Characterization of Interleukin-33 and the IL-33 Receptor Complex

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften
-Dr. rer. nat.-

Immunologie
des Fachbereiches Biologie und Chemie
der Justus-Liebig-Universität Gießen

vorlegt von
Shafaqat Ali
(M.Phil)
aus
Sialkot, Pakistan

Gießen, Juli 2009

Dekan

Prof. Dr. Volkmar Wolters

Gutachter

Prof. Dr. Michael U. Martin

Immunologie, Justus-Liebig-Universität Gießen

Prof. Dr. Alfred M. Pingoud

Institut für Biochemie, Justus-Liebig-Universität Gießen

Erklärungen

Hiermit versichere ich, die vorliegende Arbeit selbständig verfasst und keine andere als die angegebenen Hilfsmittel benutzt zu haben. Stellen, die ich anderen Arbeiten und Veröffentlichungen dem Wortlaut oder Sinn entsprechend entnommen habe, sind durch Quellenangaben gekennzeichnet.

Shafaqat Ali

Gießen, den 27 Juli 2009

Dedicated to my parents

Table of Contents

List of Figures

List of Tables

List of abbreviations

Abbreviations	Descriptions
AcPL	Accessory protein like
AP-1	Activator protein 1
BMD	Bone marrow-derived
BMDMC	Bone marrow-derived mast cells
BSA	Bovine serum albumin
CMV	Cytomegalovirus
Ct	Cycle threshold
DEAE dextran	Diethylaminoethyl-Dextran
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FI	Full length
HEV	High endothelial venule
hIL-1β	Human interleukin 1 beta
HMGB1	High-mobility group box 1
HRP	horseradish peroxidase
IFN	Interferon
IKK	Inhibitor of κB kinase
IL-18	Interleukin 18
IL-18BP	Interleukin-18 binding protein
IL-18Rα	Interleukin-18 receptor α-chain
IL-18Rβ	Interleukin-18 receptor β-chain
IL-1R	Interleukin-1 receptor
IL-1RAcP	Interleukin 1 receptor accessory protein
IL-1Rrp2	Interleukin-1 receptor-related protein 2
IL-33	Interleukin-33
IL-33Rα	Interleukin-33 receptor α-chain
IP	immunoprecipitation
IRAK	Interleukin-1 receptor-associated kinase
IκB	inhibitor of NF-κB
JNK	cJun-N-terminal kinase
LAF	Lymphocyte activating factor
LB medium	Luria-Bertani broth medium
LPS	Lipopolysaccharide
MAPK	mitogen-activated protein kinase
MEKK	Mitogen-activated protein kinase kinase kinase
mIL-33-Bio	Murine interleukin-33, biotinylated
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MyD88	Myeloid differentiation primary response gene 88
NEMO	NF-κB essential modulator
NF-kB	Nuclear factor-κB
NF-kB-Luc	NF-κB luciferase
PEI	Polyethyleneimine
PVDF	Polyvinylidene Fluoride

RLU	Relative light units
RPMI	Roswell Park Memorial Institute
sAcP	Soluble interleukin-1 receptor accessory protein
SEM	The standard error of the mean
SIGIRR	Single Ig-domain containing interleukin-1 receptor-related
sIL-33Rα	Soluble interleukin-33 receptor α-chain
TAB	TAK1 binding protein
TAK	TGF-β-activated kinase
TIR domain	Toll-like Interleukin-1 Receptor homology domain
TLR	Toll like receptor
TNF	Tumor necrosis factor
TNFR	TNF receptor
Tollip	Toll interacting protein
TRAF6	Tumer necrosis factor receptor associated factor-6
WB	Western blot
ΔC-AcP	C-terminally truncated interleukin 1 receptor accessory protein

Summary

Interleukin-33 (IL-1F11), the most recent addition to the IL-1 family, is a potent pro-inflammatory cytokine that stimulates the generation of Th2-associated cytokines. Two pivotal aspects of the IL-33 system are characterized in this study: First the composition and function of the IL-33 receptor complex on IL-33 responder cells and second the generation of biologically active IL-33 by the IL-33 producer cell.

IL-33 binds to the IL-33 receptor α-chain (formerly known as the orphan receptor ST2) and as is shown here, this results in the recruitment of interleukin-1 receptor accessory protein (IL-1RAcP) as the co-receptor. The IL-33-dependent interaction of membrane bound and soluble form of IL-1RAcP and IL-33Rα-chain was demonstrated in co-immunoprecipitation assays. IL-33-induced formation of the receptor heterodimer is the first necessary step for IL-33-mediated activation of T cells and mast cells. Lack of the IL-1RAcP abrogated responses to IL-33 and IL-1 in the mouse thymoma clone EL-4 D6/76. Responsiveness to IL-33 and IL-1 was restored with the expression of full length IL-1RAcP, while a mutant protein lacking the Toll/IL-1 Receptor domain (TIR) was not sufficient to restore IL-33 or IL-1 responsiveness in EL-4 D6/76 cells. Moreover the monoclonal antibody 4C5, which neutralizes IL-1β effects by blocking of murine IL-1RAcP, inhibited IL-33-stimulated signaling in mouse thymoma cells and bone marrow-derived mast cells. In these cells, IL-33 activated the classical IL-1 signaling pathway including IL-1 receptor associated kinase (IRAK-1), cJun-N-terminal kinase, p38 MAPK and the NF-κB pathway in an IL-1RAcP –dependent manner.

In search for putative further components of the IL-33 receptor complex, TIR8 / SIGIRR was studied, as it was proposed to be a candidate IL-33 receptor component. Using wild type TIR8 and chimeric fusion proteins it could be excluded that TIR8/SIGIRR can substitue for IL-1RAcP as a co-receptor. Furthermore, no experimental proof could be generated to show that this molecule is participating in the signaling IL-33 receptor complex.

Like IL-18 and IL-1β, IL-33 was found to have strong immunomodulatory functions. However, whereas IL-1β and IL-18 promotes pro-inflammatory and Th1-associated responses, IL-33 induces Th2-associated cytokines. In order to find the differential

gene regulation in response to these cytokines, IL-1-, IL-18- and IL-33 -induced gene expression profiles were compared by microarray from a triple responsive T cell line. Although a large number of genes were regulated by the IL-1 family members, no differentially regulated gene could be identified, suggesting the use of the same signaling molecules/pathways by all three cytokines, at least in this cellular system. Thus, it is proposed that the unique expression profile of the cytokines in specific cell/tissue types, and expression of specific receptors on effector cells may specifiy the differential function of these cytokines in different cell types.

Common notion in the field is that IL-33, like IL-1β and IL-18, requires processing by caspase-1 to a mature form, in order to achieve biological activity as a cytokine. Contrary to the current dogma, here it is described that IL-33 is biologically active as unprocessesed full length molecule. Full length IL-33 binds to the IL-33 receptor-α chain and mediates the recruitment of IL-1RAcP to activate cells. IL-33 is processed in cells, however not by caspase 1 to a mature, biologically active, IL-33 but instead it is cleaved by caspase 3 at aa175 to yield two products which are both unable to bind to the IL-33 receptor. Thus caspase 3 processing inactivates IL-33 as a cytokine. Full length IL-33 and its N-terminal caspase 3 breakdown product, however, translocate to the nucleus of the producing cell. Interestingly, bioactive IL-33 is not released by cells constitutively or after activation of the inflammasome as is the case with other IL-1 family members, like IL-1β and IL-18. Thus, it is tempting to speculate that IL-33 is not a classical cytokine but a molecule with dual function which normally exerts its function in the nucleus of intact cells and only activates others cells via the IL-33 receptor complex if cells are destroyed. Thus IL-33 may act as an endogenous danger signal, to alert cells of the innate immune system of the destruction of the IL-33 producing cells during hypoxia or after mechanical injury to initiate a sterile inflammation.

Zusammenfassung

Interleukin-33 (IL-1F11) der jüngste Zugang zur IL-1 Familie, ist ein potentes entzündungsförderndes Zytokin, das die Herstellung von Th2 -artigen Zytokinen stimuliert. Zwei zentral wichtige Aspekte des IL-33 Systems werden in dieser Studie charakterisiert: Zuerst der Aufbau und die Funktion des IL-33 Rezeptorkomplexes auf Zellen, die auf IL-33 reagieren und zweitens die Bildung des biologisch aktiven IL-33 durch die produzierende Zelle.

IL-33 bindet an die IL-33 Rezeptor α-Kette (früher als “Waisenrezeptor” ST2 bekannt) und, wie hier gezeigt, resultiert dies in der Rekrutierung des Interleukin-1 Rezeptor Akzessorischen Proteins (IL-1RAcP) als Korezeptor. Die IL-33- abhängige Wechselwirkung des Membran-gebundenen und löslichen IL-1RAcP und der IL-33Rα-Kette wurde in Ko-immunopräzipitationsexperimenten gezeigt. IL-33-induzierte Bildung des Rezeptor-Heterodimers ist der erste notwendige Schritt der IL-33 vermittelten Aktivierung von T-Lymphozyten und Mastzellen. Das Fehlen des IL-1RAcP verhinderte Antworten auf IL-33 and IL-1 in der Maus Thymoma Linie EL-4 D6/76. Die Fähigkeit auf IL-33 und IL-1 zu reagieren, wurde durch die Expression des intakten IL-1RAcP wieder hergestellt, während eine Mutante, welcher die Toll/IL-1 Rezeptor Domäne (TIR) fehlte, nicht in der Lage war, die IL-33- oder IL-1-Antwort in EL-4 D6/76-Zellen wieder herzustellen. Darüberhinaus inhibierte der monoklonale Antikörper 4C5, welcher IL-1β -Effekte durch das Blockieren des murinen IL-1RAcP zu neutralisieren vermag, die IL-33-stimulierte Signaltransduktion in Maus Thymoma Zellen und in Mastzellen, die frisch aus Knochenmark ausdifferenziert worden waren. In diesen Zellen aktivierte IL-33 den klassischen IL-1 Signalweg einschließlich der IL-1 Rezeptor assoziierten Kinase (IRAK-1), der cJun-N-terminalen Kinase, der p38 MAP- Kinase und des NF-κB Wegs in einer IL-1RAcP –abhängigen Weise.

Auf der Suche nach möglichen weiteren Komponenten des IL-33 Rezeptorkomplexes wurde TIR8 / SIGIRR untersucht, da vorgeschlagen worden war, dass dies ein Kandidat hierfür sein könnte. Unter Verwendung von Wildtyp-TIR8/SIGIRR und chimären Fusionsproteinen konnte ausgeschlossen werden, dass TIR8/SIGIRR IL-1RAcP als Korezeptor ersetzen kann. Weiterhin konnte kein experimenteller Nachweis erbracht werden, dass dieses Molekül ein Teil des Signal-transduzierenden IL-33 Rezepptorkomplexes ist.

Wie IL-18 und IL-1β übt IL-33 starke immunmodulatorische Funktionen aus. Während IL-1β und IL-18 jedoch proinflammatorische und Th1 -assoziierte Antworten fördern,

induziert IL-33 Th2-assoziierte Zytokine. Um eine differentielle Genregulation in Antwort auf diese Zytokine herauszufinden, wurden IL-1-, IL-18- und IL-33 –induzierte Genexpressionsprofile mittels Microarrays in einer Triple-responsiven T-Zelllinie aufgenommen und verglichen. Obwohl eine große Zahl von Genen durch die Mitglieder der IL-1 Familie reguliert wurden, konnten keine differenziell regulierten Gene identifiziert werden, was vermuten lässt, dass alle drei Zytokine denselben Signalweg nutzen, zumindest in dem untersuchten Zellsystem. Es wird daher vorgeschlagen, dass die diskreten Expressionsprofile der einzelnen Zytokine in bestimmten Zell- bzw. Gewebetypen und die Expression der spezifischen Rezeptoren auf Effektorzellen die jeweilige differentielle Funktion dieser Zytokine in unterschiedlichen Zelltypen bestimmen.

Es wird in Fachkreisen allgemein angenommen, dass IL-33, wie IL-1β und IL-18, das Prozessieren durch die Caspase-1 zur reifen Form benötigt, um als Zytokin biologisch aktiv sein zu können. Dem Dogma widersprechend, wird hier beschrieben, das IL-33 als unprozessiertes Molekül biologisch aktiv ist, also in seiner gesamten durch die Primärsequenz definierten Länge. Unprozessiertes IL-33 bindet an die IL-33 Rezeptor-α Kette und vermittelt die Rekrutierung von IL-1RAcP, um Zellen zu aktivieren. Dabei wird IL-33 in Zellen prozessiert, jedoch nicht durch Caspase 1 zu einer reifen, biologisch aktiven Form, sondern es wird durch Caspase 3 an Position175 gespalten, wobei zwei Produkte entstehen, die beide nicht in der Lage sind, an den IL-33 Rezeptor zu binden. Insofern inaktivert Caspase 3 IL-33 als Zytokin. Unprozessiertes IL-33 und sein N-terminales Caspase 3 -Abbauprodukt können jedoch in den Kern der produzierenden Zelle translozieren.

Interessanterweise wird bioaktives IL-33 nicht durch die Zellen freigesetzt, weder konstitutiv noch nach Aktivierung des Inflammasoms, wie es der Fall ist mit anderen Mitgliedern der IL-1 –Familie, z.B. IL-1β und IL-18. Daher ist es verlockend zu spekulieren, dass IL-33 gar kein klassisches Zytokin ist, sondern ein Molekül mit dualer Funktion welches normalerweise seine Aufgaben im Kern der produzierenden Zelle ausübt und nur dann andere Zellen über den IL-33 Rezeptorkomplex aktiviert, wenn die produzierenden Zellen zerstört werden. Damit mag IL-33 als ein endogenes Alarmsignal wirken, welches Zellen des angeborenen Immunsystems darauf aufmerksam macht, dass Zellen durch Zerstörung zu Grunde gegangen sind, zum Beispiel nach Sauerstoffunterversorgung oder durch mechanische Verletzung, um eine sterile Entzündung in Gang zu setzen.

Chapter 1

Introduction

Cytokines are protein mediators of the immune system that activate leukocytes and tissue cells by binding to specific plasma membrane receptors. Cytokines can be grouped in families by different criteria such as their source, e.g. lymphokines are produced by lymphocyts, or their role in the immune reponse, e.g. whether they initate and support an acute inflammation or whether they dampen and turn off inflammation. Interleukin-1 (IL-1) α and IL-1β are two of the best known and best characterized cytokines of the immune system that organize acute inflammation at the site of infection and systemically. In the last years it has become evident that a family of IL-1 like cytokines exist, which act in innate and adaptive immunity.

1.1. Biological activities of IL-1 family cytokines

Cytokines of the IL-1 family play a major role in a wide range of inflammatory, infectious, and autoimmune diseases (Dinarello, 1996). IL-1 family cytokines, such as IL-1 itself, IL-18, and IL-33 are critical for successful clearance of extra- and intracellular pathogens and they participate in mounting the host defense against malignant transformations. Normally, their production is tightly controlled in acute inflammation to avoid tissue damage due to their overall catabolic action. However, if control fails their overproduction can lead to autoimmune disorders such as rheumatoid arthritis. IL-1, IL-18 and IL-33 are potent inducers of secondary cytokine production by leukocytes or tissue cells, yet the individual profiles induced by the respective cytokine differ. IL-1α and IL-1β (IL-1) are important pro-inflammatory cytokines of the innate arm of immunity. IL-1 plays an important role in immune regulation and inflammatory processes by inducing expression of many effector proteins, e.g. cytokines/chemokines, nitric oxide synthetase and matrix metalloproteinases (MMPs) (Dinarello, 2002). In addition, IL-1 is also known as a lymphocyte activating factor (LAF) promoting proliferation of sub-optimally stimulated thymocytes (Th0) *in vitro* (LAF-assay), thus participating in adaptive immunity as well.

Excessive and/or dysregulated activity of IL-1 cytokines is associated with tissue destruction and therefore the synthesis, release and biological activity of IL-1 cytokines have been identified as therapeutic targets for common inflammatory disorders such as rheumatoid arthritis (RA) (Salvi and Lang, 2005; Burger et al., 2006).

IL-18 is also able to induce the release of pro-inflammatory cytokines, however its major feature, in collaboration with IL-12, is the induction of Interferon-γ production. (Yoshimoto et al., 1998). Using animal models, it has been shown that IL-18 is required for the IFN-γ-dependent eradication of several microbial infections. IL-18 skews Th0 cells in the beginning of the adaptive immune response towards Th1 differentiation, favoring a cellular adaptive immune response. In addition to IFN-γ production and Th1 polarization, IL-18 induces both T- and NK-cell maturation and potentiates cytotoxicity (Dinarello and Fantuzzi, 2003) and is also involved in Th17 cell responses (Harrington et al., 2006). IL-18 can induce TNF-α production both *in vitro* and *in vivo*, an effect that links this cytokine to the development of the inflammatory response and the pathogenesis of several autoimmune diseases. Altogether, it is clear that by regulating T-helper cell differentiation, B-cell activation and Immunoglobulin production as well as its activity on Dendritic cells (DC), Natural Killer (NK) cells and neutrophilic granulocytes, IL-18 is a cytokine that has an important role in both the innate as well as the adaptive immune response (reviewed in (Carroll et al., 2008).

Recently, six novel members of the IL-1 cytokine family (IL-1F5-IL-110) were identified on the basis of sequence homology, three-dimensional structure, gene location and receptor binding (Debets et al., 2001; Towne et al., 2004; Nicklin et al., 2002; Taylor et al., 2002; Sims et al., 2001; Dunn et al., 2001). Still, little is known about the biological effects of these novel members of the IL-1 family cytokines.

Interleukin-33 (IL-33, IL-1F11) is the most recently identified member of the family of IL-1-like cytokines (Schmitz et al., 2005). IL-33 activates basophils, mast cells, eosinophils, NK and NKT cells, Th2 lymphocytes, cardiomyocytes and cultured glial cells (reviewed in (Haraldsen et al., 2009). Recombinant IL-33 was found to drive production of Th2-associated cytokines in mast cells (Ali et al., 2007; Allakhverdi et al., 2007; Hayakawa et al., 2007; Ho et al., 2007; Iikura et al., 2007; Moulin et al., 2007; Schmitz et al., 2005), to induce chemotaxis of Th2 cells (Komai-Koma et al.,

2007) and to protect the heart against cardiac stress (Sanada et al., 2007) and atherosclerosis (Miller et al., 2008). Moreover, IL-33 is also able to induce pro-inflammatory cytokines suggesting its involvement in pro-inflammatory disorders (Dinarello, 2005; Moulin et al., 2007; Smithgall et al., 2008; Ali et al., 2007). Despite its involvement in the classical inflammatory diseases such as asthma, rheumatoid arthritis, atherosclerosis and urticaria, IL-33 has now also been shown to participate in cardiovascular pathophysiology (reviewed in (Kakkar and Lee, 2008; Gabay and McInnes, 2009). In addition to its clasical cytokine activity IL-33 translocates to nucleus of producing cells and acts as chromatin associated nuclear factor (Carriere et al., 2007; Roussel et al., 2008). Thus similar to IL-1α and chromatin-associated cytokine High-mobility group box 1 (HMGB1), IL-33 is also a dual function protein that may act as both a cytokine and an intracellular nuclear factor (reviewed in (Gadina and Jefferies, 2007; Haraldsen et al., 2009).

1.2. Expression, processing and release of IL-1 family cytokines

1.2.1. Expression

IL-1 family members IL-1α, IL-1β and IL-18 are released by a variety of cell types including monocytes, macrophages, dendritic cells, epithelial cells, keratinocytes endothelial cells, smooth muscle cells, and synovial fibroblasts (Dinarello, 1999; Gracie et al., 2003; McInnes et al., 2000; Dinarello and Fantuzzi, 2003). IL-1F6, 8 and 9 are expressed predominantly in the skin (Towne et al., 2004). Furthermore, IL-1F6 is also expressed in the trachea and thymus, IL-1F8 in skeletal muscle and glial cells (Wang et al., 2005), and IL-1F9 in the trachea, uterus and in bronchial epithelia (Vos et al., 2005).

Very little is known yet about the cellular sources of IL-33 *in vivo*. Expression of IL-33 was initially found in human high endothelial venules (HEV) (Baekkevold et al., 2003). These endothelial cells are specialized cells in secondary lymphatic tissues which express specific adhesion molecules allowing lymphocytes the transmigration from the blood into the lymphoid tissue. Expression of IL-33 mRNA has also been seen in endothelial cells from other lymphoid tissues, chronically inflamed rheumatoid arthritis, synovium and intestine of patients with Crohn's disease, keratinocytes, dendritic cells, activated macrophages, fibroblasts, smooth muscle cells, normal endothelial cells and epithelial cells (Carriere et al., 2007; Moussion et al., 2008;

Schmitz et al., 2005). However, whether all these different cell types actually produce protein and release biologically active IL-33 is unclear.

1.2.2. Processing

IL-1α and IL-1β are translated as 31 kDa leaderless precursor molecules. IL-1α is biologically active in this full length form (Mosley et al., 1987), whereas precursor IL-1β has to be cleaved intracellularly by caspase 1 (also known as IL-1β converting enzyme ICE) to the 17 kDa mature and active form (Dinarello, 1998). IL-18 also lacks a signal peptide and is produced as an inactive 23 kDa precursor that is cleaved by caspase 1 to form the biologically active 18 kDa species (Gu et al., 1997). Proteinase-3, caspase 3, cathepsins and elastase can also cleave the precursor polypeptide, but this can result in the production of inactive forms of IL-18 (Akita et al., 1997; Robertson et al., 2006; Sugawara et al., 2001).

IL-33 is closely related to IL-18 and IL-1α/IL-1β. Like IL-18 and IL-1β, IL-33 is synthesized as a 31 kDa precursor form lacking a leader peptide. Recently, IL-33 has been reported to be processed to the mature cytokine form by caspase 1 *in vitro* (Schmitz et al., 2005), but a more recent study failed to find any evidence for caspase-1 processing of IL-33 *in vivo* (Carriere et al., 2007). IL-1F5, -6, -8, -9 and -10 lack signal peptides and, to date, no caspase-1 cleavage sites have been identified (Kumar et al., 2000; Smith et al., 2000; Lin et al., 2001), whereas IL-1F7 contains a putative signal peptide and has been shown to be cleaved by caspase-1 (Kumar et al., 2002).

1.2.3. Release

It has been postulated that cells such as monocytes require a second stimulus to release active IL-1 cytokines. The initial stimulus, e.g. LPS, causes large accumulation of precursor IL-1β in the cytosol and only a modest IL-1β secretion (Dinarello, 1998). IL-1β release is induced strongly by extracellular adenosine triphosphate (ATP), which signals via the P2X7R receptor causing K+ efflux from cells activating procaspase-1 and hence processing of precursor IL-1β (Ferrari et al., 2006). Release of IL-18 involves a similar mechanism (Dinarello and Fantuzzi, 2003). There is evidence to suggest that IL-1β may be packaged into small plasma membrane microvesicles that are released into the extracellular space (MacKenzie et al., 2001; Andrei et al., 2004). Similar to IL-1, maturation and release of IL-33 has been proposed to be a caspase 1 -dependent mechanism (Dinarello, 2005; Schmitz

et al., 2005). Although *in vitro* evidence of caspase 1 cleavage has been published (Schmitz et al., 2005; Sharma et al., 2008), it is not yet clear how IL-33 may be released from the cells to exert its cytokine activities towards target cells expressing the IL-33 receptor. A caspase 1 cleavage sequence within the primary IL-33 protein structure is not conserved across all species (reviewed in (Kakkar and Lee, 2008) and definitive localization of precursor IL-33 within lysosomal structures has not yet been reported. However, alum-induced activation of the inflammasome resulted in release of IL-33 (Li et al., 2008). The heterochromatin-binding of IL-33 resembles the biology of IL-1α more closely than other members of the IL-1 family and, as has recently been suggested for precursor IL-1α (which is not processesed by caspase 1 but by calpain!), it is possible that in fact caspase 1 acts as a secretory targeting factor for precursor IL-33 (Keller et al., 2008).

Despite the transcriptional regulatory properties of precursor IL-1α, it is doccumented that precursor IL-1α can bind to IL-1 receptor and acts as a pro-inflammatory cytokine (Dinarello, 1998; Mosley et al., 1987). It is possible that like precursor IL-1α which binds to IL-1 receptor type 1, precursor IL-33 may also have the ability to bind to the IL-33 receptor and can exert pro-inflammatory or regulatory functions.

1.3. The interleukin-1 receptor family

The interleukin-1 receptor (IL-1R) family comprises transmembrane proteins [the type I receptors (IL-1RI, IL-18Rα, IL-33Rα, IL-1Rp2, TIGIRR and SIGIRR), the type II receptor (IL-1RII), and the receptor accessory proteins/co-receptors (IL-1RAcP, IL-18Rβ)] and their soluble forms. All transmembrane molecules are characterized by three extracellular immunoglobulin (Ig)-like domains (except SIGIRR, which contains only one Ig-like domain). Type I recptors and co-receptors possess an intracellular region comprising a Toll/IL-1 receptor (TIR) domain (Fig.1.1) which is lacking in IL-1RII. The prototype molecules of the family of IL-1 receptors are IL-1RI (Sims et al., 1988) and its co-receptor molecule IL-1RAcP (Greenfeder et al., 1995). IL-1α and IL-1β bind to IL-1RI or IL-1RII, of which only IL-1RI can initiate signal transduction where as IL-1RII serves as a regulatory molecule. IL-1 signal transduction is initiated by binding of either form of IL-1 to IL-1RI, which undergoes a conformational change allowing IL-1RAcP to recognize the ligated IL-1RI (Fig.1.2). IL-1RAcP itself does not recognize IL-1 alone. Upon ligand binding both transmembrane proteins, IL-1RI and IL-1RAcP, form a heterodimeric complex which results in the close association of the

signaling domains located in the cytoplasmic regions of the two chains (reviewed in (Martin and Wesche, 2002; O'Neill, 2008). These signaling domains are found in rather conserved form in nearly all members of the Toll-like receptor and IL-1 receptor families. This is why they were termed TIR-domains (Toll-like Interleukin-1 Receptor homology domains). It has been demonstrated that IL-1RAcP is essential for IL-1 signaling (Korherr et al., 1997; Wesche et al., 1997b; Cullinan et al., 1998) and that the C-terminal TIR-domains of both chains are required to facilitate down-stream signaling (Lang et al., 1998; Radons et al., 2002).

IL-18 signals through the IL-18 receptor complex (IL-18R), which is homologous to the IL-1RI complex, a heterodimer consisting of IL-18Rα (IL-1 receptor related protein 1 or IL-1Rrp1) and IL-18Rβ (Accessory protein like or AcPL) subunits. In agreement with the IL-1 receptor system the extracellular domains of both IL-18R chains contain three Ig-like domains and an intracellular region comprising a Toll/IL-1 receptor (TIR) domain each. In the IL-18 receptor system mature IL-18 binds to IL-18Rα and then the IL-18Rβ-chain recognizes the ligated IL-18Rα-chain to form a transmembrane heterodimer. IL-18Rβ is unable to bind to IL-18 alone. Again both chains carry a TIR-domain in their cytoplasmic parts allowing the formation of a scaffolding structure after heterodimerization which enables the recruitment of adaptor proteins to the TIR-domain dimer (Boraschi and Tagliabue, 2006; Subramaniam et al., 2004) and initiates downstream signaling.

Ligand-mediated heterodimerization with subsequent intracellular association of strongly homologous but discrete TIR domains seems to be a typical feature of the IL-1 receptor subfamily of TIR-domain containing receptors. Although the molecules of the IL-1R complex and the IL-18 receptor complex are closely related, they can not substitute for each other. The receptors are specific for their respective ligands and the co-receptors recognize only the respective ligated receptors.

A series of IL-1 receptor family members share the features of the receptors for IL-1 and IL-18 (Born et al., 2000). IL-1Rrp2 and ST2 (T1, Der4) also contain three extracellular Ig-like domains, they are type I transmembrane molecules and possess a TIR domain in their cytoplasmic part. One family member, single Ig-domain containing IL-1R related (SIGIRR), possesses a single extracellular Ig-like domain (Thomassen et al., 1999). SIGIRR or TIR8 (for Toll / IL-1R 8), also contains a TIR domain in its intracellular region, and has been discussed to compete with the other

TIR domain containg receptors for the intracellular adaptor molecule MyD88 and the downstream signaling moelcule TRAF6, thus inhibiting IL-1, IL-18 and LPS activity (Wald et al., 2003).

IL-1R-related protein 2 (IL-1Rrp2) is the receptor for three novel members of the IL-1 family, IL-1F6, IL-1F8, and IL-1F9 , which was reported to require IL-1RAcP as co-receptor for activity (Dunn et al., 2001; Kumar et al., 2000; Smith et al., 2000; Towne et al., 2004). The recently identified receptor for IL-33 (IL-1F11), ST2, has been known as a member of the IL-1 receptor family for many years (Klemenz et al., 1989; Tominaga et al., 1991) although its ligand had remained elusive until 2005 when IL-33 was identified as its ligand (Schmitz et al., 2005). As the IL-33Rα-chain is very homologous to IL-1RI and IL-18Rα-chain it was speculated that the IL-33Rα-chain also required a β-chain containing a TIR-domain for signal transduction and it was also suggested that this β-chain itself will not bind to IL-33 and that it is a member of the IL-1 receptor family (Dinarello, 2005).

IL-1RII is a transmembrane molecule with the extracellular feature of the IL-1R family lacking a cytoplasmic TIR domain (McMahan et al., 1991). Soluble versions of IL-1R family members also exists, however they do not initiate signal transduction (reviewed in (Barksby et al., 2007).

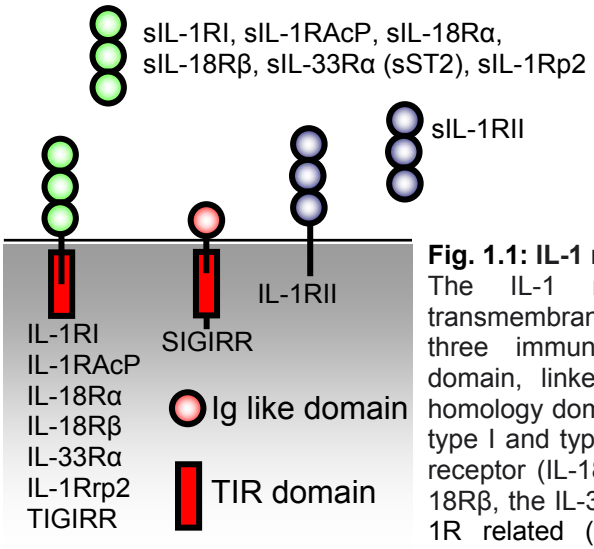


Fig. 1.1: IL-1 receptor-like family.
The IL-1 receptor-like family includes soluble and transmembrane receptors. They share the common feature of three immunoglobulin-like motifs within the extracellular domain, linked by an α-helix to an intracellular Toll/IL-1R homology domain (red box). Members of this family include the type I and type II IL-1 receptors (IL-1RI and IL-1RII), the IL-18 receptor (IL-18Rα), their accessory proteins IL-1RAcP and IL-18Rβ, the IL-33 receptor (ST2), single Ig-domain containing IL-1R related (SIGIRR) and others (such as IL-1Rrp2 and TIGIRR). Soluble receptors lacking the transmembrane and cytosolic parts of protein are also exists.

1.3.1. Regulation of the biological activity of IL-1 like cytokines

Control of IL-1 family member expression occurs primarily through processing of the precursor cytokine into the active form and its subsequent release (Novick et al., 1999; Kim et al., 2000). In addition to controlled expression and release, activities of IL-1 family members are also regulated by soluble receptors, natural antagonists and inhibitors. IL-1 activities are also regulated by IL-1RII. IL-1RII lackes a cytoplasmic TIR domain (McMahan et al., 1991) and functions as a regulatory receptor (Lang et al., 1998). IL-1RII, binds IL-1α/β as a decoy receptor and can not initiate signal transduction (Lang et al., 1998; Colotta et al., 1993). Two mechanisms have been described for IL-1RII which modulate the responsiveness of cells to IL-1: One is ligand sequestration (Colotta et al., 1994), and the other one is competition for the co-receptor (Lang et al., 1998; Mantovani et al., 2001). Soluble versions of IL-1R family members are well documented (reviewed in (Barksby et al., 2007). Soluble versions of IL-1 receptor family members exert regulatory functions by sequestering cytokines. Respective soluble receptors bind to IL-1, IL-18 or IL-33; they do not initiate signal transduction (reviewed in (Barksby et al., 2007). Moreover the soluble forms of co-receptors enhances the ability of soluble type I and soluble type II receptors to inhibit cytokine action (Lang et al., 1998; Smith et al., 2003; Smeets et al., 2003).

In addition to soluble receptors IL-1 induced signaling via the IL-1RI receptor can be blocked by the binding of the receptor antagonist, IL-1Ra. The biological activity of IL-18 is also controlled by IL-18 binding protein (IL-18BP) (Novick et al., 1999; Kim et al., 2000). IL-18 BP is a constitutively expressed and secreted natural inhibitor (not a receptor) of IL-18 activity. IL-18BP possesses a high-affinity binding site and neutralizes IL-18 (reviewed in (Dinarello et al., 2003). Interestingly, IL-18BP expression in keratinocytes and intestinal cell lines is up-regulated in response to IFN-γ (Paulukat et al., 2001), indicating a negative feedback loop for IL-18-induced IFN-γ production by Th1 cells. The IL-1 homologue, IL-1F7, also binds to IL-18BP and it has been proposed that it plays a role as a negative regulator of IL-18 activity. When bound to IL-18BP, IL-1F7 can form a complex with IL-18Rα chain preventing the formation of a functional receptor complex (Carroll et al., 2008; Bufler et al., 2002).

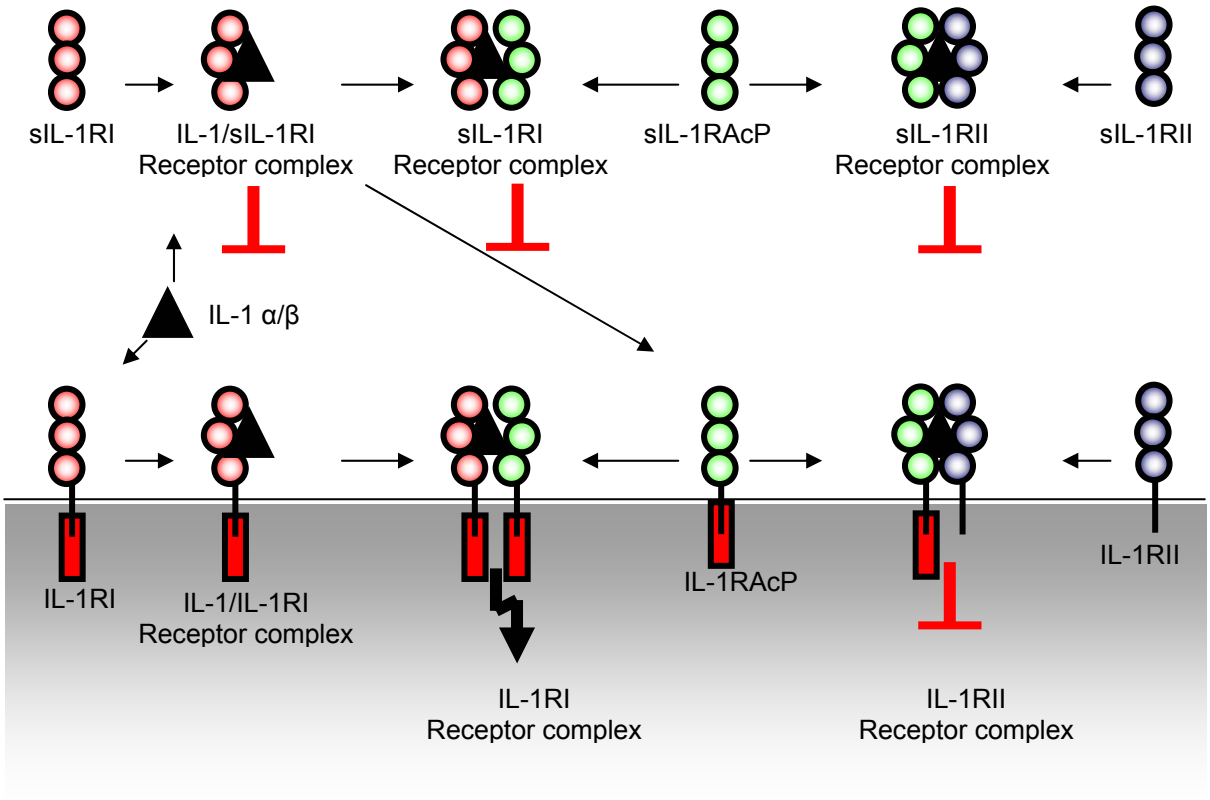


Fig. 1.2: Regulation of signaling in the IL-1 receptor system.
IL-1RI binds IL-1α or IL-1β and then interacts with IL-1RAcP to form a heterodimeric signaling receptor complex. Receptor and co-receptor each contribute an individual TIR domain. Soluble type I or type II, IL-1 receptors and membrane-inserted type II IL-1R regulate IL-1 responsiveness by sequestering ligand or IL-1RAcP.

1.3.2. Signaling of the IL-1 family: the MyD88/IRAK/TRAF6 module

1.3.2.1. IRAK recruitment to the receptor complex

After ligand binding and formation of the heterodimeric receptor complex, presumably the close spatial association of the two TIR domains allows homotypic protein–protein interactions of the TIR domains. As a consequence, probably the conformation of the TIR domains is altered, or the combination of the cytoplasmic domains creates novel scaffolds. This is allowing an interaction of the adapter proteins MyD88 with the TIR domains at conserved surface patches (reviewed in (Martin and Wesche, 2002). MyD88, mediates a homophilic interaction with the TIR domains of receptor and co-receptor complex (Muzio et al., 1997; Wesche et al., 1997a; Janssens et al., 2002), possibly as a dimer (Burns et al., 1998). Recruitment of MyD88 leeds to the recruitment and activation of down stream molecules of the IRAK family of protein kinases namly IRAK-1, 2 and 4 (reviewed in (Akira and

Takeda, 2004) via their death domains (reviewed in (Liew et al., 2005). In addition Tollip mediated translocation of IRAK-1 to the active IL-1R complex is also reported (Xiao et al., 1999; Burns et al., 2000).

1.3.2.2. IRAK phosphorylation and departure from the receptor complex

As, IRAK-1 binds MyD88 at the receptor complex, it gets phosphorylated, both by the related kinase IRAK-4 as well as by autophosphorylation. IRAK-4 mediated phosphorylation of IRAK-1 (Burns et al., 2003) triggers IRAK-1's own kinase activity, resulting in multiple autophosphorylation events (Cao et al., 1996; Kollewe et al., 2004). Both IRAK-4 and IRAK-1 then dissociate from MyD88 rapidly and interact with tumor necrosis factor receptor (TNFR)- associated factor-6 (TRAF6) (reviewed in (O'Neill, 2008).

1.3.2.3. Formation of the IRAK/TRAF6 signalosome

The interaction of the activated IRAKs with the adaptor protein TNF receptor-associated factor 6 (TRAF6, an E3 ubiquitin ligase) induces activation of TRAF6. TRAF6 is then thought to auto-ubiquinate, attaching K63-polyubiquitin to itself (reviewed in (Vallabhapurapu and Karin, 2009). K63-polyubiquitinated TRAF-6 then recruits already preexisting complexes of TGF-β-activated kinase 1 (TAK1) and two TAK1 binding proteins, TAB2 and TAB3 (Jiang et al., 2002; Takaesu et al., 2000) through the recognition of polyubiquitin chains on TRAF6 by highly conserved zinc finger domains in TAB2 and TAB3. This activates TAK1 which then couples to the inhibitor of NF-κB (IκB) kinase (IKK) complex. Phosphorylated IRAK1 is degraded by proteasomes after recognition of specific phosphorylated amino acids on IRAK-1 by ubiquitin ligases (Ordureau et al., 2008). The Pellino proteins might also be involved in degradatation of activated IRAKs resulting in termination of TLR signaling (reviewed in (Moynagh, 2009).

1.3.2.4. Activation of NF-κB and JNK/p38 MAPKs

The interaction of K63-polyubiquitinated TRAF6 and TAK1 complex is believed to induce the dimerization, autophosphorylation and activation of TAK1. Activated TAK1 then interact with the inhibitor of NF-κB (IκB) kinase (IKK), which contains the scaffold protein NF-κB essential modulator (NEMO) and IKK2, the kinase responsible for phosphorylation of IκB. TAK1 also couples to the upstream kinases for p38, JNK and ERK1/2 leading to activation of NF-κB, JNK/p38, MAPKs and ERK1/2 (reviewed in (Moynagh, 2009). Activated NF-κB and other transcription factors can translocate

to the nucleus and mediate an increase in inflammatory cytokine gene expression, leading to pro-inflammatory responses.

The role of the TAK1:TAB1/2/3 complex in IKK activation remains controversial, although it is generally agreed that this complex is needed for JNK and p38 activation. Indeed, a TAK1:TAB1 fusion protein is constitutively active and capable of stimulating AP-1 activity (reviewed in (Vallabhapurapu and Karin, 2009).

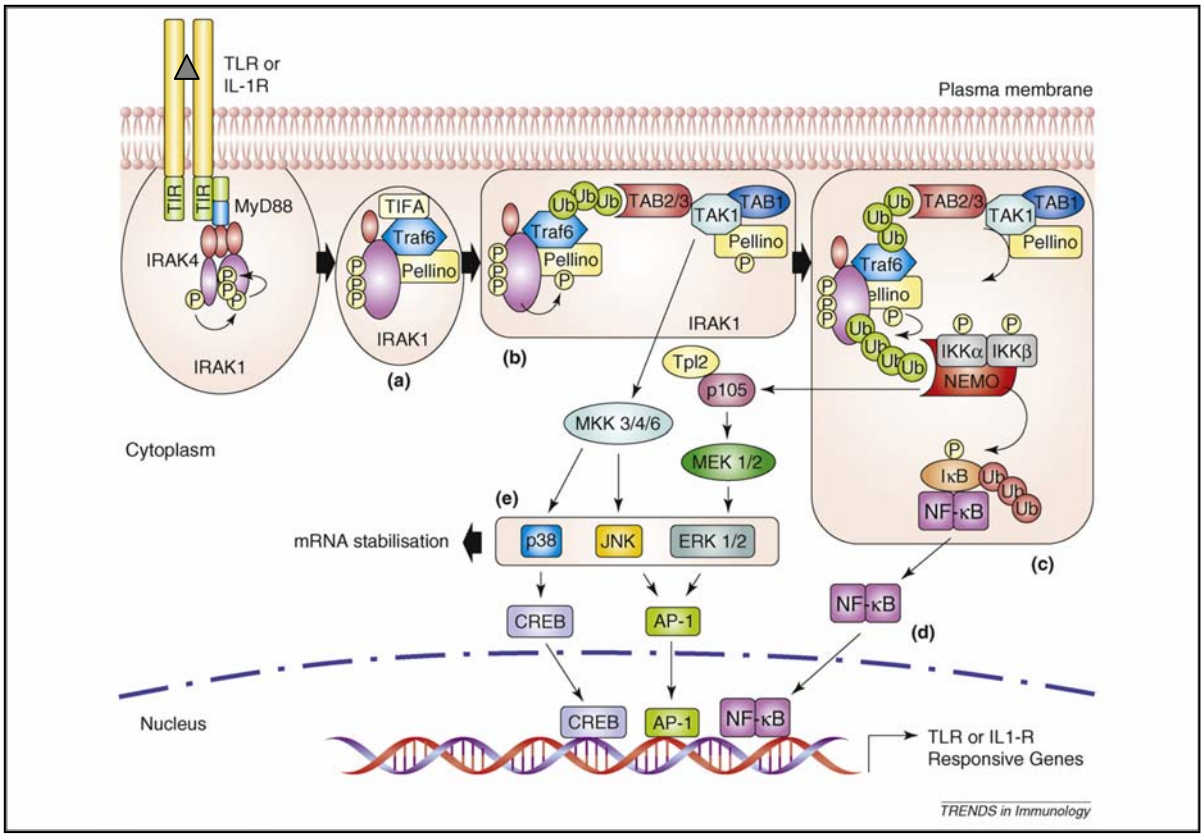


Fig. 1.3: Proposed model of TLR/IL-1R-dependent activation of NF-κB and MAPK pathways. Ligand binding to IL-1R complex triggers the recruitment of MyD88 via a homophilic TIR-TIR interaction. MyD88 brings IRAK-4 into the receptor complex. In addition, preformed Tollip/IRAK-1 complexes are recruited to the receptor, which allows IRAK-1 to bind MyD88 via its death domain. In this way, IRAK-1 and IRAK-4 come in close proximity, which allows IRAK-4 to phosphorylate IRAK-1 on critical residues that are necessary to trigger IRAK-1. **(a)** The stimulation of TLRs or IL-1R promotes the association of Pellino proteins with IRAK1 and TRAF6. The K63-linked polyubiquitylation (Ub) of TRAF6 facilitates recruitment and activation of a pre-associated TAK1–TAB complex; **(b)** IRAK1 phosphorylates (P) Pellino proteins, leading to activation of their E3 ligase activity and Pellino-mediated K63-linked polyubiquitylation of IRAK1; **(c)** The NEMO binds to polyubiquitylated IRAK1, bringing the IKK and TAK1 complexes into close proximity; **(d)** TAK1 activates the IKKs, resulting in the phosphorylation (P) of the inhibitory IκB proteins and enabling for translocation of NF-κB into the nucleus; **(e)** In addition to interacting with and activating the IKK complex, TAK1 can also trigger the ERK1/2, p38 and JNK MAPK pathways. The MAPK pathways activate several transcription factors that co-operate with NF-κB to induce the expression of a range of IL-1R/TLR-responsive genes. Figure is taken from **(Moynagh, 2009)**.

In addition to the TAK1 dependent NF-kB activation, co-existence of the parallel IL-1-mediated MEKK3-dependent signaling pathways has recently been reported for NF-kB activation (Yao et al., 2007). These two pathways are regulated at the level of IRAK modification. The TAK1-dependent pathway causes IKKα/β phosphorylation and IKKβ activation, leading to classic NF-kB activation through IκBα phosphorylation and degradation. The TAK1-independent MEKK3-dependent pathway induces IKKγ phosphorylation and IKKα activation, resulting in NF-κB activation through IκBα phosphorylation and subsequent dissociation from NF-κB but without IκBα degradation (Fraczek et al., 2008).

Presently, there is no information available that the mechanisms discussed here for the IL-1R complex are different for the IL-18 receptor complex. As far as investigated, no qualitative differences in the major signal pathways activated by IL-18 were revealed to those reported for IL-1 (Thomassen et al., 1998). The mode by which IL-33 exerts its effect has not been fully established but it probably acts similarly to other members of the IL-1 family, specifically IL-1β and IL-18 (reviewed in (Kakkar and Lee, 2008).

1.4. Aims of the study

Interleukin-33 is a member of the IL-1 family of cytokines which is involved in health and disease. Understanding the immunobiology of IL-33 promises to provide further insight not only into the normal function of this novel mediator of the immune system but also into the pathogenesis of diseases such as asthma, rheumatoid arthritis, atherosclerosis and cardiovascular disorders. This knowledge may help to identify novel therapeutic targets to treat these diseaes.

The experiments of this study address two central aspects of the biology of IL-33.

First, they focus on the cell responding to the cytokine, where it was the aim to

- identify and characterize the components of the IL-33 receptor complex required for signal transduction.
- compare signaling and gene regulation of IL-33 with that of IL-1 and IL-18 using micro-arrays.

And second, they focus on the cell producing IL-33, where it was the aim to

- clarify whether precursor-IL-33 requires caspase 1 processing to be able to bind to the IL-33R on responder cells and exert its biological functions as a classical cytokine.
- investigate whether IL-33 is released from intact cells as full length molecule or after proteolytical maturation comparable to other members of the IL-1 family.

Chapter 2

Materials and Methods

2.1. Mammalian cell culture

All murine thymoma cell lines (EL-4 6.1, D6/76 (a clone of EL-4 cells lacking IL-1RAcP, generated by von Hoegen (von Hoegen et al., 1989), 6-IRAK-19 cells (EL-4 6.1 cells stably transfected with a plasmid encoding human IRAK-1 generated by J. Knop, unpublished) and EL-4 1B1 cells (EL-4 6.1 cells stably transfected with mIL-33Rα encoding plasmid, generated in this laboratory by Jessica Endig during her Diploma thesis) were cultured in RPMI1640, with the addition of 2 mM L-glutamine, 1 mM Na-Pyruvate, non essential amino acids, and 5 % fetal calf serum (all PAA, Germany) at 5% CO_2 in a humidified incubator at 37 ºC. Cells were passaged after every 2-3 days by diluting the cultures to 5 x 10^4 cells/ml with fresh medium.

Human HEK293RI (Cao et al., 1996) cells were maintained in DMEM medium with addition of 2 mM L-glutamine and 10% FCS at 10% CO2 in humidified incubator.

Murine bone marrow derived mast cells were generated by Prof. Dr. Michael Huber (Max Plank Institute of Immunobiology, Freiburg Germany) as described recently (Gimborn et al., 2005).

Mouse keratinocytes (generated from IL-1 receptor knock out animals by Prof. Werner Falk, University of Regensburg, Germany) murine fibroblasts, NIH 3T3, mouse embryonal fibroblasts (IRAK- control) and L929 were cultured and maintained in DMEM medium with the addition of 2 mM L-glutamine and 10% FCS (all PAA, Coelbe, Germany) at 10% CO2. Cells were passaged after 2-3 days by trypsinization.

Mouse T cell lines D10G4.1 and D10N were cultured in RPMI1640, supplemented with 2 mM L-glutamine, 1 mM Sodium-Pyruvate, non essential amino acids, 2 µg/ml Concanavalin A (Pharmacia, Freiburg, Germany), 3 x 10^{-5}M β-mercaptoethanol, interleukin-2 (as 10% (v/v) conditioned medium mouse spleen cells stimulated with

Concanavalin A for 1 day), 1 ng/ml hIL-1β (a kind gift from Dr. D. Boraschi, Institute for Biomedical Technology, Pisa, Italy) and 10% fetal calf serum (PAA, Coelbe, Germany) at 5% CO_2 in humidified incubator at 37 ºC. For stimulation cells were cultured overnight without IL-2 and IL-1. Cells were passaged after every 2-3 days by diluting the cultures to 5 x 10^5 cells/ml fresh medium.

Human U937 cells, mouse B cells (WEHI 231 and 70Z/3) and macrophages (P388D1, RAW264.7) were cultured in RPMI1640, with the addition of 2 mM L-glutamine, non essential amino acids, 5 x 10^{-5} M β-mercaptoethanol, and 5% fetal calf serum (all PAA, Coelbe, Germany) at 5% CO_2 in humidified incubator. Cells were passaged after every 2-3 days.

Dendritic cells (Xs106) were cultured in IMDM medium, with the addition of 1 mM Na-Pyruvate, 5% (v/v) supernatant of NS47 cells and 10% fetal calf serum (PAA, Coelbe, Germany) at 5% CO_2 in a humidified incubator at 37 ºC. Cells were passaged after every 2-3 days.

Unstimulated and CD3- stimulated (for 6 hours) Th0, Th1, Th2 and Th17 cells were obtained from Prof. Michael Lohoff, Microbiology, Philip University Marburg, Germany, as frozen cell pellets.

2.2. Transient transfections

EL-4, EL-41B1, 6-IRAK-19 and EL-4 D6/76 cells were transiently transfected with plasmids as indicated in figure legends using the DEAE dextran-chloroquine transfection method as previously described (Knop et al., 1998). Briefly, cells were seeded at cell density of 5 x 10^5 cells/ml a day before transfection. For transfection cells were collected by centrifugation (500 x g for 8 minutes), washed with PBS (137 mM NaCl + 2.7 mM KCl +10 mM Na_2HPO_4 + 1.76 mM KH_2PO_4 and pH 7.4 with HCl, autoclaved) and resuspended in TBS (25 mM Tris + 123 mM NaCl + 5 mM KCl + 0.7 mM $CaCl_2.2\ H_2O$ + 0.5 mM $MgCl_2.6\ H_2O$ + 0.6 mM $Na_2HPO_4.\ 2H_2O$, pH 7.4 with HCl and filtre sterlized) at the cell density of 5 x 10^6 cells per ml. 15 µl DEAE dextran (10 mg/ml in H_2O and filter sterlized, Pharmacia) and 12 µl chloroquine (2 mg/ml in water and filter sterilized, Sigma) were diluted with 273 µl of TBS. One µg of required plasmid DNA (in total) as indicated in figures was diluted in 300 µl of TBS. Total amount of plasmid DNA was always adjusted to the same amount using empty vector in all assays. This DEAE dextran-chloroquine solution and diluted DNA were mixed just before addition to the cells. Cells were collected by centifugation at 500g for 4

minutes at room temperature and supernatant was discarded. Cells were resuspended in 600 µl of DEAE dextran-chloroquine-DNA solution and allowed to rotate on rotor at room temperature for 30 minutes. Cells were centrifuged as earlier, supernatant was discarded and cells were washed twice with 1 ml medium (each wash). After washing cells were cultured in 10 ml of RPMI 1640 medium with 1 x penicilline/streptomycin (100 x stock, PAA, Cölbe, Germany) untill further use as described in each experiment.

HEK293RI cells were transfected by a modified polyethylenimine (PEI) transfection method (Ehrhardt et al., 2006). Briefly 3.6 x 10^6 cells were seeded into 56 cm^2 Petri dishes in a volume of 10 ml medium. On the next day, 12 µg plasmid DNA were diluted with serum-free medium to a total volume of 330 µl and mixed with 30 µl of a 1 mg/ml (pH 7) PEI (Sigma-Aldrich) solution, and allowed to rest for 10 minutes. The cell culture medium was adjusted to 3 ml prior to addition of the DNA-PEI mixture. After the following incubation for 4 hours at 37 ºC in a CO_2 incubator, medium was topped up to 10 ml and the cells were incubated overnight.

Murine L929 cells were also transfected with PEI method as described for HEK293RI. For transfection of keratinocytes 3 x 10^6 cells / plate and 60 µl PEI was used.

2.3. Cytokines and other recombinant proteins

hIL-1α was a kind gift from Dr. J. Sims, formerly Immunex Seattle, USA. **hIL-1β** was a kind gift from Dr. D. Boraschi, Institute for Biomedical Technology, Pisa, Italy. **mIL-18** was purchased from MBL, Japan. Recombinant **mIL-33 and hIL-33** were purchased from Alexis, Lörrach, Germany. **mIL-33-Bio** was a kind gift from Prof. W. Falk, University of Regensburg, Germany. **TNFα** was a kind gift from BASF AG, Ludwigshafen, Germany. **LPS** was purchased from Difco Laboratories, USA. **mST2:hIgG(Fc),** was purchased from R & D Systems, Wiesbaden, Germany.

Full length cytokines (**IL-1α/β, IL-18, and IL-33**) and soluble receptors (**sIL-33Rα:hIgG(Fc)** and IL-33 cytokine trap, **sIL-33Rα-sIL-1RAcP:hIgG(Fc)** fusion proteins) were expressed in HEK293RI cells or keratinocytes. Cytokines were immunoprecipitated from cell lysates or supernatants (where specified) using anti-Flag M2 agarose (Sigma) or anti-Myc agarose (Sigma). Soluble IL-33Rα:hIgG(Fc) fusion protein and IL-33 cytokine trap were precipitated from cell free supernatants using protein A sepharose (Sigma). Precipitated proteins were eluted from the beads

by incubation with 100 mM glycine pH 2 and pH was neutralized with 0.1 volume of 1.5 M Tris pH 8.6.

2.4. Measurement of cytokine production

For determination of cytokine production, transfected and untransfected EL-4, EL-4 1B1 or EL-4 D6/76 cells were seeded into 96 well plates at density of 2.5×10^4 cells per well in 200 µl of culture medium. 24 hr after transfection cells were either kept unstimulated or stimulated with varying concentrations (as indicated in figures) of rmIL-33, rhIL-1β, rmIL-18 (MBL, Japan) or incubated with anti-Myc agarose (unstimulated) or indicated amounts of full length mIL-33 captured on anti-Myc agarose (Sigma) in the presence of 0.5 µM Calcium ionophore A 23187 for 16 hours at 37 ºC. Mouse IL-2 was determined in the supernatants by enzyme linked immunosorbent assay (ELISA, OptEIA mIL-2 Set, Becton Dickinson, Heidelberg, Germany).

HEK293RI cells were transfected with IL-33Rα or empty vector using the PEI transfection method. 16-20 hours after transfection, cells were seeded into 96 well plates at 2.5×10^4 cells per well, and after additional 4 hours stimulated with different concentrations of mIL-33. After 16-20 hours of stimulation human IL-8 concentration in the cell culture supernatants was determined by ELISA (hIL-8 Cytoset, Biosource / Invitrogen, Karlsruhe, Germany).

Mouse IL-6 was measured in supernatants from bone marrow-derived mast cells using an IL-6 ELISA kit (OptEIA TM mIL-6 ELISA set, Becton Dickinson Biosciences, Germany).

If the anti- mouse IL-1RAcP mAb 4C5 (kind gift of Dr. N. Dimoudis, Roche, Penzberg) was used in inhibition studies, the cells were always preincubated with the neutralizing antibody for 30 min at 37 ºC before cytokines were added. The rat IgG mAb RA3-6B2 (anti-B220) served as an isotype control.

2.5. MTT assay / D10 assay

D10 cells need IL-1 or IL-33 for proliferation. In order to measure the cell proliferation/cell viability, the MTT Formazan-assay was performed. This assay measures the activity of mitochondrial dehydrogenases in living cells. (4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is taken up by the

cells and metabolized in the presence of NADH, resulting in NAD and blue formazan crystals.

D10G4.1 cells were seeded in 96 well cell culture plates (2 x 10^4 cell in 200 µl/well RPMI medium) with or with out IL-1 or IL-33 and allowed to grow for three days at 37 ºC with 5% CO_2. On the third day medium was reduced to 100 µl and 10 µl of MTT solution (5 mg/ml, Sigma) was added. Cells were incubated in the presence of MTT for 90 minutes. Cells were spun down and supernatant was removed. The blue tetrazolium crystals were dissolved by adding 100 µl of isopropanol containing 5% formic acid to each well followed by sonication in water bath sonicator. The plates were analyzed photometrically at 570 nm excitation in an ELISA reader.

MTT assay was also performed for EL-4 cells and HEK293RI cells to normalize the difference of live cell number in different wells. For this purpose stimulated or unstimulated cells were incubated as described for ELISA. After removing the cell supernatant for determination of cytokines by ELISA, the cells were incubated with MTT and the assay was performed as described earlier.

2.6. Reporter gene assay

Cells were transfected with 3 x NF-kB-Luc plasmid (0.5 µg / 5 x 10^6 EL-4 cells/ derivatives and 1µg / 3.6 x 10^6 HEK293RI or L929 cells) along with other plasmids as indicated in each experiment. 0.5 million transfected EL-4 cells (4 hours after transfection) or 50.000 HEK293RI or L929 cells (next day) were seeded in 96 well plate and stimulated if needed in 300 µl (EL-4) / 200 µl (HEK293RI or L929) of total volume and incubated overnight at 37 ºC in CO_2 incubator. Next day cells were centrifuged in 96 well plate at 1250rpm for 8 minutes and supernatant was discarded. Cells were washed with 250 µl PBS and lysed with 35 µl / well of 1 x passive lysis buffer (Promega) directly in 96 well plate by incubation for 15 minutes on ice. Cell debries were removed by centrifugation of 96 well plate at 3500rpm for 15 minutes at 4 ºC and luciferase activity was measured 1 sec after addition of 100 µl of substrate solution (436 µM D-Luciferin, (Acid free Biomol #54568) + 436 µM NaOH (Luciferin in 5 ml Water + NaOH solution drop by drop) + 20 mM Tricin + 2.67 mM $MgSO_4 . 7 H_2O$ + 1.07 mM Magnesiumcarbonathydroxid . $5H_2O$ + 33.3 mM DTT + 530 µM ATP, Disodiumsalt Grade 1, Sigma #A2383 + 290 µM Coenzym A, Trilithiumsalt Dihydrat MP Biomedicals #100493) to 25 µl of cell lysate, Luciferase activity was measured for 10 sec using a microplate luminometer (MicroLumatePlus LB 96V, Berthold

Technologies, Bad Wildbad, Germany) using WinGlow software provided with instrument.

For activation of NF-κB in response to full length IL-33, its N and C terminal fragments transfected EL-41B1 cells (0.5 x 10^6) were incubated either with anti-Myc agarose (unstimulated), or with indicated amount, flmIL-33_{1-266}, mIL-33_{1-175}, mIL-$33_{175-266}$, flIL-33_{D175A} captured on anti-Myc agarose (Sigma) or recombinant IL-33 (Alexis). If IL-33Rα:Fc fusion protein was used, IL-33 was preincubated of 30 minutes with indicated amount of soluble IL-33Rα:Fc before stimulation.

2.7. Measurement of protein concentration from cell lysate

The protein concentrations from cell lysates were determined by the Bradford method using Bio-Rad Protein Assay reagent (dye reagent concentrate, Cat.No. 500-0006, A Coomassie Brillant Blue G-250 in Phosphoric acid and Methanol). BSA was used as standard protein and the reaction was carried out in 200 µl volume in 96 well plate. A protein standard of BSA was prepared from 80 µg / ml to 0 µg / ml (i.e. 80, 60, 40, 30, 20, 10 , 5 and 0 µg / ml) and 2 µl proteins from cell lysates were diluted with water to 100 µl. Bio-Rad Protein Assay reagent (2 volume) was also diluted with water (3 volume). 100 µl of the standards and samples were mixed with equal volume of diluted Bio-Rad Protein Assay reagent and alowed to stay for 5 minutes. The absorbance at 595 nm was measured in an ELISA reader (SPECTRAmax® 340 PC384). The protein concentrations were calculated by comparing the absorbence from test samples with that of standard using SOFTmax PRO 4.3 LS software provided with the instrument.

2.8. Western blotting

Cells were stimulated or kept unstimulated as described in figure legends and lysed by 500 µl lysis buffer (50 mM HEPES (Acid free) + 250 mM NaCl + 20 mM β-Glycerophosphat + 5 mM Na_2-4-Nitrophenylphosphate . $6H_2O$ + 1 mM Na_2EDTA . 2 H_2O pH 7.9 and 10% (w/v) Glycerol + 0.5% (w/v) IGEPAL = NP-40 (Sigma) + 1 × Protease inhibitors complete (Roche) + 5 mM DTT + 1 mM Na-Orthovanadate) (Cao et al., 1996) for immunoprecipitation and Western blots), JNK assay lysis buffer (for JNK assay and anti JNK western blots), or by Laemmli buffer (17.6 mM Tris pH 6.8 + 6% SDS + 30% Glycerol + 15% β-Mercaptoethanol + 0.024% Bromphenolblue, (Laemmli, 1970)) for p38, phospho p38, and IL-33 processing experiments). Before lysis, cells were washed twice with ice cold PBS. Cells were incubated with 500 µl of

lysis buffer for 30 min at 4 ºC with gentle rotation. For direct lysis by Laemmli buffer, cells were harvested by trypsinization, scrapping or centrifugation, washed with 1 ml PBS and heated for 20 minuts with Laemmli buffer at 95 ºC. The cell lysate was centrifuged at 13.000 rpm for 20 min before the supernatant was used for further experiment.

Proteins were immunoprecipited when needed. Proteins from cell lysates and or immunoprecipitates were seperated in 7.5%-15% discontinous SDS-PAgels (Laemmli, 1970) using denaturing electrophorsis buffer (192 mM Glycin + 25 mM Tris Base, pH ~8.5 + 0.1% SDS). Proteins were transfered to PVDF membrane in a semi dry electroblotter. For electroblotting 1 mA current / 1 cm^2 of membrane was sullplied for 2 hours in presence of blot buffer (192 mM Glycin + 25 mM Tris Base, pH ~8.5 + 10% Methanol). After electroblotting, the PVDF membrane was blocked in a buffer containg either 5% skimmed milk or 5% BSA solution in 1 x TBS/T (20 mM Tris +137 mM NaCl, pH 7.6 with HCl + 0.1% Tween-20) for 1 hour at room temperature with gentle shacking. Thereafter the membrane was washed thrice in 1 x TBS/T buffer, then incubated with the primary antibody (sources and dilutions are given in table 2.1) for overnight at 4 ºC with gentle shacking. The next day the membrane was washed thrice with 10 ml TBS/T (10 minutes each) and incubated with appropriate secondary antibody conjugated with horseraddish peroxidase for 1-2 hours at room temperature with gentle shacking. The membrane was washed thrice (10 min each) with 10 ml of TBS/T buffer and specific signals were visulized after enhanced chemiluminescence (ECL) reaction.

Table 2.1. Primary antibodies and their dilutions used in Western blotting

Epitope	Source	Species from	Dilution	Buffer
Flag (bioM2)	Sigma	Mouse	1:500	TBS/T
GAPDH	Santa Cruz sc-25778	Rabbit	1:400	5 % MP in TBS/T
JNK	Becton Dickinson BD #610627	Mouse	1:500	5 % MP in TBS/T
Myc (clone A-14)	Santa Cruz sc-789	Rabbit	1:2.000	1 % MP in TBS/T
ST2 (IL-33Rα)	MD Biosciences (DJ8)	Rat	1:1000	5 % MP in TBS/T
p38	Cell singlling CST :9212	Rabbit	1:4.000	5 % BSA in TBS/T
Phospho-p38	Cell singlling CST: 9211S	Rabbit	1:1.000	5 % BSA in TBS/T
IkB	Santa Cruz sc:371	Rabbit	1:400	5 % MP in TBS/T
Phospho IkB	Cell singlling CST: 9246	Mouse	1:1000	5 % MP in TBS/T

For ECL reaction, one ml of blot reagent A was mixed with one drop of blot reagent B (both Interchem, France) and poured directly on the PVDF membrane placed on transparent plastic sheet. The membrane was incubated for one minute at room temperature and covered with transparent plastic sheet. The membrane was exposed to camera for imaging (INTAS Chemiluminescence, Intas Science Imaging Instruments GmbH, Göttingen, Germany) untill the clear signal was observed.

For second staining of the membrane the 1st antibodies were removed by incubating the membrane in 10 ml of the stripping buffer (25 mM Glycin-HCl, pH 2.0 + 1% SDS) for 30 minutes. The membrane was washed thrice with the TBS/T and immunostained again as described above.

Table 2.2. Secondary antibodies / reagents used in Western blotting

Secondary reagent	Source (Concentration)	Dilution	Buffer
Streptavidin-HRP conjugate	Dianova (0.5 mg/ml)	1:10000	1% MP in TBS/T
HRP conjugate Mouse anti Rabbit IgG (light chain)	Dianova (0.8 mg/ml)	1:10000	1% MP in TBS/T
HRP conjugate Goat anti Rabbit IgG (heavy & light chain)	Jackson ImmunoResearch (0.8 mg/ml)	1:10000	1% MP in TBS/T
HRP conjugate Rabbit anti Mouse IgG (heavy & light chain)	Jackson ImmunoResearch (0.8 mg/ml)	1:10000	1% MP in TBS/T

2.9. Immunoprecipitations

HEK293RI or murine keratinocytes were transfected with 12 µg of mammalian expression plasmids or empty vector. Transfected cells were kept unstimulated or stimulated as described in figure legends. Cells were washed twice with 10 ml of ice cold PBS and lysed with 500 µl of lysis buffer (Cao et al., 1996) for 30 min at 4 ºC with gentle rotation. The cell lysate was centrifuged at 13,000 rpm for 20 min before the supernatant was incubated with antibodies for 16 hours at 4 ºC with gentle rotation. For anti-Flag immunoprecipitation, 15 µl of anti-Flag M2 agarose beads (Sigma) were used. For anti-Myc immunoprecipitation, 0.6 µg of anti c-Myc antibody (A-14, Santa Cruz Biotechnology, sc-789 Heidelberg, Germany) and for IL-33, 1 µg anti-IL-33 Nessy-1 (Alexis) with Protein A sepharose (GE Healthcare, Freiburg, Germany) were used instead. Beads were washed thrice with lysis buffer and once with PBS before being resuspended in 30 µl of Laemmli sample buffer, and heated to 95 ºC for 10 min. Proteins were separated on a 10%-15% discontinous SDS PAgel

and transferred to PVDF membrane in a semi-dry fashion. Proteins were detected by staining with specific antibodies (anti c-Myc A-14, Santa Cruz Biotechnologies, sc-789; anti-Flag bioM2, Sigma, F9291).

2.10. Co-immunoprecipitation and Western blotting

HEK293RI cells were transfected with 6 µg of pMyc-mIL33Rα and/or 6 µg of pFlag-mIL1RAcP or any other plasmid following the PEI transfection method. 6 µg of empty vector pMyc-Zeo were added when only one of either plasmids was transfected. Transfected cells were kept unstimulated or stimulated with 10 ng/ml rhIL-1β or 100 ng/ml rmIL-33 for 15 min at 37 ºC. Cells were washed thrice with 10 ml of ice cold PBS and lysed with 500 µl of lysis buffer (Cao et al., 1996) for 30 min at 4 ºC with gentle rotation in the absence or presence of 10 ng rhIL-1β or 100 ng rmIL-33. The cell lysate was centrifuged at 13,000 rpm for 20 min before the supernatant was incubated with 12 µl of anti-Flag M2 agarose beads (Sigma) for 16 hours at 4 ºC with gentle rotation. For anti-Myc immunoprecipitation, 0.6 µg of anti c-Myc antibody (A-14, Santa Cruz Biotechnology, sc-789 Heidelberg, Germany) with Protein A sepharose beads (GE Healthcare, Freiburg, Germany) were used instead. Beads were washed thrice with lysis buffer and once with PBS before being resuspended in 30 µl of Laemmli sample buffer, and heated to 95 ºC for 10 min. Proteins were separated on a 10 % SDS PAgel and transferred to PVDF membrane in a semi-dry fashion. Myc-tagged and Flag-tagged proteins were detected by staining with specific antibodies (anti c-Myc A-14, Santa Cruz Biotechnologies, sc-789; anti-Flag bio M2, Sigma, F9291, Anti-IL-33Rα (Anti-T1/ST2, MD Biosciences).

For binding of full length IL-33 to IL-33 receptor on intact cells, transfected cells were incubated for 1hour on ice with flmIL-33-Flag or Myc-flmIL-33-Flag (obtained from 3 cycles of freez thawing followed by sonication from Flag tagged flIL-33 over expressing HEK293RI cells or empty vector trasfected cells). For anti-Myc immunoprecipitation anti-Myc Agarose (Sigma) was used.

2.11. Interaction of sAcP and sIL-33Rα

N-terminally Flag- and His(6)-tagged recombinant mouse soluble AcP (Genbank accession no. NM_134103) was expressed in stably transfected HEK293RI cells. Flag affinity agarose (Sigma–Aldrich) was loaded with sAcP by incubation with cell supernatant and washed. Then 50 µl of loaded gel was resuspended in 1 ml of PBS with 2% BSA and incubated at room temperature for 1 h with or without rmIL-33 and

with or without mouse sIL-33Rα:Fc (1004-MR; R&D Systems) at 1 µg/ml. Agarose pellets were again washed, and bound proteins were resolved by SDS/PAGE and blotted onto PVDF membrane. Coprecipitated sIL-33Rα:Fc was visualized by using horseradish peroxidase (HRP)-coupled anti-human IgG antibody (A0170; Sigma–Aldrich). The experiment was performed in collaboration with Prof. Werner Falk at University of Regensburg.

2.12. Measurement of signaling pathways

2.12.1. cJun-N-terminal kinase (JNK) assay

EL-4 cells were transfected with mouse IL-33Rα encoding plasmid (pMyc-mIL33Rα) using the DEAE dextran method. 20 hours after transfection 2 × 10^6 cells were stimulated with rhIL-1β (10 ng / ml) or rmIL-33 (200 ng / ml) for 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 minutes at 37 ºC. Cells were harvested by centrifugation for 5 minutes at 500g at 4 ºC, washed with cold PBS and lysed with 60 µl of lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 2 mM DTT and 0.5 % Triton X-100). Protein concentration was measured with Bio-Rad Protein Assay Reagent (Bio Rad Laboratories, Munich, Germany) as described earlier. 10 µg of lysate was subjected to an *in vitro* kinase assay in the presence of kinase buffer (25 mM HEPES pH 7.5, 10 mM $MgCl_2$, and 25 µM ATP), 2 µCi γ[^{32}P]-ATP and 1.3 µg of GST-cJun substrate (amino acids 1-79, Stratagene, Amsterdam, The Netherlands) in 40 µl of total reaction volume. The assay was performed at 30 ºC for 30 min, stopped by addition of Laemmli-buffer followed by heating at 95 ºC for 10 min. Proteins were separated on a 12% SDS gel, which was subsequently dried and exposed over night to a film for autoradiography. Parallel samples were run on gels, electroblotted and total amount of JNK was measured by Western blotting.

2.12.2. IRAK-1 autophosphorylation activity

6-IRAK-19 cells (generated by J. Knop during his Ph D thesis at MH Hannover) were transiently transfected with a plasmid encoding mIL-33Rα-chain alone or in combination with a plasmid encoding ΔC-IL-1RAcP. 24 hr after transfection 2 x 10^7 cells were either left unstimulated or were stimulated for 15 min at 37 ºC with either 10 ng/ml rhIL-1β or 100 ng/ml rmIL-33. Subsequently, cells were lysed and IRAK-1 was immunoprecipitated using the mAb 2A9 (kind gift of Z. Cao and H. Wesche, formerly Tularik), and subjected to an *in vitro* kinase assay as described previously (Kollewe et al., 2004). Briefly, washed immunoprecipitates were resuspended in a

kinase reaction mixture containing 20 mM HEPES, pH 6.5, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM $MnCl_2$,1 µM ATP, and 1 µCi of [γ-^{32}P]-ATP and incubated for 20 min at 30 ºC. The kinase reaction was stopped by heating in SDS sample buffer. Phosphorylated proteins were separated by SDS-PAGE. The gels were dried, and autoradiographies were performed.

2.12.3. Measurement of phosphorylation and degradation of IκBα

EL-41B1 cells (2.5 x 10^6/point) were stimulated with full length mIL-33 (eluted with glycine-HCl after immunoprecipitation of flmIL-33 with anti-Myc Agarose) for 0, 5, 10, 15 and 30 min at 37 ºC. Phospho IκBα and total IκBα were detected by Western blot analysis using anti-Phospho IκBα (BD Biosciences) and anti- total IκBα (Santa Cruz Biotechonology) antibodies.

2.13. Heat inactivation of IL-33

Recombinant mIL-33 (Alexis, USA) was diluted in RPMI medium to 20 ng/ml and 200 ng/ml and IL-1β was diluted to 200 pg/ml. The diluted IL-33 and IL-1β were heat treated for 10 or 30 minutes at 95 ºC and used for stimulation of EL-4 cells.

2.14. Pull-down assay

IL-33Rα-IL-1RAcP:hIgG(Fc) or IL-33Rα:hIgG(Fc) fusion proteins were expressed in HEK293RI cells by transient transfection. Cells were incubated for 3 days after transfection and fusion proteins were precipitated by incubating cell supernatants with protein A agarose (1µl / ml) for overnight at 4 ºC. Precipitates were washed with lysis buffer and again incubated (20 µl / ml of cell lysate) for over night at 4 ºC with cell lysate from stimulated keratino cells, expressing N-Myc and/or C-terminaly Flag tagged mIL-33. Beads were washed thrice with lysis buffer followed by washing with PBS. All the liquid was removed, 30 µl of Laemmli buffer (3x) were added and heated for 10 minutes at 95 ºC. Proteins were separated on 12.5% SDS-PAGE, transfered to PVDF membrane and IL-33 was detected by anti-Flag and/or anti- Myc staining by Western blot.

2.15. *In vitro* caspases cleavage assays

N-terminally Myc- and C-terminally Flag-tagged flmIL-33_{1-266}, its mutant IL-33_{D175A}, flIL-1β and flIL-18 were immunoprecipitated from cell lysate of transiently transfected HEK293RI using anti-Flag agarose. Beads were washed thrice with lysis buffer followed by two washes with the caspase reaction buffer (50 mM HEPES, pH 7.4,

100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol, 10 mM DTT). flmIL-33_{1-266}, its mutant IL-33_{D175A} and flIL-1β captured on anti-Flag agarose were incubated with various amounts of caspase 1 (Calbiochem) in 20 µl caspase recaction buffer for 4 hr at 30 ºC. flmIL-33_{1-266}, IL-33_{D175A} and flIL-18 were incubated with various amounts of caspase 3 (kind gift of G. Meiss, Biochemistry, JLU Giessen). Reaction mixture was heated with 20 µl of 3x SDS-PAGE sample buffer and proteins were detected by Western blot analysis using anti-Flag (Sigma) or anti-Myc (Santa Cruz Biotechnology) antibodies.

2.16. Cleavage of IL-33 by apoptotic U937 cell lysate

Caspase 3 was activated by induction of apoptosis in U937 cells (by incubation with 1 ng/ml TNFα and 50 µg/ml cycloheximide for 1.5 h). Washed cells were lysed by 3 cycles of freeze thawing followed by sonication in caspase reaction buffer. flmIL-33 and mutant flmIL-33_{D175A} were incubated with lysates from control cells or apoptotic cells for 0, 10 or 30 minutes at 30 ºC. Proteins were denatured in sample buffer and seperated on 12.5% SDS PAGE. IL-33 was detected by anti-Flag or and anti-Myc staining by Western blotting.

2.17. Nuclear translocation studies

N-terminally Myc-tagged IL-33_{1-266}, IL-33_{1-175}, IL-$33_{176-266}$ or IL-33_{D175A} were expressed in HEK293RI or murine keratinocytes after transient transfections. Cells were lysed with lysis buffer (Cao et al., 1996) as described above. Nuclear and cytosolic fraction were separated by centrifugation at 500g for 10 minutes. The separated nuclei were washed thrice with lysis buffer and lysed by sonication in 200 µl KE buffer (20 mM HEPES, pH 7.9 + 400 mM NaCl + 1 mM EDTA, pH 8.0 + 1 mM EGTA, pH 7.6 + 1 mM DTT and 1 mM PMSF). The IL-33 was detected from cytosolic or nuclear fraction by Western blotting using anti-Myc antibodies.

2.18. RT-PCR analysis

2.18.1. RNA isolation

Total RNA from stimulated or unstimulated mammalian cells was isolated using NucleoSpin® RNA II (MACHEREY-NAGEL GmbH & Co. KG, Düren Germany) RNA extraction kit following manufactures recommendations. For RNA extraction 1 x 10^6 - 5 x 10^6 cultured cells were collected by centrifigation, washed with PBS and lysed by direct addition of the RA1 buffer (350 µl RA1 buffer + 3.5 µl β-mercaptoethanol) to

cell pellet followed by vigorous vortexing. Viscosity of the cell lysate was reduced by filtration of the cell lysate through NucleoSpin® filter columns. The binding conditions were adjusted by addition of 350 µl of the 70% ethanol and homogenization by pipetting in and out. The nucleotides were bound to silica membrane by loading the mixture to NucleoSpin® RNA II column and centrifugation for 30 sec at 11000g. The membrane was desalted by addition of 350 µl of membrane desalting buffer (MDB). DNA was digested by 15 minutes incubation of membrane at room temperature with 95 µl of DNAase reaction mixture (containing DNAse in 1 x reaction buffer). DNase was inactivated by addition of the 200 µl of RA2 buffer. The membrane was washed twice by addition of 600 µl and 250 µl of RA3 buffer and dried by an additional centrifugation of 2 minutes at 11000g. RNA was eluted with 60 µl of RNase free water.

Quantity and quality of RNA was determined by spectrophotometer by measuring OD at 260 and 280 (1 OD at 260=40 µg RNA), running on agarose gel or by Bioanalyzer (Agilent technologies). RNA was stored at -80 ºC untill further use.

2.18.2. cDNA synthesis

cDNA was synthesized by reverse transcription reaction using M-MuLV H^- reverse transcriptase (Fermentas, Germany). The reverse transcription reaction contains 1 x RT buffer (Fermentas, Germany), 100 ng/µl of RNA, 5 µM oligos (randam hexamer), 1 mM dNTPs (each) and 10 units of reverse transcriptase (Fermentas, Germany) in DEPC water. The reverse transcription reaction was carried out in 20 µl of total volume. Two µg of RNA and oligos (5 µM final) were diluted with DEPC water and incubated at 70 ºC for 5 minutes. The reaction tube was kept on ice and 4 µl of 5 x RT buffer and 2 µl of dNTPs mix (from 10 mM each) were added. The reaction mixture was incubated at 37 ºC for 5 minutes and 200 units of M-MuLV H^- reverse transcriptase was added. The reverse transcription was carried out at 42 ºC for 60 minutes followed by inactivation of reverse transcriptase at 70 ºC for 10 minutes. The cDNA was used directly for PCR or stored at –20 ºC untill further use.

2.18.3. PCR amplification

Different cell types were seeded (2 x 10^6 cells) and either kept unstimulated of stimulate with IL-1β, LPS or other stimuli as described in table 3.1. The next day cells were harvested (by centrifugation for suspenstion cells or by trypsin treatment for adherant cells) and total RNA was isolated using NucleoSpin® RNA II (MACHEREY-

NAGEL GmbH & Co. KG, Düren Germany) RNA extraction kit. cDNA was generated using random hexamer primers. IL-1 family receptors (hIL-33Rα, mIL-1R1, mIL-1RAcP, mIL-18Rα, mIL-18Rβ, mIL-33Rα) and cytokines (mIL-1α, mIL-1β, mIL-18, mIL-33) gene expression was analyzed from resting and stimulated cells by PCR using specific primers. List of primers used with target genes and annealing temperature is given in table 2.3.

All reactions were carried out in Eppendorf master cyclers (personal or gradient) in 30 µl reaction volume. Master mixtures were prepared containing, 1 x PCR reaction buffer with $MgCl_2$ (Fermentas), 200 µM dNTPs (each), 1 µM forward and reverse primers (each) and 1 unit of Taq DNA polymerase (Fermentas). Master mixture was divided in 0.2 ml PCR tubes (Thermo scientific) and 100 ng - 200 ng of template cDNA was used for each reaction. For the amplification of mIL-18Rα 200 ng of template cDNA but for all other genes 100 ng template was used. Positive (10 pg of plasmid DNA) and negative (No template and RT negative) controls were used to control the specificity and realiability of the PCR. The PCR was performed with the following profile; initial denaturation at 95 ºC for 3 minutes followed by 37 cycles for receptors and 30 cycles for cytokines of denaturation at 94 ºC for 30 seconds, annealing (temperature given in table blow) for 30 seconds and extension at 72 ºC for 30 seconds. The reaction was ended with a final extension of 5 minutes at 72 ºC and kept on hold at 4 ºC untill tubes were taken out of the cycler.

Table 2.3. List of primers for detection of gene expression

Primer Name	Target gene	Annealing Temperature	Sequence 5´-3´	Product Size
hIL-33R1_fwd	hIL-33Rα	51 ºC	ATCAAGGTACAGGGCGCACA	297bp
hIL-33R1_rev			CCATTAAGCTGCCACAGGAC	
mIL-1R1_fwd	mIL-1R1	55 ºC	TCATCCTCACGGCTACAATTG	355bp
mIL-1R1_rev			AAGCCTCCCATATCTCTCAC	
mIL-1RAcP_fwd	mIL-1RAcP	55 ºC	CTGGTTATTCCCTGCAAAGTC	509bp
mIL-1RAcP_rev			TTCTCCAAAACTCCACGCAG	
mIL-18R1_fwd	mIL-18Rα	51 ºC	ATTGGAGCATCAGGAAAGAGG	523bp
mIL-18R1_rev			CCAGGCACCACATCTCTTTC	
mIL-18RAcP_fwd	mIL-18Rβ	55 ºC	TGGGAACACAGCACAAGATG	565bp
mIL-18RAcP_rev			AATGGAGTTCTGGGCAAAGC	
mIL-33R1_fwd	mIL-33Rα	52 ºC	ACTTGTCAATTCACACACGC	290bp
mIL-33R1_rev			CTGGAACTTTCATTTCGACC	
mIL-1a-S115s_fwd	mIL-1α	52 ºC	TCAGCACCTTACACCTACCAGAG	468bp
mIL-1a-S270as_rev			TGATATCTGGAAGTCTGTCATAGAGG	
mIL-1b_fwd	mIL-1β	55 ºC	AGCAGCACATCAACAAGAGC	355bp
mIL-1b_rev			TCGTTGCTTGGTTCTCCTTG	
mIL-18_fwd	mIL-18	55 ºC	AACTTTGGCCGACTTCACTGT	377bp
mIL-18_rev			TGGCAAGCAAGAAAGTGTCC	
mIL-33_fwd	mIL-33	55 ºC	ATCGGGTACCAAGCATGAAG	321bp
mIL-33_rev			ATCACCTTCTTCCCATCCAC	

2.19. Relative quantification of gene expression

Relative mRNA expression of different genes in response to IL-1β, IL-33 and IL-18 in EL-4 1B1 cells was analyzed by quantitative PCR (Stragene). EL-41B1 cells (5 x 10^6) were kept unstimulated or stimulated with IL-1β (100 pg/ml), IL-33 (100 ng/ml) or IL-18 (100 ng/ml) for 2 hours. RNA was isolated using NucleoSpin® RNA II (MACHEREY-NAGEL GmbH & Co. KG, Düren Germany) RNA extraction kit, cDNA was generated and changes in mRNA level for *IL-2*, *Csf2, IL-22, Ccl5, Cxcl10, Tnfrsf9, Icam1* and *Serpinc1* were measured. Expression of *GAPDH* (a house keeping gene) was used to normalize the data.

Primers for the quantification of above described genes were selected from primer data bases (*mIcam1* from http://medgen.ugent.be/rtprimerdb; m*IL-2*, *mCcl5, mCxcl10* and *mTnfrsf9* from http://pga.mgh.harvard.edu/primerbank/index.html; *mCsf2, mIL-22, mSerpinc1* and *GAPDH* from http://mouseprimerdepot.nci.nih.gov) and costum synthesized (Sigma). All the quantitative PCR were performed on Stratagene quantitative PCR (Mx3005P™) and the data was analyzed using the MxPro software, provided with the instrument followig the manufacturer´s guidlines. After an initial activation step at 95 ºC for 10 min, 40 cycles (95 ºC for 30 sec and 60 ºC for 60 sec) were performed and a single fluorescence reading was obtained at 60 ºC of each cycle step. A melting/dissociation curve was determined at the end of cycling to ensure the amplification of a single PCR product.

Table 2.4. List of primers for quantification of gene expression

Primer Name	Sequence 5´-3´	Final Conc.	Gene / template Conc.	Product Size
mCxcl10-7f	CCAAGTGCTGCCGTCATTTTC	300 nM	mCxcl10 5ng	157
mCxcl10-163r	GGCTCGCAGGGATGATTTCAA	300 nM		
mIL-2176f	TGAGCAGGATGGAGAATTACA	600 nM	mIL-2 10ng	120
mIL-2-295r	GTCCAAGTTCATCTTCTAGG	600 nM		
mTnfrsf9-69f	CGTGCAGAACTCCTGTGATAAC	300 nM	mTnfrsf9 10ng	104
mTnfrsf9-172r	GTCCACCTATGCTGGAGAAGG	150 nM		
mCcl5-98f	GCTGCTTTGCCTACCTCTCC	300 nM	mCcl5 5ng	104
mCcl5-201r	TCGAGTGACAAACACGACTGC	300 nM		
mIcam1-1063f	CCGCAGGTCCAATTCACACT	150 nM	mIcam1 10ng	104
mIcam1-1205r	TCCAGCCGAGGACCATACAG	300 nM		
mCsf2-104f	CCATCAAAGAAGCCCTGAAC	300 nM	mCsf2 10ng	140
mCsf2-243r	CCGTAGACCCTGCTCGAATA	300 nM		
mIL-22-384f	GCTCAGCTCCTGTCACATCA	150 nM	mIL-22 20ng	107
mIL-22-490r	TCGCCTTGATCTCTCCACTC	150 nM		
mSerpinc1-326f	TTTTCCTGTCACCCTTGAGC	300 nM	mSerpinc1 10ng	135
mSerpinc1-460r	AGTGGATCTGGTCGGATGTC	300 nM		
mGAPDH-301f	ACCACCATGGAGAAGGC	600 nM	mGAPDH all above	234
mGAPDH-534r	GGCATGGACTGTGGTCATGA	150 nM		

All the quantitative PCR were performed in triplicate in 12 µl of the reaction volume containing 1 x master mixture with SYBR Green (Stratagene), 30 nM ROX as reference dye, 150-600 nM forward and reverse primers, template cDNA. Three kinds of assays were performed for each gene. At first the optimal primer concentrations were defined by analyzing various concentrations (150, 300 and 600 nM) of forward and reverse primers and 10 ng of template. Primer concentrations with minimum cycle threshold (ct) and single amplification product (based on dissociation curve) were considered optimal (optimum concentration and sequence of each primer with the traget gene is given in table 2.4). In the second assay optimal amount of template was determined for each gene by analysing the various amounts of template (40 ng to 0.31 ng, it was obtained with 1:1 serial dilution from maximum to minimum) and plotting the standard curve. The amount of template which results in a standard curve having 90-110% efficiency was selected for relative gene expression analysis. Finally the relative gene expression was analysed by amplification of mRNA from unstimulated or stimulated cells. The amout of cDNA templates (based on initial RNA concentration) used for final relative gene expression assay is given in table 2.4. The data obtained was normalized against expression level of *GAPDH* and calibrated against basal gene expression (unstimulated) in all relative gene expression asays.

2.20. Expression plasmids

For construction of the expression plasmids, genes were amplified or fused by PCR. Detailed PCR conditions are explained in discription of individual expression plasmids. All PCR reactions for gene amplification were performed in 50 µl volume and pfu DNA polymerase (Fermentas, having proof reading activity) was used. The primers sequences, used for gene fusion or amplification are given in table 2.5. The PCR reaction consists of 1 x PCR buffer with $MgSO_4$, 200 µM dNTPs (each), 0.4 µM forward and reverse primers (each), 2.5 units of pfu DNA polymerase, template (100 ng cDNA or 1 ng plasmid DNA) and water to make up total volume to 50 µl. All enzymes used to clone genes were purchased from Fermentas, Germany.

pFLAG-mIL33Rα was used for cell surface expression of amino acids 18–567 of mouse IL-33 receptor α chain (IL-33Rα) (without signal peptide, NM_001025602, Pro18–Phe567) with N-terminal 3 × Flag epitope tag under control of the CMV promoter. It was generated by inserting the mouse IL-33Rα coding sequences

(without signal sequence) into the HindIII and BamHI cloning site of vector p3×Flag-CMV-9 (Sigma, Deisenhofen, Germany). Mouse IL-33Rα chain was cloned from mouse mast cells (kindly provided by Prof. Dr. M. Huber, MPI Freiburg). Total RNA was isolated, cDNA was generated and full length information for the mouse IL-33Rα chain was amplified by PCR using mIL-33R1-P18s-Hind and mIL-33R1F567as-Bam primers. The PCR was performed in Eppendorf master cycler (gradient) using the following profile: initial denaturation for 5 minutes at 95 ºC followed by 35 cycles of denaturation at 94 ºC for 45 sec, primer annealing at 52 ºC for 30 sec and extension at 72 ºC for 200 sec. Programe was ended with a final extension of 10 minutes and kept on hold at 4 ºC.

The amplified gene was cloned in p3 x FLAG-CMV-9 in HindIII and BamHI cloning sites. Ligation of insert was confirmed by restriction with HindIII and BamHI.

pMyc-mIL33Rα was used for cell surface expression of amino acids 18–567 of mouse IL-33Rα (NM_001025602, Pro18–Phe567) with N-terminal c-Myc epitope tag under control of the CMV promoter. It was generated by inserting the mouse IL-33Rα coding sequence (without signal sequence) into the HindIII and BamHI cloning site of vector **pMyc-CMV-9** (kind gift from Prof. W. Falk). pMyc-CMV-9 is generated by insertion of the mouse IL-2 signal sequence and the c-Myc epitope sequence in pcDNA3.1/Zeo(+) (Invitrogen, Karlsruhe, Germany).

Murine IL-33Rα coding sequences were cut from pFlag-mIL-33Rα with HindIII and BamHI restriction enzymes. This HindIII and BamHI fragment was purified from the gel and ligated in the pMyc-CMV-9 vector digested with same set of restriction enzymes. Ligation of mIL-33α gene was confirmed by restriction of pMyc-mIL-33Rα with HindIII and BamHI.

pFlag-mIL1RAcP was used for cell surface expression of human IL-1RAcP (NM_008364, Ser21–Val570) with N-terminal Flag epitope tag under control of the CMV promoter. It was generated by inserting the human IL-1RAcP coding sequence into the multiple cloning site of vector pFlag-CMV-1 (Sigma). The vector was a kind gift of Prof. W. Falk, University of Regensburg Germany.

The 3×NF-κB-Luc reporter plasmid, expresses firefly luciferase after binding of activated NF-κB to three consecutive NF-κB binding sites in the promotor region of the plasmid. Plasmid was used for the NF-κB reporter gene assays.

ΔC-mIL-1RAcP (ΔC-AcP) encodes amino acids 1-384 of murine IL-1RAcP in pEF-Bos vector (generated by R. Hofmeister, Regensburg). This mutant is expressed on the cell surface but lacks the cytoplasmic TIR domain. The vector was a kind gift of Prof. W. Falk.

pEGFP-C1 (Clontech) plasmid was used as transfection control plasmid.

pmIL-1α-FL expresses C-terminally Flag-tagged murine interleukin-1α precursor (NM_010554) under CMV promoter. Plasmid was generated by Mr. Björn Gerlach during his diploma work by insertion of full length murine interleukin-1α encoding sequence in pC-Flag.

pmIL33-Myc/His (a kind gift from Prof Werner Falk) expresses C-terminally Myc- and 6×His-tagged mouse interleukin-33 (IL-33, NM_133775.1) under CMV promoter. Plasmid was generated by insertion of full length IL-33 information in pcDNA3.1/myc-His A (Invitrogen).

pmIL33-FL was used for mammalian expression of C-terminally Flag-tagged murine interleukin-33 precursor (NM_133775.1, Met1–Ile266). IL-33 coding sequence was amplified by PCR from pmIL33-Myc/His using mIL-33-M1s-Hind and mIL-33-1266as-Bam primers. The PCR was performed in Eppendorf master cycler (personal) using the following profile: initial denaturation for 3 minutes at 95 ºC followed by 25 cycles of denaturation at 94 ºC for 30 sec, primer annealing at 51 ºC for 30 sec and extension at 72 ºC for 120 sec. Programe was ended with a final extension of 10 minutes and kept on hold at 4 ºC untill the samples were taken out of the thermal cycler.

The amplified mIL-33 sequences was replaced with human IL-1α gene from phIL-1α-Flag using HindIII and BamHI restriction enzymes. Ligation of the IL-33 gene was confirmed by restriction with EcoRI and BamHI.

pFL-hTIR8 was used for cell surface expression of N-terminally Flag-tagged human Toll/Interleukin-1 receptor 8 (NM_021805, Pro2–Met410). This was a gift kind provided by Prof. Alberto Mantovani, Instituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano (MI), Italia.

pFL-mTIR8 was used for cell surface expression of N-terminally Flag-tagged murine Toll/Interleukin-1 receptor 8 (NM_023059, Ala2–Val409 + 132 bp of 3'-UTR). This

was a gift kind provided by Prof. Alberto Mantovani, Instituto Clinico Humanitas, Via Manzoni 56, 20089 Rozzano (MI), Italia.

Table 2.5. List of primers used for amplification/fusion of genes for cloning in expression vectors

Primer Name	Target gene	Sequence 5´- 3´
FLAGs	Flag	GCTGACTACAAAGACGATGACGAC
mAcP-S21s-Hind	mIL-1RAcP	TGTAAGCTTTCGGAGCGCTGTGATGACTG
mAcP-V570as-Bam	mIL-1RAcP	TGTGGATCCTCATACGTTTTTCAGGGATGAG
mAcP-TIR8as	mIL-1RAcP	TCAGCCGACACTTAACAAAGAGGACCATCTCCAGC
mAcP-TIR8s	mTIR8	GAGATGGTCCTCTTTGTTAAGTGTCGGCTGAACATGC
mIL-33-M1s-Hind	mIL-33	CGAAAGCTTGCCACCATGAGACCTAGAATGAAGTAT
mIL-33-1266as-Bam	mIL-33	CAAGGATCCCGGATTTTCGAGAGCTTAAACATA
mIL-33R1F567as-Bam	mIL-33Rα	CGAGGATCCTCAAAAGTGTTTCAGGTCTAAG
mIL-33R1-P18s-Hind	mIL-33Rα	GTGAAGCTTCCCATGTATTTGACAGTTAC
mTIR8-Acp-as	mTIR8	AAGTGAGCTCGGTAATAGAGCAGGGCCACCAGCAG
mTIR8-Acp-s	mIL-1RAcP	TGGCCCTGCTCTATTACCGAGCTCACTTTGGAACAG
mTIR8-V310as-Xba	mTIR8	CATTCTAGACTACACCTTCCGTGGCAGTGCT
mTIR8-V409as-Xba	mTIR8	CACTCTAGACTACACATCATCCTCAGACAC
mIL33-M1s-Sal	mIL-33	GTGGTCGACTATGAGACCTAGAATGAAGTA
FLAGas-Not	Flag	AGAGCGGCCG*CTA*GACCTTGTCATCGTC
IL33-D175as-Not	mIL-33	TATGCGGCCG*CTA*ATCCACACCGTCGCCT
IL33-G176s-Sal	mIL-33	GTGGTCGACTGGGAAGAAGGTGATGGT
mIL33-D175As	mIL-33	AGGCGACGGTGTG GCT GGGAAGAAGGTG
mIL33-D175Aas	mIL-33	CACCTTCTTCCC AGC CACACCGTCGCCT
IL1b-M1s-Sal	IL-1β	CTCGTCGAC*A*ATGGCAACTGTTCCTGAACTC
IL1b-S269as-Age	IL-1β	GTTACCGGT*GG*GGAAGACACGGATTCCATGG
IL18-M1s-Sal	IL-18	GAAGTCGACA*A*TGGCTGCCATGTCAGAAGA
IL18-S192as-Age	IL-18	AGGACCGGT*GG*ACTTTGATGTAAGTTAGTGAGA

pFL-mTIR8-AcP was generated for mammalian expression of an N-terminally 3×Flag-tagged fusion protein of extracellular & trans-membrane part of the murine TIR8 [single immunoglobulin IL-1R-related molecule (SIGIRR) (NM_023059, Ala2–Tyr138)] and the intracellular part of the murine interleukin-1 receptor accessory protein [(NM_008364, Tyr389–Val570)] on mammalian cell surface. For the construction of the TIR8-AcP chimera, the sequences encoding the extracellular and transmembrane parts (amino acid 1-138) of murine TIR8 was amplified by PCR using FLAGs and mTIR8-Acp-as. The sequence encoding the intracellular rest of mIL-1RAcP (amino acid 389-570) was amplified by PCR using mTIR8-Acp-s and mAcP-V570as-Bam primers. The PCR profile used was as follows: initial denaturation for 3 minutes at 95 ºC followed by 20 cycles of denaturation at 94 ºC for 30 sec, primer annealing 52 ºC for 30 sec and extension at 72 ºC for 110 sec. And then a final extension of 10 minutes and hold at 4 ºC.

The two amplified gene fragments were fused and again amplified by PCR using FLAGs and mAcP-V570as-Bam primers. One µl of two above described PCR

reactions were used as template for fusion and reamplification of chimeric genes. Profile used was as follows: initial denaturation for 3 minutes at 95 ºC then 5 cycles of denaturation at 94 ºC for 45 sec, primer annealing 48 ºC for 30 sec and extension at 72 ºC for 120 sec followed by 25 cycles of denaturation at 94 ºC for 45 sec, primer annealing 55 ºC for 30 sec and extension at 72 ºC for 120 sec and then a final extension of 10 minutes followed by hold at 4 ºC. This TIR8-AcP chimeric sequence was ligated in pFlag-CMV-9 in EcoR1 and BamHI cloning sites. Ligation was confirmed by restriction with EcoR1 and BamHI.

pFL-mAcP-TIR8 was used for mammalian cell surface expression of an N-terminally 3×Flag-tagged fusion protein of extracellular & trans-membrane part of the murine interleukin-1 receptor accessory protein, [(NM_008364, Ser21–Phe388)] and the intracellular part of the murine single immunoglobulin toll-interleukin-1 receptor related (SIGIRR, TIR8), [(NM_023059, Val139–Val409). *mAcP-TIR8* gene was generated by PCR based fusion of extracellular & trans-membrane part of the murine interleukin-1 receptor accessory protein, (NM_008364, Ser21–Phe388) and the intracellular part of the murine TIR8, (NM_023059, Val139–Val409). The extra-cellular and trans-membrane region coding sequences of mIL-1RAcP was amplified using mAcP-S21s-Hind and mAcP-TIR8as primers and 1 ng of pFl-mIL-1RAcP as template. The primers were allowed to anneal at 53 ºC. The cytoplasmic region of mTIR8 was amplified from pFl-mTIR8 using mAcP-TIR8s and mTIR8-V409as-Xba primers. The annealing temperature for binding of mAcP-TIR8s and mTIR8-V409as-Xba primers was 58 ºC. The rest of the profile was same for both reactions i.e. initial denaturation for 3 minutes at 95 ºC then 25 cycles of denaturation at 94 ºC for 45 sec, primer annealing as described for 30 sec and extension at 72 ºC for 90 sec. The two amplified gene fragments were fused and reamplified by PCR using mAcP-S21s-Hind and mTIR8-V409as-Xba primers. Following profile was used for fusion and amplification of mAcP-TIR8: initial denaturation for 3 minutes at 95 ºC then 5 cycles of denaturation at 94 ºC for 45 sec, primer annealing 48 ºC for 30 sec and extension at 72 ºC for 200 sec followed by 35 cycles of denaturation at 94 ºC for 45 sec, primer annealing 57 ºC for 30 sec and extension at 72 ºC for 200 seconds and then a final extension of 10 minutes.

The amplified murine AcP-TIR8 chimeric gene was ligated in the pFlag-CMV-9 in HindIII and XbaI cloning sites. Ligation of murine AcP-TIR8 chimeric gene was

verified by restriction of pFl-AcP-TIR8 plasmid with HindIII and NheI, followed by restriction with HindIII and BamHI.

pFL-mAcP-TIR8-ΔC was designed for mammalian cell surface expression of an N-terminally 3×Flag-tagged fusion protein of extracellular & trans-membrane part of the murine interleukin-1 receptor accessory protein, [(NM_008364) Ser21–Phe388] and the C-terminally truncated intracellular part of the murine TIR8, [single immunoglobulin toll-interleukin-1 receptor related, (NM_023059, Val139–Val310)]. AcP-TIR8-ΔC chimeric genes was gererated by fusion of the PCR amplified extra-cellular and trans-membrane region coding sequences of mIL-1RAcP and cytoplasmic region of mTIR8 (as described for mAcP-TIR8 section). The two amplified genes were fused by PCR and chimeic gene was amplified using mAcP-S21s-Hind and mTIR8-V310as-Xba primers. The amplification conditions were same as for mAcP-TIR8 amplification.

The amplified murine AcP-TIR8-ΔC chimeric genes was ligated in the pFlag-CMV-9 in HindIII and XbaI cloning sites. Ligation of murine AcP-TIR8-ΔC chimeric gene was verified by restriction of pFl-AcP-TIR8-ΔC plasmid with HindIII and NheI. Further confirmation was made by restriction cuts of the plasmid by HindIII and BamHI.

pFL-mTIR8-ΔC encodes a N-terminally 3×Flag-tagged C-terminally truncated murine TIR8, [single immunoglobulin toll-interleukin-1 receptor related (NM_023059, Ala2–Val310)] on mammalian cell surface. C terminally truncated version of *mTIR8* (995bp TIR8-ΔC lacking last 99 amino acids coding sequences) was amplified by PCR using FLAGs and mTIR8-V310as-Xba primers and 1 ng of pFl-mTIR8 as template DNA. PCR programe was as follows: initial denaturation for 3 minutes at 95 ºC then 25 cycles of denaturation at 94 ºC for 30 sec, primer annealing 52 ºC for 30 sec and extension at 72 ºC for 120 sec followed by a final extension of 10 minutes and hold at 4 ºC.

The amplified murine TIR8-ΔC was ligated in the pFl-CMV-9 in HindIII and XbaI cloning sites. Cloning of mTIR8-ΔC gene in pFl-CMV-9 was confirmed by restriction of plasmid DNA (pFl-TIR8-ΔC) with HindIII and NheI and restriction with HindIII and BamHI.

pFl-smIL-33Rα:Fc was used to express Flag-tagged soluble form of IL-33Rα-chain fused with the Fc fragment of human IgG. **pFL-IL-33Rα-IL-1RAcP:Fc** was used for mammalian expression of IL-33 inline cytokine trap (Economides et al., 2003) fusion

protein consisting of the extracellular part of the murine IL-33 receptor alpha chain, extracellular part of murine IL-1 receptor accessory protein and the Fc fragment of human immunoglobulin IgG1. Plasmids were prepared by Daniel Radtke and Meike Thomas during their bachelor thesis work.

pMyc-mIL-1β-Fl, **pMyc-mIL-18-Fl** and **pMyc-mIL-33$_{1-266}$-Fl** encode full length murine IL-1β, IL-18 and IL-33, respectively with N-terminal Myc tag and C-terminal Flag tag. pMyc-mIL-33$_{1-266}$-Fl was generated by cloning of full length IL-33-Flag peptide encoding sequences from pmIL-33-Fl using mIL33-M1s-Sal and FLAGas-Not primers in pRK7 N-Myc (Schall et al., 1990). pMyc-mIL-1β-Fl, pMyc-mIL-18-Fl were generated by replacing full length IL-33 encoding sequence with PCR amplified IL-1β and IL-18 encoding sequences from cDNA obtained from murine macrophage cell line, RAW264.7. IL-1β was amplified using IL1b-M1s-Sal and IL1b-S269as-Age primers. For the amplification of mIL-18 IL18-M1s-Sal and IL18-S192as-Age primers were used. Cloning of full length IL-33 was confirmed by restriction of the recombinant plasmids with Sal1 and Not1 where as full length IL-1β and IL-18 were confirmed by restriction with Sal1 and Age1 restriction enzymes.

pMyc-mIL-33$_{176-266}$-Fl encodes N-terminally Myc- and C-terminally Flag-tagged mIL-33 aa 176-266. pMyc-mIL-33$_{176-266}$-Fl was generated by cloning mIL-33 aa 176-266-Flag peptide amplified from pmIL-33-Fl using mIL33-G176s-Sal and FLAGas-Not primers in pRK7 N-Myc. **pMyc-mIL-33$_{1-175}$** was used for expression of N-terminally Myc-tagged mIL-33 aa 1-175. mIL-33 aa 1-175 was amplified from pmIL-33-Fl using mIL33-M1s-Sal and IL33-D175as-Not primers and ligated in pRK7 N-Myc in Sal1 and Not1 cloning sites. Ligation of mIL-33$_{1-175}$ and mIL-33$_{176-266}$ was confirmed by restriction with Sal1 and Not1 enzymes.

Full length IL-1β, IL-18 and IL-33, IL-33$_{1-175}$, and IL-33$_{176-266}$ all were amplified in Mastercycler (Eppendorf, Germany) using amplification program: initial denaturation for 3 minutes at 95 ºC then 25 cycles of denaturation at 94 ºC for 30 sec, primer annealing 52 ºC for 30 sec and extension at 72 ºC for 120 sec followed by a final extension of 10 minutes and hold at 4 ºC.

pMyc-mIL-33$_{D175A}$-Fl encodes flIL-33 with mutation D175A. pMyc-mIL-33$_{D175A}$-Fl was generated by PCR based point mutation D175A in pMyc-mIL-33$_{1-266}$-Fl using mIL33-D175As and mIL33-D175Aas primes. Mutation was generated using QuikChange

Site-Directed Mutagensis Kit (Stratagene, USA) following manufacturers recomendations.

The nucleotide sequences of all above described genes was confirmed by sequencing (done commercially by Eurofins MWG Operon Sequencing Department, Martinsried Germany).

2.21. DNA isolation and purification

One colony or 5 µl of glycerol culture of transfected bacterial cells were cultured in 3-5 ml of LB medium (171 mM NaCl +10 g/l Bacto-Trypton (Roth, Germany) + 5 g/l yeast extract (Roth, Germany), pH 7.0 with 5 M NaOH, autoclaved) containing appropriate antibiotic (50 µg/ml Ampicillin, sodium salt (Roth, Germany) or 30 µg/ml kanamycin sulphate (Roth Germany), 0.2 µm sterile filtered) at 37 ºC with shacking (180rpm). Bacterial cultures were allowed to grow overnight for miniprep or 6-8 hours and subcultured for midi or maxi preps.

Bacterial cells were collected by centrifugation at 5000rpm for 5 minutes. Small amounts of plasmid DNA (miniprep) was purified from 1.5 ml bacterial cultures using JETQUICK Plasmid Spin Kit (GENOMED GmbH Löhne Germany). The midi preps was performed for plasmid purification from 50-100 ml bacterial cultures using Jetstar 2.0 plasmid purification Midi kit, (GENOMED GmbH, Löhne Germany) and larger amouts of plasmids were purified using Jetstar 2.0 plasmid purification Maxi kit (GENOMED GmbH, Löhne Germany). All the kits were used following the protocol described by the manufacturer. After purification plasmid DNA was air dried and dissolved in 10 mM Tris/HCl pH 8 and stored at –20 ºC.

DNA from PCR products and from gel slices were purified using NucleoSpin® Extract II (MACHEREY-NAGEL GmbH & Co. KG, Düren Germany) kit following the manufacturer´s recomendations.

DNA concentrations and quality was determined by spectrophotometer by measuring OD at 260 and 280 (from 1:50 diluted DNA). The quantity of DNA was calculated as: 1 OD_{260}= 50 µg / ml DNA. The ratio OD 260/280 should be 1.8 - 2 for pure DNA.

2.22. DNA digestion with restriction endonucleases

For the digestion of DNA with restriction endonucleases, 1-5 µg of plasmid DNA or purified PCR product was incubated with 5 - 10 units of restriction endonucleases (Fermentas, Germany) in appropiate buffer system (as recommended by Fermentas)

in 20 µl reaction volume. The reaction mixture was incubated at the recommended temperature (usually at 37 ºC) for 2 hours and restriction endonucleases were inactivated by heating at 65 ºC - 80 ºC (specific for endonucleases).

2.23. Agarose gel electrophoresis

Agarose gels (1% to 2%) were used to resolve DNA fragments. The agarose was dissolved in 1 x TAE buffer (40 mM Tris + 1 mM EDTA + 20 mM Acetic acid) by boiling in a microwave oven. The solution was allowed to cool down to about 50 ºC – 60 ºC (approximately). Agarose was poured in gel tray fixed in gel casting plate (PeQLab Biotechnology GmbH, Germany) and equipped with desired comb. The gel was allowed to stay untill solidified. DNA samples were mixed with 1/10 volume of 10 x loading buffer (5 mM Tris-HCl, pH 8.0 + 0.0025% Bromphenolblue + 0.0025% Xylen Cyanol + 5% Glycerol) and loaded on agarose gel. Gel was run at 100 - 120 Volts in 1 x TAE buffer in gel tank (PeQLab Biotechnology GmbH, Germany). DNA was stained by dipping gel in ethidium bromide solution (1µg / ml in water, Roth, Germany) for 15 minutes and visualized using UV transilluminator in gel doccumentation system (Syngene, UK).

2.24. Ligation

The vector (plasmid DNA) and/or PCR amplified fragments digested by restriction endonuclases were separated on agarose gel, stained with ethidium bromid and desired fragments were purified from gel slices using NucleoSpin® Extract II kit (MACHEREY-NAGEL GmbH & Co. KG, Düren Germany). The purified vectors and inserts were ligated in 20 µl reaction volume containing, 100 – 250 ng linear plasmid DNA, 3 M ratio of insert (compared to plasmid) and 5 units of T4 DNA ligase (Fermentas) in 1 x T4 DNA ligase reaction buffer (Fermentas). The reaction contents were mixed and incubated for one hour at 22 ºC in Eppendorf thermal cycler (Personal). And 5 µl of the ligation mixture was transformed to the competent E-Coli XL1 blue cells by heat shock method.

2.25. Bacterial transformations

2.25.1. Preparation of competent cells

One colony or 5 µl of glycerol culture of E-Coli XL1blue strain **(**recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lac1q ZΔM15 Tn10 (TetR) – Stratagene, USA) was cultured overnight in 3 ml of LB medium at 37 ºC with shacking (180rpm).

The next day 500 µl of this overnight culture was subcultured in 50 ml LB medium at 37 ºC with shacking until the OD_{600nm} was reached between 0.3 to 0.4. The bacteria were harvested by centrifugation at 5000 rpm for 5 minutes at 4 ºC and resuspended in 5 ml of ice cold TSS (50 mM $MgCl_2 \cdot 6\ H_2O$ + 10% Polyethylenglycol (6000 or 8000, Serva Finbiochemica, GmbH & Co, Heidelberg Germany) + 5% DMSO in LB medium, 0,2 µm sterile filtered). Cells were aliquoted (100µl) in 1.5 ml Eppendorf cups, frozen directly in liquid nitrogen and stored at – 80 ºC untill further use.

2.25.2. Transformation by heat shock

Competent bacterial cells were thawed on ice and 5 µl of ligation mixture or 50-100 ng of plasmid DNA was mixed by pipetting in and out and allowed to stay on ice for 30 minutes. A heat shock of 2 minutes at 42 ºC was given to this transformation mixture and again kept on ice for 2 minutes. 900 µl of LB medium containing 20 mM glucose, 12.5 mM $MgCl_2$ and 12.5 mM $CaCl_2$ was added in each tube and incubated at 37 ºC for 1 hour with shacking. A part (100 µl) of this cell suspension was spread on LB-Agar plates (LB-Medium + 1.5% Bacto-Agar (Roth, Germany), autoclaved + Antibiotic) containing the appopriate selection antibiotic. The rest of the cells were collected by centrifugation at 5000 rpm for 5 minutes, resuspended in 100 µl of the LB medium and spread on separate plate. Plates were incubated for 16-20 hours at 37 ºC.

2.26. Microarray analysis

In order to identify differentially regulated genes by IL-1, IL-18, and IL-33 stimulation, microarrays were performed on the triple responsive cell line EL-41B1. EL-41B1 cells were either kept unstimulated or stimulated with 100 pg/ml IL-1β, 100 ng/ml IL-33 or 100 ng/ml IL-18 for 2 hours. Total RNA isolated as described above and cDNA was prepared, labelled with flourescent dyes and microarray was performed (all arrays were perfomed at the Institute of Pharmacology, Hannover Medical School by Dr. Oliver Dittrich-Breiholz).

At first the gene expression was determined using the inflammation array (Inflamus, 2nd version inflamation array) developed by the cytokine microarray project at the Institute of Pharmacology, Hannover Medical School (Dr. Oliver Dittrich Breiholz and Prof. Michael Kracht). Arrays contained 160 selected gene probes including cytokines, chemokines and their receptors, receptor associated factors, growth factors, acute phase proteins, adhesion molecules, signal transduction proteins,

transcription factors, matrix metallo proteases, enzymes, matrix proteins, some housekeeping genes and some control oligos. Array data was normalized by comparing the average signal obtained by house keeping genes expression and control oligos.

Whole mouse genome oligo microarrays (4 x 44 k, Agilent Technologies) were performed using same RNA from unstimulated and IL-1, IL-33 or IL-18 stimulated EL-41B1 cells. RNA was converted to cRNA and labelled with Cy5 or Cy3 for unstimulated or stimulated cells respectively. Labelled cRNAs of stimulated or unstimulated cells were mixed and co-hybridized on to whole mouse genome oligo microarrays (4 x 44 k, Agilent Technologies), which carry oligonucleotide probes for the mouse genome. Flourescent intensity values of all spots were measured from sacnned microarrays using MAVI software (MWG Biotech, Germany). The ratio of the stimulated and unstimulated signal was derived and data was analyzed using Excel (Microsoft office, 2001).

2.27. Radiolabelling of IL-33 and IL-1α

In order to measure the binding affinity of IL-33 to the IL-33 receptor 3 µg of IL-33 (a kind gift from Prof. W. Falk, University of Regensburg, Germany) was labelled with ^{125}I (1 mCi) using the chloramine T method. IL-1α (5 µg, a kind gift from Dr. J. Sims, formerly Immunex Seattle, USA) was also labelled in parallel as control.

2.27.1. Preparation of column

Sephadex G25 (2.5 g) was soaked in 20 ml of PBS for 30 minutes at room temperature. Column was prepared from 10 ml plastic pipette by positoning a small amount of cotton at the bottom of the pipette. A small piece of silicon tube (2-3 cm) was applied to the tip of the pipette with stopper. Presoaked Sephadex G25 was poured air-free into the column slowly up to 10 ml by allowing the liquid to flow through until homogenious Sephadex G25 gel reached the 10 ml mark. Unspecific protein binding sites of the column were saturated by incubating with 0.25% gelatine in PBS (preboiled and cooled to room temperature) for 10 minutes and allowed to stay until use.

2.27.2. Iodination of IL-33 and IL-1α

Carrier protein-free recombinant cytokines were diluted with 0.5 M phosphate buffer (pH 7.5) up to 25 µl volume. 1 mCi of Na^{125}I (I-RB-31 74TBq/mmol in 40 mM NaOH,

Hartmann Analytic, Braunschweig, Germany) was added along with the 40 µl of freshly prepared 1 mg/ml solution of Chloramine-T and mixed. The mixture was allowed to react at room temperature for 2 minutes. Then the reaction was stopped by adding 30 µl freshly prepared $Na_2S_2O_5$ solution (10 mg/ml), followed by addition of 50 µl of 10 mg/ml KI solution. The whole reaction mixture was mixed with 300 µl of 0.25% gelatin, loaded on Sephadex G25 column and 15 fractions of one ml were collected. During this size exclusion chromatography column was not allowed to dry by slowly adding PBS on the top of colume. 10 µl from each fraction was analyzed for the presence of radioactive material on liquid scientilation counter (Beckman Coulter, USA) and another 10 µl were mixed with Laemmli buffer for SDS-PAGE and rests were frozen at –20 ºC.

2.28. Crosslinking of IL-1α and IL-33 to respective receptors

IL-33 and IL-1α, ligand recepetor complexes were cross linked with BS3 (Pierce). For crosslinking studies HEK293RI cells (3.6 x 10^6 cells in 56cm^2 plates) were transfected with Flag tagged IL-33Rα encoding plasmid alone or in combination with Flag-IL-1RAcP encoding plasmid. The next day the transfected cells were washed twice with PBS and incubated either with radiolabelled cytokines (12 µl IL-1α-^{125}I or 50 µl IL-33-^{125}I) for 15 minutes at 37 ºC in 1 ml PBS. In order to prove the specificity of the binding of radiolabelled cytokines a part of the transfected cells were also pre incubared with the nonradiolabelled cytokines (10 ng/ml IL-1α or 100 ng/ml IL-33). The cells were washed twice with PBS (pH 7.5) and incubated with freshly prepared 5 mM BS3 (Pierce) in PBS at 4º C for one houre in 1 ml volume. Thereafter 50 µl of 1 M Tris (pH 7.5) were added to quench the reaction and allowed to stay for 15 minutes on ice. Cells were washed with PBS and lysed. Flag tagged proteins were immunoprecipitated using anti-Flag M2 agarose. For IL-1α treated cells 2 µg/ml anti-IL-1R1 (12A6, Pharmingen, USA) was also used for immunoprecipitation. Proteins from cell lysates and immunoprecipitates were separated on 15% SDS/PAGE. The gel was dried in gel drier and exposed to the photographic film overnight. Molecules crosslinked with radiolabbled cytokines were visualized from developed photographic film.

2.29. Saturation and competition studies

EL-41B1 (5 x 10^6 / point, EL-4 cells stably transfected with IL-33Rα) cells were incubated with increasing amounts of either radio-labelled IL-1α (0.31, 0.62, 1.25,

2.5, 5 or 10 ng / ml) or radio-labelled IL-33 (3.13, 6.25, 12.5, 25, 50, 100 ng / ml) for 15 minutes at 37 ºC followed by 45 minutes on ice in 200 µl RPMI medium. For competition studies EL-41B1 cells were incubated with radiolabelled IL-1α (10 ng / ml) or IL-33 (100 ng / ml) with or without 100 fold excess of unlabelled ligand. Cells and supernatant were separated by centrifugation at 1250rpm for 5 minutes in the presence of 200 µl of silicon oil (Wacker Chemie AR20/AR200 1:1). Radioactivity in the fractions (cells = bound; supernatant = unbound) was determined via liquid scintillation in a counter in the presence of 1 ml scintillator.

2.30. Statistical analysis

The data were analysed using Excel software, from Microsoft Office 2001. The quantitative PCR data were analyzed using MxPro software (stratagene, USA) provided with the thermal cycler.

Chapter 3

Results

The aim of this study was to characterize in detail the IL-33 / IL-33 receptor system and compare it with the well known and closely related IL-1 / IL-1 receptor system and IL-18 / IL18 receptor system. Cytokine production, processing to biologically active forms and release from producing cells was one aspect to be investigated. The other one was to elucidate the receptor system on the responding cell and to compare the signal transduction mechanisms initiated by binding of the cyotkines to their respective receptor complexes. While well-characterized expression plasmids encoding IL-1 and IL-18 receptor components were already used in the laboratory, no tools were available to study IL-33 production and IL-33 signaling. At the onset of this work, only the sequence for ST2 was published, a beta-chain was proposed but not demonstrated experimentally. Thus the first task was to create tools in order to investigate the IL-33 receptor complex and allow the search for a beta-chain.

3.1. Characterization of the IL-33 receptor complex

3.1.1. Cloning of the IL-33 receptor α-chain

As ST2 had been identified as the IL-33 receptor, the sequence was available. With this information mouse IL-33 receptor alpha-chain was cloned using mRNA from murine bone marrow derived mast cells and a RT-PCR approach as described in material and methods. For easy handling of the proteins the sequences encoding N-terminal Myc- or Flag-epitope tags were added in the expression plasmids.

The cloned IL-33Rα was expressed in mammalian cells upon transient transfection (Fig.3.1A). HEK293RI cells do not express endogenous IL-33Rα, and thus do not respond to IL-33 but they readily respond to IL-1 (data not shown).

Expression of IL-33Rα-molecules rendered HEK293RI cells responsive to IL-33. This was true for either Flag-tagged IL-33Rα-chain (Fig.3.1B left) or Myc- tagged IL-33Rα-

chain (Fig.3.1B right). Stimulation of transfected HEK293RI cells with IL-33 resulted in the activation of the transcription factor NF-κB in dose dependent manner with respect to receptor and ligand concentration (Fig.3.1B).

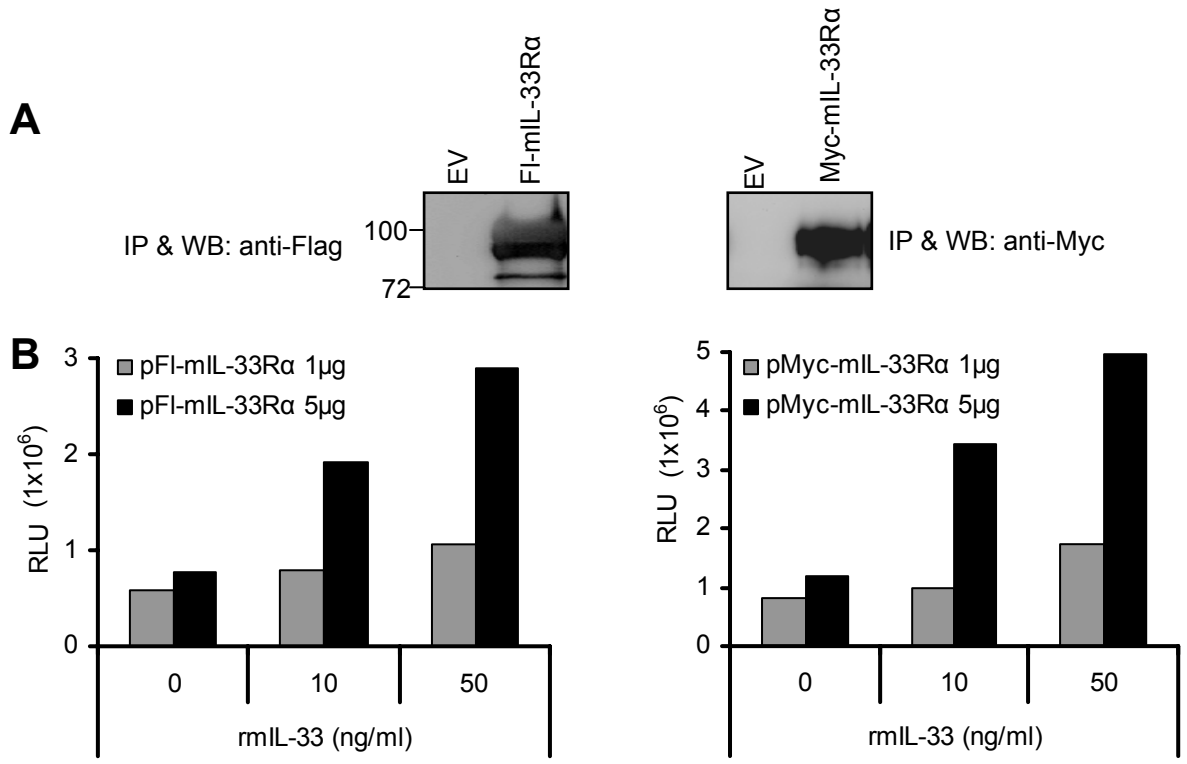


Fig. 3.1: Expression and biological evaluation of mouse IL-33Rα (ST2).
(A). HEK293RI cells express IL-33Rα upon transient transfection with a plasmid encoding mIL-33Rα. Flag-tagged and Myc-tagged mIL-33Rα-chain was expressed in HEK293RI cells by transient transfection of pFl-mIL-33Rα and pMyc-mIL-33Rα, respectively. The tagged mIL-33Rα was detected by Western blotting after immunoprecipitation using anti-Flag M2 or antiMyc (A14) antibodies.
(B). Flag-tagged and Myc-tagged mIL-33Rα chain is functional. HEK293RI cells were transiently transfected with different amounts of Flag-mIL-33Rα (left) or Myc-IL-33Rα encoding expression plasmid along with 1 µg of the NF-κB driven luciferase reporter gene plasmid, cells were stimulated with rmIL-33 as indicated and NF-κB driven reporter gene activity (luciferase) was measured (RLU = relative light units). Cells respond to mIL-33 upon transfection with pFl-mIL-33Rα plasmid or pMyc-mIL-33Rα in dose dependent fashion.

3.1.2. Identification of IL-33 receptor β-chain

All known receptor complexes in the family of IL-1R proteins consist of heterodimers. One chain recognizes the corresponding IL-1 cytokine family member, this being the formal receptor, and the second chain recognizes the ligated receptor but not the lignad itself. In these heterodimers both chains each contribute a TIR-domain. In contrast to the Toll-like receptors where homodimers are able to transduce a signal,

homodimers have not been reported in the IL-1 receptor family to date. Given the very close relationship of IL-1 and IL-33 it was tempting to speculate that IL-1RAcP was able to function as beta-chain not only in the IL-1 receptor complex but also in the IL-33 receptor complex.

3.1.2.1. Murine EL-4 D6/76 cells do not respond to IL-33 due to the lack of IL-1RAcP

The murine thymoma cell line EL-4 6.1 (EL-4) responds to IL-1 (Zubler et al., 1985), IL-18, and IL-33 by release of IL-2 (Fig.3.2A). This finding demonstrates that EL-4 cells express functional receptors and all other molecules required for signal transduction for all three members of the IL-1 cytokine family. Although picomolar concentrations of rhIL-1β were sufficient to induce IL-2 synthesis, lower nanomolar concentrations were required of either rmIL-18 or rmIL-33. The different levels of mIL-2 production in response to IL-1, IL-18 and IL-33 may relate to different affinities of the three cytokines for their specific receptor components and/or to different receptor numbers for IL-1β, IL-18 and IL-33 expressed on the cell surface.

EL-4 D6/76 cells are derived from EL-4 cells (von Hoegen et al., 1989). Although these cells express functional IL-1RI that can bind IL-1 they do not respond to IL-1 due to lack in IL-1RAcP expression (Hofmeister et al., 1997; Korherr et al., 1997; Wesche et al., 1997b). Transient or stable reconstitution of EL-4 D6/76 with full length IL-1RAcP restored completely the IL-1 responsiveness (Fig.3.2B) (Hofmeister et al., 1997; Korherr et al., 1997; Wesche et al., 1997b). While EL-4 D6/76 cells responded to IL-18 (Fig.3.6A), they did not respond to IL-33 (Fig.3.2B left). Reconstitution with full length mIL-1RAcP restored responsiveness not only to IL-1, but also to IL-33 (Fig.3.2B left), demonstrating that lack of IL-1RAcP is the reason for the inability of D6/76 to respond to both cytokines. It was not possible to reconstitute responsivenss to IL-1 or IL-33 by using a C-terminally truncated version of mIL-1RAcP (ΔC-IL-1RAcP), which lacks the TIR domain, indicating that the TIR domain is required for IL-1 and IL-33 signaling. The response to either IL-33 or IL-1β could be increased by overexpressing mIL-1RAcP (Fig.3.2B right). This result demonstrates that full length IL-1RAcP is equally indispensable for IL-33- and IL-1-induced signaling by their respective receptor complexes.

IL-33 did not induce the same level of IL-2 production in EL-4 or transfected D6/76 cells as was reached with IL-1. To investigate whether the expression of endogenous

mIL-33Rα-chain was limiting in these cell lines, EL-4 cells were transiently transfected with increasing concentrations of plasmid encoding full length mIL-33Rα-chain. In addition, D6/76 cells were transfected with a constant concentration of plasmid encoding full length mIL-1RAcP. In both situations, the response to IL-33 was enhanced approaching the IL-1 response with respect to IL-2 production (Fig.3.2B left**)** and NF-κB activation (Fig.3.6B). This finding suggests that low expression of the endogenous IL-33Rα-chain is limiting IL-33 responses in this murine thymoma cell line.

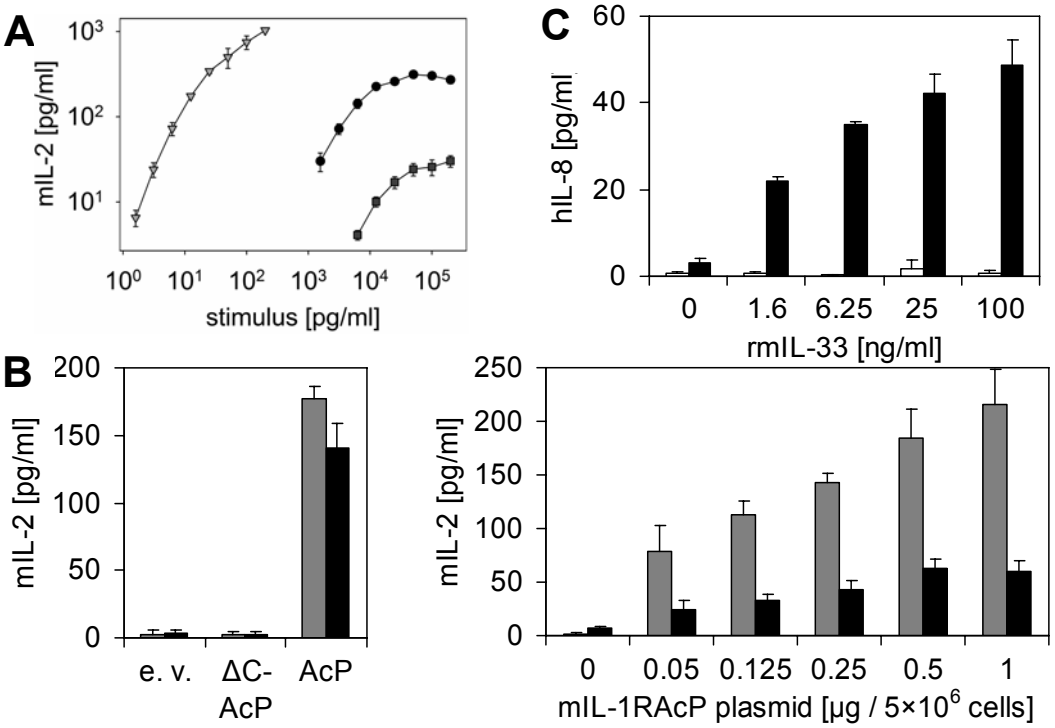


Fig. 3.2: Cytokine production is induced by IL-33 and IL-1 only in the presence of IL-1RAcP.
(A). Murine EL-4 6.1 cells respond to IL-33, IL-18 and IL-1 by producing IL-2 in a concentration dependent manner. 2.5 x 10^4 EL-4 6.1 cells were stimulated with the concentrations of rhIL-1β (grey diamonds), mIL-18 (black squares) or rmIL-33 (black circles) as indicated on the abscissa for 18 hr in the presence of 50 nM Calcium ionophore A23187 in a total volume of 200 µl. Mouse IL-2 was measured in cell-free supernatants by ELISA. Recombinant mouse IL-33 was a thousand-fold less efficient than rhIL-1β in stimulating mIL-2 production.
(B). The EL-4 D6/76 cell line responds to IL-33 or IL-1 only if reconstituted with full-length IL-1RAcP. 5 x 10^6 EL-4 D6/76 cells were transiently co-transfected with 0.2 µg of plasmid encoding mIL-33Rα-chain, and either empty vector or C-terminally truncated mIL-1RAcP (ΔC-IL-1RAcP) or full length mIL-1RAcP. Cells were subsequently stimulated with either 100 ng/ml rmIL-33 or 100 pg/ml rhIL-1β. 18 hr later mIL-2 was measured in cell-free supernatants by ELISA. Only full-length IL-1RAcP allowed a response to rhIL-1β (grey bars) or rmIL-33 (black bars) while the mutant form of mIL-1RAcP lacking the TIR-domain had no effect (left). Over expression of mIL-1RAcP resulted in an increase in IL-1 -(grey bars) or IL-33- induced IL-2 production (right).
(C). After transfection with mIL-33Rα-chain the human embryonal kidney cell line HEK293RI responds to mIL-33 in a concentration dependent manner by producing IL-8. Transiently tranfected HEK293RI cells expressing mIL-33Rα were stimulated with the concentrations of rmIL-33 as indicated for 18 hr and hIL-8 was detected by ELISA in the cell-free supernatants.
Data depicted are means +/- standard deviations from one experiment (carried out in triplicates or quadruplicates) out of a series of three similar ones with comparable results.

The human embryonic kidney cell line HEK293RI responds readily to IL-1 with the production of IL-8 due to the fact that it expresses endogenous IL-1RAcP and that it was stably transfected with IL-1RI (Cao et al., 1996). HEK293RI cells do not respond to either murine or human IL-33 since they do not express the endogenous human IL-33Rα-chain as measured by RT-PCR (data not shown). Murine IL-33 stimulated HEK293RI cells to IL-8 production when these cells were transiently transfected with murine IL-33Rα-chain (Fig.3.2C). These results demonstrate that murine IL-33Rα-chain ligated with murine IL-33 is recognized by the endogenous human IL-1RAcP of HEK293RI cells. Thus, one must conclude that there is no species restriction in this direction, i.e. human IL-1RAcP can recognize murine IL-33Rα-chain when occupied by murine IL-33. Similar findings were also observed in EL-4 D6/76 cells transfected with either human or murine full length IL-1RAcP with respect to both IL-1 and IL-33 responsiveness (data not shown).

3.1.2.2. IL-1RAcP is required for IL-33 induced cytokine production

The previous experiments proved that full length IL-1RAcP restored responsiveness to IL-1β and IL-33 in EL-4 D6/76 cells. In order to complement these gain-of-function studies upon full length IL-1RAcP expression, the loss-of-function studies were performed by either neutralizing IL-1RAcP with antibodies (Fig.3.3) or transfection of a dominant negative version of IL-1RAcP (ΔC-IL-1RAcP) which lacks the TIR domain (Fig.3.4) known to be required for IL-1 signal transduction.

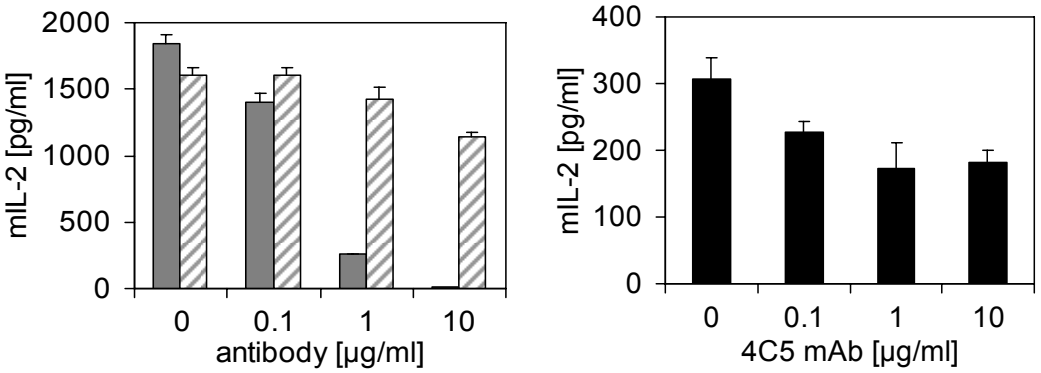


Fig. 3.3: The response to IL-33 in EL-4 cells is dependent on IL-1RAcP.
IL-1 and IL-33 -stimulated IL-2 release in murine EL-4 6.1 cells is abrogated by the neutralizing anti-mIL-1RAcP mAb 4C5. EL-4 cells were transiently transfected with mIL-33Rα-chain. A day after transfection 2.5 x 10^4 cells were preincubated for 30 minutes in the absence or presence (as indicated in bar chart) of neutralizing antibody 4C5 (anti mIL-1RAcP mAb, full bars) or the isotype control antibody RA3-6B2 (rat IgG anti mouse B200 antibody, hatched bars) and stimulated with either 100pg/ml hIL-1β (left gray bars) or 50 ng/ml IL-33 (right black bars). mIL-2 was measured by ELISA from cell free supernatant 18 hours after stimulation (Data shown here is from EL-4 6.1 cells). Data shown here are means +/- standard deviations from one experiment (carried out in triplicates or quadruplicates) out of a series of at least three similar ones with comparable results.

For neutralization studies the anti-mouse IL-1RAcP monoclonal antibody 4C5 was used. This antibody was described to neutralize IL-1β activity (Greenfeder et al., 1995; Hestdal et al., 1994) by blocking IL-1RAcP. The pre-incubation of EL-4 6.1 and EL-4 D6/76 cells expressing mIL-33Rα-chain and full length mIL-1RAcP with different concentrations of mAb 4C5 (anti mIL-1RAcP mAb, Roche) abrogated IL-1β (Fig.3.3 left) and IL-33 (Fig.3.3 right) induced mIL-2 production in a 4C5-concentration dependent manner. This effect was specific for the neutralizing anti-mIL-1RAcP 4C5 mAb because the isotype control antibody RA3-6B2 (rat IgG anti- mouse B220 antibody) neither affected hIL-1β (Fig.3.3 left and 3.7 left) nor mIL-33 (Fig.3.7 right) induced mIL-2 release.

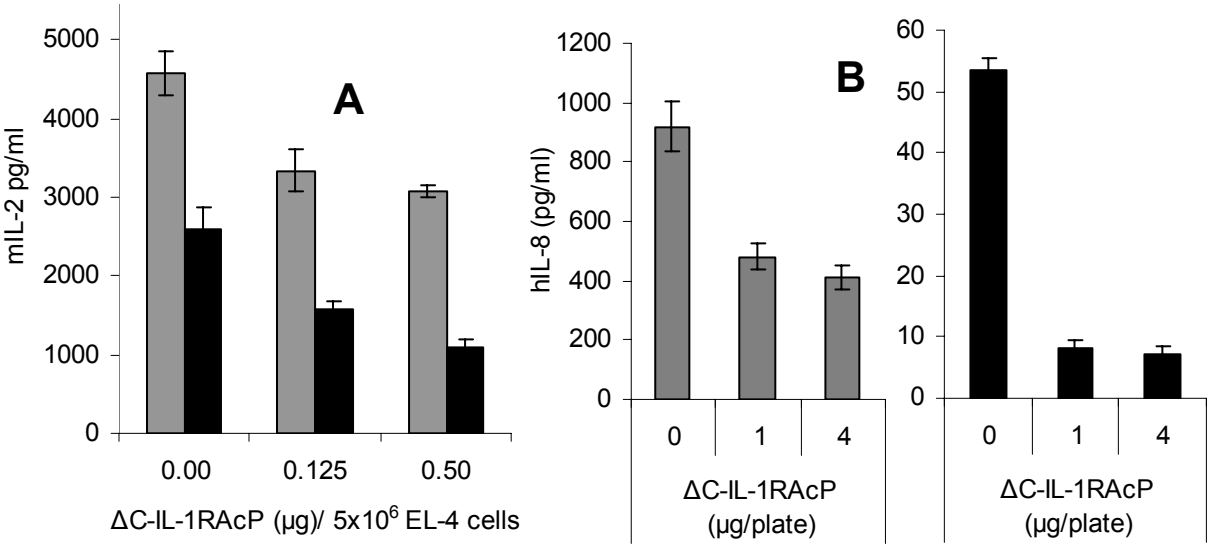


Fig. 3.4: The response to IL-33 in EL-4 or HEK293RI cells is dependent on IL-1RAcP.
(A). IL-1 and IL-33 stimulated IL-2 release by EL-4 cells is inhibited by the over-expression of a C-terminally truncated IL-1RAcP molecule. 5 x 10^6 EL-4 6.1 cells were transiently transfected with 0.5 μg of mIL-33Rα-chain encoding plasmid and plasmid containing C-terminally truncated mIL-1RAcP (ΔC-IL-1RAcP) lacking the TIR domain, which was not sufficient to allow signal transduction (as indicated). Cells (2.5 x 10^4) were stimulated with 100 pg/ml rhIL-1β or 50 ng/ml rmIL-33 and mIL-2 was measured from cell free supernatant by ELISA 18h after transfection. The answer to either rhIL-1β (grey bars) or rmIL-33 (black bars) was significantly reduced in EL-4 6.1 cells by over expressing the ΔC-IL-1RAcP in a concentration-dependent manner.
(B). IL-1 and IL-33 -stimulated IL-8 release by HEK293RI cells is inhibited by the over-expression of a C-terminally truncated IL-1RAcP molecule. 3.6 x 10^6 HEK293RI cells were transiently transfected with 2 μg of mIL-33Rα-chain encoding plasmid and plasmid containing C-terminally truncated mIL-1RAcP (ΔC-IL-1RacP), as indicated. Cells (2.5 x 10^4) were stimulated with either 100 pg/ml rhIL-1β (grey bars, left) or 50 ng/ml rmIL-33 (black bars, right) and hIL-8 was measured from cell free supernatant 18h after stimulation. The over expression of ΔC-IL-1RAcP in HEK293RI cells reduce hIL-8 production in a concentration-dependent manner. Data shown here (for A, and B) are means +/- standard deviations from one experiment (carried out in triplicates or quadruplicates) out of a series of at least three similar ones with comparable results.

In order to support the result achieved by the administration of the neutralizing anti-mIL-1RAcP (4C5) mAb, a dominant negative version of IL-1RAcP that does not contain the TIR domain was used. Titration of a plasmid encoding ΔC-IL-1RAcP into

EL-4 6.1 and HEK293RI cells (that were additionally transfected with a plasmid encoding mIL-33Rα) decreased the mIL-2 production in response to both IL-1β and IL-33 (Fig.3.4A) in EL-4 6.1 cells and hIL-8 production in response to IL-1β (Fig.3.4B left) and IL-33 (Fig.3.4B right) in HEK293RI cells in dose dependent manner. These results are consistent with the findings that IL-1RAcP is necessarily required for IL-1 or IL-33 induced cytokine production.

3.1.2.3. Activation of signaling events by IL-33 is dependent on the presence of IL-1RAcP

The IL-1 receptor family uses the MyD88/IRAK/TRAF6 module to activate downstream signaling elements, such as cJun N-terminal kinase (JNK) or the transcription factor NF-κB. IRAK-1 activity was measured in 6-IRAK-19 cells (a stably transfected EL-4 cell clone that expresses hIRAK-1 (J. Knop & M. Martin unpublished data). 6-IRAK-19 cells were transiently transfected with mIL-33Rα-chain to increase IL-33 responses. Stimulation with either rhIL-1β or rmIL-33 resulted in enhanced autophosphorylation of IRAK-1 (Fig.3.5). Cotransfection with ΔC-AcP (ΔC-IL-1RAcP) reduced IRAK-1 activation. This result demonstrates that IRAK-1 is activated by the IL-33R complex and that this activation depends on the presence of the TIR domain in full length mIL-1RAcP.

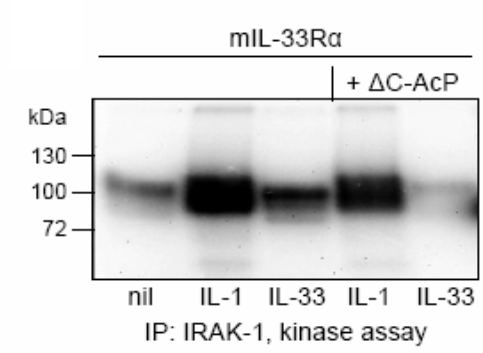


Fig. 3.5: IL-33-induced IRAK1 phosphorylation depends on IL-1RAcP. C-terminally truncated IL-1RAcP reduces IL-33 activation of IRAK-1. 6-IRAK-19 cells (a clone of EL-4 cells stably transfected with hIRAK-1) were transiently transfected with 1 µg of plasmid encoding mIL-33Rα-chain for every 10^7 cells. In addition, some of the cells were cotransfected with 1 µg of plasmid encoding C-terminally truncated mIL-1RAcP lacking the TIR domain (ΔC-AcP). One day later, transfectants were either stimulated with 10 ng/ml rhIL-1β or 100 ng/ml rmIL-33 for 15 min. IRAK-1 was immunoprecipitated from cell lysates by using mAb 2A9 (a kind gift from Z. Cao). Autophosphorylation of IRAK-1 was measured in an *in vitro* kinase assay and visualized by autoradiography. Transfection efficiency was in the order of 40% as ascertained by parallel transfections with EGFP plasmid and cytofluorometry. The data shown are of one representative experiment out of a series of three with comparable results.

The activation of NF-κB is a central element of the signaling pathways activated by the IL-1 receptor family. Although D6/76 cells responded to IL-18 and TNFα, they did not respond to IL-1 or IL-33 by activation of NF-κB. Upon transfection with full length mIL-1RAcP, NF-κB activation was restored to both cytokines with no effect on TNFα

or IL-18 response. Preincubation with mAb 4C5, which neutralizes murine IL-1RAcP, inhibited IL-1- and IL-33-stimulated NF-κB activation, but did not affect IL-18- or TNFα-stimulated activation of the transcription factor (Fig.3.6A right). ΔC-AcP was not able to restore IL-1 or IL-33 responsiveness with respect to NF-κB activation in D6/76 cells (data not shown). Transfection of EL-4 cells with increasing amounts of the plasmid-encoding ΔC-AcP resulted in a concentration-dependent reduction in IL-33-induced NF-κB activation, demonstrating that the truncated form of mIL-1RAcP behaved in a dominant-negative fashion (Fig.3.6B).

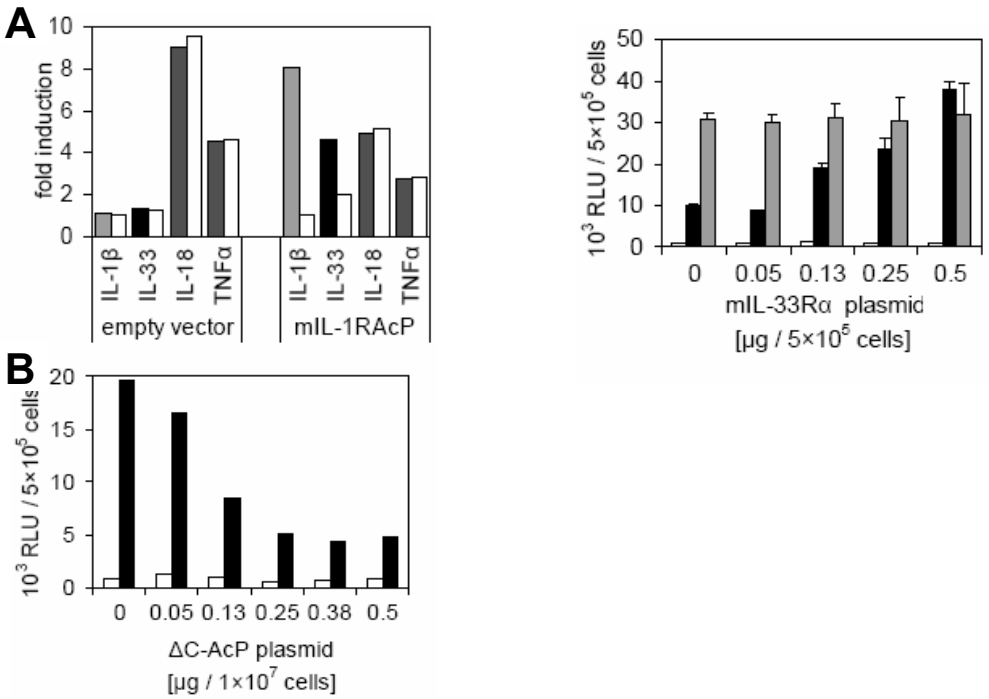


Fig. 3.6: IL-33-induced activation of NF-κB depends on IL-1RAcP.
(A). Activation of the transcription factor NF-κB by IL-33 depends on IL-1RAcP. D6/76 (*Left*) and EL-4 (*Right*) cells were transiently cotransfected with plasmids encoding mIL-33Rα-chain and an NF-κB-dependent luciferase reporter plasmid (D6/76 cells, 0.5 µg of plasmid each for 5 x 10^6 cells; EL-4 cells, 0.5 µg of reporter plasmid plus the indicated concentrations of plasmid for mIL-33Rα or empty vector). In addition, in D6/76 cells, full length mIL-1RAcP was overexpressed. Cells were stimulated with either 100 ng/ml rmIL-33 (black bars) or 100 pg/ml rhIL-1β (gray bars) and either 100 ng/ml rmIL-18 or 100 ng/ml rhTNFα, and luciferase activity was determined in the cell lysates the next day. Preincubation with 50 µg/ml mAb 4C5 (white bars) was for 1 h before stimulation. Depicted is fold induction, relative light unit (RLU) values of stimulated samples/RLU values of controls.
(B). IL-1RAcP lacking the TIR domain (ΔC-AcP) is dominant-negative in IL-33 signaling as measured in a NF-κB reporter gene assay. EL-4 cells were transiently transfected with increasing amounts of a plasmid encoding ΔC-IL-1RAcP (ΔC-AcP) in addition to an NF-κB-dependent luciferase reporter plasmid (1 µg for every 1 x 10^7 cells). The next day, cells were stimulated with 100 ng/ml rmIL-33 for a further 18 h. Luciferase activity was measured in the cell lysates.
Data shown in A and B are duplicates or tripicates of one representative experiment out of a series of three with comparable results.

Transfection of EL-4 cells with increasing amounts of the plasmid encoding full length mIL-33Rα-chain resulted in an increase in NF-κB activation after IL-33 stimulation while leaving the IL-1 response unaffected (Fig.3.6A left). This finding proves the

specificity of the observed effect for IL-33 and that the endogenous expression level of mIL-33Rα-chain limited the response to its ligand in this cell line.

Preincubation of EL-4 or IL-1RAcP transfected D6/76 cells with mAb 4C5 resulted in a reduction of IL-1β- and IL-33-stimulated NF-κB activation (Fig.3.7). NF-κB activation was nearly completely inhibited by 4C5 after stimulation with rhIL-1β, whereas rmIL-33-stimulated activation was reduced to about 50%. The isotype-matched control antibody had no effect on IL-1- or IL-33-stimulated NF-κB activation (Fig.3.6A left and Fig.3.7, open bars).

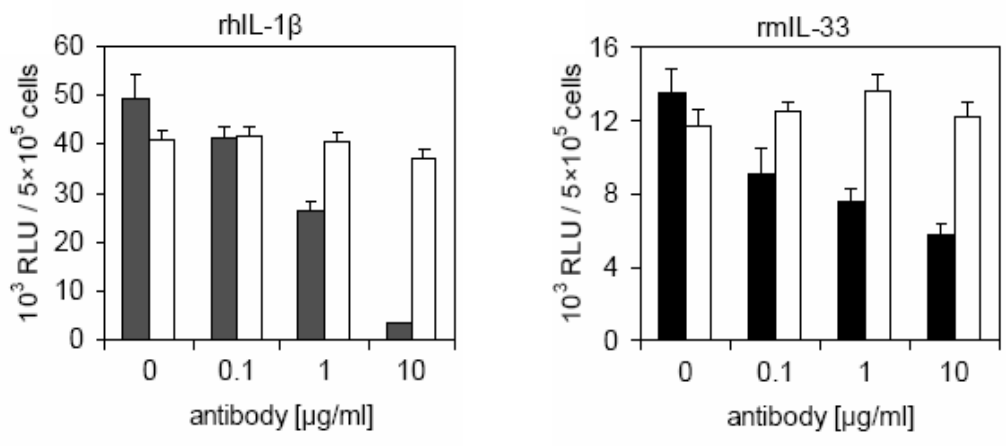


Fig. 3.7: IL-33-induced activation of NF-κB depends on IL-1RAcP.
The neutralizing anti-mIL-1RAcP mAb 4C5 reduces IL-1β- and IL-33-stimulated NF-κB activity. EL-4 cells were transiently cotransfected with a plasmid encoding mIL-33Rα-chain and an NF-κB-dependent luciferase reporter gene. Subsequently, cells were preincubated for 30 min with either 4C5 (filled bars) or the isotype control antibody RA3–6B2 (open bars) at 0, 0.1, 1, or 10 µg/ml and stimulated with either 100 pg/ml rhIL-1β (gray bars) or 50 ng/ml rmIL-33 (black bars). The next day, luciferase activity was determined in the cell lysates. Data shown are triplicates of one representative experiment out of a series of three with comparable results.

IL-33 activates cJun kinase and p38 MAP kinase

As was reported previously, the activation of JNK by IL-1 depends on the presence of IL-1RAcP in D6/76 cells (Wesche et al., 1997b). In order to investigate activation of JNK and p38 MAP kinases by IL-33, time course studies were performed. IL-33 and IL-1β induced activation of JNK was studied in EL-4 cells. IL-1 and IL-33 activated JNK in a transient fashion. The maximum activity was measured at 12–16 min in an *in vitro* kinase assay by using GST-cJun as substrate. The kinetic of JNK activation was identical between IL-1 and IL-33 (Fig.3.8A). IL-33 and IL-1β stimulate JNK activation transiently after stimulation which last up to 32 minutes with the peak of phosphorylation between 12-16 minutes.

p38 phosphorylation time course study was performed in transiently transfected HEK293RI cells expressing IL-33Rα. Western blot analysis of phoso p38 and total p38 proteins reveled that IL-33 stimulate p38 phosphorylation after 10 minutes of stimulation which lasted longer than 60 minutes with the maximum at 60 minutes where as IL-1β resulted in p38 phosphorylation just after stimulation which was detectable longer than 60 minutes with the maximum at 30 minutes (Fig.3.8B).

In summary, these results demonstrate that key elements of the classical signaling pathway used by the IL-1 receptor family such as IRAK-1, NF-κB, and JNK and p38 were specifically activated by IL-33 only in the presence of full length IL-1RAcP.

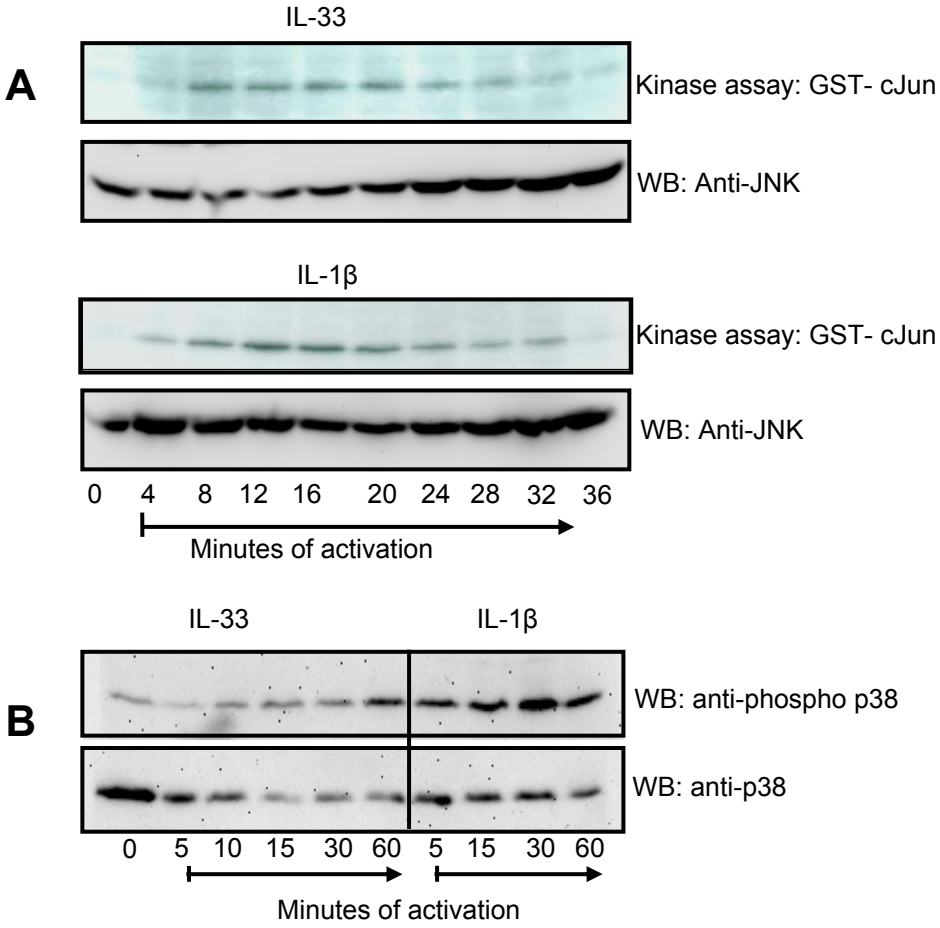


Fig. 3.8: IL-33 stimulates activation of cJun and p38 MAP kinase.
(A). IL-33 and IL-1 induced activation of EL-4 cells leeds to JNK activation. A time course study was performed to measure the activation of the cJun kinase JNK. 2.5 x 10^6 EL-4 cells transiently transfected with IL-33Rα encoding plasmid were stimulated with either 50 ng/ml rmIL-33 or 10 ng/ml rhIL-1β for indicated time points. Cells were lysed and JNK assay was performed from 10 µg of the protein. Proteins were separated on 12.5% SDS-PAGE and phosphorylated GST:cJun was visualized by autoradiography after 3 days exposure (upper). In parallel the same amount of protein was separated and total JNK protein was detectd by Western blotting using anti-JNK antibodies (lower).
(B). Activation of p38 in transiently transfected HEK293RI cells by IL-33 and IL-1. A time course study was made for activation of p38 MAP kinase. HEK293RI cells were transiently transfected with IL-33Rα encoding plasmid. Next day these transfected cells were stimulated with either 50 ng/ml rmIL-33 or 10 ng/ml rhIL-1β for 0, 5, 10, 15, 30 and 60 minutes. Cells were lysed with 100 µl of Laemmli buffer and phospho p38 and total p38 proteins were detected by Western blotting using anti-phospho p38 antibodies (upper) and anti-p38 antibodies (lower).

3.1.2.4. Demonstration of specificity of IL-33 effects

3.1.2.4.1. Heat treatment reduces IL-33 bioactivity

The data described above indicate that the response to IL-33 is specific to IL-1RAcP and IL-33Rα, as IL-33 response can be increased by either increasing the expression level of IL-1RAcP or IL-33Rα-chain (Fig.3.2B and 3.6A). However, in order to prove that IL-33 is the only causative reagent for the biological effects on cells treated with rmIL-33 and to rule out non-specific effects due to any contamination or indirect effect of IL-33, rmIL-33 and rhIL-1β were heat-treated for 10 or 30 minutes at 95 ºC to destroy the proteins. These heat-treated cytokines were used to stimulate EL-4 cells. Interestingly, rmIL-33 was rather heat stable in comparison to IL-1 as depicted in the results from the bioassay. However, by heating for 30 minutes at 95 ºC, practically all of the biological activity was destroyed when using low concentrations of IL-33 (Fig.3.9). These results proof that the effects observed here are solely due to IL-33 protein and not to a contamination e.g. by LPS.

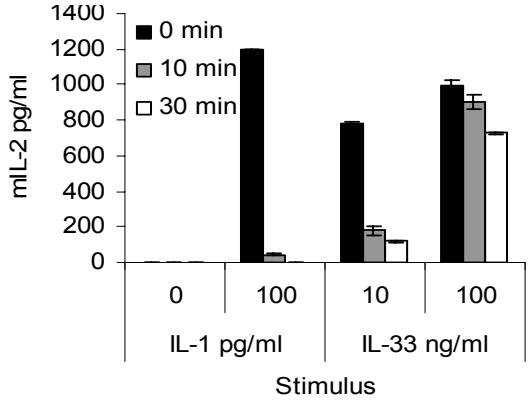


Fig. 3.9: Demonstration of specificity of IL-33 effects in EL-4 cells. Heat treatment of rmIL-33 inhibits mIL-2 production by EL-4 cells. rhIL-1β and rmIL-33 were heat treated at 95 ºC for 0, 10 and 30 minutes. EL-4 cells (2.5 x 10^4 / well) were stimulated with heat treated rhIL-1β and rhIL-33 in 200 µl total volume for over night and mIL-2 was measured by ELISA from cell free supernatant. rmIL-33 is relatively stable to heat treatment.

3.1.2.4.2. Soluble IL-33Rα-chain (ST2) inhibits IL-33 induced stimulation of EL-4 cells

In order to demonstrate the specificity of IL-33 -induced signaling, soluble mIL-33Rα:Fc (soluble ST2) was used to inhibit the IL-33 induced mIL-2 production by EL-4 cells. Soluble mIL-33Rα: Fc did not work to neutralize the IL-33 in the EL-4 system at a 100 fold molar excess when IL-33 induced IL-2 production was measured after overnight incubation (data not shown). Thus a different type of experiment was designed in which rmIL-33 was to be removed by ST2:Fc fusion protein in solution

before addition of IL-33 to the cells. In this experimental setup loss of IL-33 activity was achieved leaving unaffected the IL-1β -induced IL-2 production (Fig.3.10A), thus demonstrating the IL-33 specificity of the effect. However, due to costs of the Fc fusion protein, only a 100 fold molar excess of ST2:Fc could be tested, which did not suffice to achieve complete inhibition. In a different approach, mIL-33 or IL-1β was preincubated with IL-33Rα:Fc fusion protein and then the mixture was added to cells for 15 min to stimulate JNK activity. In this short term stimulation inhibition of IL-33 stimulated JNK activity was achieved which was not seen on IL-1 stimulated JNK activity (Fig.3.10B), indicating that all the responses observed are specific for IL-33, rather than being caused by any contamination.

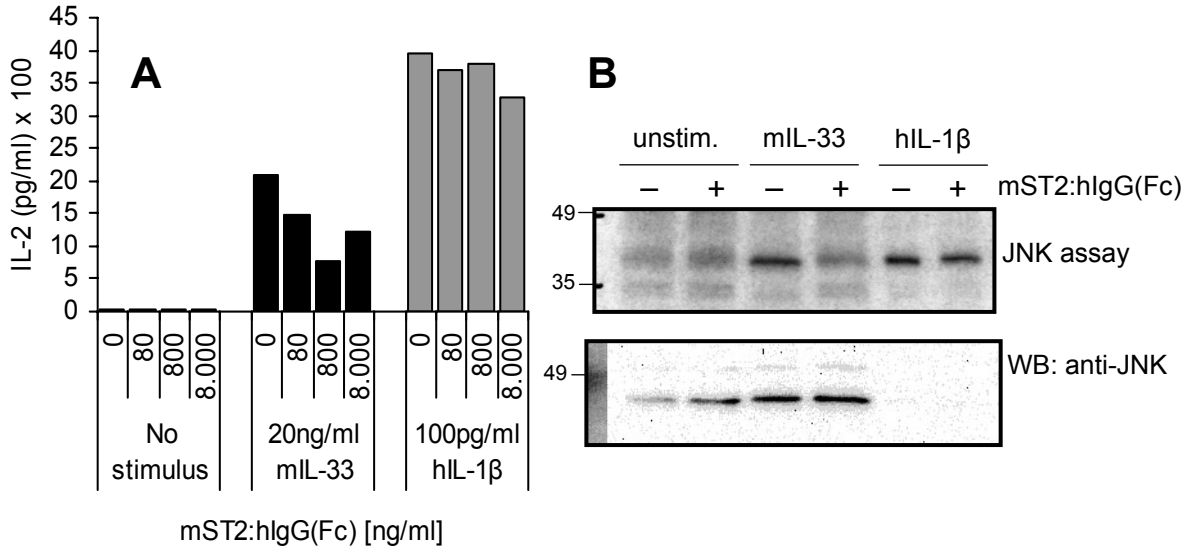


Fig. 3.10: Soluble IL-33Rα-chain inhibits IL-33 effects.
(A). Soluble mIL-33Rα-Fc (ST2) inhibits IL-33 induced mIL-2 production.
Medium, 20 ng/ml rmIL-33 or 100 pg/ml rhIL-1β were incubated with mST2:hIgG(Fc) (as indicated) in the presence of protein A sepharose for one hour at 37 ºC and supernatant and beads were separated by centrifugation. 2.5 x 10^4 EL-4 cells were stimulated with preincubated medium, rhIL-1β (100pg/ml) or rmIL-33 (20 ng/ml) from protein A free supernatant for overnight and mIL-2 was measured by ELISA from cell free supernatant. Soluble ST2 inhibits specifically mIL-33 induced mIL-2 production in dose dependent manner.
(B). Soluble mIL-33Rα-Fc (ST2) inhibits IL-33 induced activation of JNK.
2.5 x 10^6 IL-33Rα transfected EL-4 cells were kept unstimulated or stimulated for 15 minutes with either 50 ng/ml rmIL-33 or 10 ng/ml rhIL-1β in the presence or absence of 20 µg/ml mST2:hIgG(Fc) (preincubated with stimulus for 30 minutes). 10 µg of whole cell lysates were used in a kinase assay with 1 µg of GST:cJun as substrate and 2 µCi [^{32}Pγ]-ATP. Proteins were separated on 12.5% SDS-PAGE and phosphorylated GST:cJun was visualized by autoradiography after 3 days exposure. In parallel same amount of protein was separated on 12.5% SDS-PAGE and total JNK protein was visualized by Western blot/ECL reaction with anti-JNK antibodies (lower). For IL-1β only 1/4 amount of sample was loaded compared to the unstimulated or stimulated with rmIL-33.

3.1.2.5. IL-1RAcP is required for IL-33-induced cytokine release in mast cells

Freshly isolated and differentiated murine bone marrow-derived (BMD) mast cells (BMDMC) were used to confirm the results obtained with EL-4 cells on primary mast cell cultures. (BMDMC were isolated, differentiated and purity was analyzed by Prof Dr. Michael Huber (Max-Plank Institute of Immunobiology, Freiburg Germany) and most of the experiments on mast cells were also performed in Freiburg in collaboration with Dr. Huber).

BMDMC express high levels of ST2 (IL-33Rα-chains) as determined by RT-PCR (data not shown**)** and FACS analysis (Fig.3.11A). BMMC also expressed IL-1RAcP mRNA (data not shown). The IL-33 receptor is functional in that IL-33 dose-dependently stimulates production of the pro-inflammatory cytokine IL-6 to an extent comparable to LPS in BMDMC (Fig.3.11B), and this response is IL-33 specific and dependent on IL-1RAcP because it was suppressed by the neutralizing antibody (mAb) 4C5 (Fig.3.11D).

To investigate the effects of IL-33 on the production of additional cytokines and chemokines in BMDMC, the levels of TNF-α, IL-1α, IL-1β, MCP-1, IL-10, IFNγ, IL12p70, IL-2, IL-4 and IL-13 were quantified in supernatants fo BMDMC using Beadlyte MultiCytokine Flex kits in a Luminex 100 instrument in collaboration with Prof. Werner Falk, University of Regensburg (cytokines were measured at University of Regensburg). It was found that IL-1α, MCP-1, IL-10, IFNγ, IL12p70, IL-2, and IL-4 were below the detection limit for this assay in supernatants obtained from both resting and stimulated BMMC. Stimulation of BMDMC with IL-33 stimulated rapid (less than 3 hours) release of TNFα and IL-1β, but did not affect the low constitutive release of IL-13 (Fig.3.11C). Pretreatment with the monoclonal antibody (mAb) 4C5, which neutralizes IL-1RAcP (Greenfeder et al., 1995; Hestdal et al., 1994), resulted in a concentration-dependent reduction of the IL-33-induced cytokine production. If mast cells were stimulated with low concentrations of rmIL-33 (1–3 ng/ml), a complete inhibition of stimulated IL-6 production could be achieved (Fig.3.11D). TNFα release remained, 27% at 25 µg/ml or 12% at 50 µg/ml mAb 4C5, respectively.

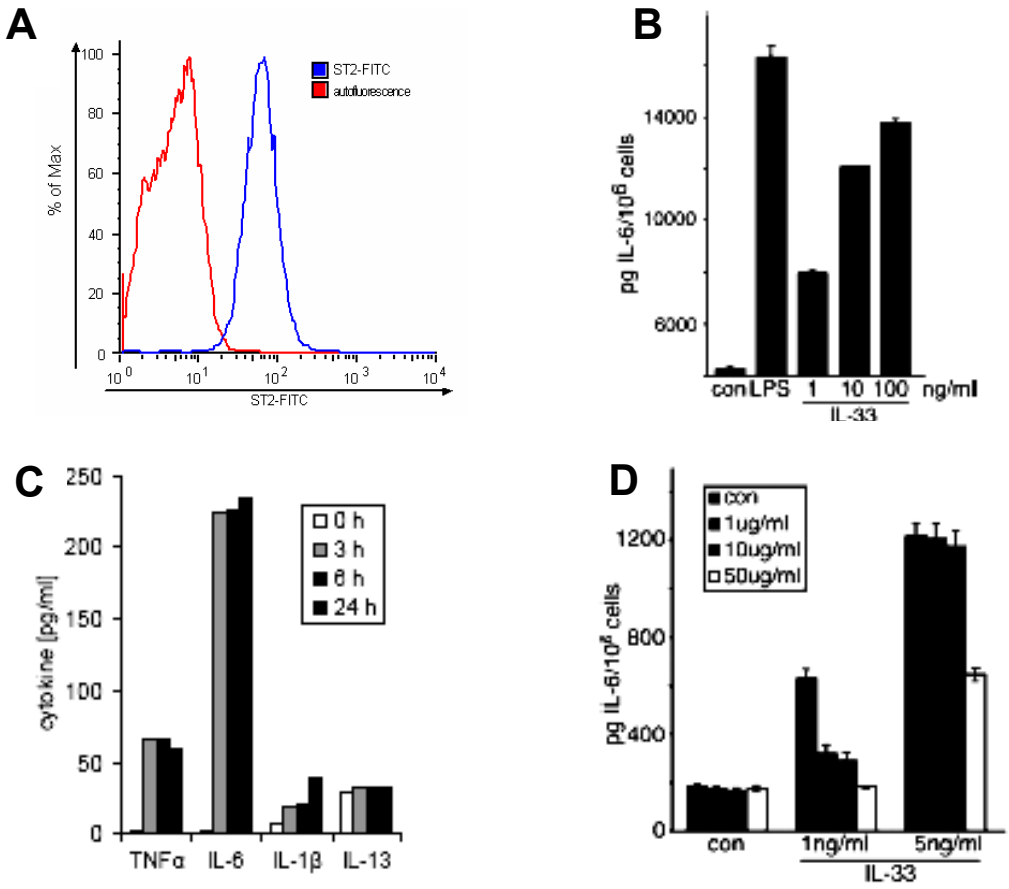


Fig. 3.11: Bone marrow derived mast cells respond to IL-33 with cytokine production in an IL-1RAcP-dependent manner (data from collaboration with M. Huber and W. Falk).
(A). Murine bone marrow derived mast cells express ST2 / mIL-33Rα-chain on their cell surface. Mast cells were incubated with an FITC-labelled anti-mouse ST2 antibody and surface expression was analysed by flow cytometry.
(B). Murine bone marrow derived mast cells produce IL-6 after stimulation with rmIL-33 in concentration dependent manner. Cells were stimulated with 0, 1, 10, 10 100 ng/ml rmIL-33 or 1 µg/ml LPS. The next day mIL-6 was detected by ELISA in cell-free supernatants. LPS served as a positive control.
(C). Mast cells produce several pro-inflammatory cytokines after stimulation with rmIL-33. The 1 x 10^6 cells per millilitre were seeded and stimulated with 2 ng/ml rmIL-33 for 0, 3, 6, and 24 h as indicated. Cytokine profiles were detected in the supernatants by using Beadlyte MultiCytokine Flex kits in a Luminex 100 instrument.
(D). IL-33 stimulated IL-6 release in murine bone marrow derived mast cells is reduced by the neutralizing anti-mIL-1RAcP mAb 4C5. Cells were pre-incubated for 30 min with three different concentrations of 4C5 mAb (black bars = no mAb, dark grey bars = 1 µg/ml, light grey bars = 10 µg/ml, open bars = 50 µg/ml 4C5 each) and then either left untreated (con) or stimulated with 1 ng/ml or 10 ng/ml rmIL-33. The next day murine IL-6 was measured by ELISA in cell free supernatants. Results are from one representative experiment of a series with comparable results (C) or are means of triplicates or quadruplicates of one typical experiment out of a series of at least three similar experiments with comparable results (B and D). For mast cell stimulation, only commercial rmIL-33 was used (LPS ≤ 0.1 units/µg protein).

3.1.2.6. IL-1RAcP is required for IL-33 -induced proliferation of D10G4.1 cells

D10G4.1 cells express functional IL-1 receptor and proliferate in response to IL-1β stimulation (Fig.3.12A). These cells also express IL-33Rα and IL-1RAcP mRNA as detected by RT-PCR (data not shown). The IL-33 receptor in D10G4.1 cells is functional in that the cells respond to IL-33 in dose dependent fashion in the D10 proliferation assay (Fig.3.12A). Although the concentrations of IL-33 required to achieve comparable responses is 100 times higher than that of IL-1β in D10 cells, the relative IL-33 requirement in D10 cells is much less than in EL-4 cells (Fig 3.2A), indicating that the expression of IL-33 receptor is not as limiting as in EL-4 cells or, alternatively, that these cells may express some additional molecules which increase the binding affinity of the IL-33 to the receptor.

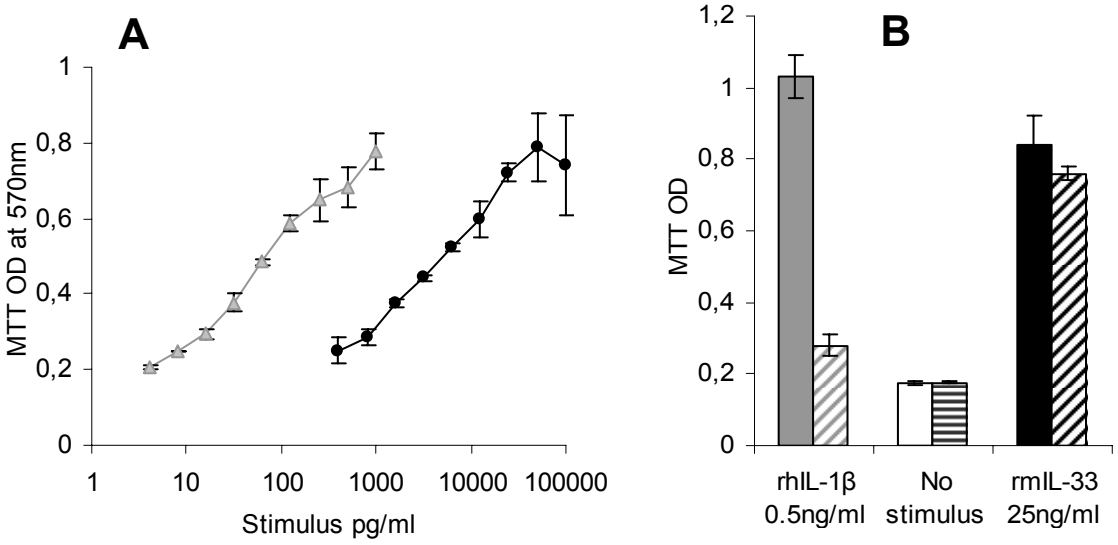


Fig. 3.12: D10G4.1 cells respond to IL-1 and IL-33 in D10 proliferation assay.
(A). Concentration curve for IL-1β and IL-33 responsiveness in D10 assay. D10G4.1 (2.5 x 10^4/ well) cells were cultured in 96 well plate in the absence or presence of increasing concentration (as indicated) of either hIL-1β (grey triangles) or mIL-33 (black circles) for 5 days and MTT assay was performed on 5th day.
(B). Neutralizing antibody inhibits D10 proliferation in response to IL-1β but not in response to IL-33. 2.5 x 10^4 cells were stimulated with indicated concentrations of IL-1 or IL-33 in the presence (hashed bars) or absence (full bars) of 10 µg/ml anti-IL-1RAcP (4C5) antibody and MTT assay was performed after 5 days of incubation. Results shown for A and B are shown as means of three replicates in one experiment.

In order to assess the role of IL-1RAcP in IL-33 -induced D10 proliferation, anti-IL-1RAcP neutralizing antibodies 4C5 were used. As shown in Fig.3.12B, the neutralizing antibodies inhibited IL-1β induced D10 proliferation considerably while leaving unaffected the IL-33 induced D10 proliferation even at lower concentrations of IL-33. The neutralizing anti-IL-1RAcP antibody 4C5 neutralizes the effect of IL-1β in all cell types (tested). It also inhibited the IL-33 induced responsiveness in EL-4

cells and mast cells but not in D10 cells. These results raise the question, whether IL-1RAcP is definitely required for IL-33 induced signaling in D10 G4.1 cells or not. In order to answer the question, NF-κB dependent reporter gene assay was performed after overexpression of ΔC-IL-1RAcP (lacking TIR domain). Results depicted in Figure 3.13 demonstrate the dominant negative effect of ΔC-IL-1RAcP molecule (lacking TIR domain) overexpression in a dose dependent fashion. This dominant negative effect was comparable for IL-1β and IL-33-stimulated NF-κB activity indicating the absolute requirment of IL-1RAcP for IL-33 induced activation of D10 G4.1 cells, demonstrating the need for IL-1RAcP also in D10 cells.

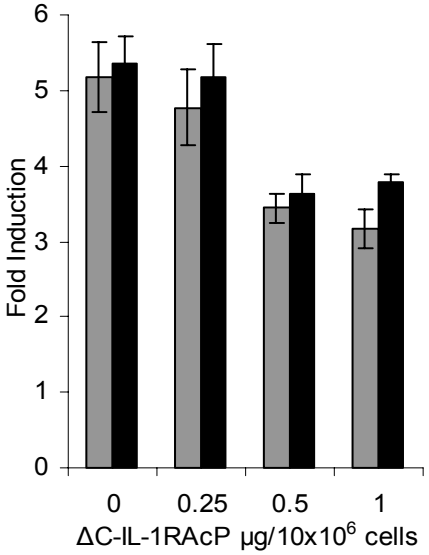


Fig. 3.13: IL-1RAcP is required for IL-33 signaling in D10 cells. 10 x 10^6 D10G4.1 cells were transiently transfected with 1 µg of plasmid containing firefly luciferase gene under NF-κB promoter along with increasing amounts (as indicated) of TIR domain lacking IL-1RAcP (ΔC-IL-1RAcP) expressing plasmid. Cells were kept unstimulated or stimulated 4 hours after transfection with either 1 ng/ml IL-1β (grey bar) or 50 ng/ml IL-33 (black bar). Next day reporter gene activity was analysed from cell lyates. Results shown here are representative of 3 independent experiments with similar results and are shown as means of three replicates in one experiment ± SEM.

3.1.2.7. The interaction of IL-33Rα-chain and IL-RAcP is dependent on IL-33

It is well known that IL-1 receptors and IL-1RAcP form heterodimeric receptor complexes on the cell surface after binding of IL-1 to either IL-1RI or IL-1RII (Lang et al., 1998). To clarify whether such a complex also forms between IL-1RAcP and IL-33Rα-chain, epitope-tagged versions of IL-1RAcP and IL-33Rα were co-expressed in HEK293RI cells. After stimulation with rmIL-33, complexes were co-immunoprecipitated. IL-1RAcP was co-precipitable with IL-33Rα-chain (Fig.3.14A), and vice versa (Fig.3.14B), only in the presence of rmIL-33, but not if the cells were incubated with rhIL-1β (Fig.3.13A & B). In some experiments, a weak interaction in the absence of ligand was also observed, most likely due to the strong protein expression in HEK293RI cells. This result demonstrates that the interaction of the membrane-inserted forms of IL-1RAcP and IL-33Rα-chain is specific for and dependent on IL-33. It has been shown that soluble IL-1 receptors and IL-1RAcP interact in the presence of IL-1 (Smeets et al., 2003; Smith et al., 2003). Employing a

soluble IL-33Rα:Fc fusion protein and an epitope-tagged soluble IL-1RAcP, it was demonstrated that a specific interaction in solution took place in the presence of IL-33, which was stable enough to be immunoprecipitated (Fig.3.14C. (The depicted experiment was performed in collaboration with Prof. Werner Falk, Regensburg, subsequent experiments were performed in Giessen after cloning of own IL-33Rα:Fc fusion protein). This result extend the finding that IL-1RAcP is the co-receptor for IL-33Rα-chain from the membrane-inserted forms to the soluble molecules.

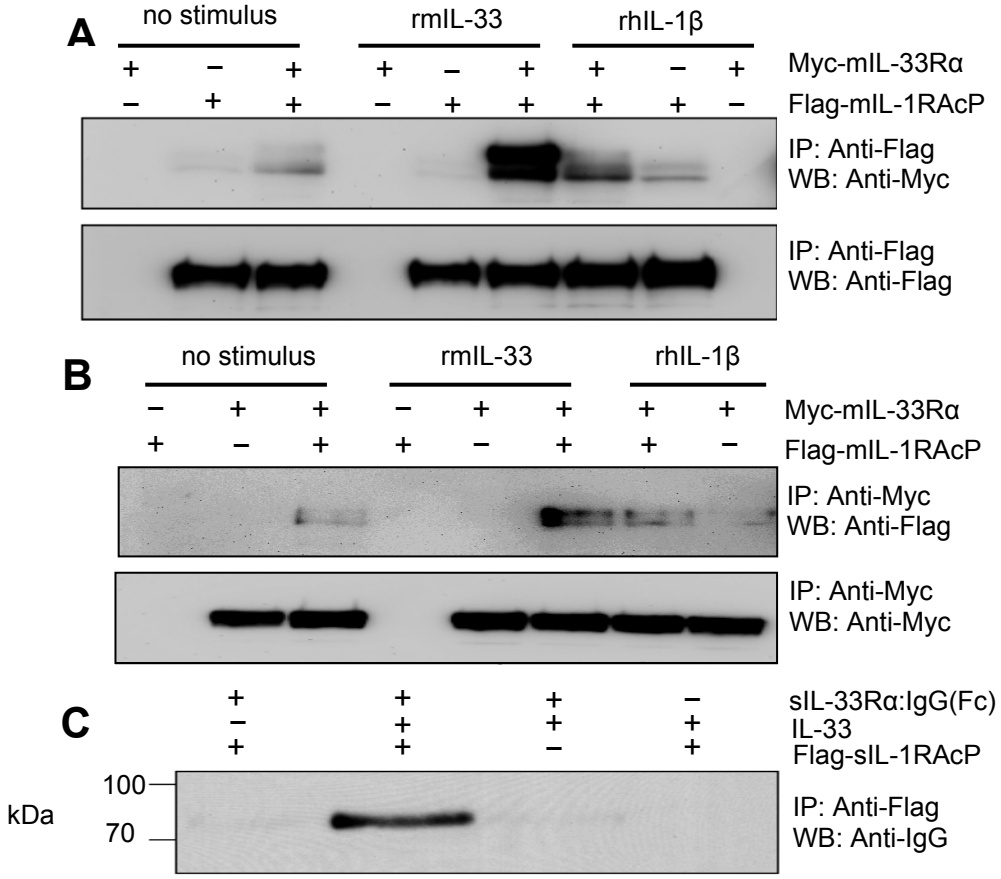


Fig. 3.14: The interaction of IL-1RAcP with the IL-33Rα chain is dependent on IL-33.
(A & B). Membrane IL-33Rα-chain and IL-1RAcP form a complex in the presence of IL-33.
HEK293R1 cells were transiently transfected with plasmids containing Flag tagged mIL-1RAcP and/or Myc tagged mIL-33Rα alone or in combination. Transfected cells (next day) were left unstimulated or stimulated with 10 ng/ml rhIL-1β or 100 ng/ml rmIL-33 (as indicated at top edge of Fig) for 15 min at 37 °C. Cells were lysed in absence or presence of 10 ng rhIL-1β or 100 ng rmIL-33. Flag tagged mIL-1RAcP (A) or Myc tagged IL-33Rα (B) molecules were immunoprecipitated for overnight at 4 °C using anti-Flag M2 agarose beads (A) or anti-Myc antibodies (B). Precipitated proteins were washed, and visualized by Western blot/ECL reaction with anti-Myc and anti-Flag antibodies. A strong band in co-immunoprecipitated Flag-mIL-1RAcP and Myc-mIL-33Rα only in IL-33 presence indicate strong physical interaction of both receptor chains. The results shown are representative of 5 independent experiments with similar results.
(C). Soluble forms of IL-33Rα-chain and IL-1RAcP form a complex in the presence of IL-33.
srmIL-33Rα:(hIgG) Fc fusion protein was incubated in the absence or presence of rmIL-33 with Flag-epitope-tagged srmIL-1RAcP fixed on Flag-affinity gel. Coprecipitated fusion protein was visualized by Western blot after SDS/PAGE by using an anti-human IgG secondary antibody (Fig. 3.14C was generated in collaboration with Prof. Werner Falk at University of Regensburg).

3.1.2.8. IL-33 receptor works in heterodimeric form

All known members of the IL-1 receptor family function as heterodimeric receptor complexes. Thus it was likely that the functional IL-33 receptor complex would also work in a heterodimeric form. However, in principle, also homodimers were possible. In order to demonstrate heterodimerization and exclude the possiblity of homodimerization of IL-33Rα upon IL-33 binding, Flag-tagged and/or Myc-tagged IL-33Rα were expressed in HEK293RI cells along with or without untagged ΔC-IL-1RAcP and incubated with IL-33. Co-immunoprecipitation results showed that the co-expressed Flag- or Myc-tagged IL-33Rα do not interact either in the absence or presence of ligand (Fig.3.15) indicating that IL-33 does not dimerize ST2 (IL-33Rα) but rather signals through recruitment of IL-1RAcP.

From the above results it can be concluded that IL-1RAcP associates with IL-33Rα in a ligand-dependent manner (Fig.3.14A & B), furthermore, these results suggest that IL-33 does not dimerize IL-33Rα (Fig.3.15) but rather signals through recruitment of IL-1RAcP. Finally, the soluble form of IL-33Rα can recruit also the soluble IL-1RAcP in the presence of IL-33 (Fig.3.14C) suggesting that the combination of soluble IL-33Rα and soluble IL-1RAcP can act in a synergistic manner to inhibit IL-33 activities.

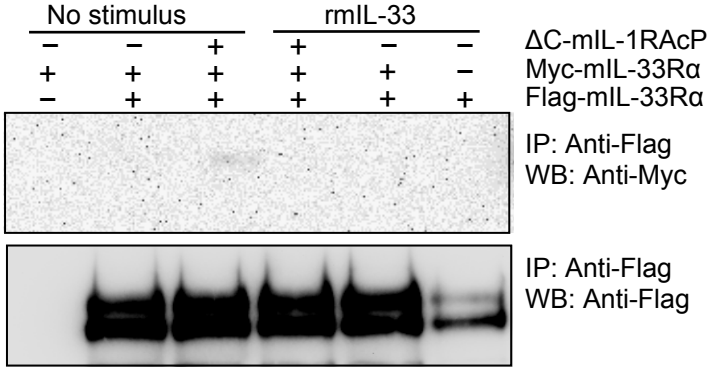


Fig. 3.15: IL-33 does not homodimerize ST2 (IL-33Rα).
Flag-tagged and/or Myc-tagged IL-33Rα were expressed along with or without untagged ΔC-IL-1RAcP in HEK293RI cells. Next day cells were either kept unstimulated or stimulated with 100 ng/ml rmIL-33 for 15 minutes. Cells were lysed and proteins were immunoprecipitated for overnight with Anti-Flag M2 agarose. Immunoprecipitated IL-33Rα proteins were visualized after staining with anti-Myc antibodies (Upper) or anti-Flag antibodies (Lower). IL-33 does not dimerize ST2 (IL-33Rα). Results shown here are representative of 3 independent experiments with similar results.

3.1.3. Investigating the role of TIR8 (SIGIRR) in IL-33 signaling

The series of experiments described above prove that IL-1RAcP is absolutely required for IL-33-induced activation of the cells. Thus, IL-1RAcP is definitely a component of the IL-33 receptor complex in addition to IL-33Rα. This does not, however, exclude that at least in some cell types additional molecules may participate in the functional IL-33R complex. It was proposed that a possible candidate was TIR8 (SIGIRR). The role of this member of the IL-1 receptor family remains unclear, although it has been published that it may function as a negative regulator of Toll like receptors.

3.1.3.1. TIR8 over expression inhibits LPS and IL-1 induced signaling

In order to define the role of TIR8 in IL-33 signaling, the expression vectors of human and murine TIR8 were obtained from Prof. Alberto Mantovani (Instituto Clinico Humanitas, Rozzano (MI), Italia). The expression of the proteins was analyzed in HEK293RI cells by Western blotting. Both expression vectors yielded high levels of Flag-tagged TIR8 proteins (Fig.3.16A).

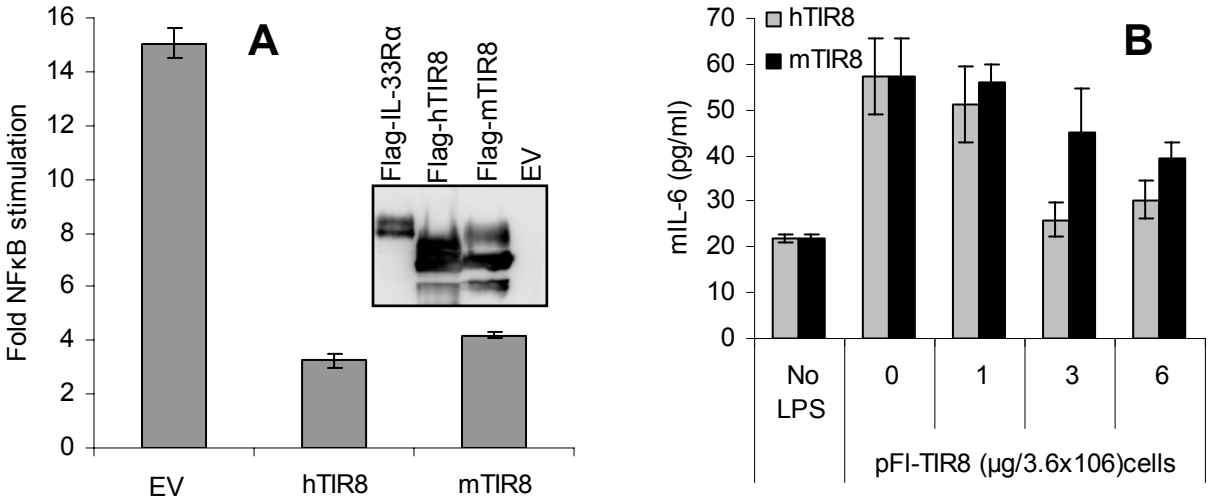


Fig 3.16: TIR8 (SIGRR) is a negative regulator of IL-1 and LPS signaling.
(A). Overexpression of human or mouse TIR8 inhibits IL-1 induced NF-κB activation in HEK293RI cells. HEK293RI cells were cotransfected with one µg reporter gene plasmid and either with 5 µg of empty vector (EV) vector, human or mouse TIR8 encoding plasmid. Next day cells were either stimulated with 100 pg/ml rhIL-1β or kept unstimulated. After overnight incubation reporter gene (luciferase) activity was measured from cell lysates. Depicted data are fold stimulations. Expression of human and mrine TIR8 is also showen by Western blot analysis from transiently transfected HEK293RI cells after immunoprecibitation with anti-Flag mAb.
(C). TIR8 inhibits LPS-induced mIL-6 production in L929 cells. L929 cells were transfected with indicated amount of hTIR8 (grey bars) or mouse TIR8 (black bars) encoding plasmid (total amount of plasmid DNA was maintained by adding empty vector). Next day 2.5 x 10^4 cells were either kept unstimulated or stimulated with 1ng/ml LPS in 96 well microtiter plate. After overnight incubation mIL-6 was measured by ELISA from cell free supernatants. Results in A and B are representative of two independent experiments and are expressed as mean of three replicates in one experiment ± SEM.

TIR8 is known as a negative regulator of Toll/Interleukin-1 receptor signaling. The overexpression of both human and murine TIR8 reduced IL-1 -induced activation of NF-κB in HEK293RI cells (Fig.3.16A) and LPS-induced production of IL-6 (Fig.3.16B) in L929 cells in dose-dependent manner. As shown in Fig.3.16A and B TIR8 overexpression inhibited the IL-1β and LPS induced signaling. However, it was observed that this inhibition was only achievable when there is very high expression of TIR8, at lower plasmid concentrations, protein expression did not affect LPS induced signaling.

3.1.3.2. TIR8 expression does not affect IL-33 signaling

In order to clarify whether IL-33Rα can use TIR8 as co-receptor for IL-33 signaling instead of IL-1RAcP murine TIR8 was overexpressed either alone or in combination with mIL-1RAcP (AcP) in EL-4 D6/76 cells. TIR8 expression was not able to reconstitute the responsiveness to IL-1β (Fig.3.17 left) and IL-33 (Fig.3.17 right). Moreover, co-expression of mTIR8 with mIL-1RAcP did not affect IL-1β or IL-33-induced mIL-2 production by EL-4 D6/76 cells, indicating that mTIR8 exerts neither stimulatory nor inhibitory effects on IL-1 and IL-33-induced IL-2 production in EL-4 D6/76 cells.

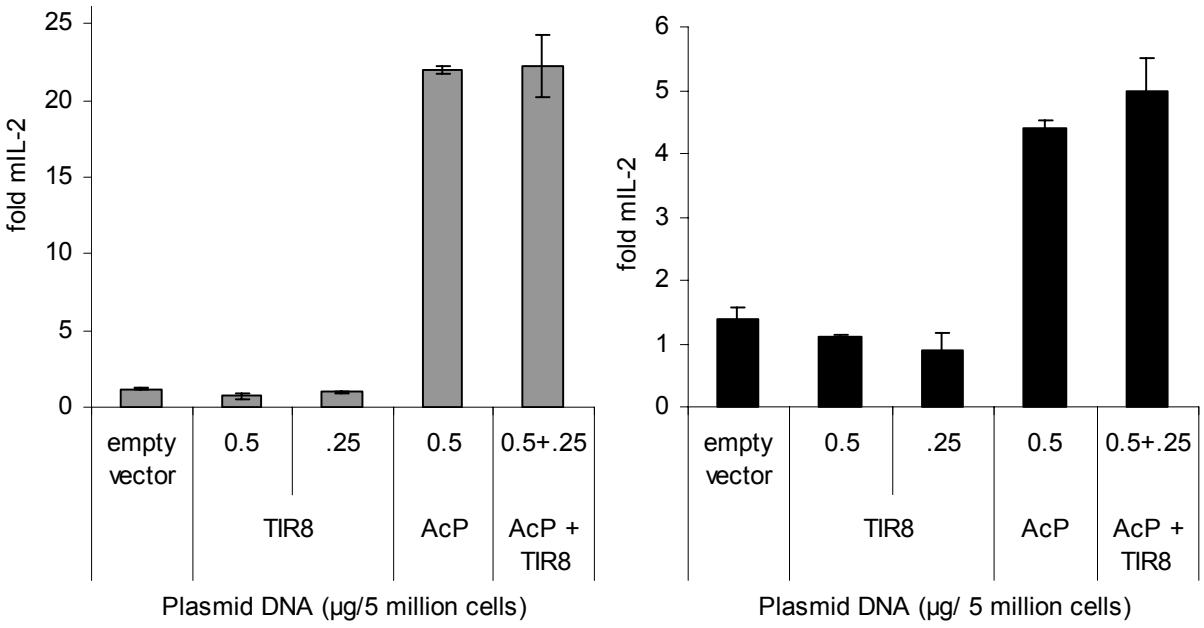


Fig. 3.17: mTIR8 expression does not reconstitute IL-1 or IL-33 signaling in EL-4 D6/76 cells. EL-4 D6/76 cells (5 x 10^6) were transiently transfected with 0.25 µg of plasmid containing mIL-33Rα gene along with indicated plasmids. Next day 2.5 x 10^4 cells/well were stimulated with either 100 pg/ml IL-1β (left, grey) or 50 ng/ml IL-33 (right, black) for overnight. mIL-2 was measured from cell free supernatant by ELISA. Results shown are representative of 2 independent experiments with similar results. Results are shown as mean of three replicates in one experiment and are expressed as fold luciferase activity (stimulated/unstimulated) ± relative error.

From the results it was clear that TIR8 expression was not able to render EL-4 D6/76 cells responsive to IL-33. In addition, the results described above demonstrate that TIR8 was unable to inhibit the IL-1 signaling which was seen in HEK293RI cells (Fig.3.16A). A possible explanation may be the rather low protein expression of ectopically expressed proteins in EL-4 D6/76 cells when compared to that of HEK293RI cells. So to demonstrate the effect of TIR8 overexpression on IL-33 signaling, HEK293RI cells were made responsive to IL-33 by transient transfection of mIL-33Rα encoding plasmid and the effect of TIR8 overpression on IL-33 signaling was analyzed in a reporter gene assay. The results of the NF-κB reporter gene showed slight inhibition of the IL-1 -induced NF-κB activation at higher concentrations of TIR8 coding plasmid (Fig.3.18A). However, overexpression of the TIR8 had no effect on IL-33 stimulated NF-κB activation in transiently transfected HEK293RI cells (Fig.3.18A).

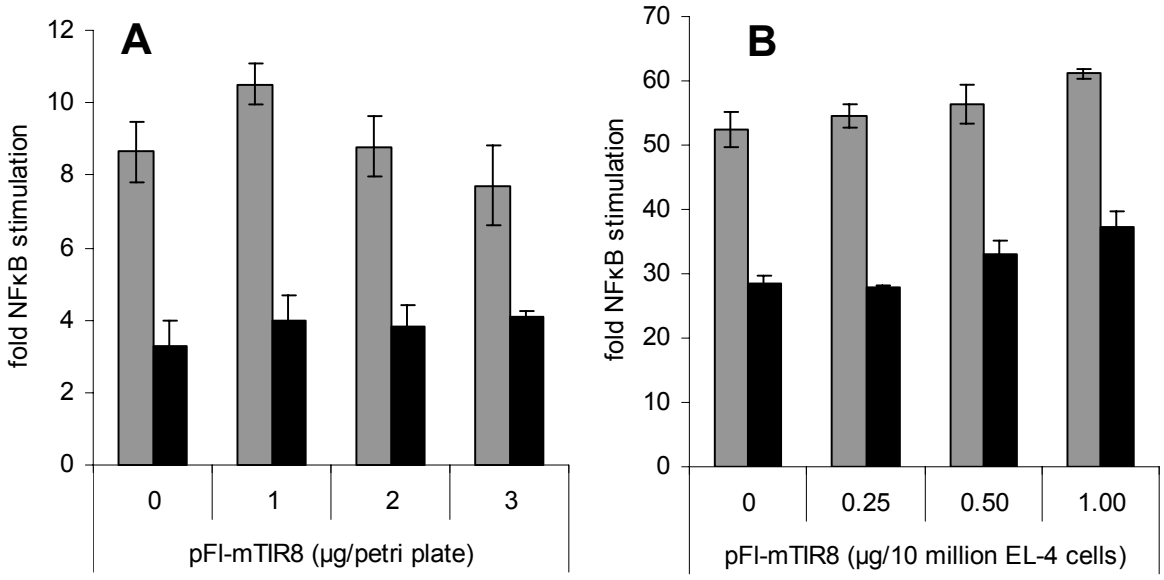


Fig. 3.18: TIR8 expression does not inhibit IL-33 signaling.
(A). TIR8 expression does not affect IL-33 induced NF-κB stimulation in HEK293RI cells. HEK293RI cells were transiently transfected with 1 µg of reporter gene plasmid, 2 µg of IL-33Rα encoding plasmid and increasing amount of mTIR8 encoding plasmid Next day 5 x 10^4 cells/well were kept unstimulated or stimulated with either 100 pg/ml IL-1β (grey bar) or 50 ng/ml IL-33 (black bar) in 96 well microtiter plate. After over night incubation reporter gene activity was measured from cell lysates. IL-33 signaling was not affected by TIR8 co-expression.
(B). IL-33 signaling in EL-4 cells was not affected by TIR8 over expression. EL-4 cells (10 x 10^6) were transiently transfected with 1 µg of reporter gene plasmid along with increasing amount of plasmid containing mTIR8 gene. 4 hours after transfection 5 x 10^5 cells/well were kept unstimulated or stimulated with either 100 pg/ml IL-1β (grey bar) or 50 ng/ml IL-33 (black bar) in 96 well microtiter plate. Next day reporter gene activity was measured from cell lysates. Results shown in A and B are representative of three independent experiments with similar results. Results are shown as mean of three replicates in one experiment and are expressed as fold luciferase activity (stimulated / unstimulated) $\pm$ relative error.

The difference in IL-1 induced NF-κB inhibition shown in Fig.3.16C and Fig.3.18A may be explained by the difference in amount of TIR8 protein expression. It was not possible to transfect with the same amount of TIR8 encoding plasmid because HEK293RI cells additionally require IL-33Rα expression to render the cells responsive to IL-33. Relatively lower response of transfected HEK293RI cells to IL-33 and lower concentrations of TIR8 expression may explain that no effect of TIR8 expression was detected on IL-33 signaling.

In order to verify these results in cells responding naturally to IL-33, a reporter gene assay was performed on EL-4 cells expressing increasing amounts of mTIR8. Expression of TIR8 showed neither inhibitory nor stimulatory effects on IL-1 or IL-33 induced NF-κB stimulation in EL-4 cells (Fig.18B). Thus it can be concluded that TIR8 expression does not affect the IL-33 signaling and that the inhibitory effect which was seen in other systems may be an overexpression artifact.

3.1.3.3. TIR8 does not recognize IL-33 receptor complex

In the past it has been shown that the replacement of the cytoplasmic domain of IL-1RI by that of other family members still resulted in NF-κB activation after IL-1 binding to the chimera (Greenfeder et al., 1995; Mitcham et al., 1996). Assuming that TIR8 might function as accessary protein for IL-33 receptor, it was necessary to ascertain whether the extracellular domain of TIR8 could recognize IL-33 bound to the IL-33 receptor α-chain. In order to answer this question a TIR8-AcP chimera (Fig.3.19A) was generated by fusion of the extracellular and trans-membrane regions of murine TIR8 with the intracellular region of mIL-1RAcP (which is able to generate signaling). If the working hypothesis was correct, the extracellular and/or transmembrane domain of TIR8 should be able to recognize the ligand receptor complex and thus the intracellular IL-1RAcP moiety should start signaling upon stimulation.

The expression of TIR8-AcP chimera could not reconstitute IL-1β (Fig.3.19C left) or IL-33 (Fig.3.19C right) responsiveness in EL-4 D6/76 cells as measured by NF-κB dependent reporter gene assay whereas wild-type IL-1RAcP was able to do so. In addition TIR8-AcP fusion gene expression did not affect the IL-1RAcP reconstituted IL-1β or IL-33 responsiveness when TIR8-AcP and IL-1RAcP were co-expressed. These findings indicate that extracellular and transmembrane parts of mTIR8 are unable to recognize IL-1 bound to IL-1 receptor and/or IL-33 bound to IL-33 receptor.

It must be concluded that TIR8 can not be a direct component of the receptor complexes for either IL-1 or IL-33.

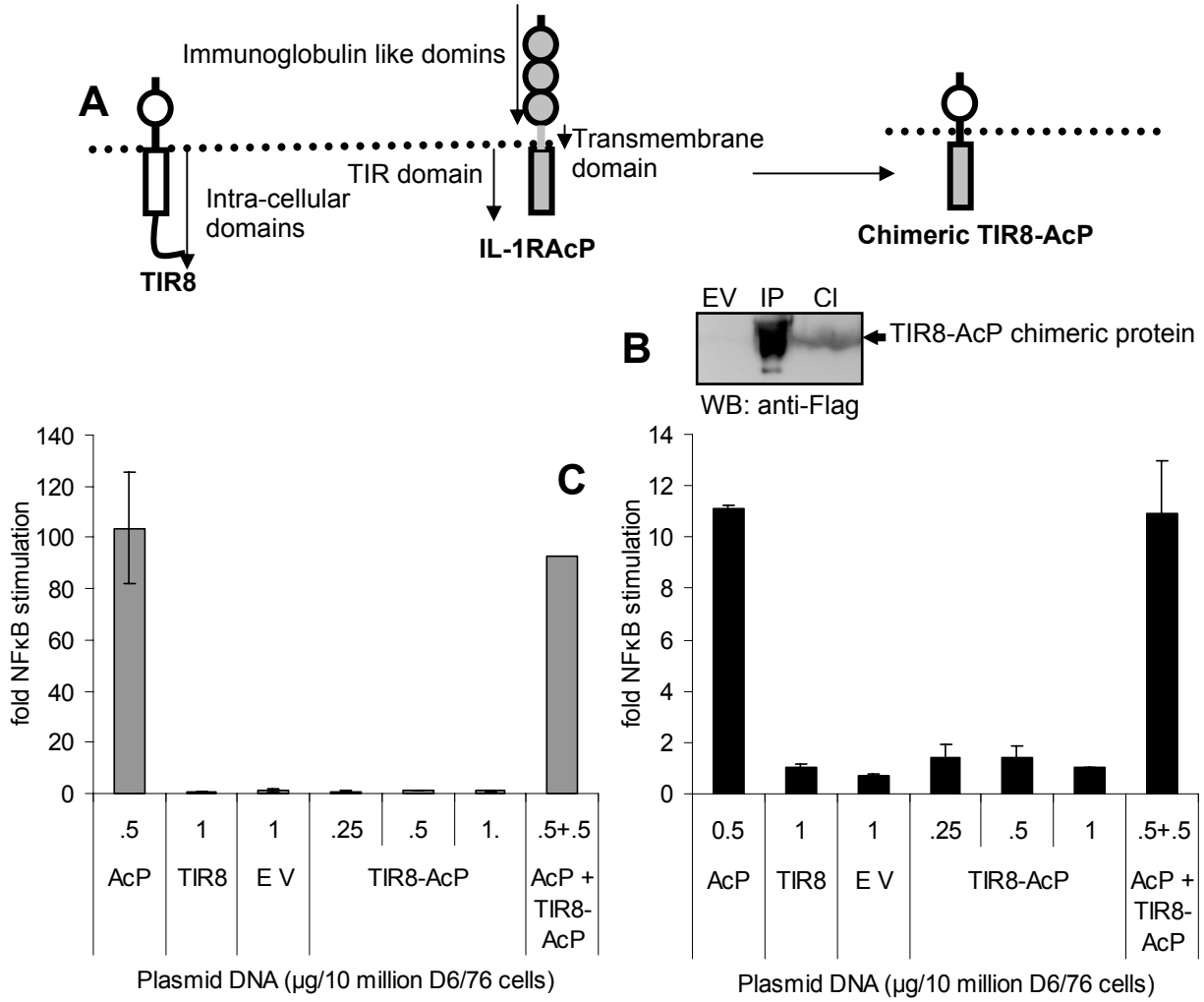


Fig. 3.19: TIR8 does not recognize IL-33 receptor complex.
(A). Schematic diagram of construction of mTIR8 (extracellular and transmembrane) and mIL-1RAcP (intracellular part, containing TIR domain) chimeric gene.
(B). Western blot analysis of mTIR8-AcP fusion gene expression. mTIR8-AcP fusion gene was expressed in HEK293RI cells after transient transfection. Flag tagged fusion protein was analyzed by Western blotting from cell lysate (CI) or after immunoprecipitation (IP) using anti-Flag M2 agarose. EV= cell lysate from empty vector transfected cells.
(C). mTIR8-AcP expression does not affect IL-33 signaling in EL-4 D6/76 cells. EL-4 D6/76 cells (10×10^6) were transiently transfected with 1 µg of plasmid containing luciferase gene under NF-κB promoter along with indicated plasmids (AcP: IL-1RAcP; EV: empty vector). Four hours later 5×10^5 cells/well were kept unstimulated or stimulated with either 100 pg/ml IL-1β or 50 ng/ml IL-33 for over night. Next day reporter gene activity was measured from cell lysates. Results shown are representative of 2 independent experiments with similar results. Results are shown as mean of three replicates in one experiment and are expressed as fold luciferase activity (stimulated/unstimulated) ± relative error.

3.1.3.4. TIR domain in TIR8 is not able to transduce IL-33 signaling

In order to investigate a possible stimulatory or inhibitory role of the cytoplasmic part of TIR8 as accessory protein two chimeric proteins were generated by fusion of coding sequences of the extracellular and transmembrane parts of mIL-1RAcP and the cytopasmic region of murine TIR8 or C-terminally truncated cytoplasmic region of mTIR8 (Fig.3.20A). In addition one C-terminally truncated version of mTIR8 (mTIR8-ΔC) was also constructed. TIR8-ΔC was generated to examine the possibility whether the 100 amino acids stretch following the last conserved motif of the cytoplasmic region mediates an inhibitory effect (as has been suggested for the analogous in the toll protein (Norris and Manley, 1995).

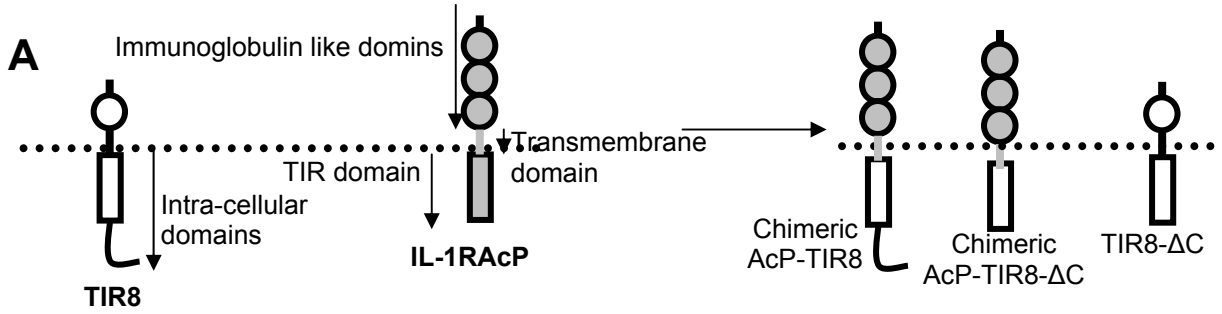
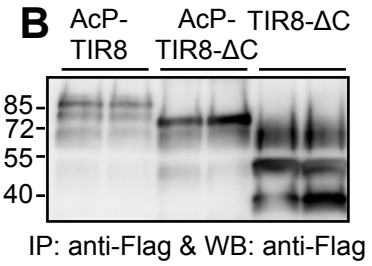


Fig. 3.20: Construction, molecular cloning and expression of mIL-1RAcP-mTIR8 chimeric gene.
(A). Schematic diagram of construction of mIL-1RAcP (extracellular and transmembrane) and mTIR8 (intracellular part containing TIR domain) chimeric gene.
(B). Expression of recombinant mAcP-TIR8, mAcP-mTIR8-ΔC and mTIR8-ΔC gene in HEK293RI cells. Flag tagged mAcP-TIR8, mAcP-mTIR8-ΔC and mTIR8-ΔC were expressed in HEK293RI cells after transient transfection. The day after transfection Flag tagged proteins were immunoprecipitated and detected by Western blotting using anti-Flag antibodies.



In order to undescore the possible stimulatory or inhibitory functions of the cytoplasmic part of the TIR8 as accessory protein, the constructed AcP-TIR8, AcP-TIR8-ΔC, TIR8-ΔC, and TIR8 genes were expressed in EL-4-D6/76 cells. As depicted in the Fig.3.21A, the expression of neither of the chimeric genes was able to activate NF-κB in response to IL-1β or IL-33 as measured by reporter gene assay. The results indicated that the cytoplasmic region of TIR8 is not able to initiate signal transduction as cytoplasmic TIR domain of accessory protein.

In theory, if the generated chimeric proteins (containg extracellular and transmembrane regions of IL-1RAcP and intracellular part of TIR8) are functional as a co-receptor, they should be able to recognize the IL-1- and IL-33- ligated receptor complexes and form heterodimeric receptor complex (because the extracellular region of IL-1RAcP is sufficient to recognize and bind on ligand receptor complexes of IL-1 and IL-33). If the intracellular TIR domain of TIR8 is functional as a TIR domain of a co-receptor, the putative hetrodimeric receptor complex should initiate the signal transduction. Alternativly the chimeric co-receptor may act as dominant negative molecule like C-terminally truncated version of IL-1RAcP.

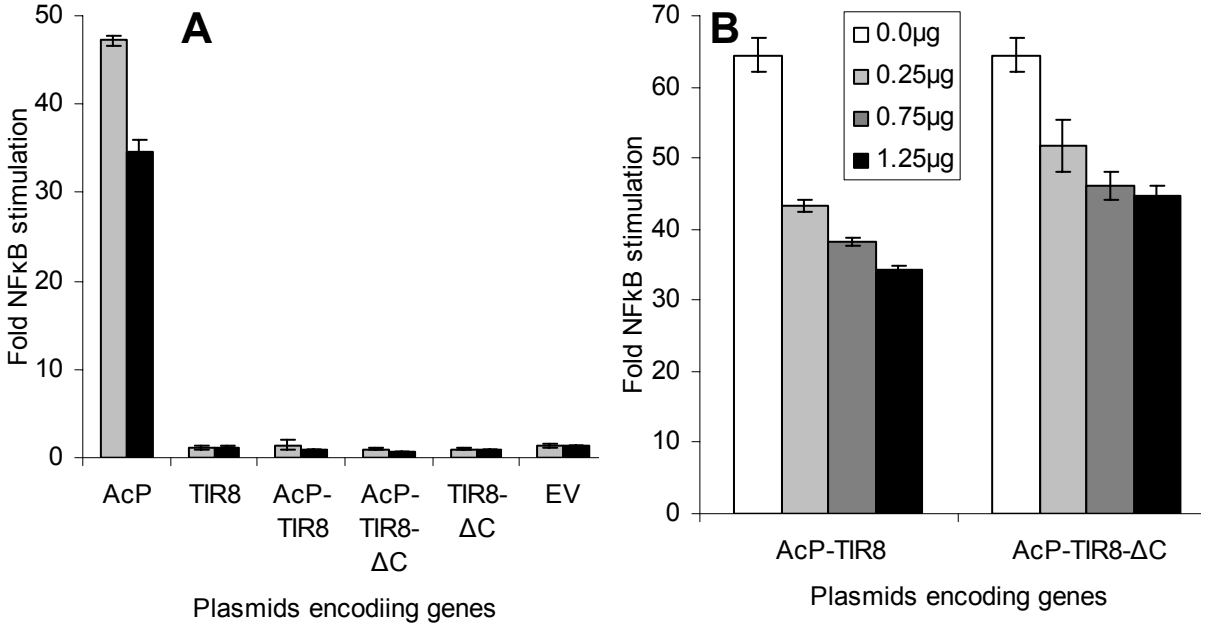


Fig. 3.21: TIR8 is not the accessory protein for IL-33 receptor.
(A). Expression of AcP-TIR8 chimeric proteins did not reconstitute IL-33 responsiveness in EL-4 D6/76 cells. AcP-TIR8, AcP-TIR8-ΔC, TIR8-ΔC, TIR8, IL-1RAcP (AcP) encoding plasmids (1 µg / 1 x 10^7 cells) or empty vector (EV) were transfacted to EL-4-D6/76 cells along with the 1µg reporter gene plasmid. Four hours later transfectents were stimulated with 100 pg/ml IL-1β or 50 ng/ml IL-33 and reporter gene activity was measured from cell lysates.
(B). AcP-TIR8 chimeric proteins expression inhibits IL-1 induced NF-κB activation. Increasing amounts of AcP-TIR8 and AcP-TIR8-ΔC genes encoding plasmids were transfected to EL-4 cells along with the reporter gene plasmid (0.75 µg/ 1 x 10^7 cells). transfectants were kept unstimulated or stimulated with 100 pg/ml IL-1β or 25 ng/ml IL-33 and NF-κB mediated reporter gene activity was measured after over night incubation. The chimeric proteins compete with functional IL-1RAcP and inhibit IL-1 and IL-33 induced signaling in dose depedent manner. The results ahown are only for IL-1 stimulation.The dipected results are representative of two independent experiments. Results are shown as mean of three replicates in one experiment and are expressed as fold luciferase activity (stimulated/unstimulated) $\pm$ relative error.

The results depicted in Fig.3.21A demonstrate that, chimeric co-receptors (AcP-TIR8 and AcP-TIR8-ΔC) are not able to initiate signaling, because IL-1 and IL-33 responsiveness was not reconstitued in EL-4 D6/76 cells by expression of these chimeric proteins. In order to confirm the recuritment of the chimeric co-receptor proteins (AcP-TIR8 and AcP-TIR8-ΔC) on ligand bound receptor complexes, increasing amounts of AcP-TIR8 and AcP-TIR8-ΔC were expressed in EL-4 cells. Expression of AcP-TIR8 and AcP-TIR8-ΔC resulted in dose dependent inhibition in IL-1 and IL-33 induced NF-κB activation, as measured in reporter gene assay (Fig.3.21B). These data confirm that the extracellular region of IL-1RAcP in chimeric proteins recognizes the IL-1 and IL-33 ligated receptor complexes. However the cytoplasmic region of the TIR8 is not able to initiate the IL-1 or IL-33 induced signal transduction, indicating that TIR8 can not be the accessory protein for IL-33 receptor.

3.1.4. Binding affinity of IL-33 to IL-33 receptor

3.1.4.1. Radiolabelling of IL-33 and IL-1α

In order to obtain a comparable biological response in EL-4 cells, a 500-1000 fold higher concentration of IL-33 was required compared to IL-1β (shown in Fig.3.2A). One possible explanation may be differential expression of the respective receptor molecules on the cells. A second one may be a difference in binding affinities of the two cytokines to their respective receptor complexes. To measure the binding affinity of IL-33 to the IL-33 receptor 3 µg of IL-33 were labelled with ^{125}I (1 mCi) using the chloramine T method. IL-1α (5 µg) was also labelled in parallel as control. The cytokines were purified by size exclusion chromatography using Sephadex G25 columns (Fig.3.22A). A single strong band for IL-1α-^{125}I (Fig.3.22A upper) and IL-33-^{125}I (Fig.3.22A lower) showed the success of radiolabelling and demonstrated the purity of the cytokines used.

The biological activity of radio-labelled cytokines was determined by reporter gene assay in EL-4-1B1 cells. As shown in Fig.3.22B both labelled and unlabelled forms of IL-33 or IL-1 stimulated NF-κB activity in a dose dependent fashion, suggesting that the labelling with chloramine T did not abrogate the biological activity of the cytokines.

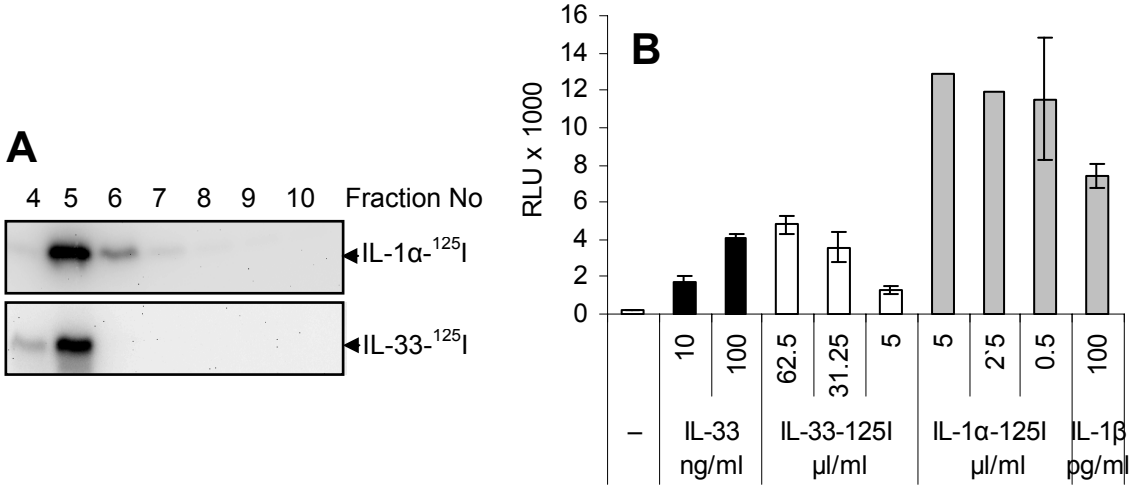


Fig. 3.22: Radiolabelled (^{125}I) IL-1α and IL-33 stimulate EL-4 1B1 cells.
(A). Radiolabelling of IL-1α and IL-33 by chloramine T method. 5 µg of IL-1α and 3 µg of rmIL-33 were radiolabelled with 1 mCi (each) ^{125}I by chloramine T method. Radiolabelled cytokines were separated from free ^{125}I by G25 columns and 10 (1 ml each) fractions were collected. 10 µl of each fraction was run on 15% gel and radioactive cytokines were visualized by autoradiography.
(B). Radiolabelled (^{125}I) IL-1α and IL-33 stimulate EL-4 1B1 cells. EL-4-1B1 cells transfected with reporter gene plasmid were stimulated with IL-33, IL-33-^{125}I, IL-1α-^{125}I, or IL-1β as indicated in figure and reporter gene activity was measured from cell lysates after over night incubation. Results shown are expressed as mean RLU ± SEM calculated from three replicates of one experiment.

3.1.4.2. Crosslinking of IL-1α and IL-33 to their respective receptors

In order to see the binding of the radiolabelled IL-33 to IL-33 receptor, crosslinking of the ligand and the receptor complex was attempted. For this purpose Flag-tagged versions of IL-33Rα and/or IL-1RAcP were expressed in HEK293RI cells by transient transfection. After incubating these transfectants with radioactive IL-33 or IL-1 for 15 minutes, ligand-receptor complexes were crosslinked using the BS3 crosslinker. Results showed that BS3 crosslinked radiolabelled IL-1α to IL-1R1 and/or IL-1RAcP (Fig.3.23) but no crosslinking was observed in cells incubated with radiolabelled IL-33, indicating that either radio-labelled IL-33 did not bind to the receptor or that the BS3 arm length (11Aº) is not suitable to crosslink the ligand receptor complex. Crosslinking was also tried in EL-4 cells with similar results.

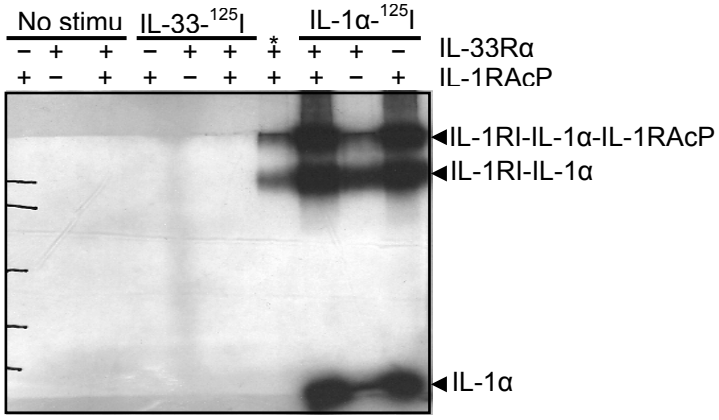


Fig. 3.23: Cross linking of IL-1α and IL-33 to respective receptors.

HEK293RI cells were transfected with Flag tagged -IL-33Rα and/or -IL-1RAcP encoding plasmids. Transfectants were incubated either with IL-1α (cold *), IL-1α-^{125}I or IL-33-^{125}I for 15 minutes at 37 ºC and ligand receptor complex was cross linked with BS3. Cells were washed and lysed. Flag tagged proteins were immunoprecipitated using anti-Flag M2 agarose. For IL-1α anti-IL-1R1(Rockland) was also used for immunoprecipitation. Proteins from cell lysate and immunoprecipitates were separated on 7.5% SDS/PAGE and radiolabelled molecules were visualized by autoradiography. BS3 cross linked radiolabelled IL-1α to IL-1R1 and/or IL-1RAcP but not IL-33 to IL-33 receptor. Results shown are representative of 2 independent experiments with similar results.

3.1.4.3. Saturation studies

In order to find the optimal amount required for binding and determination of binding affinity of IL-33 to IL-33 receptor in EL-4 cells, saturation studies were performed. EL-41B1 cells (EL-4 cells stably transfected with IL-33Rα) were incubated with increasing amounts of either radio-labelled IL-1α or radio-labelled IL-33. As depicted in Fig.3.24 radiolabelled IL-1α bound to the receptor in dose dependent fashion up to 5 ng/ml concentration. Then saturation was reached at which further addition of

labelled IL-1α did not increase the binding of cytokine to the cells. On the other hand, by addition of increasing amount of radiolabelled IL-33, there was no significant increase in the cell-bound IL-33 to be observed, whereas the unbound ligand increased in the supernatant in dose dependant manner, indicating that radiolabelled IL-33 practically did not bind to the receptor.

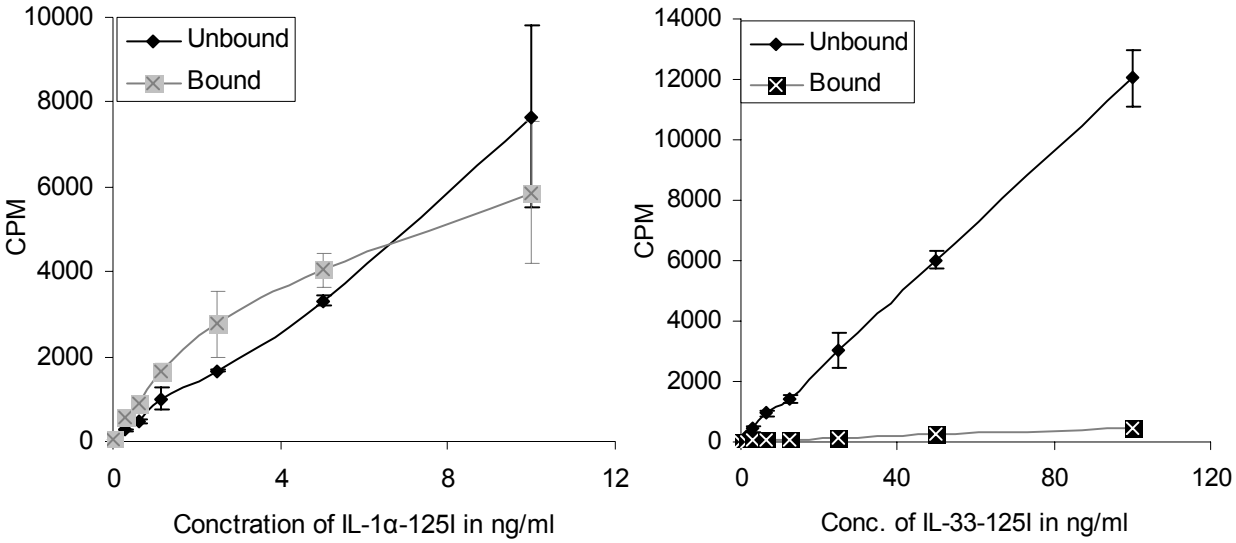


Fig. 3.24: Saturation studies of ^{125}I labelled IL-1α and IL-33.
Saturation studies of ^{125}I labelled IL-1α (left) and IL-33 (right). EL-4 1B1 (5 x 10^6) cells were either kept untreated or incubated with either increasing concentration IL-1α-^{125}I (radiolabelled) or IL-33-^{125}I (radiolabelled) for 15 minutes at 37 ºC followed by 45 minutes incubation on ice. Cells were separated from supernatant in the presence of 200 µl silicon oil (AR20/AR200 1:1) by centrifugation and counts per minutes (CPM) were measured from cells (bound) and supernatant (unbound) in liquid scintillation counter in the presence of 1 ml scintillation solution. The radiolabelled IL-1α-^{125}I binds to cell receptor but radiolabelled IL-33-^{125}I was unable to bind in EL-4-1B1 cells. Results shown are expressed as mean ± SEM, calculated from three replicates of one experiment.

3.1.4.4. Competition studies

In order to find whether radioactivity measured on cell surface is specifically due to the binding of the radiolabelled ligand to receptor, competition studies were performed. For competition EL-41B1 cells were incubated with radiolabelled IL-1α or IL-33 with or without 100 fold excess of unlabelled ligand and cell-bound and unbound radioactivity was determined. Presence of non radio-labelled IL-1α (100 fold excess) almost completely inhibited the binding of radio-labelled IL-1α to the cells indicating that binding of the radio-labelled IL-1α is specific for the IL-1 receptor (Fig.3.25 left) whereas radio-labelled IL-33 was unable to bind the IL-33 receptor (Fig.3.25 right). The weak signal obtained with radio-labelled IL-33 was most likely due to non-specific binding of the labelled protein to the cells.

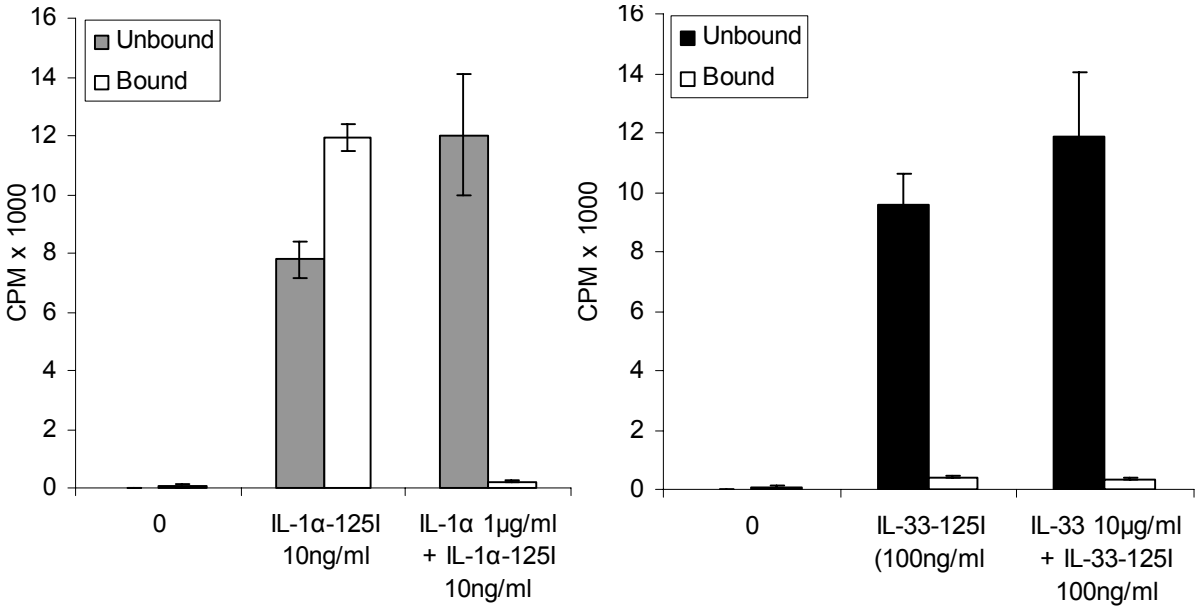


Fig. 3.25: Competition studies of ^{125}I labelled IL-1α and IL-33.
Competition studies of ^{125}I labelled IL-1α (left) and IL-33 (right) on EL-4 1B1 cells. EL-4 1B1 (5 x 10^6) cells were either kept untreated or pre-incubated for 30 minutes at 37 ºC with 1 µg/ml IL-1α (non-radiolabelled) or 10 µg/ml IL-33 (non-radiolabelled). These untreated or pre-treated cells were further incubated with either 10 ng/ml IL-1α-^{125}I (radiolabelled) or 100 ng/ml IL-33-^{125}I (radiolabelled) for one hour on ice. A part of the cells were also incubated without any cytokine as negative control. Cells were separated from supernatant in the presence of 200 µl of silicon oil (AR20/AR200 1:1) by centrifugation and CPM were measured from cells (bound, filled columns) and supernatant (unbound, open columns) in liquid scintillation counter. IL-1α competes with radiolabelled IL-1α-^{125}I for binding to cell receptors but radiolabelled IL-33-^{125}I was unable to bind to EL-4 cells. Results shown are representative of two independent experiments with similar results and are expressed as mean ± SEM, calculated from three replicates of one experiment.

One must conclude from the obtained results that IL-33 can be labelled with ^{125}I with chloramine T method, however labelling by this method actually inactivated the labelled molecules, thus abrogating binding to the receptor. The biological activity of the radio-labelled IL-33 sample shown in Fig.3.22B can be explained if only a part of the IL-33 protein is labelled with the ^{125}I (inactive) while the rest is not labelled (active). So, chloramine T labelling does not seem to be a suitable technique for labelling IL-33 in order to perform binding studies. In the meantime Palmer et al., 2008, published the binding affinity of human and murine IL-33 to IL-33 receptors in Biacore assay so the own binding studies were not followed by alternative method.

3.2. Characterization of the processing and release of IL-33

IL-33 is an interleukin-1-like cytokine that signals via the IL-33Rα-chain (ST2) (Schmitz et al., 2005) and the co-receptor IL-1RAcP as shown above (Ali et al., 2007; Chackerian et al., 2007; Palmer et al., 2008). Like IL-1α, full length IL-33 also translocates to nucleus and may act as chromatin-associated nuclear factor. mRNA expression of IL-33 is reported from a range of cell types including, endothelial cells, fibroblasts, macroghages, B cells, T cells, mast cells and dendretic cells (Schmitz et al.,2005 and table 3.1). Like other members of the family, IL-33 is produced as a precursor form, which is reported to be processed by caspase 1 to the mature form. Although Schmitz et al., (2005) showed *in vitro* cleavage of full length IL-33 by caspase 1, there is no clear evidence that IL-33 is indeed processed by this enzyme and that it is released by any cellular system. In addition the suggested caspase 1 cleavage site in IL-33 primary structure is not conserved. Thus it seemed necessary to explore whether caspase 1 mediated cleavage is required *in vivo* for biological activity of interleukin-33.

3.2.1. IL-33 is biologically active as full length molecule

Certain members of the IL-1 family, such as IL-1β and IL-18, undergo restricted proteolysis to convert their inactive precursors into the active cytokine, also called mature cytokine. However, other cytokines in the same family, such as IL-1α, display biological activity irrespective of whether they are proteolytically processed or not (Mosley et al., 1987). Because all previous studies on IL-33 have used the mature form of this cytokine ($IL\text{-}33_{109\text{-}266}$) no information is available whether the full length form (precusor) of IL-33 can bind to its receptor and exert biological actions.

3.2.1.1. Full length IL-33 can bind to IL-33 receptor

If the IL-33 is biological active as cytokine in full length molecular form, it must bind to the IL-33 receptor and recruit the accessory protein to initiate signal transduction. Full length murine IL-33 (flmIL-33, aa1-266) was cloned with a C-terminal Flag-epitope and a N-terminal Myc-epitope tag and expressed in keratinocytes (Fig.3.26A left). In keratinocytes it is processed constitutively to a faintly detectable breakdown product corresponding to the size of mature IL-33 (migrating around 20 kDa) and two major breakdown products of smaller sizes (only the C-terminal product is visible in Fig.3.2A as the Western-blot was developed with anti-Flag antibodies). Upon incubation of cell lysate form keratinocytes expressing double-tagged full length mIL-

33 with an IL-33 trap (an IL-33Rα-IL-1RAcP-hIgG fusion protein), full length form of IL-33 bound to this soluble receptor fusion protein (Fig.3.26A left). In this experiment the mature IL-33 was also pulled down. Full length mIL-33 also bound to the IL-33Rα-chain on intact cells where it allowed association of IL-1RAcP as demonstrated by co-immunoprecipitation experiments with epitope-tagged receptor components (Fig.3.26B) indicating that IL-33 initiates IL-33 receptor complex formation as full length molecule.

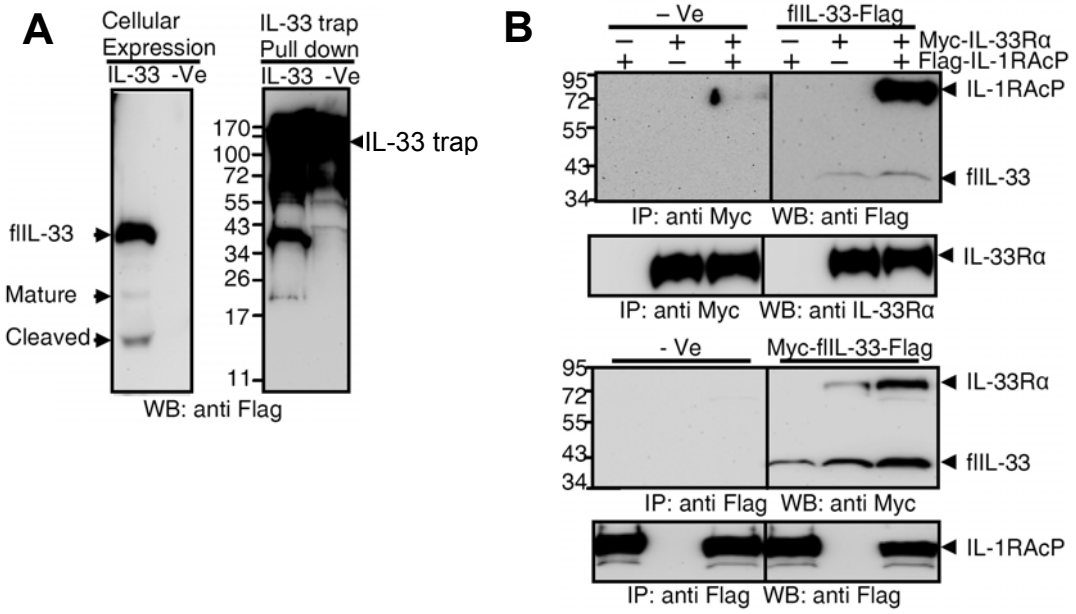


Fig. 3.26: Full length murine IL-33 binds to IL-33 receptor and allows recruitment of IL-1RAcP.
(A). Full length mIL-33 and mature IL-33 bind to an IL-33 cytokine trap. Full length mIL-33 (flmIL-33) was produced by transfecting keratinocytes with a plasmid encoding full length mIL-33 (aa 1-266) carrying a N-terminal Myc- and C-terminal Flag-tag. After 28 hr cells were lysed and full length mIL-33 and proteolytic fragments detected by Western blot (WB) using anti-Flag mAb (left). IL-33Rα-IL-1RAcP:IgG(Fc) fusion protein bound to Protein A beads was incubated with cell lysate from keratinocytes overexpressing full length mIL-33. Beads were washed, and specifically bound proteins detected by Western blot (WB) using anti-Flag mAb (right).
(B). Full length murine IL-33 binds to IL-33Rα on intact cells and allows recruitment of IL-1RAcP. Myc-tagged IL-33Rα or Flag-tagged IL-1RAcP were expressed alone or in combination in HEK293RI cells. Transfected cells were incubated for 1 hr on ice without (-ve) or with full length murine IL-33 (Myc- and/or Flag-tagged). Cells were washed thrice with ice cold PBS and lysed. IP was performed with anti-Myc (upper two panels) or anti-Flag agarose (lower two panels) and co-immunoprecipitated proteins were detected by Western blot (WB). anti-IL-33Rα (Anti-T1/ST2, MD Biosciences) was used for detection of IL-33Rα. The results shown (in A and B) are representative of two independent experiments with similar results.

3.2.1.2. Full length IL-33 stimulates T lymphocytes

To confirm the biological activity of full length IL-33, N terminally-Myc and C-terminally Flag tagged IL-33 form was expressed in mammalian cells and purified via N-terminal Myc tag immunoprecipitation. Purification via N terminal Myc tag

precipitation was performed to exclude the presence of C-terminal cleavage products. EL-4 1B1 cells were stimulated with this purified (after elution) full length IL-33 in the presence of protinase inhibitor cocktail (to avoid any cleavage in cell supernatant). Stimulation of cells with full length mIL-33 resulted in a time-dependent appearance of phospho-IκBα and disappearance of total IκBα (Fig.3.27A). In addition concentration dependent activation of NF-κB was also observed in reporter gene assay upon stimulation of cells with full length mIL-33 bound to the anti-myc agarose (Fig.3.27B). Stimulation of EL-41B1 cells with full length IL-33 bound to anti-Myc agarose also induced the cytokine production and release (Fig.3.27C). The results described here proved that full length IL-33 is able to bind to the IL-33 receptor and activate cells.

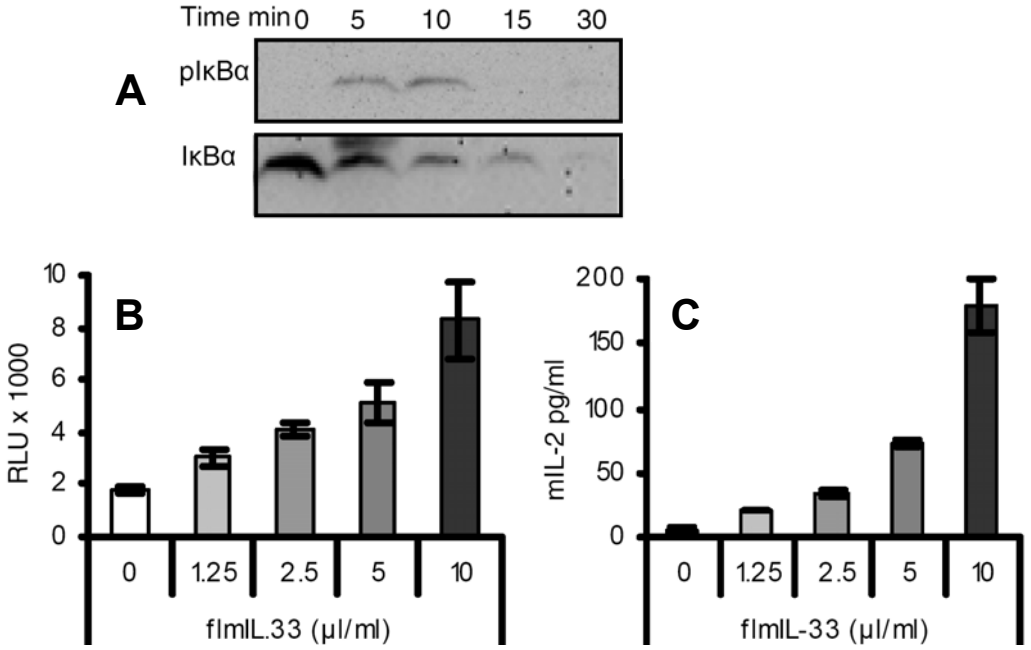


Fig. 3.27: Full length murine IL-33 activates cells.
(A). Full length murine IL-33 (flmIL-33) stimulates IκBα phosphorylation and degradation. EL-41B1 cells were stimulated with anti-Myc affinity-purified flmIL-33 for the times indicated in the presence of proteinase inhibitors. After stimulation cells were immediately denatured in SDS-PAGE sample buffer and phospho-IκB and total IκBα were detected by Western blotting. The results shown are representative of two independent experiments with similar results.
(B). Full length mIL-33 activates the transcriptional activity of NF-κB. EL-41B1 cells were transfected with a plasmid encoding a NF-κB dependent luciferase reporter gene and stimulated with increasing amounts of anti-Myc affinity-purified full length mIL-33 for 18 hr. Subsequently luciferase activity (RLU) was measured.
(C). Full length mIL-33 stimulates IL-2 production in EL-41B1 cells. EL-41B1 cells were stimulated with increasing amounts of anti-Myc affinity-purified full length mIL-33. The next day IL-2 release was measured by ELISA. Data depicted (in B and C) are means +/- standard deviations from one experiment (triplicates) out of two similar ones with comparable results.

3.2.2. Full length IL-33 is processed by caspase 3

As described above it was reported that full length IL-33 is processed to mature IL-33 by caspase 1 cleavage at aa 111 in human IL-33 precursor corresponding to aa 108 in the murine sequence. When N-terminally Myc- and C-terminally Flag-tagged full length IL-33 was expressed in keratinocytes and these cells were stimulated with LPS to induce processing to mature IL-33 via the inflammasome two major breakdown products were observed which did not correspond in size to the expected mature form (109-266) or the remainder (1-108) (Fig.3.27A left and 3.28A). In control experiments keratinocytes were able to process precursor IL-1β and IL-18 to respective mature forms, both well characterized substrates for caspase 1 (Fig.3.32), indicating that caspase 1 is active in this cellular system. Therefore, in search for caspase 1 cleavage sites, the peptide sequences of full length IL-33 was re-analyzed *in silico*. As already suggested in the literature (Carriere et al., 2007) no caspase 1 cleavage site was found in the primary structure of full length IL-33. However, a non-classical caspase 3 cleavage site ($DGVD_{175}$*G) was identified in the mouse IL-33 sequence at aa175, corresponding to aa178 in human IL-33 sequence. This sequence had been identified previously in PKCζ (Frutos et al., 1999) to be accepted by caspase 3. The calculated fragments fitted much better to the bands observed in the gels after digestion of full length mIL-33.

In order to confirm this finding plasmids encoding N- and/or C-terminally epitope-tagged versions of full length mIL-33 were generated. This was done for the wild-type form and, in addition, a point mutation was introduced at the putative caspase 3 cleavage site (D175->A) with the aim of destroying it. In addition, the epitope- tagged N-terminal (aa1-175) and C-terminal (aa176-266) fragments were generated to allow clear identification of the proteolytical products. Full length mIL-33 was processed in intact cells to two major products (Fig.3.27A and 3.28A) corresponding in size exactly to the recombinant fragments aa1-175 and aa 176-266, indicating that in cells full length IL-33 is predominantely cleaved at position 175 rather than at position 108. A minor product in the size of the postulated mature IL-33 (109-266) was also observed (best visible in Fig.3.27A).

This result shows that full length murine IL-33 is not predominately cleaved at position 108 by caspase 1 but rather at position 175 by caspase 3 in kerationcytes. In order to support this, a caspase 3-specific inhibitor was added to the cells.

Processing of full length mIL-33 was inhibited almost completely in keratinocytes by this caspase 3-specific inhibitor (Fig.3.28D).

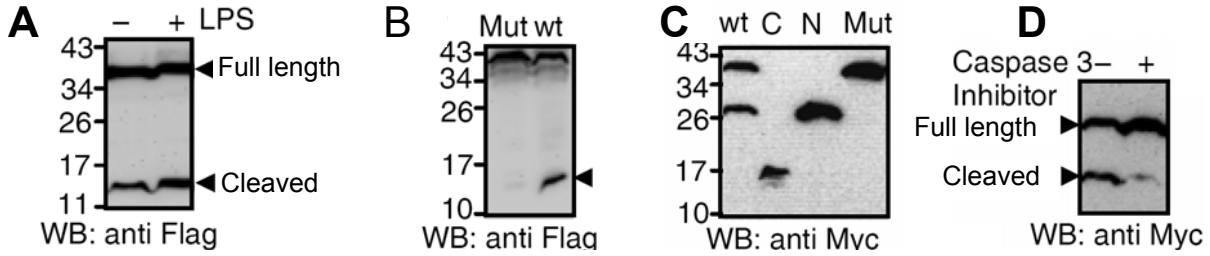


Fig. 3.28: Full length murine IL-33 (flmIL-33) is processed in keratinocytes at position D175.
(A). Processing of flmIL-33 is stimulated by LPS in keratinocytes. Keratinocytes were transfected with a plasmid encoding C-terminally Flag-tagged flmIL-33. Cells were kept unstimulated (-) or were stimulated with 500 ng/ml LPS (+) for 28 hr. Expression and processing of flmIL-33 were analysed in total cell lysates by Western blotting (WB) using anti-Flag mAb, detecting flmIL-33 and the C-terminal product.
(B). D175A point mutation abrogates IL-33 processing in keratinocytes. Wild-type flmIL-33 (wt) or mutant flmIL-33_{D175A}(mut) were expressed in keratinocytes and constitutive processing measured in cell lysates by Western blotting (WB) using anti-Flag mAb. D175A mutation abrogated constitutive processing while wt flmIL-33 was cleaved to two products (only Flag-tagged C-terminal product visible in anti-Flag WB: arrow head).
(C). Identification of caspase 3 processing products using recombinant N-terminal and C-terminal fragments. The sizes of the rec. Myc-mIL-$33_{176-266}$-Flag (C) and rec. Myc-mIL-33_{1-175} (N) confirm that processing of Myc-flmIL-33-Flag (wt) was mainly at the $DGVD_{175}$*G caspase 3 cleavage site.
(D). Inhibition of caspase 3 reduces processing of IL-33 in cells. Keratinocytes over expressing flmIL-33 were incubated with 50 µM cell-permeable (DEVD-CHO) caspase 3 inhibitor (Calbiochem) for 28 hr. Processing of Myc-flmIL-33-Flag to Myc-tagged N-terminal cleavage product was detected in cell lysates by anti-Myc WB. The results shown in A-D are representative of three different experiments with similar results.

To identify which of the two caspases actually cleaves full length mIL-33 recombinant full length mIL-33 (wild-type and D175A mutant) was incubated with recombinant caspase 1 or capase 3 *in vitro*. In this type of experiments caspase 1 did not accept full length mIL-33 as a substrate while it readily processed full length mIL-1β (Fig.3.29). Caspase 3 however, cleaved wild-type full length mIL-33 (and precursor IL-18 as a control) in a concentration- dependent manner (Fig.3.29), while mutation at aa 175 completely abrogated processing by recombinant caspase 3 *in vitro* (Fig.3.29) and upon overexpression also in keratinocytes (Fig.3.28B). The products resulting from caspase 3 processing corresponded exactly to the recombinant N- and C-terminal pieces of aa 1-175 and aa 176-266 (Fig.28C). These data show that full length mIL-33 is processed predominantely by caspase 3 at aa175 to yield two fragments. In these *in vitro* processing experiments a breakdown product corresponding to the “mature” form of aa109-266 was not observed. This was only observed as a minor processing product in intact cells. This suggests that at least one protease distinct from caspases 1 and 3 processes full length mIL-33 to mature

IL-33 in intact cells. However, it is likely that mature IL-33 is only a transient product in cells as the caspase 3 cleavage site at aa175 is present in mature IL-33. This might explain why this product appears only as a minor band in cells overexpressing full length mIL-33 as it will be further processed to the C-terminal product of aa 176-266 and to a small piece of about 70 aa.

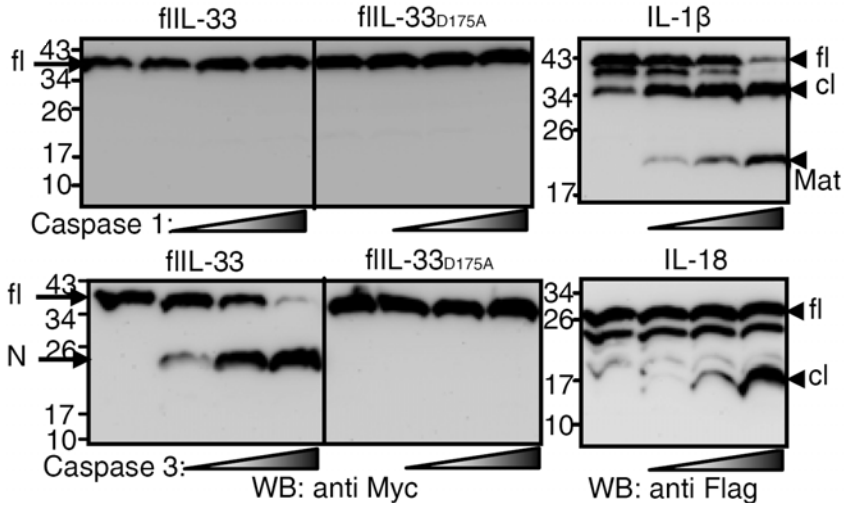


Fig. 3.29: Full length murine IL-33 (flmIL-33) is processed by caspase 3 at D175 but not by caspase 1 *in vitro*. Affinity-purified full length mIL-33 (flmIL-33) or full length flmIL-33_{D175A} (flmIL-33_{D175A}) were incubated with recombinant caspases 1 or 3. Full length mIL-1β (flmIL-1β) and full length mIL-18 (flmIL-18) were used as control substrates for caspases 1 and 3, respectively. Caspase 1 cleaved flmIL-1β at two positions but did not affect wild-type or mutant form of flmIL-33 (upper panel). Incubation of wild type Myc-flmIL-33-Flag with caspase 3 yielded a product corresponding in size to Myc-IL-33_{1-175} (N). D175A mutation abrogated processing of flmIL-33, while flmIL-18 was also cleaved (lower panel) (fl: full length; N: N terminal; cl: cleaved; Mat: mature).

3.2.3. Full length IL-33 is inactivated by caspase 3

In order to clarify whether the caspase 3 breakdown products were able to bind to the receptor, pull down assays were performed using a soluble IL-33Rα-chain:Fc-fusion protein with wild-type full length IL-33, the D175A mutant, or the N- and C-terminal caspase 3 breakdown products, respectively. Wild-type and mutated flmIL-33 bound to the IL-33Rα-chain (Fig.3.30A) and activated NF-κB in cells (Fig.3.30B), but neither the N-terminal product of aa 1-175 nor the C-terminal product of aa 176-266 were able to bind to the receptor (Fig.3.30A) or activate cells (Fig.3.30B).

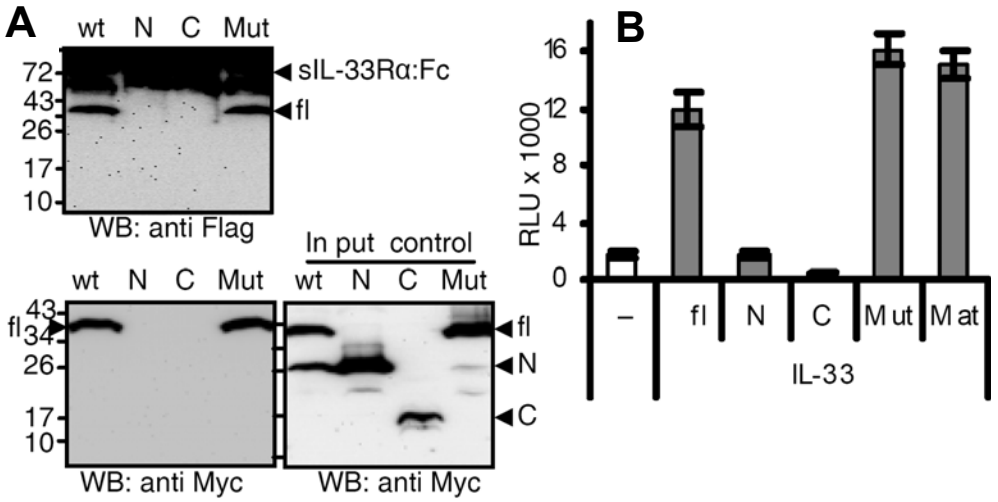


Fig. 3.30: Full length murine IL-33 (flmIL-33) is inactivated by caspase 3.
(A). Caspase 3 cleavage products do not bind to IL-33R, while flmIL-33 and flmIL-33$_{D175A}$ do. IL-33Rα: (hIgG) Fc fusion protein bound to Protein A sepharose was incubated over night at 4 ºC with lysates from keratinocytes overexpressing flmIL-33 (wt), IL-33$_{1-175}$ (N), IL-33$_{176-266}$ (C) or IL-33$_{D175A}$ (Mut). Beads with bound proteins were washed, and IL-33 forms bound to IL-33Rα were detected by Western blot (WB) using anti-Flag (upper) or anti-Myc mAb (lower), Expression of different fragments was confirmed by WB in lysates (in put control, lower right). The results shown are representative of three different experiments with similar results.
(**B). Caspase 3 cleavage products do not activate NF-κB.** EL-41B1 cells transfected with a NF-κB dependent reporter gene were stimulated with IL-33 forms purified via their N-terminal Myc-tag (fl: IL-33$_{1-266}$, N: IL-33$_{1-175}$, C: IL-33$_{176-266}$, Mut: IL-33$_{D175A}$) or commercial mIL-33$_{109-266}$ (Alexis, Mat). After overnight incubation with stimuli luciferase activity (RLU) was measured in cell lysates. Data depicted are means +/- standard deviations from one experiment (carried out in triplicates) out of two with comparable results.

3.2.4. Induction of apotosis in cells inactivates IL-33

Caspase 3 is well-knwon as a protease activated in the course of apoptosis. Thus it was asked whether induction of apoptosis would initiate breakdown of full length mIL-33 and result in its inactivation. Apotosis was induced in U937 cells by TNFα treatment in the presence of the protein biosynthesis inhibitor cycloheximide. Induction of apotosis resulted in proteolysis of procaspase 3 (Fig.3.31A) leading to enzymatically active caspase 3. Incubation of full length mIL-33 with lysates from these cells in which apoptosis was induced resulted in proteolysis of full length mIL-33 in a few minutes. The IL-33 form with the D175A mutation, however, was not cleaved (Fig.3.31B). The data suggest that induction of apoptosis leads to inactivation of full length mIL-33 as a cytokine by proteolytic cleavage.

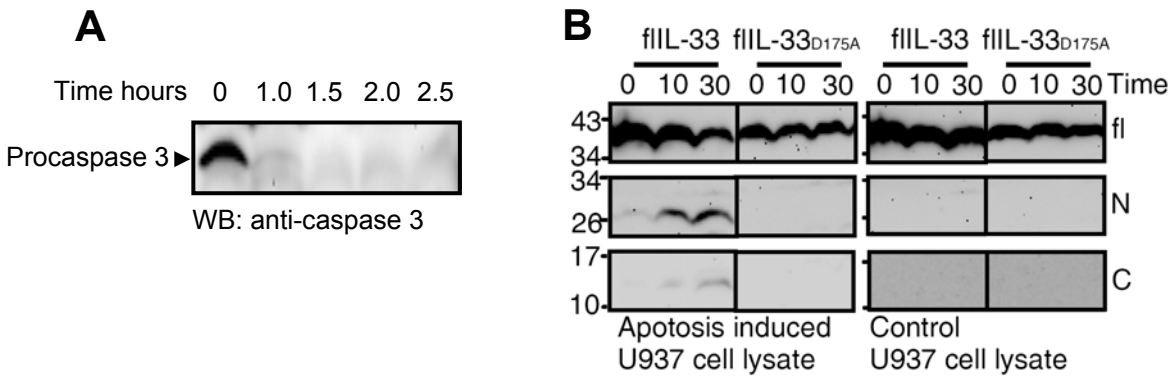


Fig. 3.31: Induction of apoptosis in cells results in processing of full length murine IL-33.
(A). Induction of apoptosis in cells cleave procaspase 3. Apoptosis was induced in U937 cells by incubation with 1 ng/ml TNFα, and 50 µg/ml cycloheximide for indicated time points. Procaspase 3 was detected by Western blotting from cell lysate obtained from apoptosis induced U937 cell.
(B). Induction of apoptosis in cells results in processing of full length mIL-33. Apoptosis was induced in U937 cells (1 ng/ml TNFα, 50 µg/ml cycloheximide, 1.5 hr). Washed cells were lysed by 3 cycles of freeze thawing followed by sonication in caspase reaction buffer. flmIL-33 and flmIL-33_{D175A} were incubated with lysates from control cells (right panel) or apoptotic cells (left panel). flmIL-33 was cleaved in a time-dependent manner, while the D175A mutant was unaffected. Full length molecules (fl, upper panel) and the N-terminal fragment (N, middle panel) were detected by anti-Myc Western blot (WB), the C-terminal fragment (C, lower panel) by anti-Flag WB. The results shown are representative of three independent experiments with similar results.

3.2.5. Caspase 3 cleavage affects the dual functions of IL-33 differently

IL-33 was originally identified as a nuclear factor in specialized endothelial cells (Baekkevold et al., 2003) before it was designated a cytokine. Therefore IL-33, like IL-1α and HMGB1, was called a dual function cytokine (reviewed in (Haraldsen et al., 2009). The biological activity of IL-33 has to be separated into the classical cytokine function which requires release and interaction with the IL-33 receptor in a paracrine

fashion and its intracrine gene regulatory function which requires its nuclear translocation.

3.2.5.1. IL-33 is not released as a classical cytokine after activation of cells

Activation of the inflammasome results in the processing of precursor IL-1β or precursor IL-18 in cells and the release of biologically active cytokines into the extracellular milieu (Fig.3.32). In order to find out whether biologically active IL-33, i.e. the classical cytokine form, is also released by cells constitutively or after activation, immunoprecipitation of biologically active IL-33 was attempted from supernatants collected from cells transiently transfected with the different variants of mIL-33. Surprisingly, no detectable bioactive IL-33 in supernatants of intact cells was immunoprecipitated, although these cells produced readily detectable amounts of IL-33 inside of the cell. Even stimulation by LPS did not result in the release of bioactive IL-33. In contrast to IL-33, bioactive IL-1β, or IL-18, and even IL-1α (which is processed by calpain not by capase 1), were easily detectable in supernatants indicating that the inflammasome had been activated successfully (Fig.3.32). Thus, it must be concluded that classical activation of the inflammasome (and thus caspase 1) does not result in the release of bioactive IL-33.

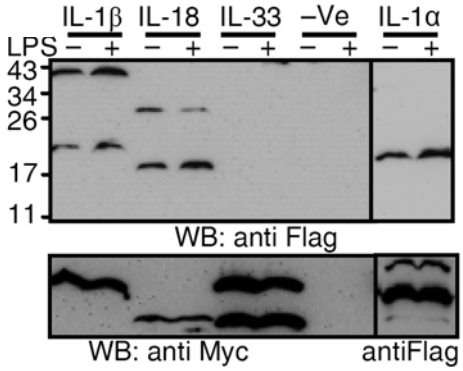


Fig. 3.32: Bioactive IL-33 is not released by intact cells upon overexpression after stimulation. N-terminally Myc- and C-terminally Flag- tagged full length versions of IL-1α, IL-1β, IL-18 or IL-33 were overexpressed in keratinocytes. Cells were either kept unstimulated (-) or stimulated with 500 ng/ml LPS (+) for 28 hr (-ve: empty vector). Released Flag-tagged molecules (C-termini, identifying mature forms) were precipitated from cell free supernatants and detected by anti-Flag Western blot (WB) (upper). The expression of the cytokines was confirmed in cell lysates by anti-Myc WB or anti-Flag for IL-1α (lower). The results shown are representative of three independent experiments with similar results.

3.2.5.2. Nuclear translocation of full length mIL-33 is not abrogated by caspase 3 processing

Finally, the issue whether proteolytical processing of full length murine IL-33 (flmIL-33) affects its ability to translocate to the nucleus was addressed. Upon overexpression full length IL-33 translocates to the nucleus (Fig.3.33), a feature it shares with IL-1α and IL-1F7b (reviewed in (Dinarello, 2009). Interestingly, not only full length IL-33 translocated to the nucleus but also the N-terminal fragment aa1-175 (Fig.3.33). The C-terminal breakdown product did not translocate to the nucleus and remained completely in the cytosol (Fig.3.33). Presently it is not known whether this N-terminal fragment regulates gene transcription comparable to full length mIL-33, but it seems that one has to separate the classical cytokine function of IL-33, i.e. via receptor interaction in an auto- or paracrine manner, from its intracrine function in the nucleus. While processing of full length murine IL-33 at aa175 destroys its cytokine property it does not abrogate nuclear translocation, presumably still allowing gene regulation.

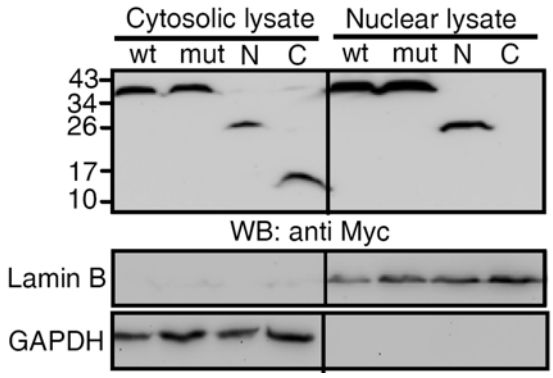


Fig. 3.33: Processing of full length mIL-33 does not abrogate nuclear translocation.
Constitutive nuclear translocation of full length murine IL-33 (wt), IL-$33_{1\text{-}175}$(N), IL-$33_{176\text{-}266}$ (C) and IL-33_{D175A}(mut) was measured in transfected HEK293RI. Cytosolic and nuclear fractions were prepared and the presence of IL-33 was detected by anti-Myc Western blot (upper). Purity of cytosolic and nuclear fractions was confirmed by anti-GAPDH and anti-Lamin B WB (lower). The results shown are representative of three independent experiments with similar results. These data were generated in collaboration with Dang Quan Nguyen.

3.3. Comparison of signaling induced by IL-1, IL-18 and IL-33

Among the well known members of the IL-1 family of cytokins, IL-1α and IL-1β are well characterized pro-inflammatory cytokines. IL-18 is a Th1 driving pro-inflammatory cytokine with the unique capacity of inducing the production of INFγ. IL-33 drives Th2 responses and was suggested to act as anti-inflammatory cytokine. Although little is known about IL-33 activites some evidence suggests that IL-33 may have a dual role of pro-inflammatory and anti-inflammatory activities. All three cytokines (IL-1, IL-18 and IL-33) are member of same IL-1 family and are closly related with respects to their structure, receptor binding, and signal transduction. All three share a large part of signal transduction pathways. However they also have unique functions. Therefore it is important to compare the signaling induced by all three cytokines and to identify the unique molecules/pathways which are responsible for the discrete biological functions. This cytokine-specific signaling molecules would be attractive targets to develop highly selective drugs useful for effective anti-cytokine therapies.

3.3.1. Screening of triple responsive cell lines

In order to compare the signaling pathways induced by each of the above said cytokines under similar conditions, a suitable cell line was required, which expresses all receptor components and subsequent downstream molecules needed for signaling for these cytokines. To find such a triple-responsive cell line, steady state mRNA levels coding for IL-1RI, IL-1RAcP, IL-33Rα, IL-18Rα, and IL-18Rβ, were detected by RT-PCR analysis from mRNA isolated from a set of stimulated and unstimulated cell lines. Cell lines included in the screening were from different immunologically important lineages including B cell lines, T cell lines and freshly differentiated T cells (a kind gift of Prof. M. Lohoff, University of Marburg), dendritic cells line and fibroblasts. mRNA from these different cell lines were analysed by RT-PCR for the expression of the above described membrane molecules. As shown in table 3.1 only a few cell types express all known receptor components of the three receptor complexes. These inculded IL-1 stimulated and unstimulated EL-4 cells (Th0 cell line), unstimulated Xs106 cells (dendritic cell line) and freshly differentiated unstimulated Th0 cells, unstimulated and anti-CD3 -stimulated Th1 cells, as well as anti-CD3 stimulated Th2 cells. Some of the cell lines lacked the expression of one of

Table 3.1. Expression of different IL-1 family receptors and cytokines in diffteren cell types at mRNA level. Cell were either kept unstimulated or stimulated as described for overnight for IL-1β and LPS and for 6 hours for anti-CD3. RNA was isolated, cDNA was generated and expression of given molecules was detected by PCR. Each PCR reaction was performed under controled conditions including positive and negative controles and each reaction was repeated at least once from same cDNA.

Cell Type	Stimu	IL-1R1	IL-1RAcP	IL-33Rα	IL-18Rα	IL-18Rβ	IL-1α	IL-1β	IL-18	IL-33	Actin
EL-4	–	+	+	+	+	+	–	–	+	+	+
EL-4	IL-1β	+	+	+	+	+	+	+	+	+	+
D10G4.1	–	+	+	+	–	+		–	+	+	+
D10G4.1	IL-1β	+	+	+	–	+	+	+	+	+	+
D10N	–	+	+	–	–	+	+	+	+	+	+
Xs106	–	+	+	+	+	+	+	+	+	+	+
Xs106	LPS	+	–	+	+	+	+	+	+	+	+
RAW264.7	–	+	+	+	–	+	+	+	+	+	+
RAW264.7	LPS	+	+	+	–	+	+	+	+	+	+
Wehi	–	+	+	–	–	–	+	+	+	+	+
Wehi	LPS	+	+	+	–	–	+	+	+	+	+
70Z/3	–	+	+	+	–	+	–	–	+	+	+
70Z/3	LPS	+	+	+	–	+	–	–	+	+	+
P388D1	–	+	+	+	–	+	+	+	+	–	+
P388D1	LPS	+	+	+	–	+	+	+	+	–	+
RGK	–	+	+	+	–	+	–	–	+	–	+
RGK	LPS	+	+	+	–	+	+	+	+		+
Keratino	–	+	+	+	–	+	+	+	+	–	+
Keratino	LPS	+	+	+	–	+	+	+	+	+	+
NIH 3T3	–	+	+	+	–	+	–	+	+	–	+
NIH 3T3	LPS	+	+	+	–	+	+	+	+	–	+
Fibroblast IRAK cont.	–	+	+	+	–	+	+	–	+	+	+
Fibroblast IRAK cont.	LPS	+	+	+	–	+	+	+	+	+	+
L929	–	+	+	–	–	+	–	+	+	+	+
L929	LPS	+	+	–	–	+	–	–	+	+	+
Th0	–	+	+	+	+	+	+	–	+	–	+
Th0	CD3	+	+	+	–	+	+	+	+	+	+
Th1	–	–	+	+	+	+	+	+	+	–	+
Th1	CD3	+	+	+	+	+	–	–	+	+	+
Th2	–	+	+	+	–	+	+	+	+	+	+
Th2	CD3	+	+	+	+	+	+	+	+	+	+
Th17	–	+	+	+	–	+	+	+	+	–	+
Th17	CD3	+	+	+	–	+	+	–	+	+	+

the required receptor component and thus are good candidates to generate a triple responsive cell line by stable transfection of the required receptor component.

EL-4 cells responded readily to IL-1β, IL-33 and IL-18 by producing mIL-2 (Fig.3.2A) and stimulating NF-κB (Fig.3.6A left). Although EL-4 cells respond to all three cytokines, more than 100 times higher concentrations of IL-18 and IL-33 were required compared to IL-1β to achieve comparable bio responses. Response to IL-33 could be enhanced by over-expressing the IL-33Rα chain (Fig.3.6A right).

Stable transfectants of EL-4 cells were generated which expressed higher numbers of Flag-tagged IL-33Rα molecules (cell lines were generated by Jessica Endig during her Diploma thesis in this laboratory). Characterization of cell responsiveness (to IL-1, IL-18 and IL-33) of the EL-41B1 clone showed that it responded much better than the parent EL-4 cells not only to IL-33 but also to IL-1 and IL-18 by producing mIL-2 as measured by ELISA (Fig.3.34A) and and by stimulating NF-κB as measured by reporter gene assay (Fig.3.34B). Thus, the EL-41B1 cell line was selected as a triple-responsive cells for analyzing differential mRNA expression by microarray analysis.

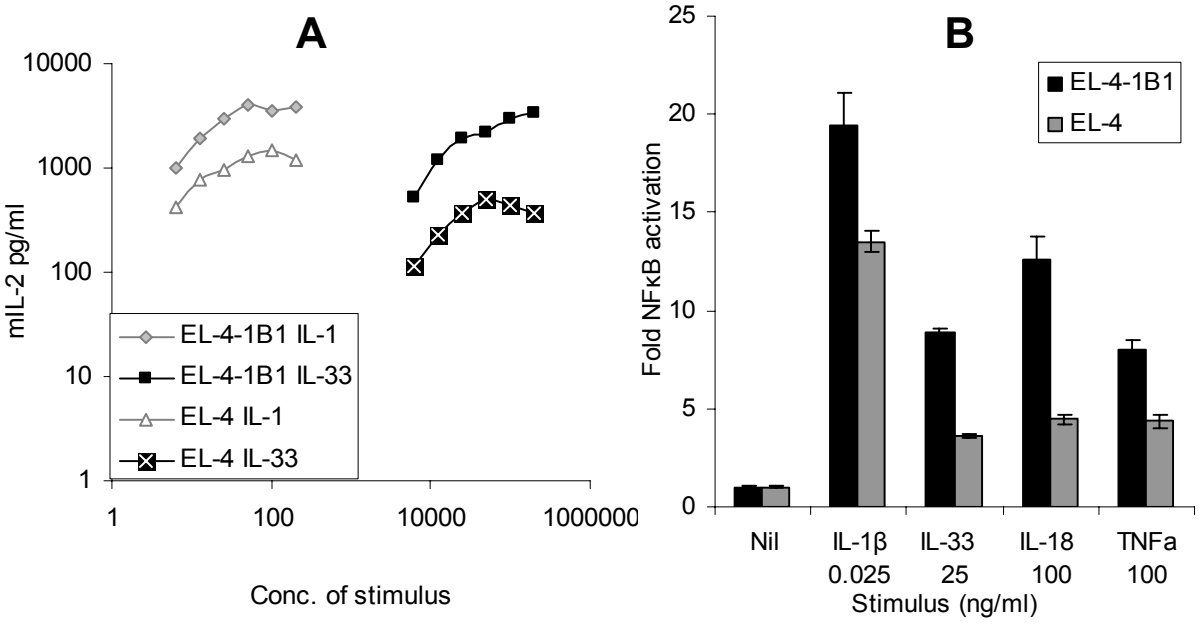


Fig. 3.34: EL-4-1B1 responds better to IL-1 family of cytokines than the parent EL-4 cells.
(A). EL-4-1B1 produce more IL-2 than parent EL-4 cells. EL-4 and EL-4-1B1(EL-4 cells stably transfected with mIL-33Rα) (2.5×10^4) cells were stimulated for overnight with increasing concentrations of rhIL-1β (left, grey) or rmIL-33 (right, black) and mIL-2 was measured with ELISA from cell free supernatants. Results shown are mean of two replicates in one experiment.
(B). IL-1 family cytokines induced NF-κB activation is more in EL-4-1B1 than parent EL-4 cells. Reporter gene transfected EL-4 (grey bars) and EL-4-1B1 (black bars) cells were either kept unstimulated or stimulated with indicated concentrations of rhIL-1β, rmIL-33, rmIL-18 or rTNFα for over night and reporter gene activity was measured from cell lysates. Data depicted are representative of 2 experiments and are expressed as fold increase in signal ± relative error.

3.3.2. Genome wide investigations of IL-1, IL-33 or IL-18 regulated genes

Microarrays were performed on the triple responsive cell line EL-41B1 to identify genes differentially regulated by IL-1, IL-18, and IL-33. EL-41B1 cells were either kept unstimulated or stimulated with 100 pg/ml IL-1β, 100 ng/ml IL-33 or 100 ng/ml IL-18. After 2 hours stimulation cells were lysed for RNA extraction. This RNA was used for microarray analysis (all arrays were perfomed at the Institute of Pharmacology, Hannover Medical School by Dr. Oliver Dittrich-Breiholz).

At first the gene expression was determined using the inflammation array (Inflamus, 2nd version inflamation array) developed by the cytokine microarray project at the Institute of Pharmacology, Hannover Medical School (Dr. Oliver Dittrich Breiholz and Prof. Michael Kracht). In these inflammatory array seven upregulated genes were identified. As shown in the Fig.3.35 the expression of all genes was stimulated by all three stimuli in the similar fashion and none of the genes was differentially regulated by any particular cytokine, indicating that the same signaling pathways were activated by IL-1, IL-33, and IL-18. However the expression intensities of the genes were different in response to different stimulus, which may be explained by the difference in receptor expression and binding affinity of the individual cytokines to their respective receptors.

In order to find more differentially regulated genes whole mouse genome oligo microarrays (4x44k, Agilent Technologies) were performed on unstimulated and IL-1, IL-33 or IL-18 stimulated EL-41B1 cells. Flourescent intensity values of all measured spots are plotted in a scatter plot (Fig.3.36). Spots outside of the grey line represent differentially expressed genes between stimulated and unstimulated cells. As shown in Fig.3.36 based on the primary data obtained from the assay IL-1 stimulation increased the expression of 794 gene entries (based on 2 fold increase as upregulated) and downregulated the expression of 629 gene entries. Similarly, IL-33 and IL-18 upregulated 669 and 872 genes and decreased the expression, up to equal or more than 2 fold of 752 and 788 gene entries, respectively.

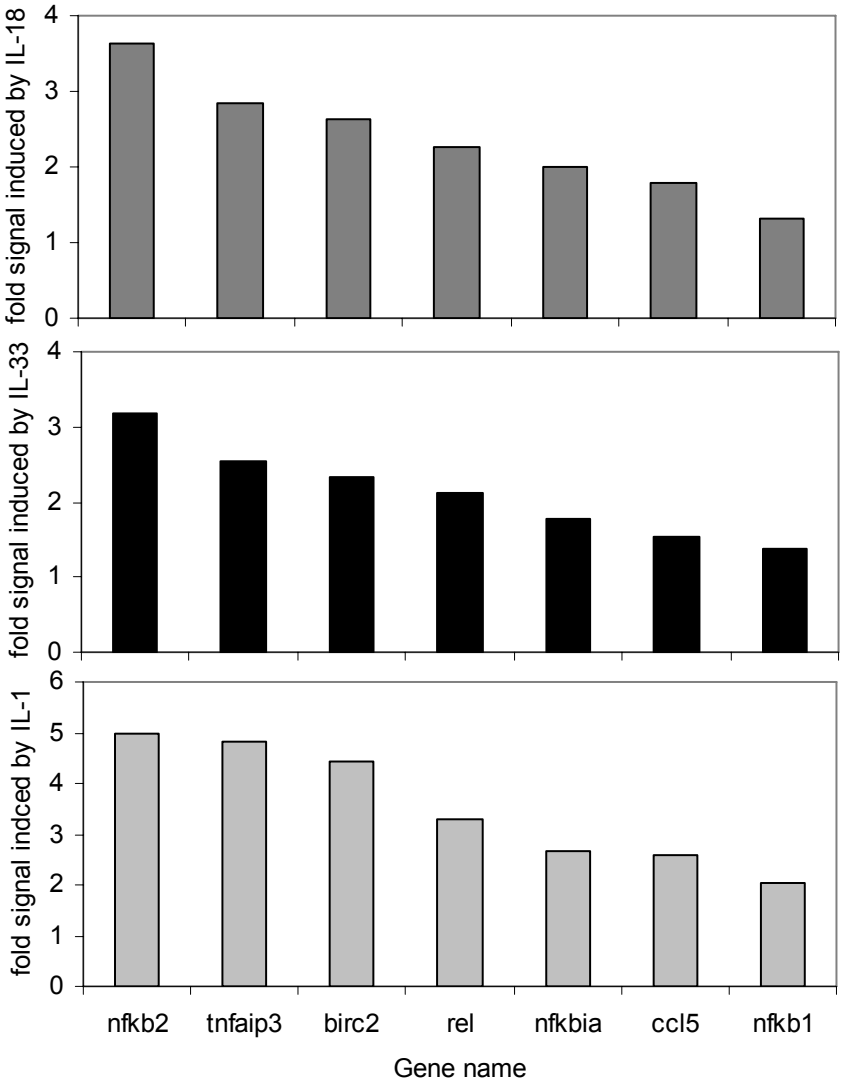


Fig. 3.35: Similarities and differences of inflammatory gene expression in response to IL-1, IL-33 and IL-18 in inflammation gene array.
EL4-1B1 cells were either kept unstimulated or stimulated with IL-1β (100 pg/ml, lower light grey), IL-33 (100 ng/ml, middle black) or IL-18 (100 ng/ml, upper dark grey) for 2 hours and gene expression was determined using Inflammation array (Inflamus, 2nd version inflamation array) developed by cytokine microarray project (SFB566) at Institute of Pharmacology Medical School Hannover. (nfkb1, nfkb2, and rel: transcription factor; tnfaip3 (TNFα inducing protein 3), nfkbia (nuclear factor of kappa light chain gene enhancer and birc2 (baculoviral iap repeat containing 2 protein), all play role in signal transduction; ccl5, (a chemokine) were up regulated by IL-1, IL-18 and IL-33 stimulation. Results shown are the mean of two different arrays with similar results from the same RNA samples and are presented as relative induction compared to unstimulated controls. Only the regulated genes by at least one of these stimuli (2 fold induction by any of the cytokine) are shown in the figure.

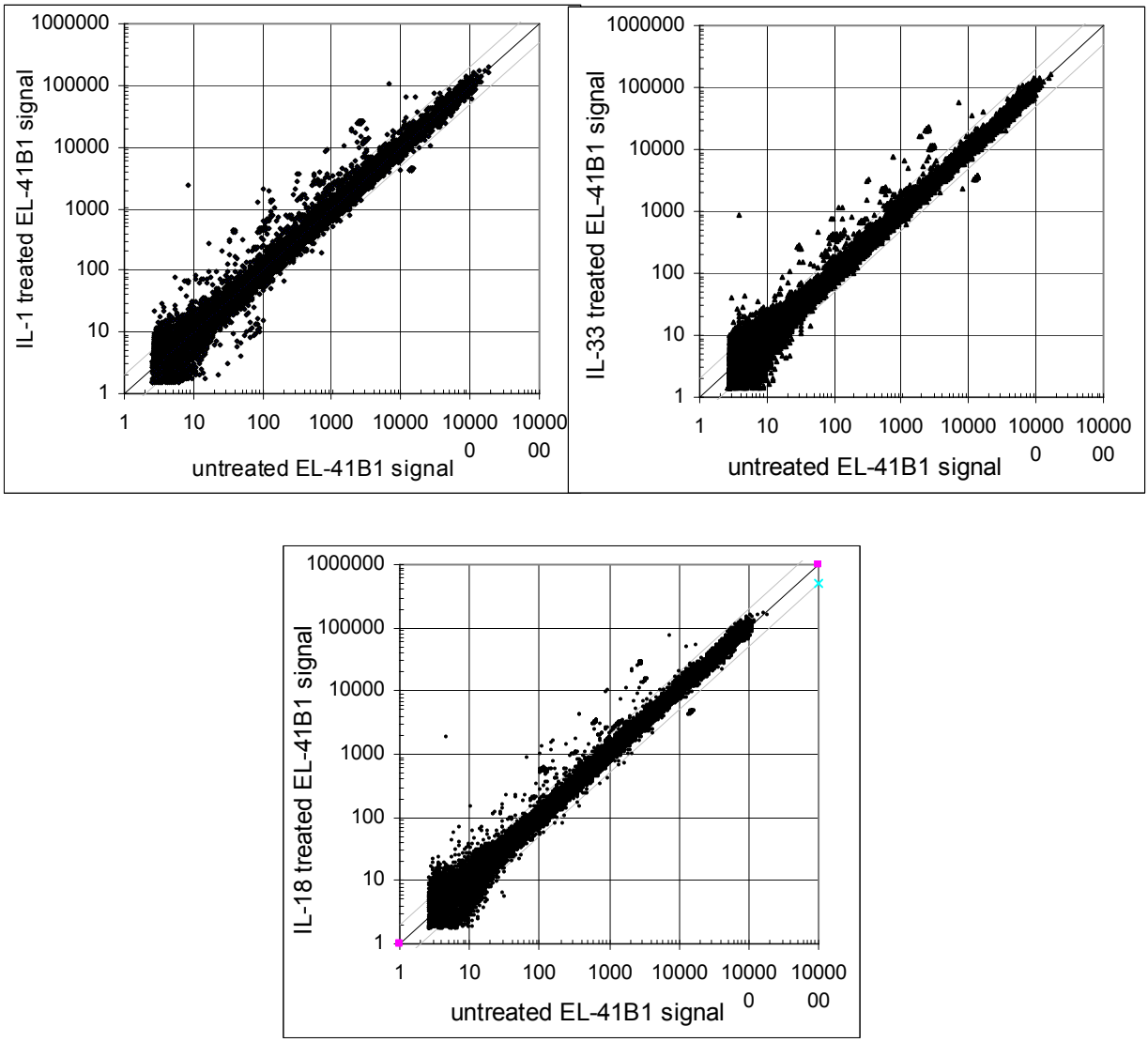


Fig. 3.36: High density (whole genome mouse microarray) microarray analysis of the genes regulated by IL-1, IL-33 and IL-18.
Murine EL-41B1 cells were stimulated for 2 hours with IL-1β (100 pg/ml), IL-33 (100 ng/ml) or IL-18 (100 ng/ml) or were left untreated. Subsequently total RNA was isolated, converted to cRNA and labelled with Cy5 or Cy3 respectively. Labelled cRNAs of stimulated or unstimulated cells were mixed and co-hybridized on to whole mouse genome oligo microarrays (4x44k, Agilent Technologies), which carry oligonucleotide probes for mouse genome. Depicted graffically are the flouriscent intensity values of all measured spots. Spots out side from liner line represents differentialy expressed genes between stimulated and unstimulated cells. Results shown are from internally controled single array. Spots above and below grey lines indicates 2 fold up and down regulated genes respecetively.

Data depicted in Table 3.2 and Fig.3.37 are obtained after filtration and analysis considering pValue log ratio ≥ 0.0001 significant. Considering 2 fold increase or decrease in mRNA transcripts as upregulation and downregulation, 74 genes were found to be upregulated (Fig.3.37) and 15 genes down regulated (Table 3.1) by stimulation with one or more cytokine. There are some quantitative differences is mRNA transcripts of some of these genes upon stimulation with different cytokines (Fig.3.37 and table 3.2) however no qualitative difference among mRNA expression in response to IL-1β, IL-18 and IL-33 was observed.

Among the upregulated genes *Ccrl2, Nfkbia*, *Serpinc1*, *IL-2*, *Icam1*, *LOC620807, Cd69*, *Gbp4*, *Slfn2*, and *A_52_P1173559* were more than 2 fold upregulated by IL-1 stimulation compared to IL-33 or IL-18 stimulation. *Cxcl10* (chemokine) gene was most strongly upregulated by IL-18 followed by IL-1 and by IL-33 (387, 274 and 217 fold respecetively) and *IL-22* gene was most strongly upregulated by IL-33 (13.47 fold) compared to the IL-1 (7.83 fold) and IL-18 (9.13 fold). Expression of a set of genes was almost similarly increased, expression of most up regulated genes, however, was higher in IL-1 -treated cells followed by IL-18 and IL-33 (Fig.3.37).

Table 3.2. Down regulated genes in mouse whole genome microarray from EL-4 1B1 cells stimulated with either IL-1β, IL-33 or IL-18. Genes with 2 fold signal reduction were considered down regulated**.** Results expressed are fold signal change calculated by dividing signal by stimulated cells/unstimulated control.

GeneName	Reference	Description/function	IL-1	IL-33	IL-18
Sbsn	NM_172205	Suprabasin, epidermal differentation	0.41	0.38	0.18
Nrp2	BC057028	Axon guidance receptor, development	0.28	0.59	0.45
Nr2c2	AK036508	Nuclear receptor, growth	0.73	0.73	0.38
AF315352	AF315352	Leucine zipper protein, pituitary development	0.74	0.72	0.46
B3galt5	NM_033149	Enzyme, metabolism	0.40	0.73	0.63
Tgfb3	NM_009368	Growth factor	0.43	0.70	0.56
Ccr2	NM_009915	Chemokine receptor	0.43	0.64	0.69
4833431D13Rik	AK029407	Riken, unknown	0.58	0.73	0.50
AK040293	AK040293	Riken, unknown	0.62	0.71	0.49
Spon2	NM_133903	Extracellular matrix protein, immune-enhancing agent	0.34	0.50	0.37
Sall4	AK017633	Transcription factor, development	0.44	0.70	0.60
Marveld3	NM_028584	Unknown	0.49	0.69	0.63
Aph1a	NM_146104	Role in embryogenesis	0.58	0.54	0.50
2310005P05Rik	NM_026189	Eepd1, DNA binding	0.48	0.50	0.37
Txnip	NM_023719	Enzyme inhibitor, protein binding	0.50	0.58	0.54

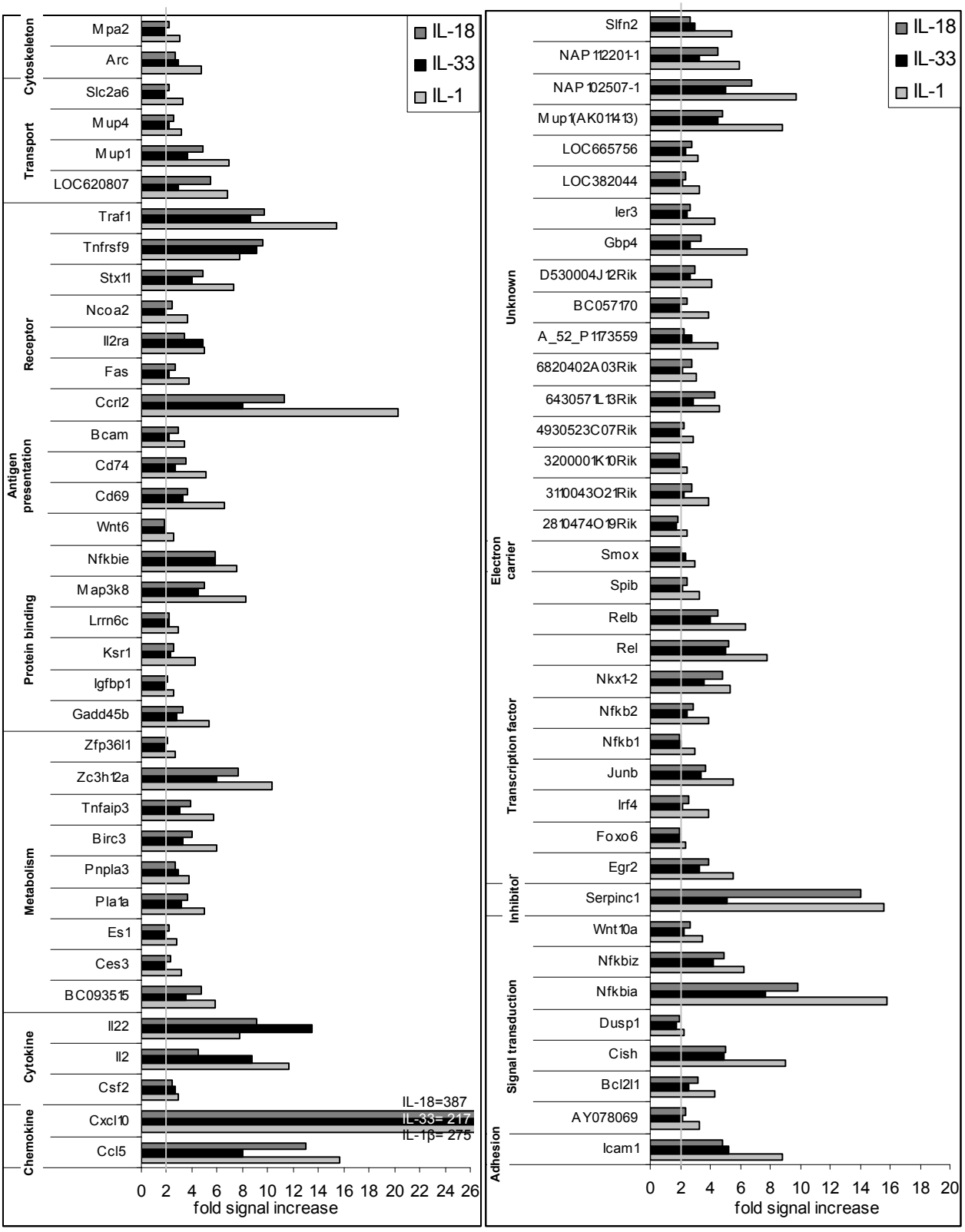


Fig. 3.37: Similarities and differences of IL-1, IL-33 and IL-18 induced gene expression by whole genome microarray. Murine EL-41B1 cells were stimulated for 2 hours with IL-1β (100 pg/ml), IL-33 (100 ng/ml) or IL-18 (100 ng/ml) or were left untreated. Total RNA was isolated, converted to cRNA and labelled with Cy5 or Cy3 respectively. Labelled cRNAs of stimulated or unstimulated cells were mixed and co-hybridized on to whole mouse genome oligo microarrays (4x44k; Agilent Technologies), which carry oligonucleotide probes for mouse genome. Depicted graffically are the fold change in flouriscent intensity values (P value log $\geq$0.0001) of all upregulated genes. Genes with 2 fold increase in flouriscent value were considered upregulated. Results shown are from internally controled single array. Genes are grouped with respect to the functions. Values above grey line are more than 2 fold signal increase.

3.3.2.1. Confirmation of gene expression by quantitative PCR

To validate the results obtained in microarray analysis, quantative-RT-PCR assays were carried out for representative genes. Changes in mRNA level for *IL-2*, *Csf2*, *IL-22*, *Ccl5*, *Cxcl10*, *Tnfrsf9*, *Icam1* and *Serpinc1* genes were measured in EL-4-1B1 without addition of cytokines (control) and after stimulation with IL-1, IL-33 or IL-18 for 2 h (Fig.3.38). In quantitative -RT-PCR analysis, master mix with SYBR Green was used and the obtained data were normalized against expression level of *GAPDH*. Data shown in Fig.3.38 for qantitaive-RT-PCR and Fig.3.37 for microarray, showed the similar trend in the expression level of selected genes in response to IL-1, IL-33 and IL-18 for both methods. However, in most cases the changes in gene expression observed in quantative -RT-PCR are more pronounced than in microarray analysis. Since in the two methods the same conditions for cell stimulation, RNA templates isolation were employed the differences in the obtained results may be explained with following, 1: Slight difference in biological conditions, which can not be controlled; 2: Different reverse transcriptases, with different efficiencies in cDNA synthesis, are being used; 3: Normalization of data for quantitative-RT-PCR was done for one gene (*GAPDH*) whereas in microarray a global normalization was carried out.

A slight discrepancy in the expression level of some genes in response to different cytokines was also observed in the two methods. Data depicted in Fig.3.38 show that IL-1, IL-33 and IL-18 stimulated the expression of a set of genes (*IL-22, Csf2, and Tnfrsf9*) almost at the same level where as the expression of *Serpinc1* (a protinase inhibitor) transcripts is different in response to all three cytokins. No difference in the expression of IL-2 transcripts was observed in response to IL-1 and IL-33 but IL-18 stimulated the expression of IL-2 mRNA significantly lower than IL-1 and IL-33. Upregulation of chemokine (*Cxcl10 and Ccl5*) and adhesion protein (*Icam1*) genes expression was almost similar both in response to IL-33 and IL-18 where as IL-1β induced expression of these genes was higher than that of IL-33 and IL-18. Although IL-1, IL-33 and IL-18 stimulated the expression of a similar set of genes qualitatively, clear differences in the quantitative expression of genes in response to these cytokines were observed.

In summary, the microarray data show that IL-1, IL-18 and IL-33 induce the regulation of the same set of genes, suggesting that the signal transduction pathways

used are identical for all three cytokine / cytokine receptor systems, at least in this cell line.

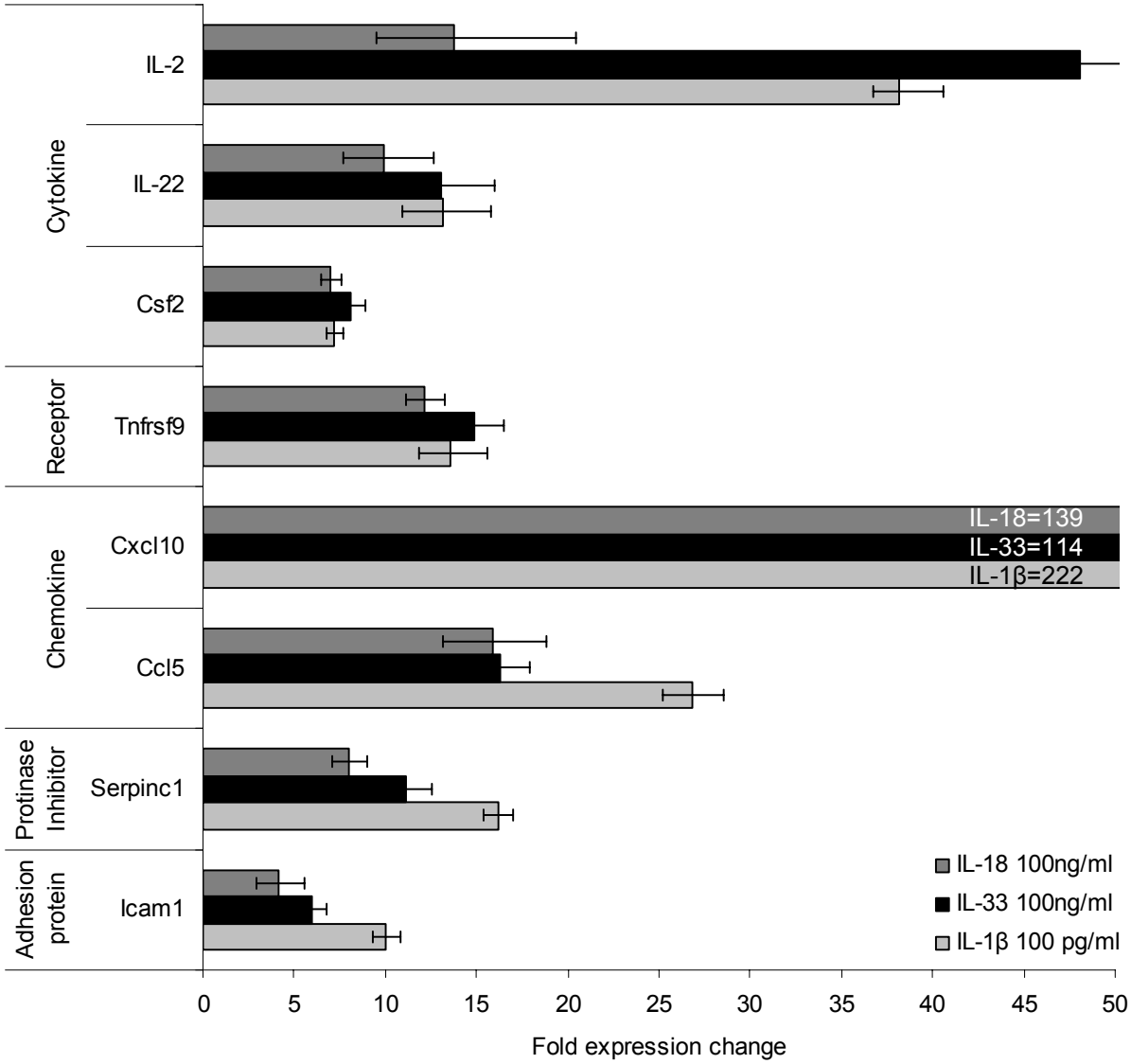


Fig. 3.38: Confirmation of microarray results by quantitative PCR.
Murine EL-41B1 cells were stimulated for 2 hours with IL-1β (100 pg/ml), IL-33 (100 ng/ml) or IL-18 (100 ng/ml) or were left untreated. Total RNA was isolated, converted to cDNA and gene expression was analyzed by quantitative PCR using specific primers and cyber green master mix. Depicted graffically are the mean fold increase in gene expression compared to the unstimulated control ± relative error. Results shown are representative of three biologically independent experiments with similar results. Genes are grouped with respect to the gene functions.

Chapter 4

Discussion

The IL-33Rα-chain, formerly known as ST2/T1, is a member of the IL-1 receptor family of TIR-domain-containing receptors. IL-33Rα-chain (ST2) possesses a TIR domain in its cytoplasmic moiety, which is required for signaling (Sims, 2002). The IL-33Rα-chain interacts with IL-33, which results in the recruitment of signaling molecules also found in the IL-1RI complex (Schmitz et al., 2005). This demonstrates the close relationship of IL-33R and IL-1R complexes as also suggested by the use of chimeric molecules (Born et al., 2000). Thus, it has been proposed that the IL-33Rα-chain also requires a β-chain containing a TIR domain and that this may be a member of the IL-1 receptor family (Dinarello, 2005; Schmitz et al., 2005). The aim of this thesis was to identify and characterize the components of the functional IL-33 receptor complex, which are required for IL-33 mediated biological activities. In addition the processing of IL-33 itself needs to be clarified. Only Schmitz et al., 2005 data suggests the caspase-1 dependent maturation of IL-33 *in vitro* however, no evidence of *in vivo* processing of IL-33 is published. Moreover it is not yet clear how IL-33 may be released from the cells to exert its cytokine activities towards target cells expressing the IL-33 receptor. These questions are addressed in this thesis.

4.1. IL-33Rα needs IL-1RAcP for signal transduction

IL-1 family cytokines undergoes ligand-mediated heterodimerization of the respective receptor and co-receptor for initiation of signal transduction. IL-1 signals through a heterodimer receptor complex consisting of IL-1RI and IL-1RAcP. IL-1R1 binds to IL-1 and IL-1RAcP, recognizes the ligated IL-1RI (Greenfeder et al., 1995; Hofmeister et al., 1997; Korherr et al., 1997; Wesche et al., 1997b). The signaling IL-18 receptor complex is also a heterodimer in which the IL-18 binds to IL-18Rα-chain (Born et al., 1998; Torigoe et al., 1997) and the IL-18Rβ-chain (Born et al., 1998) serves as a co-receptor. Two additional heterodimeric receptor complexes in the IL-1R system have also been described. IL-1F6, IL-1F8, and IL-F9 bind to IL-1Rrp2 and induce signaling

events practically identical to IL-1 (Towne et al., 2004; Debets et al., 2001) only in the presence of functional IL-1RAcP (Towne et al., 2004).

Here it is shown that IL-33Rα (ST2) requires IL-1RAcP for initiation of IL-33-induced signaling in different types of cells. In addition, it is also shown that central signaling pathways activated by IL-33 are strictly dependent on IL-1RAcP and are comparable to those also stimulated by IL-1. IL-33 was able to activate NF-κB similar to IL-1 which is in accordance with previous report from transiently transfected HEK293 cells (Schmitz et al., 2005), In present work a transient JNK activation by IL-33 was detected in a time frame comparable to the transient activation of these protein kinases by IL-1. Schmitz et al., 2005 observed a slight shift in JNK activation compared with IL-1 in transiently transfected HEK293 cells (Schmitz et al., 2005). In EL-4 cells this effect was not seen, suggesting subtle differences in different cell types. Although the affinity of mIL-33 for ST2 (616 pM KD) is higher than the affinity of the IL-1β to IL-1R1 (1 nM KD) (Palmer et al., 2008), interestingly, rhIL-1β was active in the picomolar range in EL-4 or transfected D6/76 cells, where as nanomolar concentrations of rmIL-33 (or rmIL-18) were required to achieve a comparbale effect. This result demonstrates the great potency of IL-1 when compared to its sister cytokines, IL-18 and IL-33.

The essential requirment of IL-1RAcP in IL-33-mediated signaling was also proved in loss of function experiments using monoclonal antibody 4C5, which recognizes mIL-1RAcP (Greenfeder et al., 1995; Hestdal et al., 1994) and neutralizes IL-1β-mediated signal transduction. 4C5 behaved similarly to the mAb M49, which neutralizes hIL-1RAcP and was used to inhibit IL-1RAcP-dependent signaling of IL-1F6 by IL-1Rrp2 in human Jurkat and NCI/ADR-RES cells (Towne et al., 2004). If 4C5 recognizes mIL-1RAcP and functions by inhibiting the association of the co-receptor with the IL-1-ligated IL-1RI, it also should work in the IL-33 receptor system. And indeed, a concentration-dependent inhibition of IL-33 effects was observed in the T cell lines EL-4 or D6/76 (after reconstitution with mIL-1RAcP). The study was extended to murine BMD mast cells, which are nontransformed and nontransfected primary cells, in a close collaboration with Prof. Huber at the MPI in Freiburg. These cells responded readily to IL-33 by producing IL-6, comparable to IL-6 produced by LPS stimulation. In these primary cells mAb 4C5 inhibited IL-33 induced IL-6 production in dose dependent mannar.

It was observed that 4C5 was less effective in IL-33 stimulation compared to IL-1β. It is difficult to explain why 4C5 was less effective in inhibiting IL-33-stimulated responses when compared with IL-1β. However, it has been described that this anti-IL-1RAcP antibody differs in its neutralizing capacity between IL-1α or IL-1β containing receptor complexes (Hestdal et al., 1994). Thus, it is conceivable that this antibody also distinguishes between IL-1β in IL-1RI and IL-33 in IL-33Rα.

The D10 cell line is classified as a Th2 type T lymphocyte and expresses ST2 (Yanagisawa et al., 1997). D10 cells respond to IL-33 in proliferation assay. A 100-fold excess of IL-33 was sufficient to achieve response comparable to IL-1β in D10 cells, where as 1000 fold excess of IL-33 was needed in EL-4 cells. This indicates that the expression of IL-33 receptor is not as limiting as in EL-4 cells or that the cells may express some additional molecules, which results in an increase in the binding affinity of the IL-33 to its receptor or the efficacy of IL-33 signaling. The absolute requirement of IL-1RAcP as accessory protein for IL-33 receptor was also proved in D10 cells by competition experiments. Here, a truncated version of the IL-1RacP which lackes the TIR domain was overexpressed in order to compete with endogenous IL-1RAcP. And indeed, this resulted in an inhibition of IL-33 effects, due to the dominant negative effect of the co-receptor lacking the signaling domain. Interestingly however, the result obtained employing the neutralizing antibody 4C5 in D10 cells was in contrast to that obtained in EL-4 or in mast cells. 4C5 did not affect IL-33 induced signaling in D10 cells although it neutralized the effect of IL-1β completely. This observation strengthens the hypothesis (concept) of involvement of a putative additional chain in the IL-33 receptor system of D10 cells. Interaction of this postulated additional chain in the IL-33 receptor system may result in a change in the conformation of the IL-1RAcP, so that the 4C5 antibody is no more able to recognize/ gain access to IL-1RAcP. Alternatively, presence of an additional chain may allow the recruitment of IL-1RAcP in a way that the epitope region recognized by 4C5 antibody is not involved in the interaction of IL-1RAcP with IL-33 ligated IL-33 receptor α-chain. Further experiments in that direction are required to identify such a third chain participating in the IL-33 receptor complex formation.

The requirement of the TIR domain for IL-33-induced signal transduction in the EL-4 cell system, was investigated by over-expressing a C-terminally truncated version of IL-1RAcP, lacking the TIR domain. IL-33 signaling was totally dependent on the presence of the TIR domain of the IL-1RAcP. Similar findings have also been

reported in a recent study by Chackerian et al., 2007. In fact TIR domains of both receptor and co-receptor are essential for IL-33 signaling. A C-terminally truncated version of the IL-33Rα-chain was not produced to prove this stringently in present study. However, the essencial requirment of TIR domain of ST2 and IL-1RAcP for IL-1 induced signaling has been shown by generating chimeric receptor (Brint et al., 2002; Towne et al., 2004; Thomassen et al., 1999). Therefore, it must be concluded that the TIR domains of the receptor and co-receptor are necessarly required for signal initiation.

Here It is shown that the binding of IL-33 to the IL-33Rα chain allows the formation of a complex with IL-1RAcP in the plasma membrane, as described for IL-1 binding to IL-1RI or IL-1RII (Brint et al., 2002; Lang et al., 1998; Towne et al., 2004). Furthermore, results suggest that similar to IL-1α/β and IL-18, IL-33 does not dimerize IL-33Rα but rather signals through recruitment of accessory protein. These findings are in agreement with recently published data indicating that IL-33 and IL-33Rα form a complex with IL-1RAcP *in vitro*, that IL-1RAcP is required for IL-33 induced *in vivo* effects and that IL-33 induced signal transduction is inhibited by dominant negative IL-1RAcP (Chackerian et al., 2007). Similarly another recent study described that IL-1RAcP associates with IL-33Rα in ligand dependent manner and that the affinity of IL-33 binding to IL-33Rα increased by 4 fold in the presence of IL-1RAcP (Palmer et al., 2008).

It has been shown in several studies that soluble forms of cytokine receptors function as positive and negative regulators in the signaling of cytokines and growth factors. The existence of soluble ST2 (IL-33Rα) has been well documented and circulating levels of IL-33Rα (soluble ST2) are increased under different pathological situations (Kuroiwa et al., 2000; Oshikawa et al., 2001). In the present study the antagonistic effect of soluble IL-33Rα on IL-33 signaling is demonstrated in EL-4 cells. Antagonistic effects of soluble IL-33Rα on IL-33 signaling have also recently been described in EL-4 cells and murine model of asthma (Hayakawa et al., 2007). In addition, it is shown here that a soluble IL-33Rα-chain can interact with soluble IL-1RAcP in the presence of IL-33. The formation of such soluble receptor complexes in the presence of ligand has been reported in the IL-1 system (Smith et al., 2003), where soluble IL-1RAcP seems to stabilize the ligated soluble IL-1R, allowing neutralization and sequestration of the cytokine also *in vivo*. Moreover, it has been proposed that soluble IL-1RAcP may be helpful to neutralize IL-1β in chronic

inflammatory diseases such as rheumatoid arthritis (Smeets et al., 2003; Smith et al., 2003). It is tempting to speculate that the combination of soluble IL-33Rα and soluble IL-1RAcP can act in synergistic manner to inhibit IL-33 activities. In that context soluble IL-1RAcP could also be used to neutralize and sequester IL-33 in the presence of soluble IL-33Rα-chain in Th2-biased diseases (e.g., IgE-mediated allergic reactions).

In conclusion, the results obtained in this study demonstrate that the IL-33 does not induce dimerization of IL-33Rα but rather IL-33 biology is mediated through a hetrodimeric receptor complex. The functional IL-33R complex consists of IL-33Rα and IL-1RAcP. In addition to the membrane-inserted forms, also the soluble forms of IL-33Rα and IL-1RAcP can form complexes in the presence of IL-33 (depicted in Fig.4.2) however these soluble receptor complexes are not able to initiate signal transduction. These findings suggest the novel role of soluble form of IL-1RAcP in modulating the IL-33 activities.

In summary of the results presented here and published in the literature, IL-1RAcP can act as a common β-chain for four discrete members of the IL-1 receptor family: IL-1RI, IL-RII, IL-1Rrp2, and IL-33Rα. This situation is reminiscent of the role of common receptor chains in other cytokine families, like gp130 in the IL-6 receptor system or the common γ-chain in the IL-2 receptor family. In addition, the sharing of IL-1RAcP and the fact that it also can interact with receptors in its soluble form opens new levels of cross talking among the different members of the IL-1 receptor family (schematically depicted in Fig.4.1).

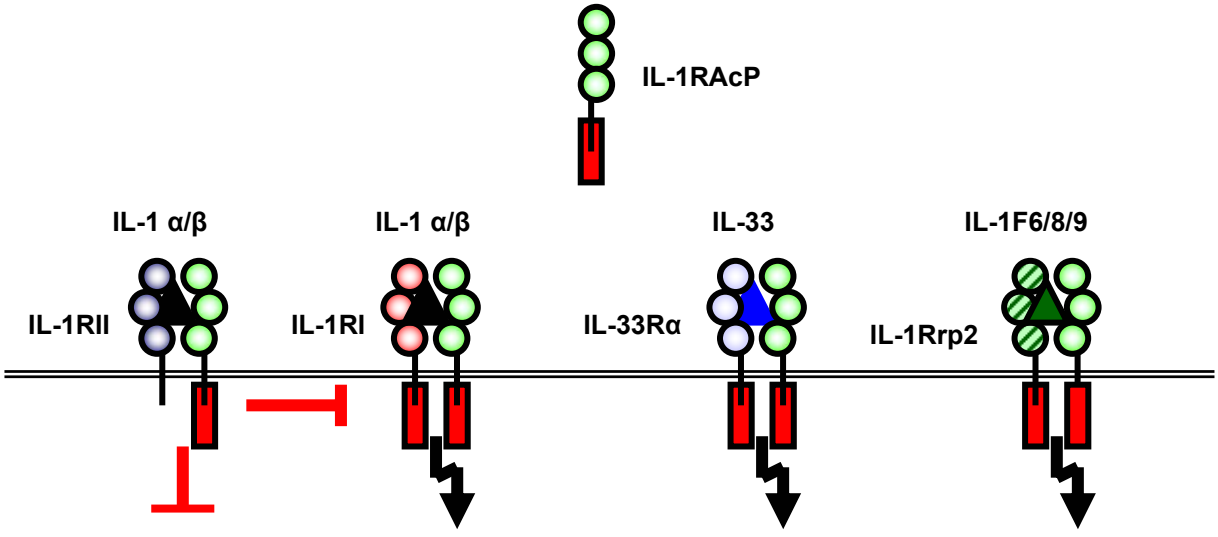


Fig. 4.1: Summary of usage of IL-1RAcP as co-receptor by the receptor-like members of the IL-1 receptor family of TIR receptors. IL-33 like IL-1 (α and β), IL-1F6, IL-1F8 and IL-1F9 uses IL-1RacP as second component of its signaling complex.

4.2. IL-33 induces pro-inflammatory cytokine release

IL-33 has been previously described to drive essentially Th2 responses and production of Th2-associated cytokines from *in vitro* polarized Th2 cells (Kondo et al., 2008; Schmitz et al., 2005). *In vivo*, IL-33 injection induced the expression of IL-4, IL-5, and IL-13 and led to severe pathological changes in the lung and the digestive tract (Kondo et al., 2008; Schmitz et al., 2005). In this thesis, it is reported that in addition to Th2 cytokine production, IL-33 is capable to stimulate secretion of pro-inflammatory mediators in IL-1RAcP dependent mannar. IL-33 induces IL-6, IL-1β, and TNFα in bone marrow derived mast cells, IL-2 in EL-4 cells, IL-6 in fibroblasts and IL-8 production in transiently transfected HEK293RI cells. In addition, IL-33 was also able to induce mRNA expression of cytokines and chemokines such as IL-22, Csf2, Cxcl10 and Ccl5 in EL-4 cells. In accordance with the findings reported here production of IL-6, IL-1β, TNFα and PGD2 in response to IL-33 in murine mast cells (Moulin et al., 2007) and pro-inflammatory cytokine and chemokines in human mast cells (Allakhverdi et al., 2007; Iikura et al., 2007) have recently been reported. In addition, IL-33 induced interferon-γ production in NK and NKT cells has also been described in recent reports (Smithgall et al., 2008; Bourgeois et al., 2009). Taken together it seems that similar to the other members of family, IL-33 can contribute as a co-stimulatory factor to innate cellular immune responses and might also be involved in pathogenesis of the inflammatory disorders including arthritis.

3.3. TIR8 (SIGIRR) is not a component of the IL-33 receptor complex

It has been shown exprimentally that IL-1RAcP is absolutely required for IL-33-induced activation of the cells (Ali et al., 2007; Chackerian et al., 2007; Palmer et al., 2008). Thus IL-1RAcP is definitely a component of the IL-33 receptor complex, however this does not exclude that at least in some cell types additional molecules may participate in the IL-33R complex. TIR8 (SIGIRR) was also a proposed candidate that may be a component of the IL-33 receptor complex (Dinarello, 2005; Schmitz et al., 2005; Barksby et al., 2007; Gadina and Jefferies, 2007). To understand the precise role of IL-33 and IL-33 receptor in the immune response, it is necessary to identify and define all the components of the IL-33 receptor complex and its regulatory molecules. It has been published that TIR8 may function as a negative regulator of Toll like receptors because TIR8 deficient mice are reported to

be more susceptible to the systemic toxicity of bacterial lypopolysaccharide (Wald et al., 2003) and intestinal inflammation (Garlanda et al., 2004).

TIR8 possesses all of the conserved motifs found in the cytoplasmic domain of the IL-1R superfamily. In addition, TIR8 contains distinct structural and functional features that are not found in other IL-1R homologous proteins. Its extracellular domain, is very short, however it appears long enough to fold into one Ig-like domain (Mantovani et al., 2004; Martin and Wesche, 2002). The peptide sequence of the conserved cytoplasmic regions is closely related to that of the other members of family. However, similar to the drosophila Toll, its long cytoplasmic tail extends beyond the last conserved region by 98 amino acids compared to the other type 1 members of the IL-1 receptor family. It was shown that this region in drosophila Toll contains inhibitory activity (Norris and Manley, 1995).

In the experiments performed within this thesis, it was possible to reproduce the inhibitory function of TIR8 in IL-1- and LPS- induced activation of cells in over-expression system. The over-expression of both human and murine TIR8 inhibited LPS- induced production of IL-6 in L929 cells and IL-1- induced NF-κB activation in HEK293RI cells. This observation is consistent with the previously published data suggesting that TIR8 is an inhibitor of IL-1R/TLR signaling (Polentarutti et al., 2003; Wald et al., 2003). This effect of TIR8 overexpression was also measured on IL-33 induced NF-κB stimulation in a reporter gene assay. A slight inhibition of IL-33 induced NF-κB was also observed in some experiments. However, this inhibition was only achievable when there was very high expression of TIR8.

It has been shown that TIR8 exerts its negative regulatory function in IL-1/TLR mediated signaling pathways through its direct effect on the immediate signaling events, including NF-κB and JNK activation (Wald et al., 2003). Detailed molecular mechanism, how TIR8 negatively regulate IL-1/LPS mediated signaling have not yet been described. It has been reported that TIR8 associates with IL-1R1 and TLR4 and also led to the formation of complex between TIR8, MyD88, IRAK4, IRAK and TRAF6 (Qin et al., 2005; Wald et al., 2003). TIR8 may negatively regulate IL-1 and LPS pathways by interfering with the appropriate recruitment and activation of the receptor- proximal signaling components (such as MyD88, IRAK molecules or TRAF6) or, alternatively, may attenuate the dissociation of the activated signaling components from the receptor complex.

By BIAcore analaysis it has been demonstrated that TIR8 is not able to bind either IL-1α, IL-1β or IL-1Ra (Thomassen et al., 1999). Its single Ig-domain is too short to fold around an IL-1-like ligand in the same manner as the three Ig folds of the IL-1R (Vigers et al., 1997). It was assumed that TIR8 could function as part of a ligand binding complex in a way similar to the IL-1RAcP or the common β chain of the IL-3, IL-5 and GM-CSF receptor complexes (Tavernier et al., 1991). These proteins can not bind a ligand on their own but bind to the high affinity receptor plus bound ligand. The involvement of TIR8 as a putative third part of the IL-33 complex or as a substitute for IL-1RAcP was tested by transfecting it with/or without IL-1RAcP in IL-1RAcP- negative cells (EL-4 D6/76). Wild type TIR8 and truncated versions of it did not have an effect on IL-1, or IL-33-dependent NF-κB activation. In addition, IL-33 stimulated EL-4 cells did not alter NF-κB activitation in presence or absence of TIR8 overexpression, which suggests that TIR8 does not function as a silencing or stimulating part of IL-33 receptor complex.

The direct interaction of TIR8 with IL-33 receptor was not investigated by co-immunoprecipitation. But the results from biological assays suggests that, TIR8 can not be used as co-receptor by IL-33Rα, as transiently transfected EL-4 D6/76 cells are not able to restore IL-33 induced signaling despite TIR8 over expression. EL-4-D6/76 expresses enough of IL-33Rα, as these cells readily reconstitute IL-33 responsiveness upon IL-1RAcP expression. Moreover, expression of TIR8 with IL-1RAcP did not affect IL-33-induced signaling in EL-4 and EL-4 D6/76 cells expressing mIL-1RAcP, indicating that mTIR8 exerts neither stimulatory nor inhibitory effects on IL-33-induced signaling in EL-4 and EL-4 D6/76 cells. The inhibitory effect which was observed in some experiments most likely is due to the strong over expression.

In the past it has been shown that replacement of the cytoplasmic domain of IL-1R1 by that of other family members still resulted in NF-κB activation after IL-1 binding to the chimeric molecule (Greenfeder et al., 1995; Mitcham et al., 1996; Thomassen et al., 1999). Assuming that TIR8 might function as accessory protein for the IL-33 receptor α-chain, it was necessary to ascertain whether the extracellular domain of TIR8 could recognize IL-33 bound to the IL-33 receptor α chain. It was observed that extracellular and/or transmembrane domain of TIR8 was not able to recognize the IL-1 or IL-33 ligand receptor complex, as the expression of a TIR8-AcP chimera (extracellular and transmembrane part of TIR8 and cytoplasmic part of IL-1RAcP)

could not reconstitute IL-33 responsiveness in IL-1RAcP lacking cells (EL-4 D6/76). Thus it must be concluded that TIR8 can not be a direct component of the receptor complexes for either IL-1 or IL-33. Results reported here are in contradiction with the previously published data showing that TIR8 can recognize IL-1R1 in IL-1 dependent manner (Qin et al., 2005; Wald et al., 2003) and ST2 in IL-33 dependent mannar (Bulek et al., 2009) but are in agreement with the data generated in BIAcore and reporter gene assays indicating that TIR8 is not able to bind to IL-1, IL-1R/IL-1 complex or IL-1RI/IL-1/IL-1RAcP complex (Thomassen et al., 1999).

Inability of involvement of cytoplasmic part of TIR8 in IL-33 or IL-1 signaling was proved by replacement of the cytoplasmic part of the IL-1RAcP with that of TIR8. After expression, this chimera was able to recognize IL-1 and IL-33 bound to respective receptors, as extracellular three Ig like domains of IL-1RAcP are sufficient to recognize the ligand bound IL-1 receptor and IL-33 receptor. Similar findings are repoted by others for IL-1 receptor. (Huang et al., 1997; Jensen et al., 2000; Neumann et al., 2000). However expression of AcP-TIR8 was unable to replace the function of full length IL-1RAcP for initiating IL-1 or IL-33 mediated signal transduction. If TIR8 is functional as the accessary protein for IL-33 receptor, the intracellular TIR8-moiety in the chimeric proteins should be able to start signaling upon interaction of Ig-like domains of IL-1RAcP with IL-33 bound IL-33 receptor.

In summary, TIR8 (SIGIRR) is neither the accessory protein for IL-1 receptors nor for the IL-33 receptor. In addition evidence for a regulatory function in those two cytokine receptor complexes could not be produced in this work. There are some contradictory reports of modulatory role of TIR8 in IL-1R/Toll like receptors, however the exact function of TIR8 needs to be investigated in more detail.

4.4. IL-33 does not need processing for bioactivity

Like IL-1α IL-33, is a dual function cytokine (reviewed in (Haraldsen et al., 2009). IL-33 exerts its function as a classical cytokine in a receptor-mediated fashion, normally in a paracrine manner, on target cells. In addition, IL-33 is able to translocate to the nucleus of the producing cell (Carriere et al., 2007) where it exerts gene regulatory functions. Similar to IL-1β and IL-18, IL-33 is produced as a precursor molecule which is reported to be cleaved by caspase 1 to yield a biologically active mature form (Schmitz et al., 2005). In contrast to previous report by Schmitz et al., 2005, this thesis demonstrates that IL-33 does not require caspase 1 mediated maturation for biological activity as a cytokine. Here it is shown that full length IL-33 can bind to the IL-33 receptor *in vitro* and on intact cells. Similar to the mature form reported by Scmitz et al., 2005, full length IL-33 bound to IL-33Rα allowed association and recuritment of IL-1RAcP which resulted in concentration- and time-dependent activation of NF-κB, as well as induction of cytokine production. In agreement with these findings activation of cells with full length IL-33 is also reported in three very recent publications (Cayrol and Girard, 2009; Luthi et al., 2009; Talabot-Ayer et al., 2009).

It was reported that caspase 1 cleaves full length human IL-33 at aa111 (corresponding to aa108 in full length murine IL-33)(Schmitz et al., 2005). Over expression of C-terminally tagged full length IL-33 in murine keratinocytes resulted two different C-terminal processing products. A faint band of processed IL-33, fits the mature form described by Schmitz, et al., 2005 however, major processing product was around 12 kDa protein which did not correspond in size to the expected mature form (aa109-266). *In silico* re-analysis of IL-33 sequences did not reveal a caspase 1 cleavage site, as suggested by some others as well (Carriere et al., 2007; Haraldsen et al., 2009; Talabot-Ayer et al., 2009), however, a non-classical caspase 3 cleavage site ($DGVD_{175}G$) located in the middle of the IL-1-like domain was found in the IL-33 sequence. This ($DGVD_{175}G$) sequence motif is conserved in mouse, rat and human IL-33 and had been previously identified in PKCζ (Frutos et al., 1999) to be accepted by caspase 3. The calculated fragments fitted much better to the bands observed in the gels after digestion of full length mIL-33. Caspase 3 cleavage sequence in full length IL-33 at aa175, was also identified recently by (Cayrol and Girard, 2009; Luthi et al., 2009).

Using N- and/or C-terminally epitope-tagged versions of full length murine IL-33 as wild-type form and with a point mutation at D175 (D175->A) it is confirmed that full length murine IL-33 was mainly processed at aa175 ($DGVD_{175}G$) in intact cells and *in vitro* by apoptotic caspase 3. However no cleavage (neither at aa108 nor at aa175) was observed by pro-inflammatory caspase 1. These results are in part contradictory to those reported recently in which it was shown that caspase 1 cleaved full length murine IL-33 at aa 108 (Schmitz et al., 2005) or at the caspase 3 cleavage site (aa,175) (Cayrol and Girard, 2009). Talabot-Ayer et al., 2009 and Luthi et al.,2009 however, reported that caspase 1 is not responsible for processing of full length IL-33 (Luthi et al., 2009; Talabot-Ayer et al., 2009). This discripincy in the results may be because of the contaminating proteases which are known to be present in reticulocyte lysates used by Schmitz et al., 2005 and Cayrol and Girad, 2009.

Caspase 3 cleavage at aa 175 inactivated the full length IL-33 bioactivity as a cytokine, while neither the N-terminal product of aa 1-175 nor the C-terminal product of aa 176-266 were able to bind to the receptor or activate cells. This finding is also reported very recently by Cayrol and Girad, 2009 and Luthi et al.,2009. Similar to IL-33 inactivation of IL-18 has also been reported by caspase 3 cleavage (Akita et al., 1997). In agreement with the recent reoprt by Cayrol and Girad, 2009 full length IL-33 with a point mutation at aa175 (D175->A) was able to bind and activate IL-33Rα in experiments performed in present work however, a D175->A mutant generated by Luthi et al., 2009 was inactive as acytokine. It is difficult to explain the difference in results however use of different epitope tags may explain the discrepancy in results.

Induction of apoptosis is known to activate apoptotic caspases including caspase-3 (Creagh et al., 2003). Here it was investigated that induction of apoptosis could initiate processing of full length IL-33 by endogenous caspases in cells undergoing apoptosis. Similar findings have been reported very recently by Cayrol and Girad, 2009 and Luthi et al., 2009. In addition caspase 7-mediated inactivation of IL-33 is also demonstrated in a recent report (Luthi et al., 2009). IL-33 has diverse target cell populations, including mast cells, CNS glia, basophils, eosinophils, NK, iNKT and others cells of the innate immune system (Ali et al., 2007; Allakhverdi et al., 2007; Iikura et al., 2007; Moulin et al., 2007; Pecaric-Petkovic et al., 2008; Smithgall et al., 2008; Suzukawa et al., 2008). Processing of IL-33 by caspase 3 may therefore provide a mechanism to inactivate pro-inflammatory cytokine activities of IL-33 during apoptosis, a process that does not trigger inflammation *in vivo*.

IL-33 was originally identified as a nuclear factor in specialized endothelial cells (Baekkevold et al., 2003) before it was designated a cytokine. Therefore IL-33, like IL-1α, was called a dual function cytokine (reviewed in (Haraldsen et al., 2009)). IL-33 is reported to be located in the nucleus of a variety of cell types, including endothelial and epithelial cells from different sources, glial cells, and skin associated keratinocytes (Carriere et al., 2007; Hudson et al., 2008; Kuchler et al., 2008; Moussion et al., 2008; Schmitz et al., 2005). It is not yet clear how IL-33 is released from cells. In the experiments depicted in this thesis, release of any bioactive IL-33 form was not detected in supernatants of intact cells, although the cells produced amounts of IL-33 readily detectable inside of the cell. In contrast to IL-33, bioactive IL-1β, or IL-18, even IL-1α, were easily detectable in supernatants indicating that the inflammasome had been activated and release mechanism worked. In consistant with these observations Cayrol and Girad, 2009 were also unable to find any evidence of release of IL-33 from endothelial cells under physiological conditions. However very recently release of bioactive IL-33 have been identified from glial cells (Hudson et al., 2008), and THP-1 cells (Li et al., 2008; Talabot-Ayer et al., 2009). Two mechanism of the release of prototypical alarmin HMGB1 have previously been shown, secretion by activated macrophages after hyperacylation of the lysine residues (Bianchi, 2007) or by passive release during cell damage or tissue injury (Scaffidi et al., 2002). Passive release of IL-1α has also been reported (Chen et al., 2007; Rao et al., 2007; Eigenbrod et al., 2008). In addition, IL-1α is also known to be released by an unconventional caspase 1-dependent mechanism which needs inflammasome activation (Keller et al., 2008). Keeping in view the result depicted in present work and some recent reports (Hudson et al., 2008; Kuchler et al., 2008; Li et al., 2008; Moussion et al., 2008; Cayrol and Girard, 2009; Luthi et al., 2009; Talabot-Ayer et al., 2009) it is highly likely that similar to IL-1α, IL-33 is released from cells both by cell or tissue damage and by inflammasome-dependent mechanism (Keller et al., 2008). Thus, similar to IL-1α (Dinarello, 1996; Chen et al., 2007; Rao et al., 2007; Eigenbrod et al., 2008) and the prototypical alarmin HMGB1 (Klune et al., 2008), IL-33 may also function as an endogenous danger signal (Matzinger, 2002) or alarmin (Bianchi, 2007), to alert cells of the innate immune system after tissue injury.

Upon overexpression full length IL-33 translocates to the nucleus, a feature it shares with IL-1α and IL-1F7b (reviewed in (Dinarello, 2009)). Interestingly, not only full length IL-33 translocated to the nucleus but also the caspase 3 cleaved N-terminal

fragment aa1-175, which is in accordance to previous reports stating that the sequences responsible for nuclear translocation reside in the N-terminus of full length IL-33 (Carriere et al., 2007). The C-terminal breakdown product did not translocate to the nucleus and remained completely in the cytosol. It remains to be demonstrated that this N-terminal fragment regulates gene transcription comparable to full length IL-33. While processing of full length murine IL-33 at aa175 destroys its cytokine function it does not abrogate nuclear translocation, presumably still allowing gene regulation.

In summary, IL-33 is functionally closer related to IL-1α than to IL-1β or IL-18, as it is also a member of the IL-1 family with dual function. IL-33 is able to interact with its receptor complex as a full length molecule, and it may also be processed to a mature bioactive form. In addition, the full length molecule and its N-terminal caspase 3 processing product translocate to the nucleus where they may exert gene regulatory functions. However, in contrast to IL-1α which is released from activated cells after processing by calpain, IL-33 does not seem to be released from intact cells after activation. Like full length IL-1α, full length IL-33 may act as an alarmin after cellular destruction or necrosis, e. g. after mechanical injury or hypoxia, which is released by dying cells into the environment where it acts as an alarm mediator signaling cellular damage, rather than cellular activation.

4.5. Comparison of IL-1, IL-18 and IL-33 induced gene expression

Among the well known members of the IL-1 family of cytokins, IL-1α and IL-1β are well characterized pro-inflammatory cytokines (Dinarello, 2006). IL-18 is a Th1 driving pro-inflammatory cytokine with the unique capacity of inducing the production of IFNγ (Dinarello, 1999). IL-33 drives Th2-responses (Schmitz et al., 2005) and was suggested to act as anti-inflammatory cytokine (Gadina and Jefferies, 2007). Although little is known about IL-33 activites some evidence suggests that IL-33 may have a dual role of pro-inflammatory and anti-inflammatory activities (Gadina and Jefferies, 2007). All three cytokines (IL-1, IL-18 and IL-33) are member of same IL-1 family and are closly related with respects to their structure, receptor binding, and signal transduction (reviewed in (Dinarello, 2005). All three share a large part of signal transduction pathways. However, they also have unique functions. Therefore it was important to compare the signaling induced by all three cytokines.

By global analysis of gene expression in EL-41B1 cells, significant induction of expression of a number of genes by IL-1, IL-18 and IL-33 was observed. The expression profile obtained by these stimuli suggests identical gene expression profile induced by IL-1, IL-18 and IL-33. No really differentially regulated gene was found, which can clearly suggest the involvement of unique molecules/pathways, that can be considered as target candidate to inhibit the signaling of one of these cytokines (other than specific receptor). Results reported here complements other recent studies that IL-1, IL-18 and IL-33 utilise similar signaling molecules/pathways (Schmitz et al., 2005; Dinarello, 2005; Barksby et al., 2007).

Receptors serve as gateways to pro-inflammatory signaling pathways. IL-1 receptor are expressed predominantly on T cells, fibroblasts, epithelial and endothelial cells (reviewed in (Boraschi and Tagliabue, 2006). IL-18 receptor are expressed mainly in mononuclear phagocytes, neutrophils, Th1 cells, NK cells, endothelial cells and smooth muscle cells (reviewed in (Boraschi and Tagliabue, 2006) where as the expression of IL-33 receptor is predominantly restricted to Th2 mast cells and fibroblasts (Lohning et al., 1998; Xu et al., 1998; Yanagisawa et al., 1997; Boraschi and Tagliabue, 2006; Kakkar and Lee, 2008). In addition the expression of cytokines is also restricted to the specified tissues. Thus, it is highly likely that the biological activities of IL-1 family members are mainly modulated in certain cell types according to the unique expression profile of the cytokines in specific cell/tissue types and expression of specific receptors on effecter cells. Detailed studies are needed to clarify the question of differential biological functions of these cytokines, when they seem to follow the same signaling pathway.

4.6. A model of IL-33 signaling

Taken together the data presented in this thesis and published in recent reports, a working model of IL-33 signaling is depicted in Fig.4.2. IL-33 is expressed in the variety of cell types constitutively and/or upon stimulation with pro-inflammatory molecules (Carriere et al., 2007; Hudson et al., 2008; Kuchler et al., 2008; Moussion et al., 2008; Schmitz et al., 2005). Similar to IL-1α it translocates to the nucleus of producing cells, associates with heterochromatin and in contrast to IL-1α exhibits transcriptional repressor properties (Roussel et al., 2008; Mizel et al., 1987; Gadina and Jefferies, 2007; Carriere et al., 2007). IL-33 does not need processing by caspases for this activity. Probably it is released from the cells by cell lysis during tissue injury. Upon release it can work as a classical cytokine via interleukin-33 receptor complex formation (comprising IL-33 receptor α-chain and IL-1RAcP) on cell surface of target cells.

In intracellular spaces IL-33 binds ST2/IL-33Rα (Schmitz et al., 2005), which in turn interact with IL-1RAcP and heterodimerize (Palmer et al., 2008; Chackerian et al., 2007; Ali et al., 2007). Similar to other members of the family recruitment of accessory protein is necessarily required for initiation of the signal transduction. Moreover, the TIR domains of receptor and co-receptor are crucial for initiation of signal transduction (Ali et al., 2007). Soluble form of IL-33 receptor also exists which can neutralize IL-33 activities (Hayakawa et al., 2007) by competing with the membrane IL-33 receptor α-chain for IL-33 binding and/or by formation of soluble-IL-33Rα-IL-33-membrane IL-1RAcP complex on cell surface. Existence of soluble form of IL-1RAcP is also well documented (Smith et al., 2003; Smeets et al., 2003; Lang et al., 1998). Soluble IL-1RAcP binds to ligand bound IL-33 receptor and may synergize the moudlatory activities of soluble IL-33 receptor (Palmer et al., 2008; Ali et al., 2007). However whether sIL-1RAcP can bind to the ligand receptor complex on cell suface is still to be investigated.

Like the other members of the family the activated IL-33 receptor complex has the ability to recruit and interact with MyD88, IRAK4, IRAK1 and TRAF6 (Schmitz et al., 2005). Similar to IL-1 and IL-18, IL-33 also activate the NF-κB, ERK1/2, JNK and p38 MAPK (Schmitz et al., 2005; Sanada et al., 2007; Kurowska-Stolarska et al., 2008; Ali et al., 2007). The ERK1/2, JNK and p38 MAPK activated transcription

factors and active NF-κB translocate to the nucleus and regulate the expression of various genes.

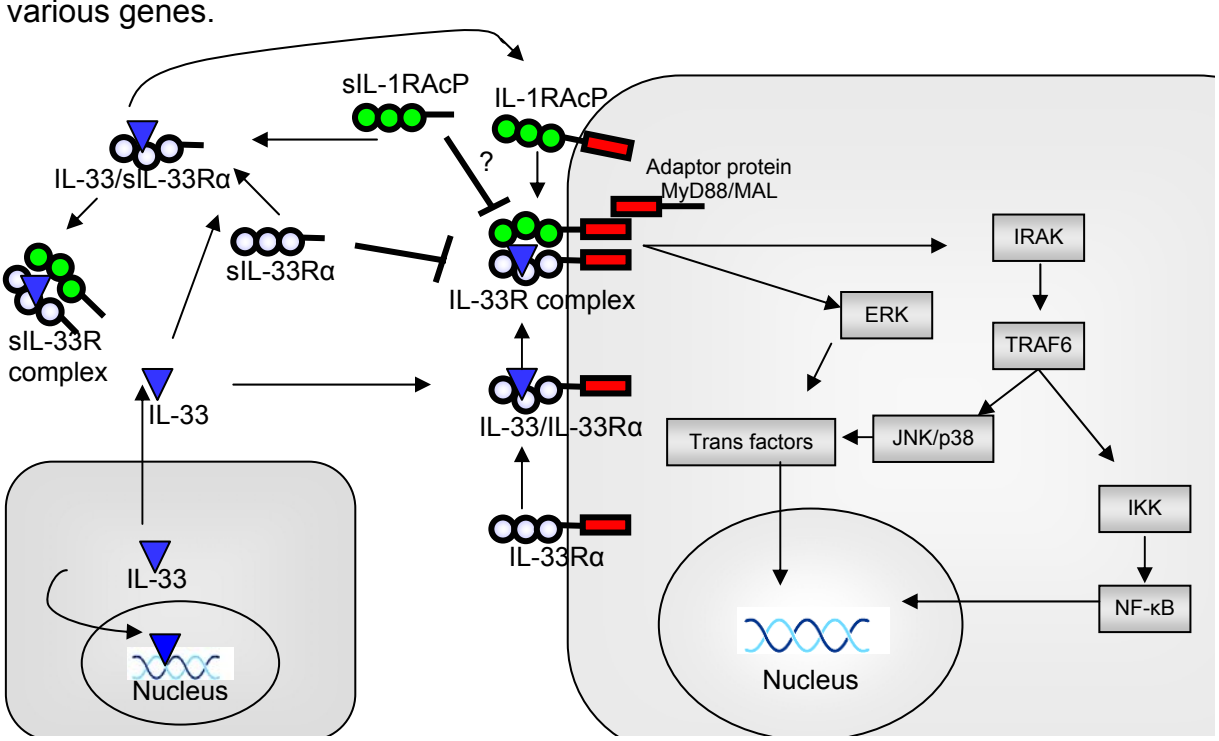


Fig. 4.2: A model for IL-33/ST2 signaling.
IL-33 does not need processing by caspase-1 for activation. It is likely that IL-33 may be released by cell damage during tissue injury. IL-33 binds to its receptor and generates a complex composed of IL-33Rα and the IL-1RAcP. Subsequent recruitment of the adaptor proteins MyD88 and MAL results in modulation of IRAK mediated TRAF6 activation and subsequent activation of JNK, p38 MAPK, ERK1/2 and IKK/NF-kB. Interestingly, although TRAF6 appears to be required for IL-33 mediated NF-kB activation and downstream induction of cytokines, IL-33 mediated ERK activation might be TRAF6 independent. Furthermore, the regulation of IL-33 signal modulation is still unclear. However, before IL-33 binds to its receptor, its action could be altered by the decoy receptor soluble ST2 (sST2). sST2 in the extracellular environment might bind free IL-33, thereby effectively decreasing the concentration of IL-33 that is available for IL-33Rα binding and reducing the biological effect of IL-33. Soluble form of IL-1RAcP may also contribute to neutralization of IL-33 activity.

It is generally accepted that the adaptor proteins MyD88 and the IL-1R-associated protein kinase (IRAK) activate downstream mitogen-activated protein kinase (MAPK) kinases through TRAF6, which activates TAK-1 which in turn activates JNKs and p38 MAPK. TRAF6 also activates the inhibitor of NF-κB (IKK) complex, leading to downstream liberation of active NF-kB from the complex (reviewed in (Kakkar and Lee, 2008; Dinarello, 2005; Martin and Wesche, 2002). Studies from mouse embryonic fibroblasts (MEFs) obtained from MyD88 and TRAF-6 knockout animals showed that p38, JNK and NF-kB activation induced by IL-33 is completely dependent on MyD88 and TRAF6, however IL-33-induced ERK activation might be TRAF6 independent (Funakoshi-Tago et al., 2008).

4.7. Perspectives

In summary IL-33 is an extremly interesting member of the IL-1 family of cytokines with distinct properties *mainly its role in Th2 and mast cells activation and maturation and cardiovascular pathophysiology*. Pharmacological intervension seems desirable in diseases in which IL-33 plays an essential role such as asthma, rheumatoid arthritis, atherosclerosis and cardiovascular disorders.

Given the fact that signal transduction pathways activated by IL-33, IL-1 and IL-18 are more or less identical, the IL-33 receptor complex appears to be the most prominent target structure to achieve IL-33 -specific inhibition. This may be achieved by soluble receptors or anti -IL-33 receptor or anti-IL-33 antibodies.

However, before IL-33 is neutralized in disease its definite biological role should be absolutely clear. Is it produced after tissue –damage or does it enhance pathogenicity after necrosis?

The coming months and years will provide the necessory informations to confirm IL-33 and its receptor complex as therapeutic target structures for pharmacological intervention.

References

Akira,S. and Takeda,K. (2004). Toll-like receptor signalling. Nat. Rev. Immunol. *4*, 499-511.

Akita,K., Ohtsuki,T., Nukada,Y., Tanimoto,T., Namba,M., Okura,T., Takakura-Yamamoto,R., Torigoe,K., Gu,Y., Su,M.S., Fujii,M., Satoh-Itoh,M., Yamamoto,K., Kohno,K., Ikeda,M., and Kurimoto,M. (1997). Involvement of caspase-1 and caspase-3 in the production and processing of mature human interleukin 18 in monocytic THP.1 cells. J. Biol. Chem. *272*, 26595-26603.

Ali,S., Huber,M., Kollewe,C., Bischoff,S.C., Falk,W., and Martin,M.U. (2007). IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells. Proc. Natl. Acad. Sci. U. S. A *104*, 18660-18665.

Allakhverdi,Z., Smith,D.E., Comeau,M.R., and Delespesse,G. (2007). Cutting edge: The ST2 ligand IL-33 potently activates and drives maturation of human mast cells. J. Immunol. *179*, 2051-2054.

Andrei,C., Margiocco,P., Poggi,A., Lotti,L.V., Torrisi,M.R., and Rubartelli,A. (2004). Phospholipases C and A2 control lysosome-mediated IL-1 beta secretion: Implications for inflammatory processes. Proc. Natl. Acad. Sci. U. S. A *101*, 9745-9750.

Baekkevold,E.S., Roussigne,M., Yamanaka,T., Johansen,F.E., Jahnsen,F.L., Amalric,F., Brandtzaeg,P., Erard,M., Haraldsen,G., and Girard,J.P. (2003). Molecular characterization of NF-HEV, a nuclear factor preferentially expressed in human high endothelial venules. Am. J. Pathol. *163*, 69-79.

Barksby,H.E., Lea,S.R., Preshaw,P.M., and Taylor,J.J. (2007). The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders. Clin. Exp. Immunol. *149*, 217-225.

Bianchi,M.E. (2007). DAMPs, PAMPs and alarmins: all we need to know about danger. J. Leukoc. Biol. *81*, 1-5.

Boraschi,D. and Tagliabue,A. (2006). The interleukin-1 receptor family. Vitam. Horm. *74*, 229-254.

Born,T.L., Smith,D.E., Garka,K.E., Renshaw,B.R., Bertles,J.S., and Sims,J.E. (2000). Identification and characterization of two members of a novel class of the interleukin-1 receptor (IL-1R) family. Delineation of a new class of IL-1R-related proteins based on signaling. J. Biol. Chem. *275*, 29946-29954.

Born,T.L., Thomassen,E., Bird,T.A., and Sims,J.E. (1998). Cloning of a novel receptor subunit, AcPL, required for interleukin-18 signaling. J. Biol. Chem. *273*, 29445-29450.

Bourgeois,E., Van,L.P., Samson,M., Diem,S., Barra,A., Roga,S., Gombert,J.M., Schneider,E., Dy,M., Gourdy,P., Girard,J.P., and Herbelin,A. (2009). The pro-Th2 cytokine IL-33 directly interacts with invariant NKT and NK cells to induce IFN-gamma production. Eur. J. Immunol.

Brint,E.K., Fitzgerald,K.A., Smith,P., Coyle,A.J., Gutierrez-Ramos,J.C., Fallon,P.G., and O'Neill,L.A. (2002). Characterization of signaling pathways activated by the interleukin 1 (IL-1) receptor homologue T1/ST2. A role for Jun N-terminal kinase in IL-4 induction. J. Biol. Chem. *277*, 49205-49211.

Bufler,P., Azam,T., Gamboni-Robertson,F., Reznikov,L.L., Kumar,S., Dinarello,C.A., and Kim,S.H. (2002). A complex of the IL-1 homologue IL-1F7b and IL-18-binding protein reduces IL-18 activity. Proc. Natl. Acad. Sci. U. S. A *99*, 13723-13728.

Bulek,K., Swaidani,S., Qin,J., Lu,Y., Gulen,M.F., Herjan,T., Min,B., Kastelein,R.A., Aronica,M., Kosz-Vnenchak,M., and Li,X. (2009). The essential role of single Ig IL-1 receptor-related molecule/Toll IL-1R8 in regulation of Th2 immune response. J. Immunol. *182*, 2601-2609.

Burger,D., Dayer,J.M., Palmer,G., and Gabay,C. (2006). Is IL-I a good therapeutic target in the treatment of arthritis? Best Practice & Research in Clinical Rheumatology *20*, 879-896.

Burns,K., Clatworthy,J., Martin,L., Martinon,F., Plumpton,C., Maschera,B., Lewis,A., Ray,K., Tschopp,J., and Volpe,F. (2000). Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nat. Cell Biol. *2*, 346-351.

Burns,K., Janssens,S., Brissoni,B., Olivos,N., Beyaert,R., and Tschopp,J. (2003). Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. J. Exp. Med. *197*, 263-268.

Burns,K., Martinon,F., Esslinger,C., Pahl,H., Schneider,P., Bodmer,J.L., Di Marco,F., French,L., and Tschopp,J. (1998). MyD88, an adapter protein involved in interleukin-1 signaling. J. Biol. Chem. *273*, 12203-12209.

Cao,Z., Henzel,W.J., and Gao,X. (1996). IRAK: a kinase associated with the interleukin-1 receptor. Science *271*, 1128-1131.

Carriere,V., Roussel,L., Ortega,N., Lacorre,D.A., Americh,L., Aguilar,L., Bouche,G., and Girard,J.P. (2007). IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc. Natl. Acad. Sci. U. S. A *104*, 282-287.

Carroll,H.P., Paunovic,V., and Gadina,M. (2008). Signalling, inflammation and arthritis: Crossed signals: the role of interleukin-15 and -18 in autoimmunity. Rheumatology (Oxford) *47*, 1269-1277.

Cayrol,C. and Girard,J.P. (2009). The IL-1-like cytokine IL-33 is inactivated after maturation by caspase-1. Proc. Natl. Acad. Sci. U. S. A *106*, 9021-9026.

Chackerian,A.A., Oldham,E.R., Murphy,E.E., Schmitz,J., Pflanz,S., and Kastelein,R.A. (2007). IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. J. Immunol. *179*, 2551-2555.

Chen,C.J., Kono,H., Golenbock,D., Reed,G., Akira,S., and Rock,K.L. (2007). Identification of a key pathway required for the sterile inflammatory response triggered by dying cells. Nat. Med. *13*, 851-856.

Colotta,F., Dower,S.K., Sims,J.E., and Mantovani,A. (1994). The type II 'decoy' receptor: a novel regulatory pathway for interleukin 1. Immunol. Today *15*, 562-566.

Colotta,F., Re,F., Muzio,M., Bertini,R., Polentarutti,N., Sironi,M., Giri,J.G., Dower,S.K., Sims,J.E., and Mantovani,A. (1993). Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science *261*, 472-475.

Creagh,E.M., Conroy,H., and Martin,S.J. (2003). Caspase-activation pathways in apoptosis and immunity. Immunol. Rev. *193*, 10-21.

Cullinan,E.B., Kwee,L., Nunes,P., Shuster,D.J., Ju,G., McIntyre,K.W., Chizzonite,R.A., and Labow,M.A. (1998). IL-1 receptor accessory protein is an essential component of the IL-1 receptor. J. Immunol. *161*, 5614-5620.

Debets,R., Timans,J.C., Homey,B., Zurawski,S., Sana,T.R., Lo,S., Wagner,J., Edwards,G., Clifford,T., Menon,S., Bazan,J.F., and Kastelein,R.A. (2001). Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2. J. Immunol. *167*, 1440-1446.

Dinarello,C.A. (1996). Biologic basis for interleukin-1 in disease. Blood *87*, 2095-2147.

Dinarello,C.A. (1998). Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme. Ann. N. Y. Acad. Sci. *856*, 1-11.

Dinarello,C.A. (1999). Interleukin-18. Methods *19*, 121-132.

Dinarello,C.A. (2002). The IL-1 family and inflammatory diseases. Clinical and Experimental Rheumatology *20*, S1-S13.

Dinarello,C.A. (2005). An IL-1 family member requires caspase-1 processing and signals through the ST2 receptor. Immunity. *23*, 461-462.

Dinarello,C.A. (2006). Interleukin 1 and interleukin 18 as mediators of inflammation and the aging process. Am. J. Clin. Nutr. *83*, 447S-455S.

Dinarello,C.A. (2009). Immunological and inflammatory functions of the interleukin-1 family. Annu. Rev. Immunol. *27*, 519-550.

Dinarello,C.A. and Fantuzzi,G. (2003). Interleukin-18 and host defense against infection. Journal of Infectious Diseases *187*, S370-S384.

Dinarello,C.A., Novick,D., Rubinstein,M., and Lonnemann,G. (2003). Interleukin 18 and interleukin 18 binding protein: possible role in immunosuppression of chronic renal failure. Blood Purif. *21*, 258-270.

Dunn,E., Sims,J.E., Nicklin,M.J.H., and O'Neill,L.A.J. (2001). Annotating genes with potential roles in the immune system: six new members of them IL-1 family. Trends in Immunology *22*, 533-536.

Economides,A.N., Carpenter,L.R., Rudge,J.S., Wong,V., Koehler-Stec,E.M., Hartnett,C., Pyles,E.A., Xu,X., Daly,T.J., Young,M.R., Fandl,J.P., Lee,F., Carver,S., McNay,J., Bailey,K., Ramakanth,S., Hutabarat,R., Huang,T.T., Radziejewski,C., Yancopoulos,G.D., and Stahl,N. (2003). Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nat. Med. *9*, 47-52.

Ehrhardt,C., Schmolke,M., Matzke,A., Knoblauch,A., Will,C., Wixler,V., and Ludwig,S. (2006). Polyethylenimine, a cost-effective transfection reagent. Signal Transduction *6*, 179-184.

Eigenbrod,T., Park,J.H., Harder,J., Iwakura,Y., and Nunez,G. (2008). Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells. J. Immunol. *181*, 8194-8198.

Ferrari,D., Pizzirani,C., Adinolfi,E., Lemoli,R.M., Curti,A., Idzko,M., Panther,E., and Di Virgilio,F. (2006). The P2X7 receptor: a key player in IL-1 processing and release. J. Immunol. *176*, 3877-3883.

Fraczek,J., Kim,T.W., Xiao,H., Yao,J., Wen,Q., Li,Y., Casanova,J.L., Pryjma,J., and Li,X. (2008). The kinase activity of IL-1 receptor-associated kinase 4 is required for interleukin-1 receptor/toll-like receptor-induced TAK1-dependent NFkappaB activation. J. Biol. Chem. *283*, 31697-31705.

Frutos,S., Moscat,J., and Diaz-Meco,M.T. (1999). Cleavage of zetaPKC but not lambda/iotaPKC by caspase-3 during UV-induced apoptosis. J. Biol. Chem. *274*, 10765-10770.

Funakoshi-Tago,M., Tago,K., Hayakawa,M., Tominaga,S., Ohshio,T., Sonoda,Y., and Kasahara,T. (2008). TRAF6 is a critical signal transducer in IL-33 signaling pathway. Cell Signal. *20*, 1679-1686.

Gabay,C. and McInnes,I.B. (2009). The biological and clinical importance of the 'new generation' cytokines in rheumatic diseases. Arthritis Res. Ther. *11*, 230.

Gadina,M. and Jefferies,C.A. (2007). IL-33: a sheep in wolf's clothing? Sci. STKE. *2007*, e31.

Garlanda,C., Riva,F., Polentarutti,N., Buracchi,C., Sironi,M., De Bortoli,M., Muzio,M., Bergottini,R., Scanziani,E., Vecchi,A., Hirsch,E., and Mantovani,A. (2004). Intestinal inflammation in mice deficient in Tir8, an inhibitory member of the IL-1 receptor family. Proc. Natl. Acad. Sci. U. S. A *101*, 3522-3526.

Gimborn,K., Lessmann,E., Kuppig,S., Krystal,G., and Huber,M. (2005). SHIP down-regulates FcepsilonR1-induced degranulation at supraoptimal IgE or antigen levels. J. Immunol. *174*, 507-516.

Gracie,J.A., Robertson,S.E., and McInnes,I.B. (2003). Interleukin-18. J. Leukoc. Biol. *73*, 213-224.

Greenfeder,S.A., Nunes,P., Kwee,L., Labow,M., Chizzonite,R.A., and Ju,G. (1995). Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J. Biol. Chem. *270*, 13757-13765.

Gu,Y., Kuida,K., Tsutsui,H., Ku,G., Hsiao,K., Fleming,M.A., Hayashi,N., Higashino,K., Okamura,H., Nakanishi,K., Kurimoto,M., Tanimoto,T., Flavell,R.A., Sato,V., Harding,M.W., Livingston,D.J., and Su,M.S. (1997). Activation of interferon-gamma inducing factor mediated by interleukin-1beta converting enzyme. Science *275*, 206-209.

Haraldsen,G., Balogh,J., Pollheimer,J., Sponheim,J., and Kuchler,A.M. (2009). Interleukin-33 - cytokine of dual function or novel alarmin? Trends Immunol. *30*, 227-233.

Harrington,L.E., Mangan,P.R., and Weaver,C.T. (2006). Expanding the effector CD4 T-cell repertoire: the Th17 lineage. Current Opinion in Immunology *18*, 349-356.

Hayakawa,H., Hayakawa,M., Kume,A., and Tominaga,S. (2007). Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. J. Biol. Chem. *282*, 26369-26380.

Hestdal,K., Ruscetti,F.W., Chizzonite,R., Ortiz,M., Gooya,J.M., Longo,D.L., and Keller,J.R. (1994). Interleukin-1 (IL-1) directly and indirectly promotes hematopoietic cell growth through type I IL-1 receptor. Blood *84*, 125-132.

Ho,L.H., Ohno,T., Oboki,K., Kajiwara,N., Suto,H., Iikura,M., Okayama,Y., Akira,S., Saito,H., Galli,S.J., and Nakae,S. (2007). IL-33 induces IL-13 production by mouse mast cells independently of IgE-FcepsilonRI signals. J. Leukoc. Biol. *82*, 1481-1490.

Hofmeister,R., Wiegmann,K., Korherr,C., Bernardo,K., Kronke,M., and Falk,W. (1997). Activation of acid sphingomyelinase by interleukin-1 (IL-1) requires the IL-1 receptor accessory protein. J. Biol. Chem. *272*, 27730-27736.

Huang,J., Gao,X., Li,S., and Cao,Z. (1997). Recruitment of IRAK to the interleukin 1 receptor complex requires interleukin 1 receptor accessory protein. Proc. Natl. Acad. Sci. U. S. A *94*, 12829-12832.

Hudson,C.A., Christophi,G.P., Gruber,R.C., Wilmore,J.R., Lawrence,D.A., and Massa,P.T. (2008). Induction of IL-33 expression and activity in central nervous system glia. J. Leukoc. Biol. *84*, 631-643.

Iikura,M., Suto,H., Kajiwara,N., Oboki,K., Ohno,T., Okayama,Y., Saito,H., Galli,S.J., and Nakae,S. (2007). IL-33 can promote survival, adhesion and cytokine production in human mast cells. Lab Invest *87*, 971-978.

Janssens,S., Burns,K., Tschopp,J., and Beyaert,R. (2002). Regulation of interleukin-1- and lipopolysaccharide-induced NF-kappaB activation by alternative splicing of MyD88. Curr. Biol. *12*, 467-471.

Jensen,L.E., Muzio,M., Mantovani,A., and Whitehead,A.S. (2000). IL-1 signaling cascade in liver cells and the involvement of a soluble form of the IL-1 receptor accessory protein. J. Immunol. *164*, 5277-5286.

Jiang,Z., Ninomiya-Tsuji,J., Qian,Y., Matsumoto,K., and Li,X. (2002). Interleukin-1 (IL-1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol. Mol. Cell Biol. *22*, 7158-7167.

Kakkar,R. and Lee,R.T. (2008). The IL-33/ST2 pathway: therapeutic target and novel biomarker. Nat. Rev. Drug Discov. *7*, 827-840.

Keller,M., Ruegg,A., Werner,S., and Beer,H.D. (2008). Active caspase-1 is a regulator of unconventional protein secretion. Cell *132*, 818-831.

Kim,S.H., Eisenstein,M., Reznikov,L., Fantuzzi,G., Novick,D., Rubinstein,M., and Dinarello,C.A. (2000). Structural requirements of six naturally occurring isoforms of the IL-18 binding protein to inhibit IL-18. Proc. Natl. Acad. Sci. U. S. A *97*, 1190-1195.

Klemenz,R., Hoffmann,S., and Werenskiold,A.K. (1989). Serum- and oncoprotein-mediated induction of a gene with sequence similarity to the gene encoding carcinoembryonic antigen. Proc. Natl. Acad. Sci. U. S. A *86*, 5708-5712.

Klune,J.R., Dhupar,R., Cardinal,J., Billiar,T.R., and Tsung,A. (2008). HMGB1: endogenous danger signaling. Mol. Med. *14*, 476-484.

Knop,J., Wesche,H., Lang,D., and Martin,M.U. (1998). Effects of overexpression of IL-1 receptor-associated kinase on NFkappaB activation, IL-2 production and stress-activated protein kinases in the murine T cell line EL4. Eur. J. Immunol. *28*, 3100-3109.

Kollewe,C., Mackensen,A.C., Neumann,D., Knop,J., Cao,P., Li,S., Wesche,H., and Martin,M.U. (2004). Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. J. Biol. Chem. *279*, 5227-5236.

Komai-Koma,M., Xu,D., Li,Y., McKenzie,A.N., McInnes,I.B., and Liew,F.Y. (2007). IL-33 is a chemoattractant for human Th2 cells. Eur. J. Immunol. *37*, 2779-2786.

Kondo,Y., Yoshimoto,T., Yasuda,K., Futatsugi-Yumikura,S., Morimoto,M., Hayashi,N., Hoshino,T., Fujimoto,J., and Nakanishi,K. (2008). Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. Int. Immunol. *20*, 791-800.

Korherr,C., Hofmeister,R., Wesche,H., and Falk,W. (1997). A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling. Eur. J. Immunol. *27*, 262-267.

Kuchler,A.M., Pollheimer,J., Balogh,J., Sponheim,J., Manley,L., Sorensen,D.R., De Angelis,P.M., Scott,H., and Haraldsen,G. (2008). Nuclear interleukin-33 is generally expressed in resting endothelium but rapidly lost upon angiogenic or proinflammatory activation. Am. J. Pathol. *173*, 1229-1242.

Kumar,S., Hanning,C.R., Brigham-Burke,M.R., Rieman,D.J., Lehr,R., Khandekar,S., Kirkpatrick,R.B., Scott,G.F., Lee,J.C., Lynch,F.J., Gao,W., Gambotto,A., and Lotze,M.T. (2002). Interleukin-1F7B (IL-1H4/IL-1F7) is processed by caspase-1 and mature IL-1F7B binds to the IL-18 receptor but does not induce IFN-gamma production. Cytokine *18*, 61-71.

Kumar,S., McDonnell,P.C., Lehr,R., Tierney,L., Tzimas,M.N., Griswold,D.E., Capper,E.A., Tal-Singer,R., Wells,G.I., Doyle,M.L., and Young,P.R. (2000). Identification and initial characterization of four novel members of the interleukin-1 family. J. Biol. Chem. *275*, 10308-10314.

Kuroiwa,K., Li,H., Tago,K., Iwahana,H., Yanagisawa,K., Komatsu,N., Oshikawa,K., Sugiyama,Y., Arai,T., and Tominaga,S.I. (2000). Construction of ELISA system to quantify human ST2 protein in sera of patients. Hybridoma *19*, 151-159.

Kurowska-Stolarska,M., Kewin,P., Murphy,G., Russo,R.C., Stolarski,B., Garcia,C.C., Komai-Koma,M., Pitman,N., Li,Y., McKenzie,A.N., Teixeira,M.M., Liew,F.Y., and Xu,D. (2008). IL-33 induces antigen-specific IL-5+ T cells and promotes allergic-induced airway inflammation independent of IL-4. J. Immunol. *181*, 4780-4790.

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

Lang,D., Knop,J., Wesche,H., Raffetseder,U., Kurrle,R., Boraschi,D., and Martin,M.U. (1998). The type II IL-1 receptor interacts with the IL-1 receptor accessory protein: a novel mechanism of regulation of IL-1 responsiveness. J. Immunol. *161*, 6871-6877.

Li,H., Willingham,S.B., Ting,J.P., and Re,F. (2008). Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J. Immunol. *181*, 17-21.

Liew,F.Y., Xu,D., Brint,E.K., and O'Neill,L.A. (2005). Negative regulation of toll-like receptor-mediated immune responses. Nat. Rev. Immunol. *5*, 446-458.

Lin,H., Ho,A.S., Haley-Vicente,D., Zhang,J., Bernal-Fussell,J., Pace,A.M., Hansen,D., Schweighofer,K., Mize,N.K., and Ford,J.E. (2001). Cloning and characterization of IL-1HY2, a novel interleukin-1 family member. J. Biol. Chem. *276*, 20597-20602.

Lohning,M., Stroehmann,A., Coyle,A.J., Grogan,J.L., Lin,S., Gutierrez-Ramos,J.C., Levinson,D., Radbruch,A., and Kamradt,T. (1998). T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. Proc. Natl. Acad. Sci. U. S. A *95*, 6930-6935.

Luthi,A.U., Cullen,S.P., McNeela,E.A., Duriez,P.J., Afonina,I.S., Sheridan,C., Brumatti,G., Taylor,R.C., Kersse,K., Vandenabeele,P., Lavelle,E.C., and Martin,S.J. (2009). Suppression of Interleukin-33 Bioactivity through Proteolysis by Apoptotic Caspases. Immunity.

MacKenzie,A., Wilson,H.L., Kiss-Toth,E., Dower,S.K., North,R.A., and Surprenant,A. (2001). Rapid secretion of interleukin-1beta by microvesicle shedding. Immunity *15*, 825-835.

Mantovani,A., Locati,M., Polentarutti,N., Vecchi,A., and Garlanda,C. (2004). Extracellular and intracellular decoys in the tuning of inflammatory cytokines and Toll-like receptors: the new entry TIR8/SIGIRR. J. Leukoc. Biol. *75*, 738-742.

Mantovani,A., Locati,M., Vecchi,A., Sozzani,S., and Allavena,P. (2001). Decoy receptors: a strategy to regulate inflammatory cytokines and chemokines. Trends Immunol. *22*, 328-336.

Martin,M.U. and Wesche,H. (2002). Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. Biochim. Biophys. Acta *1592*, 265-280.

Matzinger,P. (2002). The danger model: a renewed sense of self. Science *296*, 301-305.

McInnes,I.B., Gracie,J.A., Leung,B.P., Wei,X.Q., and Liew,F.Y. (2000). Interleukin 18: a pleiotropic participant in chronic inflammation. Immunol. Today *21*, 312-315.

McMahan,C.J., Slack,J.L., Mosley,B., Cosman,D., Lupton,S.D., Brunton,L.L., Grubin,C.E., Wignall,J.M., Jenkins,N.A., Brannan,C.I., and . (1991). A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. EMBO J. *10*, 2821-2832.

Miller,A.M., Xu,D., Asquith,D.L., Denby,L., Li,Y., Sattar,N., Baker,A.H., McInnes,I.B., and Liew,F.Y. (2008). IL-33 reduces the development of atherosclerosis. J. Exp. Med. *205*, 339-346.

Mitcham,J.L., Parnet,P., Bonnert,T.P., Garka,K.E., Gerhart,M.J., Slack,J.L., Gayle,M.A., Dower,S.K., and Sims,J.E. (1996). T1/ST2 signaling establishes it as a member of an expanding interleukin-1 receptor family. J. Biol. Chem. *271*, 5777-5783.

Mizel,S.B., Kilian,P.L., Lewis,J.C., Paganelli,K.A., and Chizzonite,R.A. (1987). The interleukin 1 receptor. Dynamics of interleukin 1 binding and internalization in T cells and fibroblasts. J. Immunol. *138*, 2906-2912.

Mosley,B., Urdal,D.L., Prickett,K.S., Larsen,A., Cosman,D., Conlon,P.J., Gillis,S., and Dower,S.K. (1987). The interleukin-1 receptor binds the human interleukin-1 alpha precursor but not the interleukin-1 beta precursor. J. Biol. Chem. *262*, 2941-2944.

Moulin,D., Donze,O., Talabot-Ayer,D., Mezin,F., Palmer,G., and Gabay,C. (2007). Interleukin (IL)-33 induces the release of pro-inflammatory mediators by mast cells. Cytokine *40*, 216-225.

Moussion,C., Ortega,N., and Girard,J.P. (2008). The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells in vivo: a novel 'alarmin'? PLoS. ONE. *3*, e3331.

Moynagh,P.N. (2009). The Pellino family: IRAK E3 ligases with emerging roles in innate immune signalling. Trends Immunol. *30*, 33-42.

Muzio,M., Ni,J., Feng,P., and Dixit,V.M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. Science *278*, 1612-1615.

Neumann,D., Kollewe,C., Martin,M.U., and Boraschi,D. (2000). The membrane form of the type II IL-1 receptor accounts for inhibitory function. J. Immunol. *165*, 3350-3357.

Nicklin,M.J.H., Barton,J.L., Nguyen,M., FitzGerald,M.G., Duff,G.W., and Kornman,K. (2002). A sequence-based map of the nine genes of the human interleukin-1 cluster. Genomics *79*, 718-725.

Norris,J.L. and Manley,J.L. (1995). Regulation of dorsal in cultured cells by Toll and tube: tube function involves a novel mechanism. Genes Dev. *9*, 358-369.

Novick,D., Kim,S.H., Fantuzzi,G., Reznikov,L.L., Dinarello,C.A., and Rubinstein,M. (1999). Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. Immunity *10*, 127-136.

O'Neill,L.A. (2008). The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunol. Rev. *226*, 10-18.

Ordureau,A., Smith,H., Windheim,M., Peggie,M., Carrick,E., Morrice,N., and Cohen,P. (2008). The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys63-linked polyubiquitination of IRAK1. Biochem. J. *409*, 43-52.

Oshikawa,K., Kuroiwa,K., Tago,K., Iwahana,H., Yanagisawa,K., Ohno,S., Tominaga,S.I., and Sugiyama,Y. (2001). Elevated soluble ST2 protein levels in sera of patients with asthma with an acute exacerbation. Am. J. Respir. Crit Care Med. *164*, 277-281.

Palmer,G., Lipsky,B.P., Smithgall,M.D., Meininger,D., Siu,S., Talabot-Ayer,D., Gabay,C., and Smith,D.E. (2008). The IL-1 receptor accessory protein (AcP) is required for IL-33 signaling and soluble AcP enhances the ability of soluble ST2 to inhibit IL-33. Cytokine *42*, 358-364.

Paulukat,J., Bosmann,M., Nold,M., Garkisch,S., Kampfer,H., Frank,S., Raedle,J., Zeuzem,S., Pfeilschifter,J., and Muhl,H. (2001). Expression and release of IL-18 binding protein in response to IFN-gamma. J. Immunol. *167*, 7038-7043.

Pecaric-Petkovic,T., Didichenko,S.A., Kaempfer,S., Spiegl,N., and Dahinden,C.A. (2008). Human basophils and eosinophils are the direct target leukocytes of the novel IL-1-family member IL-33. Blood.

Polentarutti,N., Rol,G.P., Muzio,M., Bosisio,D., Camnasio,M., Riva,F., Zoja,C., Benigni,A., Tomasoni,S., Vecchi,A., Garlanda,C., and Mantovani,A. (2003). Unique pattern of expression and inhibition of IL-1 signaling by the IL-1 receptor family member TIR8/SIGIRR. Eur. Cytokine Netw. *14*, 211-218.

Qin,J., Qian,Y., Yao,J., Grace,C., and Li,X. (2005). SIGIRR inhibits interleukin-1 receptor- and toll-like receptor 4-mediated signaling through different mechanisms. J. Biol. Chem. *280*, 25233-25241.

Radons,J., Gabler,S., Wesche,H., Korherr,C., Hofmeister,R., and Falk,W. (2002). Identification of essential regions in the cytoplasmic tail of interleukin-1 receptor accessory protein critical for interleukin-1 signaling. J. Biol. Chem. *277*, 16456-16463.

Rao,D.A., Tracey,K.J., and Pober,J.S. (2007). IL-1alpha and IL-1beta are endogenous mediators linking cell injury to the adaptive alloimmune response. J. Immunol. *179*, 6536-6546.

Robertson,S.E., Young,J.D., Kitson,S., Pitt,A., Evans,J., Roes,J., Karaoglu,D., Santora,L., Ghayur,T., Liew,F.Y., Gracie,J.A., and McInnes,I.B. (2006). Expression and alternative processing of IL-18 in human neutrophils. Eur. J. Immunol. *36*, 722-731.

Roussel,L., Erard,M., Cayrol,C., and Girard,J.P. (2008). Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. EMBO Rep. *9*, 1006-1012.

Salvi,G.E. and Lang,N.P. (2005). Host response modulation in the management of periodontal diseases. Journal of Clinical Periodontology *32*, 108-129.

Sanada,S., Hakuno,D., Higgins,L.J., Schreiter,E.R., McKenzie,A.N., and Lee,R.T. (2007). IL-33 and ST2 comprise a critical biomechanically induced and cardioprotective signaling system. J. Clin. Invest *117*, 1538-1549.

Scaffidi,P., Misteli,T., and Bianchi,M.E. (2002). Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature *418*, 191-195.

Schall,T.J., Lewis,M., Koller,K.J., Lee,A., Rice,G.C., Wong,G.H., Gatanaga,T., Granger,G.A., Lentz,R., Raab,H., and . (1990). Molecular cloning and expression of a receptor for human tumor necrosis factor. Cell *61*, 361-370.

Schmitz,J., Owyang,A., Oldham,E., Song,Y., Murphy,E., McClanahan,T.K., Zurawski,G., Moshrefi,M., Qin,J., Li,X., Gorman,D.M., Bazan,J.F., and Kastelein,R.A. (2005). IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity. *23*, 479-490.

Sharma,S., Kulk,N., Nold,M.F., Graf,R., Kim,S.H., Reinhardt,D., Dinarello,C.A., and Bufler,P. (2008). The IL-1 family member 7b translocates to the nucleus and down-regulates proinflammatory cytokines. J. Immunol. *180*, 5477-5482.

Sims,J.E. (2002). IL-1 and IL-18 receptors, and their extended family. Curr. Opin. Immunol. *14*, 117-122.

Sims,J.E., March,C.J., Cosman,D., Widmer,M.B., MacDonald,H.R., McMahan,C.J., Grubin,C.E., Wignall,J.M., Jackson,J.L., Call,S.M., and . (1988). cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily. Science *241*, 585-589.

Sims,J.E., Nicklin,M.J.H., Bazan,J.F., Barton,J.L., Busfield,S.J., Ford,J.E., Kastelein,R.A., Kumar,S., Lin,H.S., Mulero,J.J., Pan,J., Pan,Y., Smith,D.E., and Young,P.R. (2001). A new nomenclature for IL-1-family genes. Trends in Immunology *22*, 536-537.

Smeets,R.L., van de Loo,F.A., Joosten,L.A., Arntz,O.J., Bennink,M.B., Loesberg,W.A., Dmitriev,I.P., Curiel,D.T., Martin,M.U., and van den Berg,W.B. (2003). Effectiveness of the soluble form of the interleukin-1 receptor accessory protein as an inhibitor of interleukin-1 in collagen-induced arthritis. Arthritis Rheum. *48*, 2949-2958.

Smith,D.E., Hanna,R., Della,F., Moore,H., Chen,H., Farese,A.M., MacVittie,T.J., Virca,G.D., and Sims,J.E. (2003). The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. Immunity *18*, 87-96.

Smith,D.E., Renshaw,B.R., Ketchem,R.R., Kubin,M., Garka,K.E., and Sims,J.E. (2000). Four new members expand the interleukin-1 superfamily. J. Biol. Chem. *275*, 1169-1175.

Smithgall,M.D., Comeau,M.R., Yoon,B.R., Kaufman,D., Armitage,R., and Smith,D.E. (2008). IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. Int. Immunol. *20*, 1019-1030.

Subramaniam,S., Stansberg,C., and Cunningham,C. (2004). The interleukin 1 receptor family. Dev. Comp Immunol. *28*, 415-428.

Sugawara,S., Uehara,A., Nochi,T., Yamaguchi,T., Ueda,H., Sugiyama,A., Hanzawa,K., Kumagai,K., Okamura,H., and Takada,H. (2001). Neutrophil proteinase 3-mediated induction of bioactive IL-18 secretion by human oral epithelial cells. J. Immunol. *167*, 6568-6575.

Suzukawa,M., Iikura,M., Koketsu,R., Nagase,H., Tamura,C., Komiya,A., Nakae,S., Matsushima,K., Ohta,K., Yamamoto,K., and Yamaguchi,M. (2008). An IL-1 cytokine member, IL-33, induces human basophil activation via its ST2 receptor. J. Immunol. *181*, 5981-5989.

Takaesu,G., Kishida,S., Hiyama,A., Yamaguchi,K., Shibuya,H., Irie,K., Ninomiya-Tsuji,J., and Matsumoto,K. (2000). TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol. Cell *5*, 649-658.

Talabot-Ayer,D., Lamacchia,C., Gabay,C., and Palmer,G. (2009). Interleukin-33 is biologically active independently of caspase-1 cleavage. J. Biol. Chem.

Tavernier,J., Devos,R., Cornelis,S., Tuypens,T., Van der,H.J., Fiers,W., and Plaetinck,G. (1991). A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. Cell *66*, 1175-1184.

Taylor,S.L., Renshaw,B.R., Garka,K.E., Smith,D.E., and Sims,J.E. (2002). Genomic organization of the interleukin-1 locus. Genomics *79*, 726-733.

Thomassen,E., Bird,T.A., Renshaw,B.R., Kennedy,M.K., and Sims,J.E. (1998). Binding of interleukin-18 to the interleukin-1 receptor homologous receptor IL-1Rrp1 leads to activation of signaling pathways similar to those used by interleukin-1. J. Interferon Cytokine Res. *18*, 1077-1088.

Thomassen,E., Renshaw,B.R., and Sims,J.E. (1999). Identification and characterization of SIGIRR, a molecule representing a novel subtype of the IL-1R superfamily. Cytokine *11*, 389-399.

Tominaga,S., Jenkins,N.A., Gilbert,D.J., Copeland,N.G., and Tetsuka,T. (1991). Molecular cloning of the murine ST2 gene. Characterization and chromosomal mapping. Biochim. Biophys. Acta *1090*, 1-8.

Torigoe,K., Ushio,S., Okura,T., Kobayashi,S., Taniai,M., Kunikata,T., Murakami,T., Sanou,O., Kojima,H., Fujii,M., Ohta,T., Ikeda,M., Ikegami,H., and Kurimoto,M. (1997). Purification and characterization of the human interleukin-18 receptor. J. Biol. Chem. *272*, 25737-25742.

Towne,J.E., Garka,K.E., Renshaw,B.R., Virca,G.D., and Sims,J.E. (2004). Interleukin (IL)-1F6, IL-1F8, and IL-1F9 signal through IL-1Rrp2 and IL-1RAcP to activate the pathway leading to NF-kappaB and MAPKs. J. Biol. Chem. *279*, 13677-13688.

Vallabhapurapu,S. and Karin,M. (2009). Regulation and function of NF-kappaB transcription factors in the immune system. Annu. Rev. Immunol. *27*, 693-733.

Vigers,G.P., Anderson,L.J., Caffes,P., and Brandhuber,B.J. (1997). Crystal structure of the type-I interleukin-1 receptor complexed with interleukin-1beta. Nature *386*, 190-194.

von Hoegen,I., Falk,W., Kojouharoff,G., and Krammer,P.H. (1989). Internalization of interleukin 1 (IL 1) correlates with IL 1-induced IL 2 receptor expression and IL 2 secretion of EL4 thymoma cells. Eur. J. Immunol. *19*, 329-334.

Vos,J.B., van Sterkenburg,M.A., Rabe,K.F., Schalkwijk,J., Hiemstra,P.S., and Datson,N.A. (2005). Transcriptional response of bronchial epithelial cells to Pseudomonas aeruginosa: identification of early mediators of host defense. Physiol Genomics *21*, 324-336.

Wald,D., Qin,J., Zhao,Z., Qian,Y., Naramura,M., Tian,L., Towne,J., Sims,J.E., Stark,G.R., and Li,X. (2003). SIGIRR, a negative regulator of Toll-like receptor-interleukin 1 receptor signaling. Nat. Immunol. *4*, 920-927.

Wang,P., Meinhardt,B., Andre,R., Renshaw,B.R., Kimber,I., Rothwell,N.J., and Pinteaux,E. (2005). The interleukin-1-related cytokine IL-1F8 is expressed in glial cells, but fails to induce IL-1beta signalling responses. Cytokine *29*, 245-250.

Wesche,H., Henzel,W.J., Shillinglaw,W., Li,S., and Cao,Z. (1997a). MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity *7*, 837-847.

Wesche,H., Korherr,C., Kracht,M., Falk,W., Resch,K., and Martin,M.U. (1997b). The interleukin-1 receptor accessory protein (IL-1RAcP) is essential for IL-1-induced activation of interleukin-1 receptor-associated kinase (IRAK) and stress-activated protein kinases (SAP kinases). J. Biol. Chem. *272*, 7727-7731.

Xiao,T., Towb,P., Wasserman,S.A., and Sprang,S.R. (1999). Three-dimensional structure of a complex between the death domains of Pelle and Tube. Cell *99*, 545-555.

Xu,D., Chan,W.L., Leung,B.P., Huang,F., Wheeler,R., Piedrafita,D., Robinson,J.H., and Liew,F.Y. (1998). Selective expression of a stable cell surface molecule on type 2 but not type 1 helper T cells. J. Exp. Med. *187*, 787-794.

Yanagisawa,K., Naito,Y., Kuroiwa,K., Arai,T., Furukawa,Y., Tomizuka,H., Miura,Y., Kasahara,T., Tetsuka,T., and Tominaga,S. (1997). The expression of ST2 gene in helper T cells and the binding of ST2 protein to myeloma-derived RPMI8226 cells. J. Biochem. *121*, 95-103.

Yao,J., Kim,T.W., Qin,J., Jiang,Z., Qian,Y., Xiao,H., Lu,Y., Qian,W., Gulen,M.F., Sizemore,N., DiDonato,J., Sato,S., Akira,S., Su,B., and Li,X. (2007). Interleukin-1 (IL-1)-induced TAK1-dependent Versus MEKK3-dependent NFkappaB activation pathways bifurcate at IL-1 receptor-associated kinase modification. J. Biol. Chem. *282*, 6075-6089.

Yoshimoto,T., Takeda,K., Tanaka,T., Ohkusu,K., Kashiwamura,S., Okamura,H., Akira,S., and Nakanishi,K. (1998). IL-12 up-regulates IL-18 receptor expression on T cells, Th1 cells, and B cells: Synergism with IL-18 for IFN-gamma production. Journal of Immunology *161*, 3400-3407.

Zubler,R.H., Erard,F., Lees,R.K., Van Laer,M., Mingari,C., Moretta,L., and MacDonald,H.R. (1985). Mutant EL-4 thymoma cells polyclonally activate murine and human B cells via direct cell interaction. J. Immunol. *134*, 3662-3668.

Acknowledgements

All praises and thanks for Almighty **ALLAH** who is the ultimate source of all knowledge to mankind, All respects are for the Holy **Prophet Muhammad** (Peace be upon him) who is the symbol of guidance.

Heartiest thanks for my worth Doctor Father **Prof. Michael U. Martin** for his personal supervision, kind cooperation, constructive criticism, valuable suggestions, sympathetic attitute, freedom of work, and ever inspiring guidance throughout the progress of this work. He not only guided me to complete my PhD project but also facilitated me to make my social life comfortable and enjoyful.

I am also greatly thankful to **Prof. Dr. Alfred Pingoud** who readily agreed to review my work.

I am greatly indebted to **Dr. Christian Kollewe** for providing valuable suggestions, demonstrations, guidance and kind help to solve scientific problems throughout my study. Thanks for **Dr. Ralf Ross** for valuable disscussions and inspirations.

Cordial thanks for all my lab fellows particularly **Thomas Kirchner, Christoph Linke**, **Thomas Körner**, and **Dang Quan Nguyen** for their genial company, time devotion, synergistic help and cooperation during my stay in the lab. Cordial acknowledgements are for all technical and official staff at the institute particularly **Mrs. Bedriska Reitz, Mrs. Adelheid Müller, Mrs. Siegrid Franke, Mrs. Janine Steinmetz, Mr. Stefan Scheld** and **Mrs**. **Grazia Grubinski.** Their support and interesting working environment made me able to complete this task in time.

I would also like to thank the collaboration partners: **Prof. Werner Falk** (University of Regensburg, Germany), **Dr. Gregor Meiss** (Institut für Biochemie, JLU Gießen) **Prof. Michael Huber** (Max-Plank Institute of Immunobiology, Freiburg Germany) and **Dr. Oliver Dittrich-Breiholz** (Medizinische Hochschule, Hannover, Germany)

Thanks my **parents, brother, bhabi, sisters** and **friends** for your prayers, spirit of sacrifice, love and support. Smiles and intersting talks of my dearest **Subhan** and **Usman** are also not forgetable. They keep me fresh and inspired for the work.

Special gratitudes for **Prof. W. Falk** (Department of Internal Medicine I, University of Regensburg, Germany) and PD **Dr. D. Neumann** (Medizinische Hochschule, Hannover) for writting reference letters for my stipend. Without stipend it would not have been possible for me to stay and study in Germany.

I am also thankful to Higher Education Commission (**HEC**), Pakistan and Deutscher Akademischer Austausch Dienst (**DAAD**) for financial support and facilitation to stay and study in Germany.

Shafaqat Ali

Gießen, July 2009

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

Publications

Shafaqat Ali, Michael Huber, Christian Kollewe, Stephan C. Bischoff, Werner Falk, and Michael U. Martin. **IL-1 receptor accessory protein is essential for IL-33-induced activation of T lymphocytes and mast cells.** *Proc Natl Acad Sci U S A.* 2007; 104(47): 18660–18665.

Shafaqat Ali, Dang Quan Nguyen, Werner Falk,and Michael U. Martin. **Caspase 3 inactivates biologically active full length interleukin-33 as a classical cytokine but does not prohibit nuclear translocation.** Submitted for publication in *Journal of Immunology***.**

Muhammad A. Aslam, Fazali R. Awan, Isfhan Tauseef, **Shafaqat Ali**, Nasir Ahmad, Naveed A. Malik, Mumammad N. Riaz and Javed A.Qureshi. **Identification of hepatitis B virus core mutants by PCR-RFLP in chronic hepatitis B patients from Punjab, Pakistan.** *Archives of Virology*. 2008;153(1):163-70.

Amjad Hameed, Salman Ahmad Malik, Fazli-Rabbi, Aysha Sharif, Nasir Ahmad, Wajahat Hussan, Hassan Akthar Bokhari, **Shafaqat Ali,** Farwa Nurjis, Ahmad Mukhtar Khalid and Javed Anver Qureshi. **Epidemiology of Diabetes Mellitus in and Around Faisalabad, Pakistan**. *Pakistan Journal of Biological Sciences* 2002; 5(8): 878-880.

Amjad Hameed, Salman A. Malik, Fazli-Rabbi, Aysha Sharif, Nasir Ahmad, Farwa Nurjis, **Shafaqat Ali** and Javed Anver Qureshi. **Diabetic Complications: Influence of Age, Sex, Family History, Duration, Glycemic Control and Obesity.** *OnLine Journal of Biological Sciences* 2002; 2(10): 710-714.

Amjad Hameed, Tahira Iqbal, Muhammad Nawaz, Faiz Batool Tayyba Tabassum Baig, **Shafaqat Ali**, and Farwa Nurjis. **Pharmacokinetics of Ofloxacin in Male Volunteers Following Oral Administration.** *Pakistan Journal of Biological Sciences* 2002; 5(10): 1098-1100.

Tayyba Tabassum Baig, Munir Ahmad Sheikh, Amjad Hameed, Amer Jamil, Faiz Batool, Shazia Muhammad Ali, and **Shafaqat Ali**. **Bioconversion of Filter Press Cake (Mud) of Sugar Cane to Biomass Protein and its Biological Evaluation**. *Pakistan Journal of Biological Sciences* 2002; 5(10): 1052-1055.