1 of isoform 3. Moreover, the three isoforms present common exons and there are homology between the C-term of isoform 2 and isoform 3. In fig. 4B, is shown the schematic representation of the three different proteins that are composed, respectively, by 729, 628 and 428 aa. Only the isoform 1 contained

the nuclear localization signal (NLS) and the RNA-binding domain. All isoforms contained coiled-coil domain essential of interaction with other proteins.

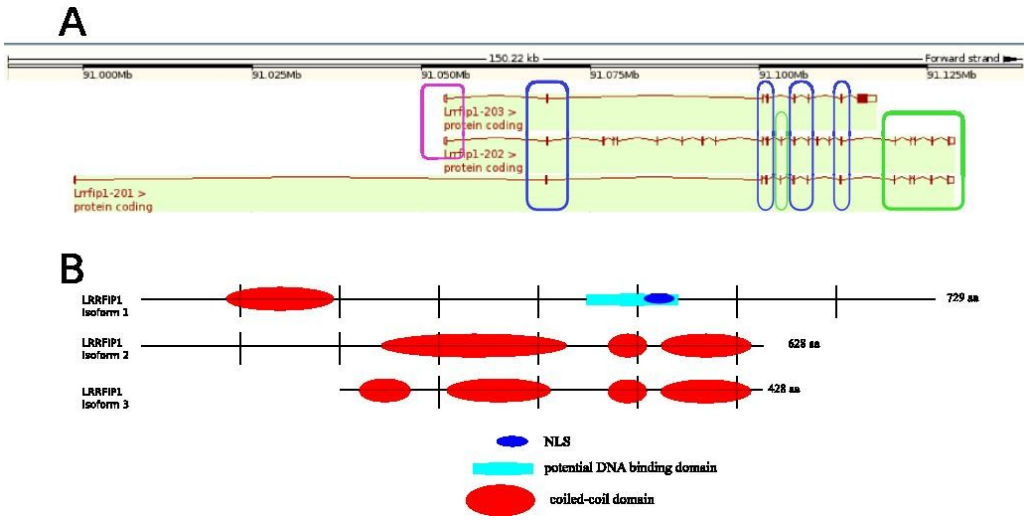

Figure 4:A, schematic rappresentation of murine LRRFIP1 transcrip variants. Exons are shown as vertical bars. In blue are indicated the portions common to the three isoforms,in pink the exons common to isoform 1 and isoform 2 while in green are showed the exons common to isoform2 and isoform 3. B, schematic rappresentation of murine LRRFIP1 proteins. Isoform 1 is 729 aa and presents a coiled-coil domain, a NLS and a DNA-binding domain. Isoform 2 is 628 aa long and presents only a coiled-coil domain. Most of this domain is also present in isoform 3 that is 428 aa long..

Therefore, LRRFIP1 shows itself to be an interesting gene through which different isoforms may modulate multiple signaling pathways involved in different biological functions.

AIM OF THE STUDY

IL-17 plays a critical role in host defense against different pathogens in mucosal epithelium. IL-17 induces and sustains tissue inflammation and drives the immune responses, mainly by two different mechanisms. The first is transcription dependent and involves transcription of genes controlling inflammation, the second is dependent upon the stabilization of different mRNA induced by other inflammatory cytokines, such as TNFα.

In the last few years some of the mechanisms regulating these two functions have been elucidated. However, much remains to be understood about this molecular pathway. The present study aims to indentify new protein involved in the IL-17 signaling, in order to unravel the molecular mechanism regulating the IL-17 dependent inflammatory responses.

MATERIAL AND METHODS

Reagents, cell lines, and constructs.

Recombinant IL-17 and TNF-alpha were from Peprotech, and were used at 200ng/ml and 2000U/ml, respectively. Anti-M2 (Flag) was from Sigma-Aldrich.

HEK293, wild type mouse embryonic fibroblast (MEF), CIKS$^{-/-}$ MEF, were maintained in Dulbecco modified Eagle medium (DMED) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), antibiotics (100 μg/mL penicillin, 100 μg/mL streptomycin), and 1 mM *L*-glutamine (Invitrogen). WT and CIKS$^{-/-}$ MEF were a gift of U. Siebenlist (NIAID, NIH).

Single stable transfected clones were generated transfecting plasmid encodes HA-LRRFIP1 isoform3 and the single clones were isolated after selection with 5μg/ml of puromycin.

The cDNA encoding CIKS and its mutants were previously described (PNAS, BBRC). The plasmids expressing LRRFIP1 isoform 2 or LRRFIP1 isoform 3 were from OriGene . LRRFIP1 isoform 2 and LRRFIP1 isoform 3 mutants were generated by PCR and cloned in pCDNA3.1-HA or FLAG (Invitrogene).

RNA-interference (RNAi) and transfection.

Cells were transfected with small interfering RNA oligonucleotides (20nM final concentration) and Interferin (PolyPlus) according to manufacturer's instruction. The siRNA sequences used are:

Mouse LRRFIP1 5′-GGACCAGAUUCAGGAUGUA-3′

The scrambled control was from Thermo Scientific (siRNA ON-TARGETplus Non-targeting Pool #D-001810-10-05).

Seventy two hours after transfection cells were collected for RNA extraction.

Lentivirus production and infections.

FLAG-CIKS cDNA was subcloned into pWPT lentiviral vector at BamHI/SalI sites. The construct was sequenced to confirm correct DNA sequence and orientation. Subconfluent 293T lentivirus packaging cells were cotransfected with either pWPT-GFP or CIKS-FLAG-pWPT and pMD2G and pCMV-R8.91 by calcium phosphate precipitation. After 24h medium was changed, and supernatant was harvested after 48 and 72 hours. Lentiviral supernatant, cleared of cell debris, was concentrated by centrifugation for 90 min at 23000 rpm at 4°C. For transduction, $CIKS^{-/-}$ MEFs were plated on 12-well plate, infected with lentiviruses in the presence of 10% foetal bovine serum and 8µg/ml polybrene (Hexadimethrine bromide; Sigma-Aldrich, Milano, ITALY).

Coimmunoprecipitation and immunoblot analysis.

$CIKS^{-/-}$ MEF reconstituted with FLAG-CIKS or with empty vector were left untreated or treated with IL-17 at different time points, were harvested by washing with ice-cold PBS, and then lysed with Lysis buffer (20 mM HEPES pH7.5, 150 mM NaCl, 1% Triton X-100, 10% Glycerol, 10mM NaF, 1mM Na_3VO_4) freshly supplemented with protease inhibitors cocktail (Roche). Nuclear and cellular debris were removed by centrifugation at 14,000 x *g* for 30 min at 4°C. Cell extracts were normalized for protein content, and incubated with 20 µl of M2 beads for 3 hours at 4°C. Beads were washed five times with Lysis buffer, and boiled in SDS sample buffer, and the resulting supernatants were subjected to 10% SDS/PAGE.

For identification of the CIKS-interacting proteins, 100mg of cell extract from $CIKS^{-/-}$ and reconstituted MEF were incubated with 500 µl of M2 beads (Sigma) pre-washed with Glycine pH3.0 to eliminate not covalently bound antibody. After 3 hours incubation at 4°C, beads were washed four times with Lysis buffer, 2 times with High Salt (1M NaCl) Lysis buffer. Proteins were eluted from the beads by using 500µl of 3xFLAG peptide (Sigma) 200mg/ml. Eluated proteins were sent to Scaloni Andrea (CNR) for mass spectrometry.

RNA isolation and Real-Time PCR.

Total RNA was extracted by using TRIZOL reagent according to manufacturer's instruction (Invitrogene). Real-time RT-PCR was carried out with cDNAs reverse-transcribed from total RNA by using GoTaq qPCR Master Mix (Promega, Madison, WI, USA) and Bio-Rad CFX Manager software (Bio-Rad, Segrate, MI, ITALY). The primers used were:

LRRFIP1 isoform 3:	FW: 5'-GATGGACATGGGCACGCA-3'
	REV: 5'-GTTACGGGAGCCCTTCTCAG-3'
GAPDH:	FW: 5'-ATGGTGAAGGTCGGTGTGAAC-3'
	REV: 5'-CCATGTAGTTGAGGTCAATGAAG-3'
Cxcl1:	FW: 5'-AGCCACCCGCTCGCTTCTCT-3'
	REV: 5'-GTCCCGAGCGAGACGAGACCA-3'
IL6:	FW: 5'-AAAGCCAGAGTCCTTCAGAGAGA-3'
	REV: 5'-GGTCCTTAGCCACTCCTTCTGTG-3'

Luciferase assay.

Either the Ig-κB-Luc or AP-1-Luc or ISRE-Luc were used as a reporter plasmids. Cells were cotransfected with a mixture of luciferase reporter plasmid, the thymidine kinase promoter-renilla luciferase plasmid pRL-TK and various amount of CIKS, LRRFIP1 isoform3 constructs by using Lipofectamine reagent (Invitrogen). 24h after transfection, cells were harvested or stimulated with IL-17 or TNF-α for 4h and cell extracts were prepared. Luciferase activity were measured with Dual-Luciferase Reporter Assay system according to the manufacturer's instruction (Promega). Data were normalized for transfection efficiency by dividing the firefly luciferase activity by the renilla luciferase activity.

RESULTS

Identification of LRRFIP1 as a specific CIKS-interacting protein.

In order to clarify the molecular mechanism regulating IL-17 signaling we decided to isolate new CIKS interactors.

We set up an experimental system constituted by MEF (Mouse embryonic fibroblast) isolated from CIKS$^{-/-}$ mice, and the same cells reconstituted by lentiviral infection, with FLAG-CIKS.

The level of expression of FLAG-CIKS in the reconstituted cells, was comparable to the endogenous protein. In fact, no transcription of IL-17 dependent genes was detected in unstimulated cells, while a strong transcriptional response was observed after IL-17 stimulation, comparable to that detected in wt MEF (Fig 5). In addition, when the cells were stimulated with IL-17 plus TNFα, there was a synergistic effect on mRNA levels. This effect was due to mRNA neo-synthesis through TNFα signaling, and to mRNA stabilization induced by IL-17 (Hartupee et al., 2007; Henness et al., 2004). As shown in figure 5 treatment of wt and reconstituted MEF with IL-17 results in upregulation of different IL-17 target genes: *cxcl1* (B), *ccl2* (C), *cxcl5* (D), *IL6* (E), *Lcn2* (F). In the absence of CIKS both the IL-17 dependent transcription and the IL-17 dependent mRNA stabilization were absent.

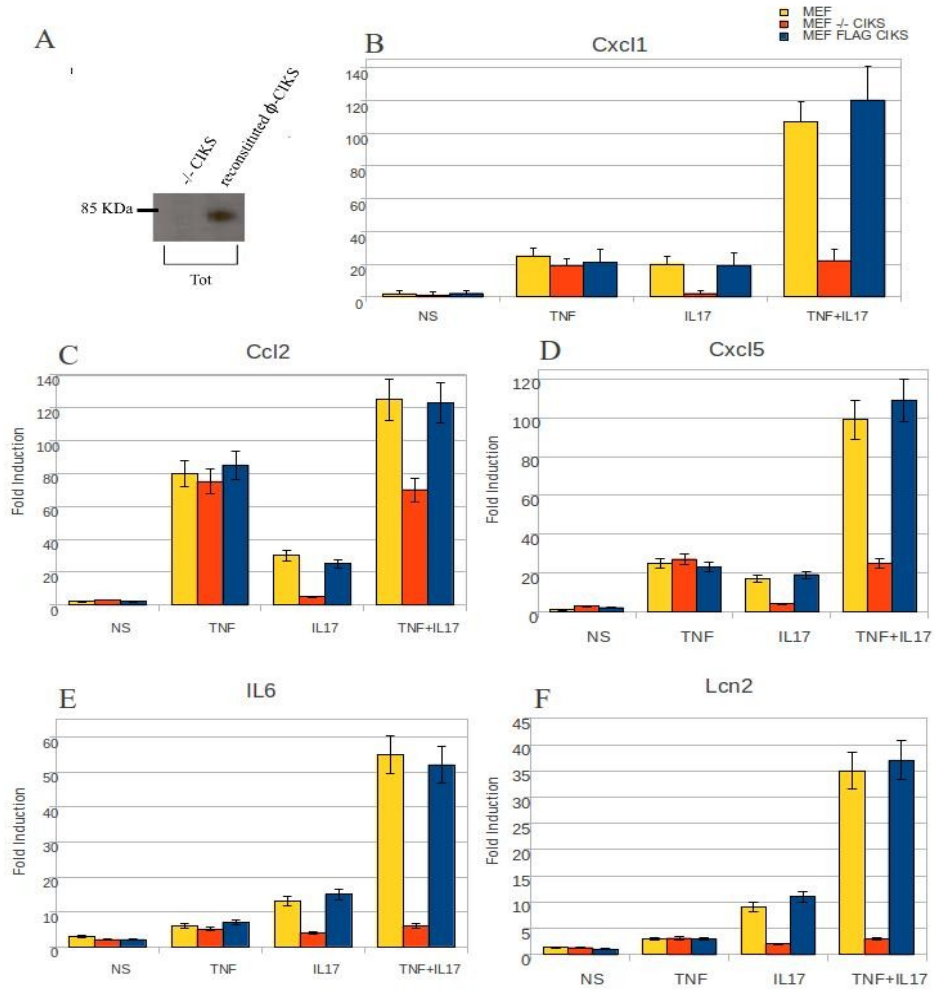

Figure 5: Functional characterization of WT, CIKS$^{-/-}$, and reconstituted MEF. Cells were stimulated with TNFα, IL-17 or both cytokines for 6 hours, RNA extracted and the level of the indicated RNAs were assessed by Real Time PCR.

To search for CIKS-interacting proteins, cell extracts from CIKS$^{-/-}$ and reconstituted MEF were immunoprecipited and analysed by mass spectrometry. We found about 30 interactors immunoprecipitated from the reconstituted cells but not in the CIKS$^{-/-}$ cells, some of them are already known as CIKS interactors, such as IKKε and SF2.

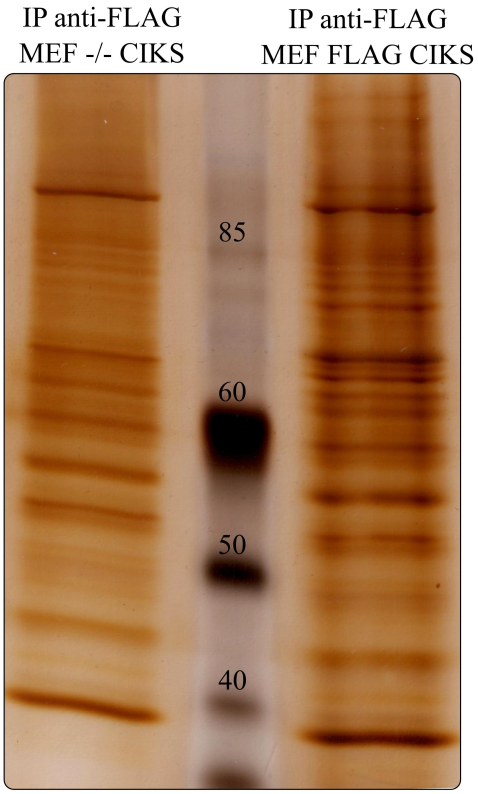

Figure 6: Immunoprecipitation of FLAG-CIKS followed by mass spectrometry analysis. Cell lysates were immunoprecipitated with anti-FLAG Ab and eluted wit 3xFLAG peptide. The eluted proteins were separated by SDS-PAGE followed by silver stain.

One of the CIKS interactors we isolated was LRRFIP1. LRRFIP1 is a leucine rich repeat interacting protein, involved in different biological functions. Three isoforms have been isolated in mice, each with substantial differences in both amino acid sequence and biological functions. We then identified which isoform was interacting with CIKS. We found that 36 peptides were common among the three different isoforms, no peptides were specific for isoform 1, 11 peptides were specific to isoform 2 and 3 and 18 peptides

belongs specifically to isoform 3 (tab. 1). This result suggest that the isoform 3, of LRRFIP1 is interacting with CIKS.

LRRFIP1 ISOFORMS	Peptides
Isoform 1	0
Isoform 2 and isoform 3	11
Isoform 3	18
All 3 isoforms	36

Table 1: peptides obtained by mass spectrometry analysis were compared with the aminoacid sequence of the 3 different isoforms of LRRFIP1. The number of peptides specific for a given isoform are indicated.

To confirm the interaction between CIKS and LRRFIP1, both isoforms 2 and 3 of LRRFIP1 were cloned in pcDNA3.1 and transiently trasfected in HEK-293 cells. As shown in figure 7 both isoforms of LRRFIP1 were co-immunoprecipitating with FLAG-CIKS, but the interaction between CIKS and LRRFIP1 isoform 3 was stronger compared with the interaction between CIKS and LRRFIP1 isoform 2. From this result we decided to investigate only LRRFIP1 isoform 3.

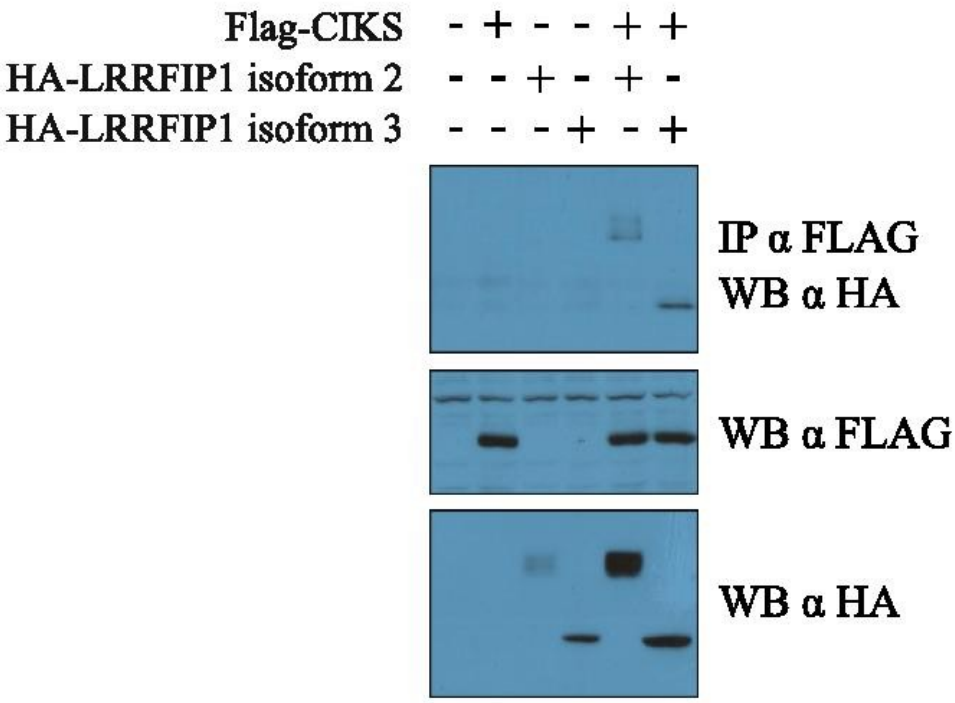

Figure 7: CIKS interacts with LRRFIP1 isoform2 and LRRFIP1 isoform 3. HEK293 were trasfected with expression plasmids encoding HA-tagged LRRFIP1 isoform2 or HA-tagged LRRFIP1 isoform3 and FLAG-tagged CIKS . The immunoprecipitates were analysed by Western blot with anti-HA (upper panel). Expression of CIKS and both isoforms of LRRFIP1 was confirmed by western blot with anti-HA (lower panel) and anti-FLAG (middle panel) Abs, respectively.

Mapping of the interaction between CIKS and LRRFIP1 isoform 3.

Next, we investigated the region of LRRFIP1 isoform 3 involved in the binding of CIKS, and the region of CIKS necessary to interact with LRRFIP1 isoform 3. HEK-293 were transfected with different deletion mutants of CIKS together with LRRFIP1 isoform 3. We immunoprecipitated full lenght FLAG-CIKS and its deletion mutants demonstrating that both N-term and C-term domains of CIKS were essential for the binding to LRRFIP1 isoform 3, as shown in fig 8.

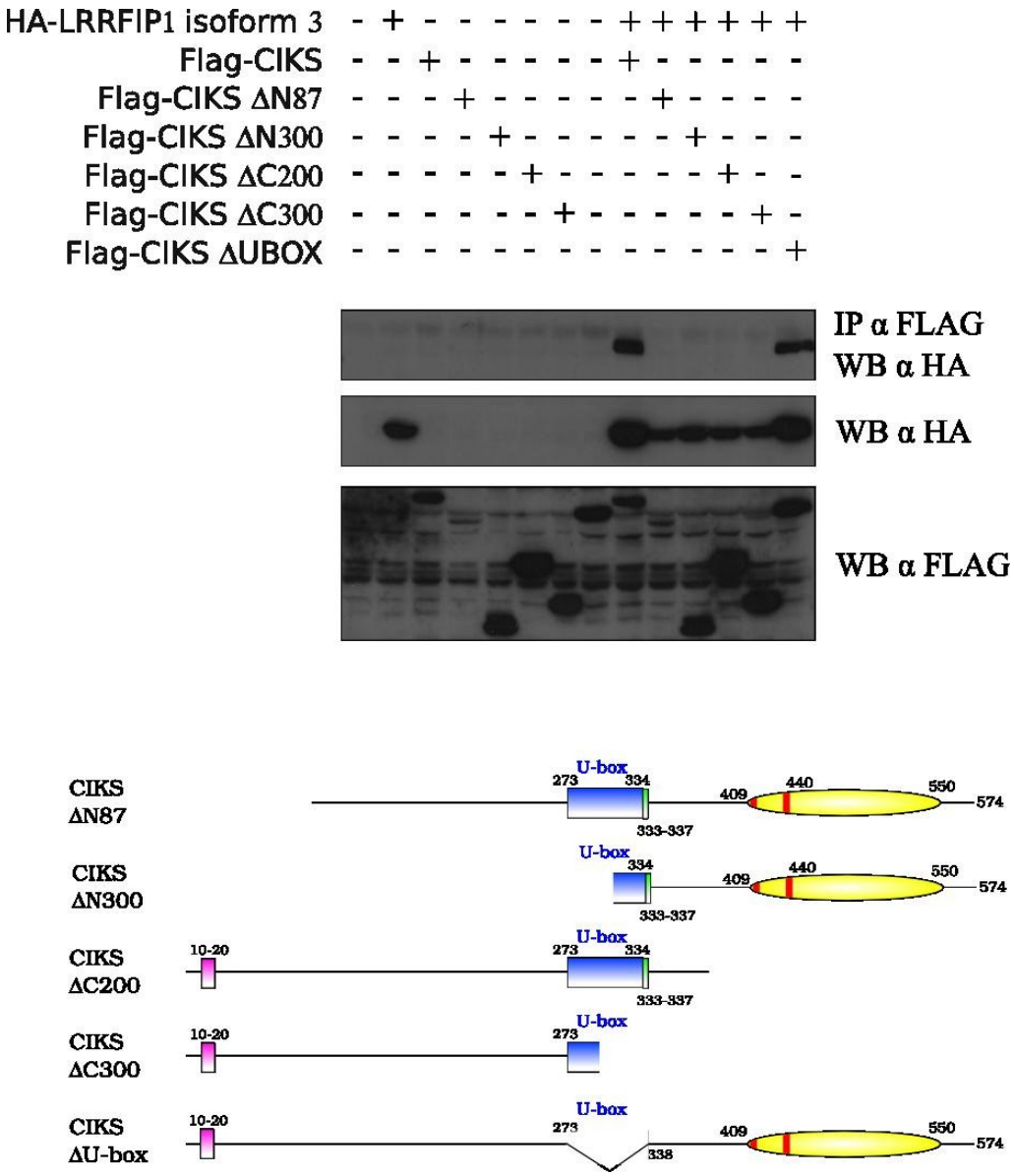

Figure 8:Identification of the domain of CIKS involved in the interaction with LRRFIP1 isoform 3. HEK 293 were trasfected with an expression plasmid encoding HA-tagged LRRFIP1 isoform 3 and FLAG-tagged CIKS full-length or different deletion mutants. The immunoprecipitates were analysed by Western blot with anti-HA (upper panel). Expression of CIKS and LRFFIP1 isoform 3 was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively.

Next, we investigated the region of LRRFIP1isoform 3 involved in the binding to CIKS. We performed a co-immunoprecipitation assay between FLAG-CIKS and HA-tagged LRRFIP1 isoform 3 full length or bearing two different mutations. Fig. 9 shown that LRRFIP1 isoform3 interacts with CIKS by its N-terminus domain.

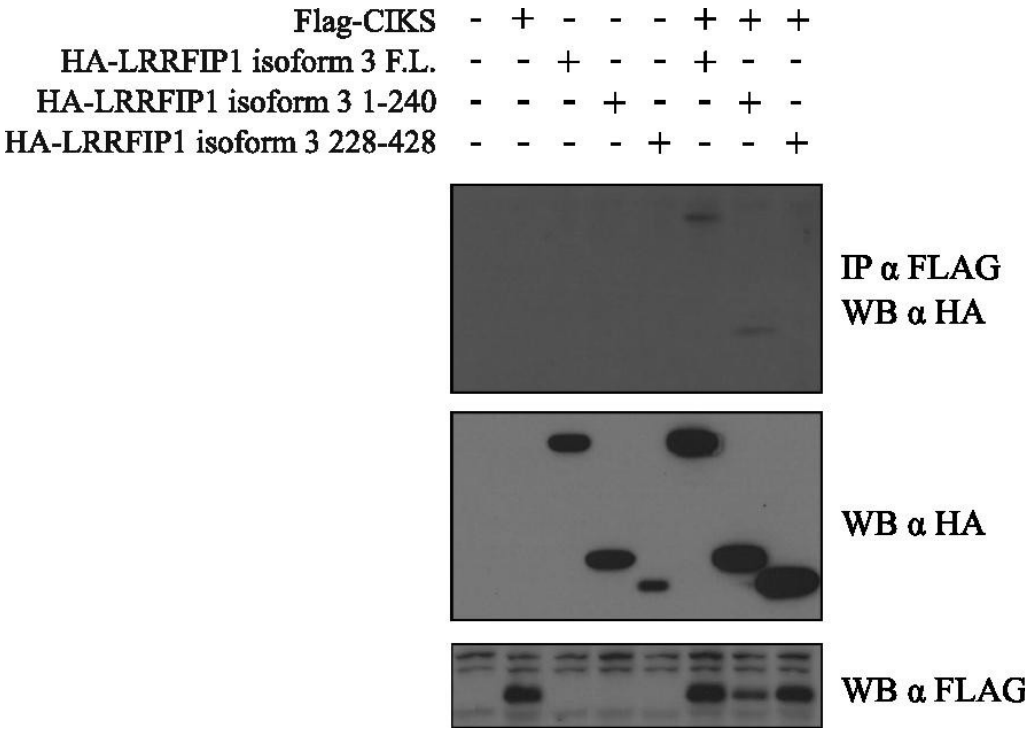

Figure 9:Mapping of LRRFIP1 isoform 3 interaction with CIKS. HEK 293 were transfected with different expression plasmids for HA-tagged LRRFIP1 isoform 3 (wt or LRRFIP1 isoform3 1-240 that lose C-terminal portion, or LRRFIP1 isoform3 228-428 that lose N-terminal portion.) and FLAG-tagged CIKS. The immunoprecipitates were analyzed by Western blot with anti-HA Ab (upper panel). Expression of CIKS and LRRFIP1 isoform3 was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively.

LRRFIP1 isoform 3 regulates NF-κB and AP1.

To investigate if LRRFIP1 was interfering with some of the CIKS-mediated functions, we performed a Luciferase assay evaluating the activity of a NF-κB- and AP-1-driven reporter plasmids. HEK-293 were trasfected with either a NF-κB reporter plasmid, or an AP-1 reporter plasmid in the presence of

a plasmids encoding CIKS with or without the plasmid encoding LRRFIP1 isoform 3. As shown in fig.10, over-expression of CIKS strongly activated the transcription activity of both NF-κB and AP-1. In contrast, over-expression of LRRFIP1 isoform 3 did not affect the activation of both reporter genes. When CIKS and LRRFIP1 isoform 3 were co-expressed, the CIKS-mediated NF-κB and AP-1 activation were not affected.

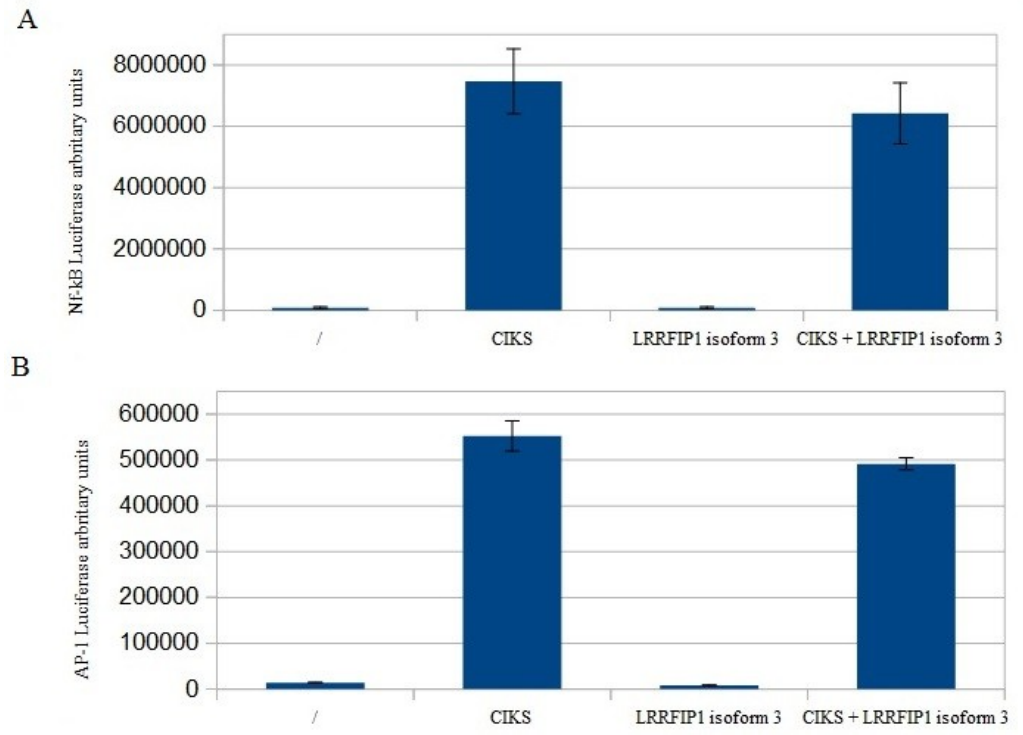

Figure 10:LRRFIP1 is unable to activate NF-ᵡ B and AP-1 after trasfection. A, Luciferase activity of HEK293 cells transfected with Ig-ᵡ B-luciferase reporter plasmid (A) or AP-1 reporter plasmid (B) and renilla luciferase palsmid, together with plasmid expressing CIKS or LRRFIP1 isoform 3.

In order to investigate if LRRFIP1 was involved in the regulation of the IL-17 signaling, we generated MEF stable clones over-expressing LRRFIP1 isoform 3. Fig11 shows two of the clones we isolated, expressing different level of LRRFIP1.

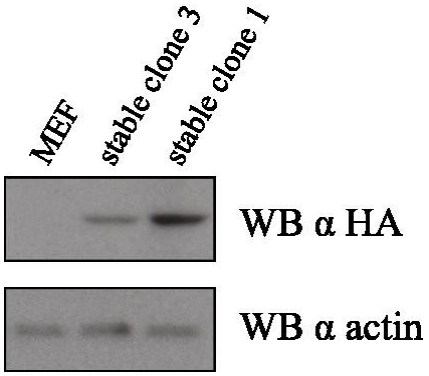

Figure 11:single stable clones of LRRFIP1 isoform3. Expression of LRRFIP1 isoform3 was confirmed by western blot with anti-HA (upper panel). The lower panel shows the normalization by β-actin.

In these cells we evaluated the promoter activity of NF-κB and AP-1 after stimulation with IL-17 and TNF-α. Cells were transfected with the indicated reporter plasmid (Fig 12), and 24h after trasfection, cells were stimulated for 4h with IL-17, TNF-α or both cytokines. As shown in fig.12, treatment of mock transfected cells with TNF or IL-17 resulted in the activation of both NF-κB and AP-1 reporter plasmid. Treatment with both cytokines further increased the reporter plasmids activation, due to the synergistic effect of TNF and IL-17. In the two clones overexpressing LRRFIP1 isoform 3, while the TNF-induced NF-κB and AP-1 activation proceeds unhampered, both the IL-17-induced and the TNF plus IL-17 induced NF-κB and AP-1 activation was almost completely blocked (Fig12A and B).

This results together with the data reported in fig 11, suggest that LRRFIP1 isoform3 is interfering with the IL-17 pathway at a level upstream of CIKS.

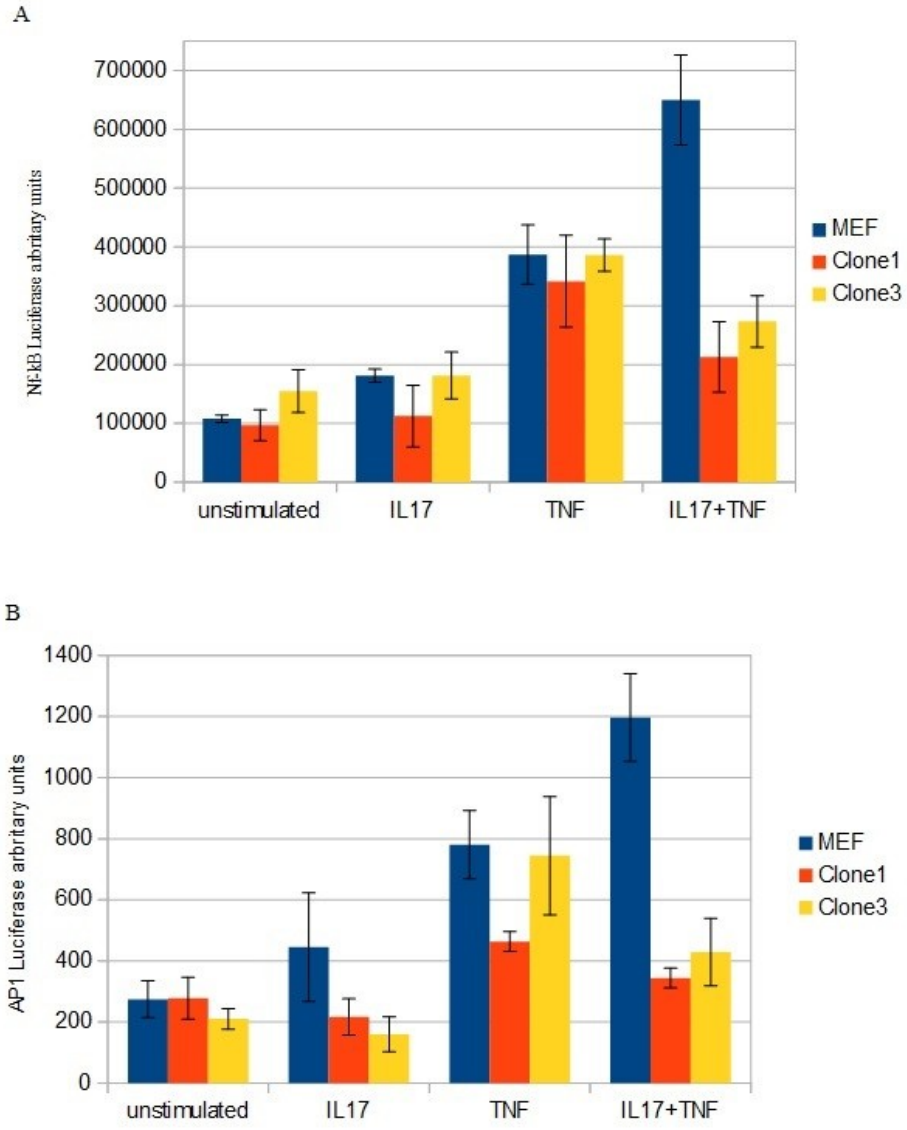

Figure 12:LRRFIP1 decreases NF-κB and AP1 activation after IL-17 stimulation. Relative reporter activity was evaluated in MEF and single stable clones after stimulation of IL-17, TNF-α or IL-17 plus TNF-α.

LRRFIP1 is involved in the IL-17-induced gene expression.

Given that LRRFIP1 interacted with CIKS and its overexpression decreased the activity of NF-κB and AP1 induced by IL-17, we sought to investigate if LRRFIP1 was able to modulate the expression levels of different IL-17-induced genes. We treated control MEF and two stable clones overexpressing LRRFIP1 with IL-17, and after 4 hours cells were harvested, RNA extracted, and analyzed by Real Time PCR. We evaluated the expression levels of CXCL1 and IL6 two of the genes induced by IL-17 stimulation. As shown in figure 13, over-expression of LRRFIP1 isoform 3 resulted in a decrease of mRNA levels of CXCL1 and IL6 compared with wild type cells.

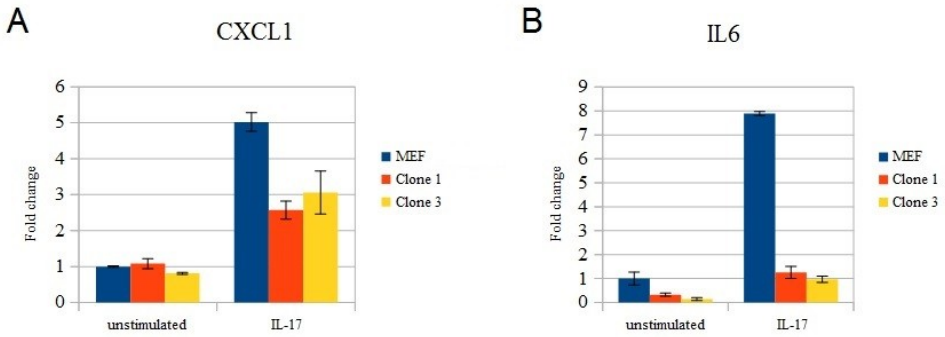

Figure 13: Over-expression of LRRFIP1 decreased mRNA levels of CXCL1 and IL6 after IL-17 stimulation. Real Time PCR analysis of CXCL1 expression (A) and IL6 (B) in MEF and in single stable clones unstimulated and stimulated for 4h with IL-17.

To confirm this result, we knocked down LRRFIP1 isoform 3 expression in MEF by using siRNA. As shown in fig.14 the expression of LRRFIP1 isoform 3 was decreased by about 60% in the interfered cells compared to the control. In these cells we evaluated the expression of CXCL1 and IL6 genes by Real Time PCR after IL-17 stimulation. As shown in figure 14 in the knocked down cells stimulation with IL-17 resulted in increased expression levels of both CXCL1 and IL6 genes compared to control cells. These results suggest that LRRFIP1 is acting as a negative regulator of the IL-17 signalling.

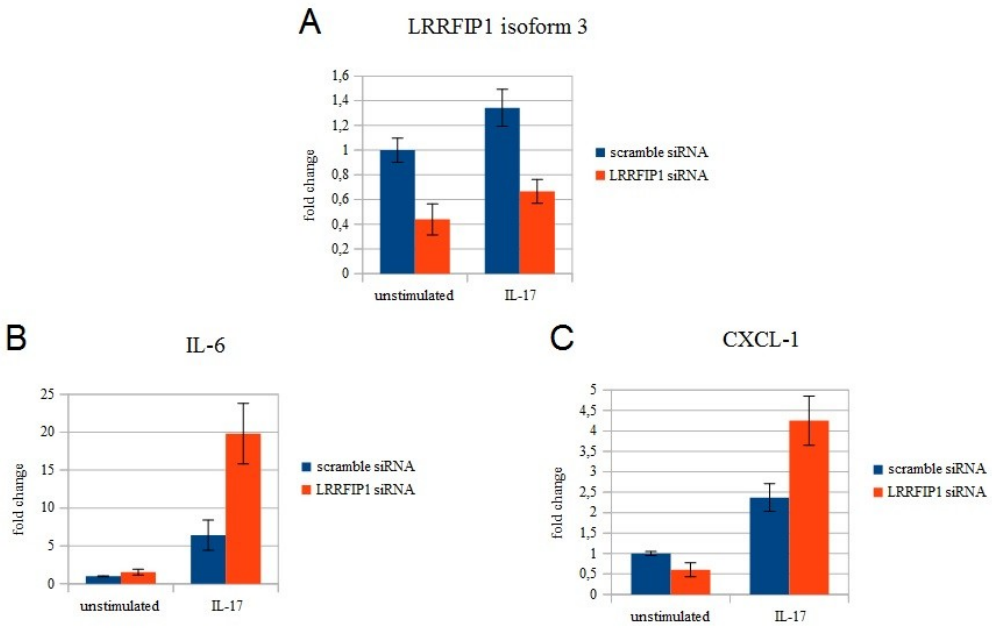

Figure 14: Knock-down of LRRFIP1 increases the expression levels of CXCL1 and IL6 after IL-17 stimulation. SiRNA scramble and siRNA LRRFIP1 were transfected in MEF cells. 72h after transfection cells were stimulated 4h with IL-17. A LRRFIP1 isoforms 3 was down-regulated assessed by Real Time PCR. B,C, down-regulation of LRRFIP1 in stimulated cells increased mRNA levels of CXCL1 and IL6.

LRRFIP1 displaces CIKS from the IL-17 receptor.

To further characterize the direct involvement of LRRFIP1 in the IL-17 pathway and to shed light on the molecular mechanism used by LRRFIP1 to negatively regulates the IL-17 pathway, we investigated the interaction between LRRFIP1 isoform 3 with CIKS and IL-17 receptor. We transfected HEK293 with the indicated expression vector (Fig. 15), immunoprecipitated FLAG-tagged IL-17R and looked for co-immunoprecipitating HA-tagged proteins. As shown in figure 15, IL-17R was not interacting with LRRFIP1 isoform 3. Interestingly, in the presence of LRRFIP1 the amount of CIKS co-immunoprecipitating with the IL-17 receptor was sharply decreased. This result suggest that LRRFIP1 is able to negatively regulates IL-17 pathway interfering with the interaction between IL-17R and CIKS.

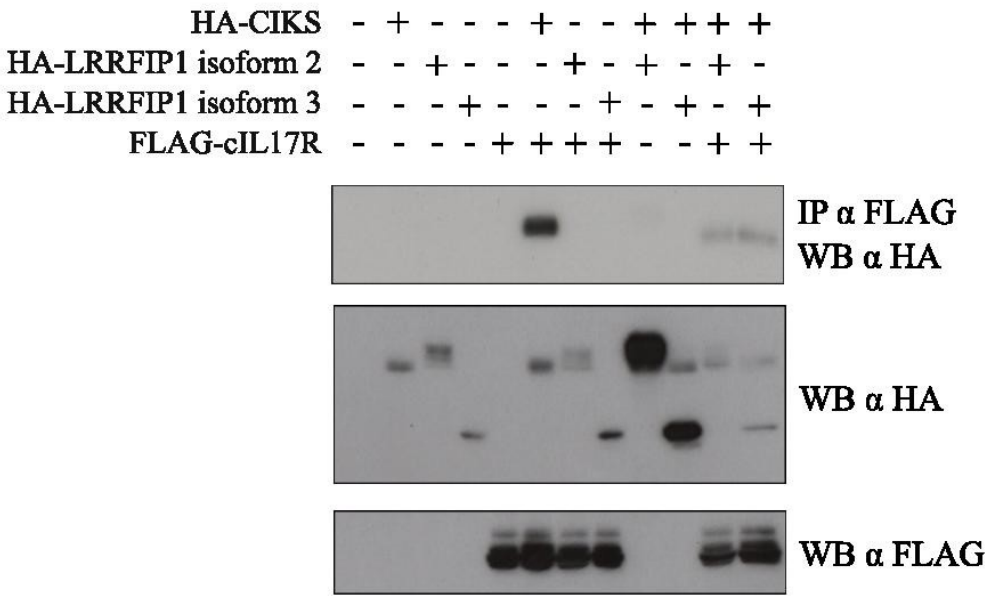

Figure 15: LRRFIP1 isoform 3 influences the interaction between IL-17R and CIKS. HEK 293 were transfected with both expression plasmids for HA-tagged LRRFIP1 isoform 3, HA-tagged CIKS and FLAG-tagged IL-17R. The immunoprecipitates were analyzed by Western blot with anti-HA Ab (upper panel). Expression of CIKS and LRRFIP1 isoforms was confirmed by western blot with anti-HA (middle panel) while the expression of IL-17R was confirmed by western blot with anti-FLAG (lower panel) Abs, respectively.

To confirm this result, we evaluated the interaction between the CIKS and the IL-17 receptor, in the presence of increasing amount of LRRFIP1. As shown in fig. 16, by in the presence of increasing amounts of LRRFIP1 isoform 3, the amount of CIKS co-immunoprecipitating with the IL-17R was gradually decreased, further suggesting that LRRFIP1 negatively regulated IL-17 signaling by interfering with the interaction between CIKS and the receptor.

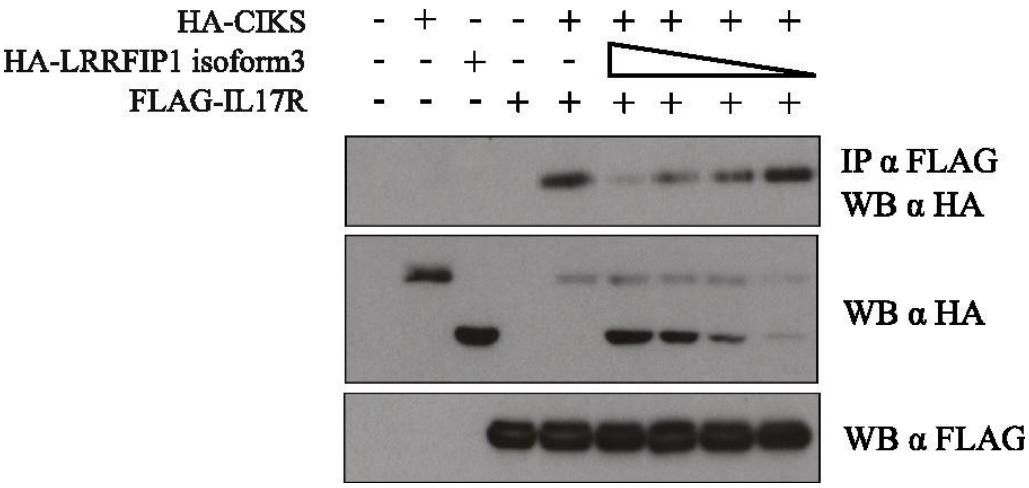

Figure 16: HEK 293 were trasfected with expression plasmids encoded for HA-tagged LRRFIP1 isoform 3, HA-tagged CIKS and FLAG-tagged IL-17R. We transfected the same quantity of FLAG-IL-17R and HA-CIKS while we transfected decreasing amount of LRRFIP1 isoform 3, from 2γ to 0,25γ. The immunoprecipitates were analysed by Western blot with anti-HA (upper panel). Expression of CIKS and LRFFIP1 isoform 3 was confirmed by western blot with anti-HA (middle panel) and anti-FLAG (lower panel) Abs, respectively.

DISCUSSION

The goal of this study was to indentify proteins interacting with CIKS to better understand the molecular mechanism regulating the inflammatory responses mediated by the IL-17. IL-17 is a pro-inflammatory cytokine that plays an essential role in host defense against microbial infections and is implicated in various inflammatory conditions such as autoimmune diseases, metabolic disorders and cancer. In the present study we present evidence identifying LRRFIP1 isoform 3 as a new CIKS-interacting protein, functioning as a negative regulator of the IL-17 pathway. Overexpression of LRRFIP1 isoform 3 blocks the IL-17 induced expression of *cxcl1* and *il-6*, two of the major IL-17 target genes, via inhibition of NF-κB and AP-1. Indeed, cells in which the expression of LRRFIP1 isoform 3 is knocked-down, the expression level of these two genes after IL-17 stimulation is strongly enhanced. This inhibitory function is possibly due to the ability of LRRFIP1 to interfere with the binding of CIKS to the IL-17 receptor. In fact, in the presence of increasing amounts of LRRFIP1 isoform 3, the binding of CIKS to the IL-17R is blocked. It is then possible to suppose that LRRFIP1 isoform 3 inhibits the interaction between CIKS and the IL-17R to block IL-17 signaling. In addition, the activation of NF-κB mediated by CIKS overexpression is not affected by LRRFIP1, further suggesting that this inhibition take place at a level upstream to CIKS, possibly at the level of the IL-17 receptor. It is tempting to speculate that upon IL-17 stimulation, CIKS is recruited to the IL-17 receptor via a SEFIR domain-mediated interaction, to initiates a cascade of events culminating in the activation of downstream effectors. After stimulation, LRRFIP1 isoform 3 displace CIKS from the cytoplasmic domain of the IL-17 receptor to block signaling. We are currently investigating this hypothesis, and we have some preliminary data suggesting that the expression of LRRFIP1 isoform 3 is induced by IL-17 (see also fig 14A).

The signaling of IL-17 is under tight control to avoid persistent inflammation. Different mechanisms negatively regulating IL-17 signaling have been described so far. Some act blocking the transcription factor C/EBPβ, that once phosphorylated is not longer able to drive the transcription of proinflammatory genes induced by IL-17. Other mechanisms inhibit the formation of the IL-17R-CIKS-TRAF6 complex. In fact, it has been demonstrated that TRAF4 and TRAF3, compete with TRAF6 for CIKS binding, thus blocking the IL-17 mediated signaling. Similarly, the IL-17RD, one of the member of the IL-17 receptor family, interacts with IL-17RA and CIKS through SEFIR-SEFIR domain interaction, to disrupt CIKS-TRAF6 binding and inhibiting NF-κB activation. Also two kinases, IKKi and TBK1, that phosphorylate CIKS on three Ser residues, are able to decrease the

interaction between CIKS and TRAF6. More recently, IL-17 signaling was shown to be negatively regulated by the ubiquitin-specific protease USP25, one subfamily member of deubiquitinating enzyme. USP25 directly removes IL-17 induced ubiquitination of TRAF5 and TRAF6, and consequently suppressed both TRAF6 dependent NF-κB activation and TRAF6 independent mRNA stabilization pathways in IL-17R signaling (Gu et al., 2013; Song and Qian, 2013) . In this study we described an additional mechanism downregulating IL-17 signaling. LRRFIP1 isoform 3 blocks the interaction between CIKS and IL-17R thereby preventing CIKS to activate the IL-17 pathway. Why different mechanisms regulating IL-17 signaling exist? One possible explanation is that the inflammatory response needs to be tightly regulated to avoid excessive inflammation then, the existence of multiple mechanisms acting at different levels, i.e. proximal to the receptor and directly into the nucleus, are needed to ensure correct regulation of the inflammatory responses. In fact, the regulation of the NF-κB activation, the master transcription factor regulating inflammation also relies on multiple mechanisms (Ruland, 2011) .

Our findings describe for the first time a function related to the isoform 3 of LRRFIP1 in innate immunity. While an involvement of the other two isoforms of LRRFIP1 in innate immunity have been reported, no function was associated to isoform 3 to date. Indeed, isoform 1 has been reported to be a cytosolic DNA and RNA sensor inducing the activation of IFN-β. It can also act as a transcriptional repressor blocking transcription of the TNF-α gene. These two functions seems to be in contrast to each other as interferone induction is a canonical response of the innate immune system, while blocking TNF transcription halts the innate response. This discrepancy might be explained by the different cell lines used in the two studies, or may simply reflects the different subcellular localization of isoform 1. When isoform 1 is in the cytosol, acts as sensor of foreign nucleic acids, activating the innate immune response, while when is in the nucleus, acts to terminate inflammation. Isoform 2 has been reported to interact with Myd88 and to positively regulate Toll-like receptor signaling. Our results assign a new function to isoform 3 and expand the functions of the LRRFIP1 family in regulating immune responses. It is interesting that different splice isoforms of the *lrrfip1* gene, give raise different proteins structurally not fully related, to regulate different pathways of the immune system.

In summary, we have identified a previously not reported association between CIKS and LRRFIP1, and we provide evidence that LRRFIP1 isoform 3 negatively regulates IL-17 pathway.

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NGAL Controls the Metastatic Potential of Anaplastic Thyroid Carcinoma Cells

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Context: We have previously identified neutrophil gelatinase-associated lipocalin (NGAL) as one of the genes mediating the oncogenic activity of nuclear factor-κB in human anaplastic thyroid carcinomas (ATCs).

Objectives: To further investigate the role of NGAL in thyroid cancer, we established NGAL knocked-down and NGAL overexpressing ATC cell lines.

Results: We found that the ability of NGAL knocked-down cells to degrade Matrigel in a transwell invasion assay and to form lung metastasis in nude mice was decreased. Because NGAL binds matrix metalloproteinase-9 (MMP-9), to form a macromolecular complex involved in the regulation of metastatic spread of cancer cells and given the strong expression of both genes in tissue specimens from human ATCs, we analyzed the MMP-9 enzymatic activity in NGAL-null ATC cells. Enzymatic immunoassays show that MMP-9 activity is reduced in NGAL-null ATC cells, even if its expression is not affected by NGAL inhibition. Ectopic expression of NGAL in an ATC cell line not expressing NGAL determines an increase of its metastatic property. The use of a mutated form of NGAL, unable to bind MMP-9, has no positive effect on the invasive potential of ATC cells and does not improve the MMP-9 enzymatic activity.

Conclusions: Our results indicate NGAL as a novel target of nuclear factor-κB prometastatic activity in thyroid cancer through enhancement of MMP-9 enzymatic activity. **(*J Clin Endocrinol Metab* 98: 0000–0000, 2013)**

One of the most problematic aspects of cancer is represented by metastatic disease that is largely incurable because of its systemic nature and the increased resistance of metastatic cells to chemo- or radiotherapy. In the vast majority of cases, the mortality from cancer is due to metastases, not to the primary tumor (1, 2). Therefore, a great effort has been attempting to understand the molecular mechanisms underlying the metastatic process to identify novel targets for more effective therapeutic intervention. Metastasis development is a very complex process which requires a number of coordinated steps to successfully promote the onset of secondary neoplastic lesions at organs distant from primary tumor. The initial event of metastatic cascade is represented by local invasion of primary tumor cells to adjacent tissues. This process is achieved by cancer cells via degradation of basement membrane, a specialized extracellular matrix that plays vital roles in organizing epithelial tissues. The degradation of basement membrane occurs by proteolysis of extracellular matrix components mediated by the activity of a family of zinc and calcium dependent endopeptidases collectively termed matrix metalloproteinases (MMPs) (3). One of the most important metalloproteinases involved in the invasion process is MMP-9, a 92-kDa protease able to

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Abbreviations: ATC, Anaplastic thyroid carcinoma; HA, hemagglutinin; HEK, human embryonic kidney; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; NF-κB, nuclear factor-κB; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; TBST, buffer of Tris-HCl, Tween 20, and NaCl.

degrade a broad range of substrates including collagens, fibronectins, and laminins (4). MMP-9 is highly expressed in many human tumors, especially of more aggressive phenotype, and its levels generally correlate with a poor prognosis (5–8). Its enzymatic activity is, at least partially, regulated by neutrophil gelatinase-associated lipocalin (NGAL), via the formation of a macromolecular complex that allows MMP-9 to preserve its proteolytic activity (9).

We have previously demonstrated that nuclear factor-κB (NF-κB) is strongly activated in human thyroid cancer in which it plays an important role, given that the inhibition of its activity in anaplastic thyroid carcinoma (ATC) cells blocks their oncogenic potential (10). In the effort to identify target genes of NF-κB activity in thyroid cancer, we found that NGAL is able to recapitulate some of protumorigenic functions of NF-κB in these tumors, such as the ability to protect ATC cells from growth factors deprivation-induced apoptosis (11). NGAL, also known as lipocalin-2, is a member of the large family of lipocalins, a group of small extracellular proteins with great functional diversity (12). It is overexpressed in a number of human tumors, including breast (13), lung (14), colon (15), ovary (16), and pancreas (17) carcinomas. Several studies have demonstrated that NGAL has protumorigenic activity that occurs through the enhancement of neoplastic cell survival and proliferation, very likely due to its iron-carrier function. In addition, NGAL contributes to cancer by promoting the metastatic potential of tumor cells because of its ability to protect MMP-9 from autodegradation (9, 18).

We found a strong coexpression of both NGAL and MMP-9 in tissue specimens from primary human ATCs so that we decided to investigate the role of NGAL in the ATC metastatic process by modulating its expression in two ATC cell lines, BHT101 and Act1.

Materials and Methods

Cell culture and biological reagents

BHT101 and Act1 cells were kindly provided by Professor M. Santoro, "Federico II" University of Naples. To knock down NGAL expression, BHT101 cells were infected with pLL 3.7 lentiviral vector containing double-stranded oligonucleotides sequences derived from the human NGAL in forward and reverse orientation, separated by a 7-bp spacer region (caagaga) to allow the formation of the hairpin structure in the expressed small interfering RNAs (siRNAs): NGAL sc-siRNA, sense strand, 5′-CCATCTATGAGCTGAAAGAcaagagaTCTTTCAGCTCATAGATGG-3′, antisense strand, 5′-CCATCTATGAGCTGAAAGAtctcttgTCTTTCAGCTCATAGATGG-3′; NGAL siRNA1, sense strand, 5′-GGGAATGCAATTCTCAGAGTTcaagagaAACTCTGAGAATTGCATTCCC-3′, antisense strand, 5′-GGGAATGCAATTCTCAGAGTTctcttgAACTCTGAGAATTGCATTCCC-3′; NGAL siRNA2, sense strand, 5′-GGACTTTTGTTCCAGGTTGTTcaagagaAACAACCTGGAACAAAAGTCC-3′, antisense strand, 5′-GGACTTTTGTTCCAGGTTGTTctcttgAACAACCTGGAACAAAAGTCC-3′. Act1 cells were infected with pLENTI CMV GFP-2A-PURO vector (ABM, Richmond, British Columbia, Canada) alone or containing the cDNA coding for human wild-type or mutated NGAL fused in frame with the hemagglutinin (HA) tag. All cell lines were grown in DMEM (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma). Anti-HA (sc-805 and sc-7392) and antiactin (sc-8432) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), anti-FLAG (F7425 and F3165) was from Sigma, and anti-NGAL (AF1757) was from R&D Systems (Minneapolis, MN).

Patients

The study has been approved by the local ethics committees. Informed consent of the patients had been obtained before their inclusion in the study. Five cases of surgically resected ATCs and two specimens of the normal controlateral thyroid lobe from surgically resected benign adenomas were analyzed for their ability to express NGAL and MMP-9 mRNAs. The clinicopathological characteristics of the ATC selected cases are shown in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

mRNA quantification by real-time RT-PCR

Real-time RT-PCR was carried out with cDNAs reverse transcribed from total RNA by using GoTaq qPCR master mix (Promega, Madison, WI) and Bio-Rad CFX Manager software (Bio-Rad Laboratories, Segrate, Milan, Italy), according to manufacturers' procedure. The primers used were: glyceraldehyde-3-phosphate dehydrogenase, forward, 5′-AAACAGAAGGCAGCTTTACGATG-3′, reverse, 5′-AAATGTTCTGATCCAGTAGCG-3′; NGAL, forward, 5′-GAAGACAAAGACCCGCAAAAG-3′, reverse, 5′-CTGGCAACCTGGAACAAAAG-3′; and MMP-9, forward, 5′-CGAACTTTGACAGCGACAAG-3′, reverse, 5′-CACTGAGGAA TGATCTAAGCCC-3′.

Immunoprecipitation and Western blots

For immunoprecipitation of transfected proteins, human embryonic kidney (HEK) 293 cells (3×10^6) were transiently transfected by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and 24 h after transfection, cells were lysed in Triton X-100 lysis buffer (20 mM HEPES, pH 7.4; 150 mM NaCl; 10% glycerol; 1% Triton X-100; and Complete protease inhibitor mixture). After an additional 15 min on ice, cell extracts were centrifuged for 10 min at 14,000 × *g* at 4 C, and supernatants were incubated for 4 h at 4 C with anti-HA antibodies bound to agarose beads (Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For Western blots, 20 μg of total proteins from cell lysates or supernatants was analyzed by 10% SDS-PAGE and blotted onto nitrocellulose membrane (Schleicher & Schuell, Whatman GmbH, Dassel, Germany). Filters were blocked for 1 h 30 min at room temperature with 5% nonfat dry milk in TBST buffer [10 mM Tris-HCl (pH 8), 0.1% Tween 20, 150 mM NaCl] and incubated with 1:2000 dilution of anti-HA, anti-FLAG, anti-NGAL, or antiactin antibodies for 1 h 30 min. After TBST washing, blots were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (NXA931; Amersham Biosciences, Buckinghamshire, UK) diluted 1:5000 in

TBST buffer and then revealed by enhanced chemiluminescence (Amersham Biosciences).

Matrigel invasion assays

Cell invasion was examined using a reconstituted extracellular matrix (Matrigel; BD Biosciences, Bedford, MA). Polycarbonate membranes (8 mm pore size) on the bottoms of the upper compartment of the transwells (6.5 mm; Costar, Cambridge, MA) were coated with 1.2 mg/ml Matrigel. The 1×10^5 cells in 100 μl of serum-free Opti-MEM I (Invitrogen), alone or added with recombinant NGAL (11), were placed on the Matrigel-coated polycarbonate membrane in the upper compartment, whereas 600 μl of Opti-MEM I supplemented with 10% fetal bovine serum was added to the lower compartment. The plates were incubated at 37 C in a 5% CO_2 atmosphere saturated with H_2O for 24 h. At the end of incubation, the cells and Matrigel at the upper side of the polycarbonate filter were mechanically removed. Cells that had invaded the Matrigel and migrated to the lower side of the filter were fixed with 11% glutheraldeyde for 15 min at room temperature, washed three times with PBS, and stained with 0.1% crystal violet-20% methanol for 20 min at room temperature. After three PBS washes and complete drying at room temperature, the crystal violet was solubilized by immerging the filters in 300 μl of 10% acetic acid. The concentration of the solubilized crystal violet was evaluated as absorbance at 590 nm. Experiments were performed at least in triplicate. Results are ±SD of at least three separate experiments.

In vivo experiments

To analyze *in vivo* the metastatic activity of neoplastic cells, 1×10^7 cells were resuspended in 200 μl of PBS and injected into the tail vein of 6-wk-old athymic mice divided into four groups of six animals each one. Groups were injected as follows: group 1, PBS alone; group 2, BHT cells; group 3, BHT pLL sc-siRNA cells; and group 4, BHT pLL siRNA2. Four weeks after injection, mice were killed and lung metastasis was analyzed by standard hematoxylin and eosin staining.

Analysis of MMP-9 activity

The quantitative determination of human active MMP-9 in conditioned media from NGAL-proficient and -deficient BHT101 and Act1 cell lines was performed by Fluorokine E immunoassay, according to the manufacturer's instructions (R&D Systems). The relative fluorescence units were determined with the PerkinElmer LS50B luminescence spectrometer (New York, NY) using an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Experiments were performed at least in triplicate. Results are ±SD of at least three separate experiments.

Statistics

Data were analyzed with ANOVA and a Student's *t* test analysis. Data are presented as the means ± SD. $P < 0.05$ was considered significant.

Results

MMP-9 and NGAL are overexpressed in human ATCs

We have previously shown (11) that NGAL is strongly expressed in human thyroid carcinomas, especially that of the anaplastic type. Because it is known that in many tumors NGAL expression parallels that of MMP-9 (19–21), we verified whether both genes were overexpressed in human thyroid tumors from the same tissue specimen. As shown in Fig. 1A, MMP-9 expression is clearly more pronounced in human ATCs than in normal thyroid tissues, as assessed by quantitative RT-PCR (qRT-PCR) analysis, and, importantly, it overlaps that of NGAL in the same tissues (Fig. 1B), indicating that both genes are highly expressed in the most aggressive and metastatic types of human thyroid carcinomas.

Establishment of knocked-down and overexpressing NGAL anaplastic thyroid carcinoma cell lines

To study the role of NGAL in the metastatic activity of ATC cells, we modulated its expression in two ATC cell lines, BHT101 and Act1, that show different levels of NGAL expression. BHT101 cells express high levels of NGAL and secrete the protein in the culture medium, whereas Act1 cells do not express NGAL that is not detectable either in the whole cell extract or in the culture medium (Fig. 2, A and B). Therefore, we knocked down NGAL in BHT101 cells by infecting cells with two different lentiviruses containing siRNA sequences that bind two different regions of NGAL mRNA (pLL siRNA1 and -2). In parallel, we ectopically expressed NGAL in Act1 cells by infecting cells with a lentivirus carrying the cDNA en-

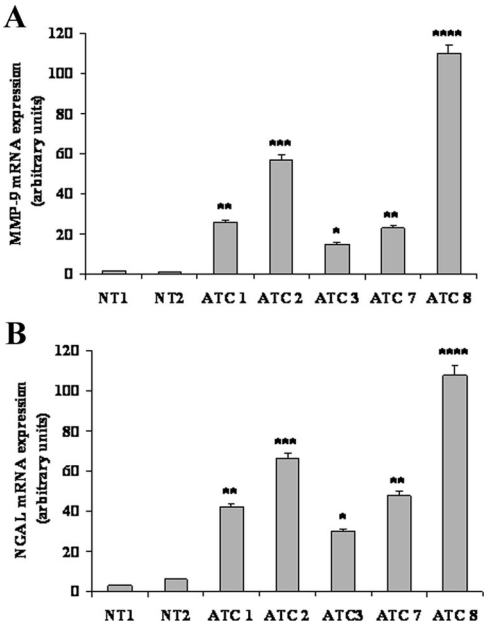

FIG. 1. MMP-9 and NGAL expression in primary human ATC specimens. qRT-PCR of MMP-9 (A) and NGAL (B) in normal thyroid (NT1 and NT2) and ATCs (ATC1, ATC2, ATC3, ATC7, and ATC8) from human specimens. The relative amount of MMP-9 and NGAL mRNA was normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA. *, $P < 0.001$; **, $P < 0.0001$; ***, $P < 0.00001$; ****, $P < 0.000001$.

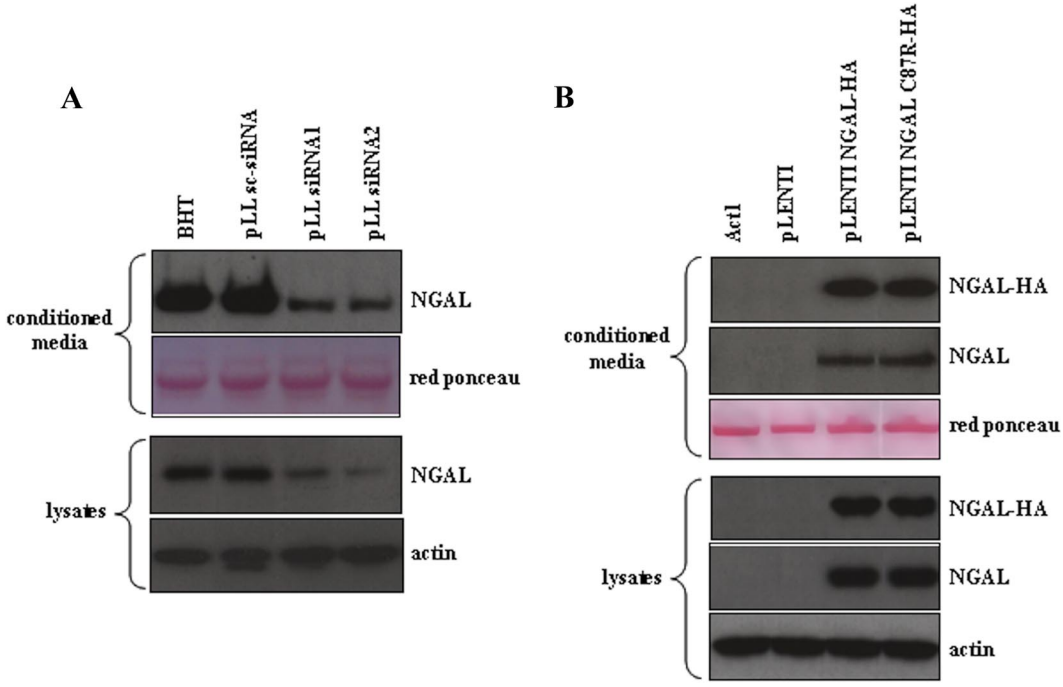

FIG. 2. Modulation of NGAL expression in ATC cell lines. Western blot of NGAL on conditioned media and lysates from BHT101 cells uninfected and infected with pLL 3.7 NGAL siRNA1, siRNA2, or scrambled (sc) siRNA (A) and from Act1 cells uninfected and infected with pLENTI alone, pLENTI NGAL-HA, or pLENTI NGAL C87R-HA (B). The expression levels of NGAL protein from lysates were normalized on actin content (A and B, *bottom panels*), whereas those of conditioned media were normalized on total protein content determined by a Bradford assay (Bio-Rad Laboratories).

coding for human NGAL. BHT101 cells infected with pLL siRNA1 and pLL siRNA2 showed a strong decrease of NGAL expression compared with uninfected cells and to control scrambled siRNA (Fig. 2A). The ectopic expression of NGAL in Act1 cells was successfully achieved both in lysates and extracellular media, as shown in Fig. 2B.

In addition, we also infected Act1 cells with a mutated form of NGAL (C87R), carrying a substitution of cysteine residue in position 87 with an arginine residue (C87R) (Fig. 2B). This mutation impairs the ability of NGAL to form the disulfide bridge with MMP-9, thereby interfering with NGAL-mediated MMP-9 protection from auto-degradation and preventing association of tissue inhibitor of metalloproteinase-1 with MMP-9 N-terminal domain (22). To test the effectiveness of this substitution, we transiently cotransfected HEK293 cells with FLAG-MMP-9 and NGAL-HA or with FLAG-MMP-9 and NGAL C87R-HA, respectively, and we coimmunoprecipitated them with anti-HA antibodies. Western blot analysis of immunoprecipitated proteins with anti-FLAG antibodies revealed that only wild-type NGAL-HA was able to bind FLAG-MMP-9, whereas the mutated NGAL C87R-HA was completely unable to interact with the metalloproteinase (Fig. 3).

NGAL enhances the invasiveness of BHT101 and Act1 cells

Both NGAL knocked-down and NGAL overexpressing cell lines were tested for their ability to degrade matrigel in a transwell invasion assays. As shown in Fig. 4, all BHT cell lines migrated through the transwell at similar extent, independently of the presence of NGAL in the culture medium (Fig. 4, A–C, *left panels*). However, they not degraded matrigel with the same efficiency (Fig. 4, A–C, *right panels*). In particular, knocking down NGAL expression determined the decrease of BHT cells invasiveness compared with that of parental and sc-siRNA BHT cells, as

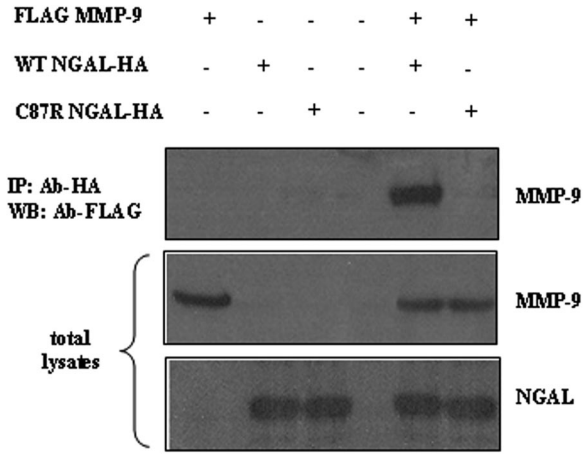

FIG. 3. The substitution of cysteine residue in position 87 with arginine (C87R) impairs NGAL binding to MMP-9. HEK293 cells were transfected with constructs encoding MMP-9, wild-type (WT), and mutated (C87R) NGAL. Cell lysates were immunoprecipitated (IP) with anti-HA antibodies (NGAL) and Western blotted (WB) with anti-FLAG to reveal the coprecipitation of MMP-9 and NGAL. The presence of HA and FLAG proteins in total lysates is shown in *lower panels*.

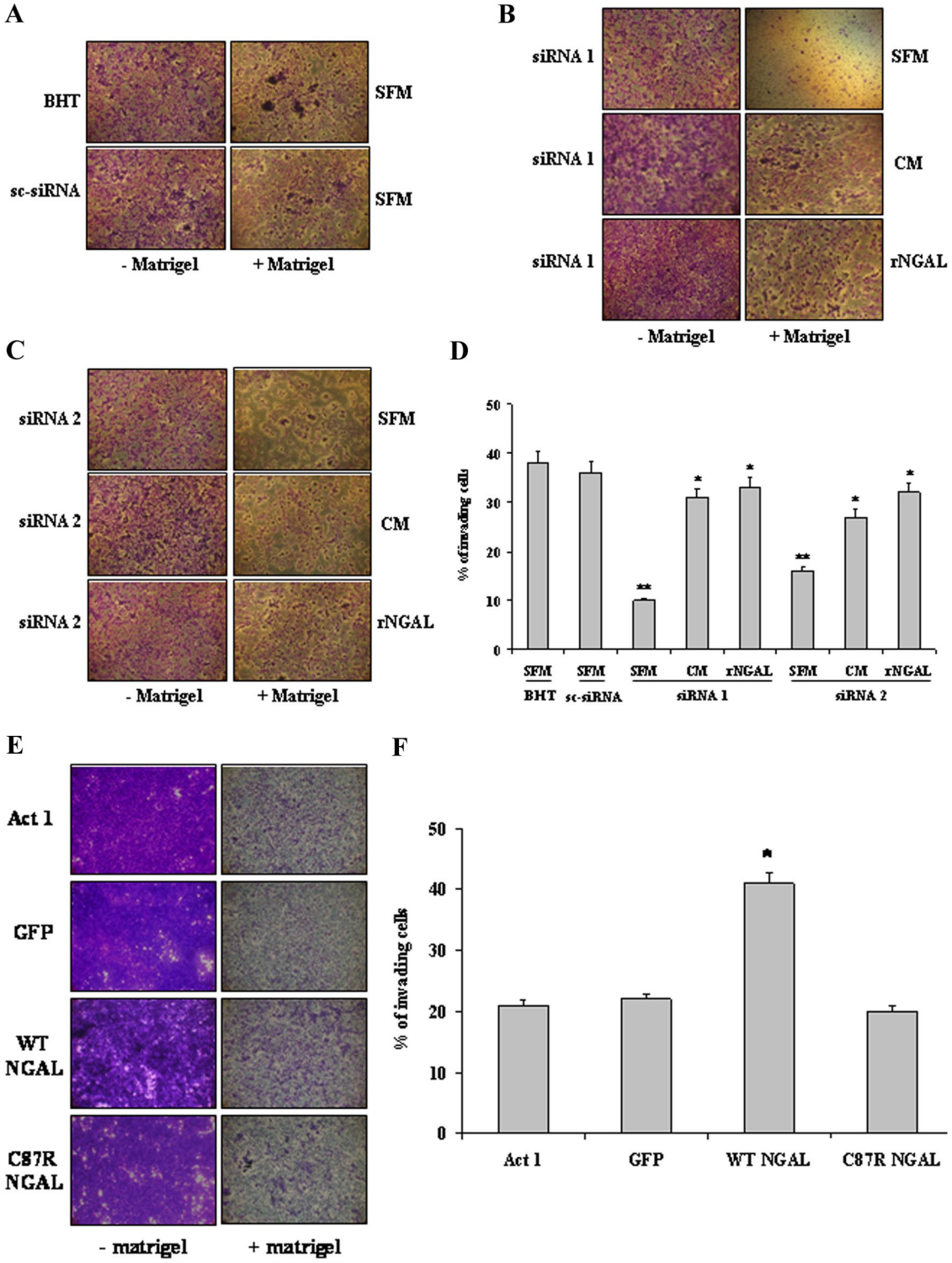

FIG. 4. *In vitro* invasive properties of NGAL-proficient and -deficient ATC cell lines. Parental and infected BHT101 (A–C) and Act1 (E) cell lines were used to analyze their ability to degrade Matrigel (BD Biosciences) in transwell assays. The extent of invasive potential was measured as the number of Matrigel degrading cells on total migrating cells (D, *, $P < 0.01$, **, $P < 0.001$; and F, *, $P < 0.001$). SFM, Serum-free medium; CM, conditioned medium; rNGAL, recombinant NGAL.

assessed by the reduced number of both BHT pLL siRNA1 and siRNA2 cells that degraded matrigel (Fig. 4D). The addition of NGAL-containing conditioned medium or recombinant NGAL to the upper chamber of transwells restored the invading activity of BHT pLL siRNA1 and siRNA2 cells (Fig. 4, B–D). On the other hand, the ectopic expression of NGAL in Act1 cells induced an increase of their invasiveness, as assessed by a more pronounced ability of NGAL-expressing Act1 cells to degrade matrigel compared with the parental and control vector cells (Fig.

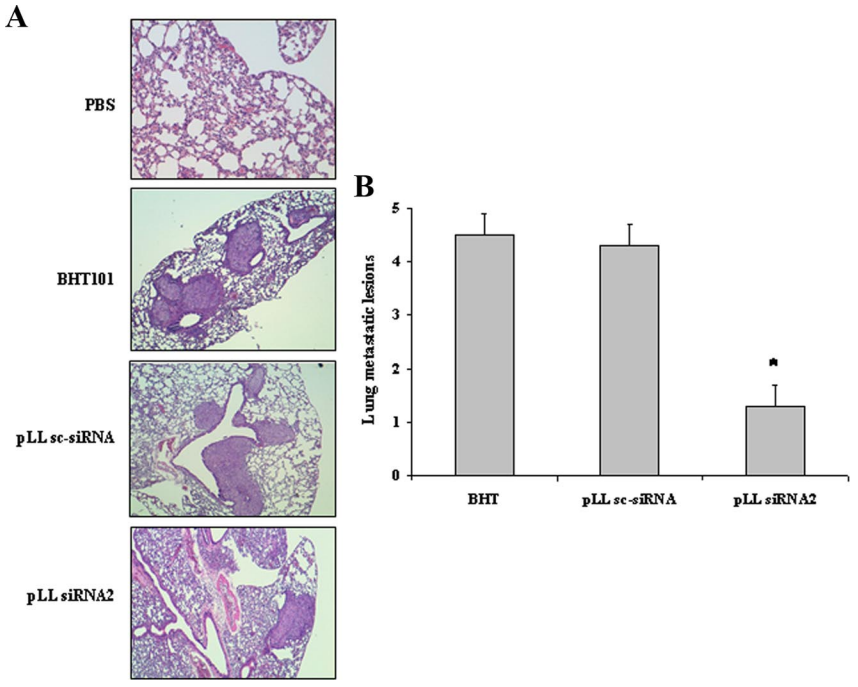

FIG. 5. *In vivo* analysis of metastatic activity of NGAL-proficient and -deficient ATC cells. Parental and infected BHT101 cells were injected in athymic mice tail vein to analyze their ability to form lung metastasis (A). The number of metastatic lesions in the lung was determined for each group of injected mice (B). *, $P < 0.0001$.

To confirm these results *in vivo*, we injected parental and NGAL knocked-down BHT101 cells in the tail vein of athymic mice, which represents a preferential way of access for tumor cells migration to future metastatic sites, *i.e.* lung and liver. Figure 5 shows the hematoxylin-and-eosin staining of lung metastases from mice injected with uninfected BHT101, BHT pLL sc-siRNA, and BHT pLL siRNA2 cells. All three cell lines gave rise to lung metastases but at different extent. In particular, the number of lung metastases from BHT101 NGAL-null cells injected mice were lower than those originated from parental and scrambled BHT101 cells (Fig. 5, A and B).

These results suggest that the presence of functional NGAL increased the metastatic potential of ATC cells, *in vitro* and *in vivo*, very likely through the improvement of MMP-9 activity.

4, E and F). Importantly, Act1 cells expressing the C87R NGAL mutant reversed the proinvading effects of wild-type NGAL, suggesting that interfering with the formation of NGAL/MMP9 complex is important in regulating the prometastatic activity of MMP-9.

MMP-9 enzymatic activity is enhanced by the presence of NGAL

To test our hypothesis, we evaluated the MMP-9 activity in the parental and infected BHT101 and Act1 cell lines by an immunoenzymatic assay. To this purpose, cells

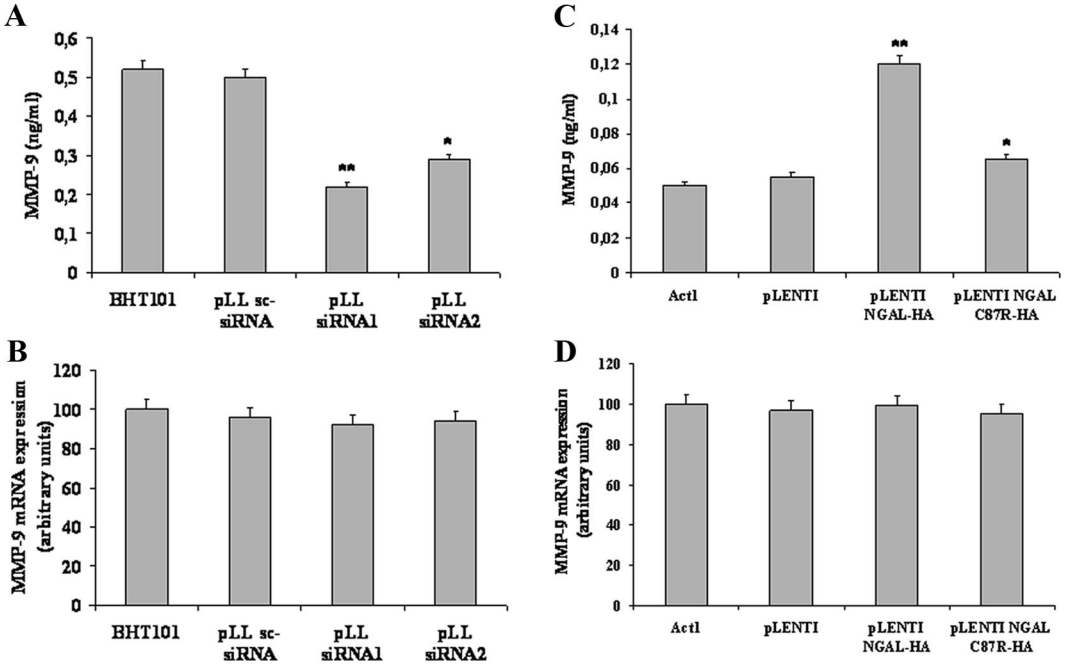

FIG. 6. Determination of MMP-9 activity in conditioned media from ATC cell lines. Conditioned media from parental and infected BHT101 (A, *, $P < 0.001$, **, $P < 0.0001$) and Act1 (C, *, $P < 0.001$, **, $P < 0.0001$) cell lines were collected to determine the concentration of active MMP-9. qRT-PCR analysis showed that no difference of MMP-9 expression was detected among cell lines (B and D).

were grown in serum-free medium, and after 48 h their conditioned media were collected to measure the levels of endogenous active MMP-9. As shown in Fig. 6A, the absence of NGAL in BHT101 cells determined a strong decrease of active MMP-9 content compared with that measured in the extracellular medium of parental and scrambled BHT101 cells. On the contrary, the concentration of active MMP-9 from the conditioned medium of NGAL-overexpressing BHT101 cells was higher than that from conditioned media of control cells (Fig. 6C). Importantly, the presence of the mutated form of NGAL in BHT101 extracellular medium had no effect on MMP-9 activity. The up- or down-regulation of enzymatic activity in the different experimental systems was not dependent on MMP-9 expression because RT-PCR analysis showed that no differences of MMP-9 mRNA levels were detectable in the various cell lines (Fig. 6, B and D).

Discussion

In the present paper, we analyzed the role of NGAL in promoting ATC tissue invasion through enhancement of MMP-9 activity. The inhibition of NGAL expression, which is strongly elevated in the same primary human ATC showing high MMP-9 expression, decreases the *in vitro* and *in vivo* metastatic potential of BHT101 ATC cell line. This effect is the consequence of the reduced enzymatic activity of MMP-9 in the absence of NGAL. However, knocking down NGAL expression in BHT101 cells does not completely abrogate the ability of these cells to invade matrigel and to form lung metastasis (Figs. 4, A and D, and 5, A and B). This is probably due to the presence of a residual NGAL expression in BHT siRNA cells, as assessed by Western blot (Fig. 2A), that still preserves MMP-9 from autodegradation, as proven by the partial but not absolute decrease of MMP-9 enzymatic activity in the extracellular medium of BHT NGAL-null cells (Fig. 6A).

The importance of NGAL as regulator of ATC cells metastatic potential is further supported by increased MMP-9 enzymatic activity in the Act1 after ectopic expression of NGAL. In this case, the secretion of extracellular NGAL improves the MMP-9 enzymatic activity of Act1 cells causing the increased invasion through matrigel in the transwell assay. This effect is clearly NGAL mediated because Act1 cells overexpressing the C87R mutant of NGAL show a MMP-9 activity and an invasive behavior similar to those of control cells. The evidence of a basal MMP-9 activity independent of NGAL expression in Act1 cells (Figs. 4, D and E, and 6C) points out that MMP-9 can function in the absence of NGAL; even so, the presence of NGAL significantly improves its enzymatic activity.

In the light of these findings, the strong coexpression of NGAL and MMP-9 in primary human ATC suggests a pro-metastatic role of NGAL/MMP-9 complex in aggressive thyroid carcinomas. Anaplastic thyroid carcinomas constitute the most undifferentiated type of thyroid cancer with marked invasive properties so that the presence of an active NGAL/MMP-9 complex could explain in part the metastatic potential of ATC cells. The biological importance of NGAL/MMP-9 complex in cancer has been extensively studied in other human tumors, such as breast cancer (18, 23), esophageal (20) and oral (24, 25) squamous cell carcinomas, cholangiocarcinomas (21), bladder cancer (26), gastric cancer (19), and rectal cancer (27). In these reports the activity of the complex generally correlates with the more aggressive behavior of neoplastic cells and, in the majority of cases, with a poor prognosis. Thus, the role of NGAL/MMP-9 complex in metastatic thyroid cancer, up to now not yet elucidated, fits with that exerted in other types of human tumors.

The identification of NGAL as a regulator of ATC-induced metastasis adds a novel piece to the puzzle of NF-κB functions in thyroid cancer (10, 11, 28–31). In fact, our previous results and the findings reported here indicate that NF-κB activity could influence the invasive potential of ATC cells through the up-regulation of NGAL other than of MMP-9, whose mRNA transcription, as it is known, is in part under NF-κB control (32). This could also explain why the expression of both genes is so elevated in ATCs in which NF-κB is strongly activated. In this way, NGAL could be viewed as a novel target gene of NF-κB-mediated metastatic activity of ATC cells and its inhibition as a potential tool for therapeutical intervention in advanced ATC therapy without altering NF-κB physiological functions.

Acknowledgments

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