

**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL**

**THE REGULATORY MECHANISMS OF MATRIX  
METALLOPROTEINASES AND TISSUE INHIBITORS OF  
METALLOPROTEINASES IN HUMAN EOSINOPHILS**



**M.Sc. THESIS**

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**Department of Molecular Biology-Genetics and Biotechnology**

**Molecular Biology-Genetics and Biotechnology Programme**

**AUGUST 2021**



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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM**  
**ENSTİTÜSÜ**

**MATRİKS METALLOPROTEİNAZ ENZİMLERİNİN VE DOKU  
İNİHİTÖRLERİNİN İNSAN EOZİNOFİL HÜCRELERİNDE  
REGÜLASYON MEKANİZMALARININ İNCELENMESİ**

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*To my grandfather Galip UYGUR and family,*



## FOREWORD

This thesis has been undertaken in Istanbul Technical University, MOBGAM during the spring semester of 2020-2021 academic year under the supervision of Assoc. Prof. Ceren ÇIRACI MUĞAN.

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## ABBREVIATIONS

<b>µg</b>	:microgram
<b>µM</b>	:micromolar
<b>mM</b>	:milimolar
<b>ASC</b>	:Apoptosis-associated speck-like protein containing a CARD
<b>APMA</b>	:4-Aminophenylmercuric acetate
<b>ATP</b>	:Adenosine Triphosphate
<b>BCA</b>	:Bicinchoninic Acid
<b>BIR</b>	:Baculovirus inhibitor of apoptosis protein repeat domain
<b>BSA</b>	: Bovine serum albumin
<b>CARD</b>	:Caspase activation and recruitment domain
<b>cDNA</b>	:Complementary deoxyribonucleic acid
<b>CLR</b>	:C type lectin receptor
<b>DAMP</b>	:Danger-associated molecular pattern
<b>DC</b>	:Dendritic cell
<b>DMSO</b>	:Dimethyl sulfoxide
<b>DNA</b>	:Deoxyribonucleic acid
<b>dNTP</b>	:Deoxyribonucleotide
<b>EDTA</b>	:Ethylenediaminetetraacetic acid
<b>ELISA</b>	:Enzyme-linked immunosorbent assay
<b>FBS</b>	:Fetal bovine serum
<b>g</b>	:Gram
<b>GM-CSF</b>	:Granulocyte macrophage colony-stimulating factor
<b>h</b>	: hour
<b>HRP</b>	:Horseradish Peroxidase



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## **THE REGULATORY MECHANISMS OF MATRIX METALLOPROTEINASES AND TISSUE INHIBITORS OF METALLOPROTEINASES IN HUMAN EOSINOPHILS**

### **SUMMARY**

The immune system is a complex and dynamic network of cells, tissues, organs, molecules and soluble factors which fight against a broad range of pathogenic microorganisms to protect the body from infections. Besides its protective roles against pathogens, the immune system has the ability to clear allergenic substances and cancer cells in the body therefore, it also plays a central role in tissue remodeling. The immune system is divided into two categories: innate and adaptive immunity which are interconnected. The innate immunity is the first line of defense mechanism which develops nonspecific and rapid responses, while adaptive immunity results in specific responses to pathogens and may develop an immunological memory for a stronger and faster response upon encounterance with the same pathogen.

The innate immune system is made up of complement proteins, specialized myeloid and lymphoid cells involving dendritic cells, macrophages, natural killer cells, eosinophils, basophils and neutrophils. These cells can recognize evolutionary conserved patterns of pathogens known as pathogen-associated molecular patterns (PAMPs) through their pattern recognition receptors (PRRs). Upon recognition of PAMPs or damage-associated molecular patterns (DAMPs), PRRs activate downstream signaling pathways that ultimately induce innate immune responses by producing inflammatory cytokines. PRRs can be divided into 4 groups: Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and RIG-I-like receptors (RLRs). Among them, TLRs and CLRs are membrane bound, while NLRs and RLRs are cytoplasmic PRRs.

TLRs are a growing family of transmembrane receptors involved in innate immunity which has 10 identified members in humans. TLR2 recognizes microbial lipoproteins and glycolipids. TLR4 can sense lipopolysaccharides of gram-negative bacteria and TLR5 recognizes bacterial flagellin. The TLRs localized in subcellular compartments of the cell recognize nucleic acid material derived from viruses and bacteria. The NLRs are a cytosolic family of PAMP and DAMP sensors which have 22 members in humans. The NLRs consist of three domains: a C-terminal series of LRRs which recognize microbial molecules or danger signals, a central NACHT domain that facilitates NLR oligomerization and an N-terminal signaling domain. A significant subset of NLRs including NLRP1, NLRP3, NLRC4, NLRP6 are reported to assemble large protein complexes "Inflammasomes" which regulate the activation of caspases 1 that leads to production of biologically inactive pro IL-1 $\beta$ , IL-18. Biologically active IL-1 $\beta$  and IL-18 promote inflammatory and antimicrobial responses and activate different helper T cell subsets such as T<sub>H</sub>1 and T<sub>H</sub>17 cells.

The NLRP3 is one of the well characterized and most studied NLR which carries a PYRIN domain and inflammasome assembly upon ligand recognition by NLRP3 is

critical for host immune defenses against bacterial, viral and fungal infections. Additionally, NLC4 has a CARD domain and forms an inflammasome complex in macrophages infected by bacterial pathogen components of type III secretion system (T3SS) or flagellin.

Eosinophils are cells of innate immune system which constitute 1-6% of the circulating leukocytes. The number of eosinophils in the blood and some tissues is known to increase during specific immune responses to parasitic infections and in allergic diseases including in different types of asthma. As intracellular organelles, eosinophils contain numerous secretory granules that are central to the functional responses of eosinophils. These granules are composed of major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase, eosinophil-derived neurotoxin (EDN), cytokines, chemokines, prostaglandins and matrix metalloproteinases (MMPs) which target parasites and potent toxins to airway epithelium. Through these granule contents, eosinophils have the potential to drive airway remodelling which is a dynamic process of extracellular matrix (ECM) remodeling. MMPs and their tissue inhibitors of metalloproteinases have central roles in ECM remodeling. Of all the MMPs, MMP-2 and MMP-9 are the most studied MMPs. MMPs have widely been studied in asthma models and, MMP-9 is the predominant one in blood and bronchoalveolar lavage fluid (BALF) of patients with asthma. Recent studies also revealed the regulation of MMP-9 through TLRs in immune cells. Even though NLRs (NLRP3 and NLRC4) and inflammasomes are well studied in other contexts, their roles in shaping immunomodulatory functions of MMPs in eosinophils are relatively unknown.

Since eosinophils constitute a minor population of circulating leukocytes, we used EoL-1 human eosinophilic leukemia cell line for the investigation of eosinophilic functions. We also expanded our work to primary human eosinophils isolated from peripheral blood which currently continues in our laboratory. To qualify the effects of PRRs in general and NLRs in particular on gelatinases, we first analyzed the activity and expression levels of MMP-2 and MMP-9 at both mRNA and protein level in EoL-1 cells upon NLRC4 and NLRP3 inflammasome activation based on the two signal rule in which the PAM3CSk4 was utilized as TLR2 ligand (signal 1) and intracellular flagella as NLRC4 ligand (signal 2). We observed that EoL-1 cells expressed MMP-2 and MMP-9 both at mRNA and protein level and NLRC4 inflammasome activation markedly elevated the expression of MMP-9, while the MMP-2 mRNA expression was slightly increased and protein expression did not significantly change. Moreover, NLRP3 inflammasome activation also increased MMP-9 mRNA and protein expressions however the effect of NLRC4 activation was more dramatic than the effects of NLRP3 activation on MMP9 expression. We also examined the enzymatic activity of gelatinases secreted from EoL-1 cells upon NLRC4 and NLRP3 inflammasome activation. MMP-9 gelatinase activity was detected in cell-free medium of EoL-1 cells and augmented with NLRC4 inflammasome activation. Interestingly, MMP-2 activity was not detected. The NLRP3 inflammasome activation also induced MMP-9 gelatinase activity to a lesser extent than NLRC4 stimulants. We also demonstrated that treatment with recombinant human IL-1 $\beta$  increased MMP-9 protein expression and MMP-9 gelatinase activity in EoL-1 cells.

Next, we assessed the tissue inhibitors of metalloproteinases (TIMPs) TIMP-1 and TIMP-2 and determined that TIMP-1 expressed in EoL-1 eosinophils, however, TLR2 stimulation rather than NLRC4 inflammasome activation may be involved in the regulation of TIMP-1

Moreover, to verify the regulation of MMP-9 through NLRC4 inflammasome, we treated EoL-1 eosinophils with caspase-1 inhibitor Z-VAD-FMK which reduced NLRC4 induced MMP-9 protein expression and activation as MMP inhibitor GM6001 did. There are studies reporting the role of MMP-9 in IL-1 $\beta$  processing. We have shown that TLR2 and NLRC4 inflammasome increased the mature IL-1 $\beta$  expression and IL-1 $\beta$  secretion was reduced by MMP and caspase-1 inhibitor indicating the importance of MMP-9 in IL-1 $\beta$  maturation.

Furthermore, cycloheximide a protein synthesis inhibitor did not alter the NLRC4 - induced MMP-9 expression and indicating that the synthesis of MMP9 is not *de novo* and it is from the pre-synthesized MMP-9 in granules. We then confirmed this observation by measuring the CD63 expression, a degranulation marker, by flow cytometer. CD63 is present within granules and undergoes secretion by piecemeal degranulation or exocytosis in response to stimuli. Additionally, the increase in MMP-9 expression and activity upon NLRC4 inflammasome activation also correlates with increase in CD147 expression known as extracellular matrix metalloproteinase inducer (EMMPRIN).

On the basis of current data, MMP-9 is involved in a wide range of cellular activities including airway inflammation, allergic responses and parasitic infections might be regulated not only via TLRs but also NLRs. Here, we report that NLRC4 inflammasome activation increased MMP-9 expression and activity, eosinophil degranulation and therefore contributed to the functions of EoL-1 cells. Taken together, our findings present potential targets for further investigations in immunomodulatory functions of MMPs in eosinophils and eosinophilic inflammation coupled with a PRRs.



# MATRİKS METALLOPROTEİNAZ ENZİMLERİNİN VE DOKU İNHİBİTÖRLERİNİN İNSAN EOZİNOFİL HÜCRELERİNDE REGÜLASYON MEKANİZMALARININ İNCELENMESİ

## ÖZET

Bağışıklık sistemi, vücudu enfeksiyonlardan korumak için çeşitli patojenik mikroorganizmalara karşı savaşan hücre, doku, organ, molekül ve çözünür faktörlerden oluşan karmaşık ve dinamik bir mekanizmalar bütünüdür. Patojenlere karşı koruyucu rollerinin yanı sıra, bağışıklık sistemi vücuttaki alerjenik maddeleri ve kanser hücrelerini temizleme yeteneğine sahiptir ve ayrıca dokuların yeniden yapılanması merkezi bir rol oynar. Bağışıklık sistemi, doğal ve edinsel bağışıklık olmak üzere birbiriyle bağlantılı iki ayrı kategoriye ayrılmıştır. Doğal bağışıklık hızlı ancak spesifik olmayan bağışıklık yanıtlarından meydana gelen vücudun ilk savunma mekanizmasıdır. Edinsel bağışıklık ise patojenlere karşı spesifik yanıt verirken daha önce karşılaştığı bir patojenle yeniden karşılaşıldığında daha hızlı yanıt verebilmek için immünolojik bellek mekanizması geliştirebilirler.

Doğal bağışıklık sistemi, kompleman proteinler, dendritik hücreler, makrofajlar, NK hücreleri, eozinofiller, bazofiller ve nötrofilleri içeren özel miyeloid ve lenfoid hücrelerden oluşur. Bu hücreler, Patern Tanıma Reseptörler (PRR'ler) aracılığıyla evrimsel olarak korunmuş patojenle ilişkili moleküler paternleri (PAMP'ler) tanıyabilirler. PAMP'lerin tanınması üzerine, PRR'ler doğal bağışıklık yanıtı oluşumunu sağlayan bir dizi işlem sonucunda enflamatuar sitokinler üretir. PRR'lar membrana bağlı ve sitozolik reseptörler olmak üzere iki gruba ayrılır. Toll benzeri reseptörler (TLR'ler) ve C-tipi lektin reseptörleri (CLR) membrana bağlıyken, NOD benzeri reseptörler (NLR) ve RIG-I benzeri reseptörler (RLR'ler) sitoplazmik PRR'ler olarak kabul edilir.

İnsanlarda TLR'lerin tanımlanmış 10 üyesi bulunmaktadır. Bunlar arasında TLR2, mikrobiyal lipoproteinleri ve glikolipitleri tanıırken, TLR4 gram-negatif bakterilerin lipopolisakkaritlerini, TLR5 ise bakteriyel flagellini tanıır. Hücre içerisinde endozomlarda lokalize olan TLR'ler, virüslerin ve bakterilerin nükleik asitlerini tanıır. Bunun yanı sıra, NLR'lerin 22 üyesi bulunmaktadır ve PAMP ya da hasar molekülleri (DAMP'ler) tarafından uyarılır. NLR'ler, proteinin C-terminalinde bulunan ve mikrobiyal molekülleri veya hasar moleküllerini tanıyan LRR domeyni, NLR proteinlerinin oligomerizasyonunu sağlayan merkezi bir NACHT domeyni ve sinyal iletiminde görev alan bir N-terminal domeyn olmak üzere 3 ayrı domeynden meydana gelmektedir. Yapılan çalışmalar NLR üyeleri NLRP1, NLRP3, NLRC4 ve NLRP6'nın "inflamazom" olarak bilinen büyük protein komplekslerini oluşturup kaspaz-1 aktivasyonunu düzenleyerek biyolojik olarak inaktif olan IL-1 $\beta$ , IL-18'in aktifleşmesine ve hücre dışına salınımına neden olduğunu göstermiştir. Biyolojik olarak aktif olan IL-1 $\beta$ , IL-18 enflamatuar ve antimikrobiyal yanıtı destekleyerek T<sub>H1</sub> ve T<sub>H17</sub> hücreleri gibi yardımcı T hücrelerini etkinleştirir. NLRP3, N-terminal domeyni PYRIN molekülünden oluşan, en çok çalışılan ve iyi karakterize edilmiş bir NLR üyesi olup NLRP3 tarafından ligand tanınması üzerine inflamazom yapısının

oluşması bakteriyel, viral ve fungal enfeksiyonlara karşı bağışıklık yanıtı oluşumu için önemlidir. Bunun yanı sıra, NLRC4'ün N-terminal domeyni CARD molekülünden oluşur ve yapılan çalışmalar bakteriyel patojenlerin tip III sekresyon sistemi (T3SS) veya flagella komponentleri ile enfekte edilmiş makrofajlarda NLRC4 proteinin inflamazom kompleksi yapısına katıldığını göstermiştir. Eozinofiller doğal bağışıklık hücresi olup, kan dolaşımındaki lökositlerin %1-6'sını oluşturur. Kandaki ve bazı dokulardaki eozinfil sayısının, parazitik enfeksiyonlara ve allerjenlere karşı immün yanıt sırasında arttığı bilinmektedir. Eozinofiller, organel olarak eozinofil fonksiyonlarının merkezinde yer alan bol miktarda sekretuar granül içerir. Bu granüller, parazitlere ve hava yolu epitellerine karşı güçlü toksikliği olan major basic protein (MBP), eozinofil katyonik protein (ECP), eozinofil peroksidaz, eozinofilden türeyen nörotoksin (EDN), sitokinler, kemokinler, prostaglandinler ve matriks metaloproteinazları (MMP) içerir. Bu granül içerikleri göz önüne alındığında, eozinofiller, hücre dışı matriksin (ECM) yeniden yapılanmasının dinamik bir süreci olan hava yolunun yeniden yapılanmasını yönlendirme potansiyeline sahiptir. MMP'ler ve MMP'lerin doku inhibitörleri, ECM'in yeniden şekillenmesinde merkezi rollere sahiptir. MMP'ler 26 bilinen üyesi ile aktif bölgesinde çinko olan, kalsiyum bağımlı endopeptidaz ailesidir. ECM ile bazal membran bileşenlerini parçalar ve böylece ECM yapısının bileşimini ve bütünlüğünü düzenlerler. Yapılan birçok çalışma MMP'lerin birçok substratı parçalayabildiğini, yara iyileşmesi, enflamasyon ve kanser metastazı gibi biyolojik ve patolojik süreçlerde önemli rolleri olduğunu göstermiştir. MMP'ler sitokinler ve kemokinler gibi enflamatuar araçları modüle ederek, iltihaplı dokularda enfeksiyon bölgelerine lökositlerin hareketlerini düzenler ve enflamatuar süreçlere katkıda bulunur. Yapılan çalışmalar MMP'ler, TIMP'ler, hava yolu enflamasyonu arasındaki ilişkiyi göstermekte olup ekstraselüler matriksin yeniden yapılanmasında ve enflamatuar süreçlerde önemli bir rolü olan MMP9'un astım hastalarının akciğer dokularındaki eozinofil hücrelerinde bulunup hava yolu enflamasyonunun gelişmesinde patojenik bir rolü olduğunu göstermektedir. Son çalışmalar ayrıca bağışıklık hücrelerinde MMP-9'un TLR'ler üzerinden regülasyonunu göstermiştir. NLR'ler (NLRP3 ve NLRC4) ve inflamazomlar diğer alanlarda akyuvar hücrelerinde çok çalışılmış olsalar da eozinofillerdeki MMP'lerin regülasyon mekanizmaları ve MMPlerin immunomodülatör fonksiyonları nispeten bilinmemektedir.

Eozinofiller, dolaşımdaki lökositlerin küçük bir popülasyonunu oluşturduğu için, bu çalışmada Eol-1 insan eozinofilik lösemi hücre hattı kullanılmıştır. Genel olarak PRR'lerin ve özellikle de NLR'lerin MMP'ler üzerindeki etkisini değerlendirmek için, ilk olarak NLRC4 ve NLRP3 inflamazom aktifleşmesine yol açan iki sinyal (1. Sinyal olarak PAM3CSK4-TLR2 ligandı, 2.sinyal olarak ise hücre içerisine transfekte edilen bakteri flagellası) ile uyarılan Eol-1 hücrelerinin MMP-2 ve MMP-9'un hem enzim aktivitesi hem de mRNA ve protein seviyeleri analiz edildiğinde, Eol-1 hücrelerinin MMP-2 ve MMP-9'u hem mRNA hem de protein seviyesinde ekspresyon ettiği gözlemlenmiştir. Eol-1 hücrelerinde NLRC4 inflamazom aktivasyonu MMP-9 ekspresyonunu anlamlı bir şekilde arttırırken, MMP-2 mRNA ve protein ekspresyonunda anlamlı bir değişiklik yapmadığı gözlemlenmiştir. Eol-1 hücrelerinde NLRC4 inflamazom aktivasyonunun neden olduğu kadar olmasa da NLRP3 inflamazom aktivasyonunun da MMP-9 mRNA ekspresyonunu arttırdığı gözlemlenmiştir. Ayrıca, hücre dışına salgılanan MMP-2 ve MMP-9'un enzim aktivitesi analiz edildiğinde, NLRC4 inflamazom aktivasyonunun hücre dışına salgılanan MMP-9 aktivitesini arttırdığı gözlemlenmiştir. Ancak ilginç bir şekilde MMP-2 aktivitesi ne bazal seviyede ne de inflamazom aktivasyonu sonrasında gözlemlenmiştir.

Rekombinant IL-1 $\beta$  (10 ng) ile stimüle olan Eol-1 hücrelerinde MMP-9 protein ekspresyonu artarken, hücre dışına salgılanan MMP-9 aktivitesinin az da olsa arttığı görülmüştür. Eol-1 hücrelerinde, TLR2 stimülasyonu MMP-9'un doku inhibitörü olan TIMP-1'in mRNA ekspresyonunu arttırırken NLRC4 inflamazom aktivasyonu TIMP-1 ekspresyonunu deęiřtirmemiřtir. Buna ek olarak, NLRP3 inflamazom aktivasyonu TIMP1 mRNA ekspresyonunu azaltmıřtır.

Bunlara ek olarak, MMP-9'un NLRC4 inflamazomu aracılıęı ile regüle olduęunu doęrulamak amacıyla, kaspaz-1 Z-VAD-FMK ile inhibe edildięinde, NLRC4 inflamazom aktivasyonunun indükledięi MMP-9 protein ekspresyonunun ve aktivasyonun azaldıęı gözlemlenmiřtir. Cevap aradıęımız sorulardan birisi NLRC4 inflamazom aktivasyonu sonucu üretilen MMP-9'un, IL-1 $\beta$  üretimi ve olgunlařmasında bir rolü olup olmadıęıdır. Bu baęlamda, TLR2 ligandı ve NLRC4 inflamazom aktivasyonunun neden olduęu olgunlařmıř IL-1  $\beta$  ekspresyonu ve hücre dışına salgılanmasının GM6001 MMP inhibitörü ile azaldıęını ve kaspaz-1'in Z-VAD-FMK ile inhibe edildięinde ise neredeyse tamamen yok olduęunu göstermiř olduk ki bu, kaspaz-1 Eol-1 hücrelerinde IL-1 $\beta$  olgunlařmasında major bir protein olmasına raęmen MMP9'un da Eol-1 hücrelerinde IL-1 $\beta$  olgunlařmasında rolü olduęunu göstermektedir.

Bunlara ek olarak, bir protein sentezi inhibitörü olan sikloheksimidin, Eol-1 hücrelerinde MMP-9 protein ekspresyonunu doza baęlı azaltıyorken hücre dışına salınan MMP-9 aktivitesini azaltmadıęı, NLRC4 inflamazom aktivasyonu kaynaklı MMP-9 protein ekspresyonunu azaltmadıęını ve Eol-1 hücrelerinden salınan MMP-9 aktivitesini tamamen ortadan kaldırmaęı da elde ettięimiz bulgulardan birisidir. Bunun da granüllerdeki MMP-9'un hücre dışına degranülasyon yolu ile salgılanmasının bir sonucu olduęu ve bir degranülasyon belirteci olan CD63'ünde NLRC4 inflamazom aktivasyonu sonucu arttıęı elde edilmiř olup bu da MMP-9'un granüllerden degranülasyon sonucu salgılandıęını doęrular nitelikte bir bulgudur.

Mevcut verilere dayanarak, hava yolu inflamasyonu, alerjik yanıtlar ve parazit enfeksiyonlarında fonksiyonel rollere sahip olan eozinofillerde MMP-9'un sadece TLR'ler aracılıęıyla deęil aynı zamanda NLR'ler aracılıęıyla da regüle olduęu, NLRC4 inflamazom aktivasyonunun Eol-1 hücrelerinde MMP-9'u, eozinofil hücre degranülasyonunu ve aktivasyonunu arttırması nedeniyle PRR'lar ile eozinofillerdeki MMP'lerin immünomodülatör iřlevleri arasındaki iliřkinin anlařılması için daha ileri arařtırmalara potansiyel hedefler sunmaktadır.



## **1. INTRODUCTION**

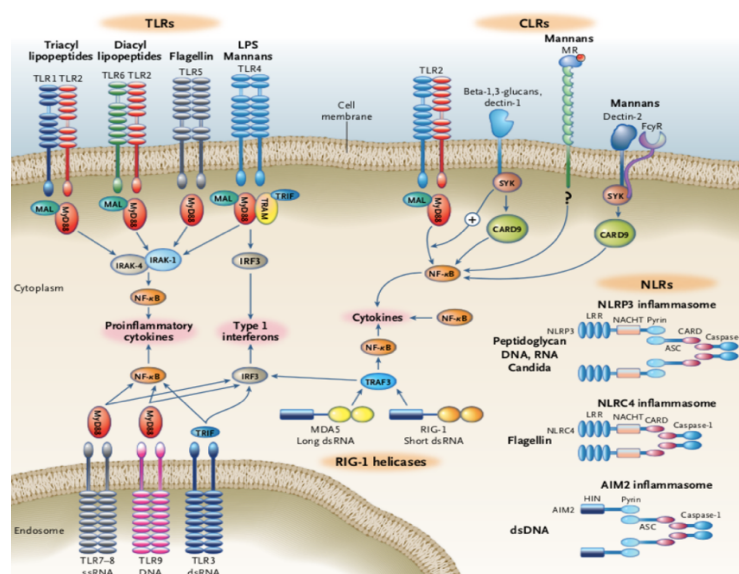
### **1.1 The Immune System and Its Components**

Humans and other mammals inhabit in a world that's heavily populated by both pathogenic and non-pathogenic microorganisms, which contains an enormous array of toxic or allergenic substances that threaten homeostasis. Pathogenic microorganisms in the community possess a mechanism by which they replicate, spread, threaten the normal host function and causes infection(Chaplin, 2010). The cells and molecules in our body that defense against those pathogenic microbes and provide protection constitute the immune system and the coordinated response of immune system to foreign substances is called the immune response. Not only the invading pathogens but also non-infectious foreign substance can elicit immune responses (Abul K. ABBAS, 2016). The human body's defense mechanism against invaders can be divided into two distinct categories which are innate and adaptive immunity. The innate immunity is the first line of defense that play important role in protection against foreign substances in concordance with adaptive immunity. Innate immunity gives much more rapid and nonspecific response when a foreign substance is detected, whereas adaptive immunity is slower because it specifically responds to pathogen or non-pathogenic foreign substances and develops immunological memory mechanism (Gasper et al., 2014) Nevertheless, chronic disorders can be occurred as a result of hyperactive immune response.

### **1.2 Innate Immune System**

The innate immune system is an evolutionary conserved defense mechanism that shares key features among plants, invertebrates and mammals (Gasteiger et al., 2017) The innate immunity augments the protection provided by means of anatomical and physiological barriers including intact skin, mucociliary clearance mechanisms, low stomach pH and bacteriolytic lysozyme in tears, saliva (Turvey & Broide, 2010) Specialized myeloid and lymphoid cells involving dendritic cells, neutrophils,

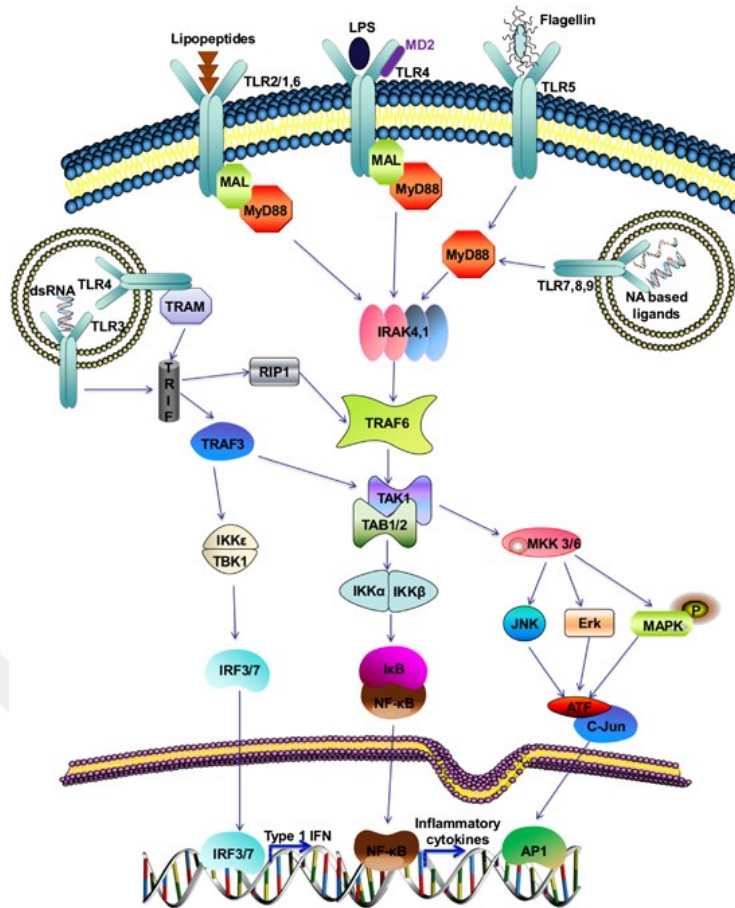
macrophages, mast cells, eosinophils and natural killer cells and also nonhematopoietic cells can elicit an immune response and become activated in response to infection, tissue damage or stresses through cell-dependent mechanisms (phagocytosis and cytotoxicity) or secreted factors including cytokines/chemokines, complements, antimicrobial peptides, alarmins, acute-phase proteins, proteases, and other less-categorized molecules (Gasteiger et al., 2017). Moreover, the innate immune cells can sense and recognize evolutionary conserved patterns of pathogens known as pathogen-associated molecular patterns (PAMPs) through their germ-line encoded pattern recognition receptors (PRRs) (Kawai & Akira, 2009). PRR have 4 subgroups; Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (Kvarnhammar & Cardell, 2012). These PRRs also recognize endogenous damage-associated molecular patterns (DAMPs). DAMPs may be produced during infection upon homeostatic disruptions such as cell injury or lysis, nevertheless these DAMPs may be caused by sterile inflammatory response (Abul K. ABBAS, 2016). Upon recognition of PAMPs or DAMPs, PRRs activate downstream signaling pathways that ultimately induce innate immune responses by producing inflammatory cytokines, type I interferon (IFN) and other mediators. These processes not only trigger immediate defensive responses such as inflammation but also prime and orchestrate antigen-specific adaptive immune responses (Kawasaki & Kawai, 2014)



**Figure 1.1 :** Pattern Recognition Receptors and Their Functional Roles (Netea & van der Meer, 2011).

### 1.3 Toll-Like Receptors (TLRs)

TLRs are type I transmembrane proteins belonging to the PRR family which consist of three major domains: 20-27 extracellular leucine-rich repeats (LRR) for the recognition of PAMPs or DAMPs, a transmembrane domain and intracellular Toll/IL-1 receptor (TIR) domain that initiates downstream signal transduction cascade (El-Zayat et al., 2019). TLRs are found at the cell surface and in subcellular compartment such as endosome and widely expressed in many cell types including epithelial and endothelial cells. Hematopoietically derived sentinel cells such as dendritic cells (DCs), neutrophils and macrophages express most of the TLRs (West et al., 2006). To date, the human TLR family has 10 identified members (TLR1 to TLR10) (Kawasaki & Kawai, 2014). Among these TLRs, TLR2 generally function as a heterodimer with TLR1 or TLR6 and involved in the recognition of a various microbial products by mediating responses to lipoproteins, peptidoglycan and lipoteichoic acids. Depending on the dimerization, TLR2 can distinguish diacyl (TLR1/2) and triacyl (TLR2/6) lipopeptides (Kawasaki & Kawai, 2014). Nevertheless, additional TLR2 ligands do not appear to require TLR1 or TLR6 for signaling, implying that TLR2 may recognize some ligands as homodimers or heterodimers with other non-TLR molecules. In addition to bacterial PAMPs, TLR2 heterodimers or homodimers recognize fungal and protozoan molecules (West et al., 2006). TLR4 and TLR5 are another TLR members that sense bacterial lipopolysaccharide (LPS) and bacterial flagellin, respectively. The TLRs localized in subcellular compartments of the cell recognize nucleic acid material derived from viruses, bacteria or host itself; for instance, TLR3 recognizes the presence of viral double-stranded RNA in the cytosol while TLR9 senses the unmethylated CpG regions present on bacterial and viral DNA (Kawasaki & Kawai, 2014).



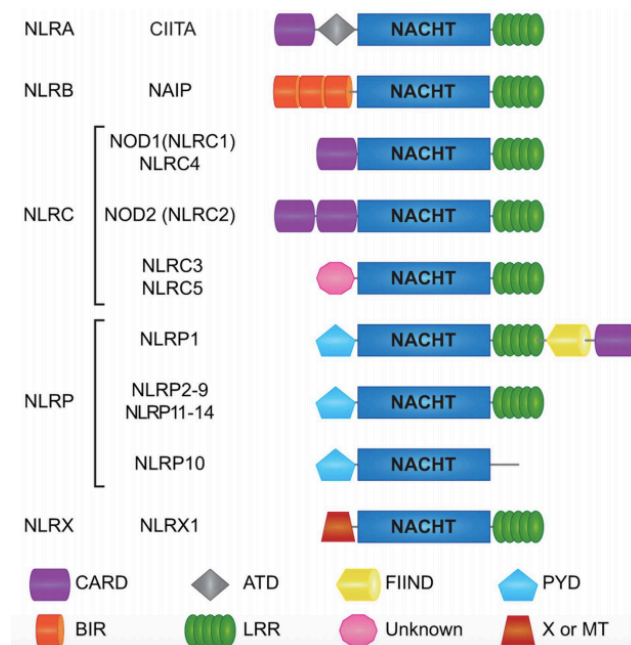
**Figure 1.2:** Overview of the Toll-like receptor (TLR) signaling pathway.

Upon stimulation of TLRs with their respective ligands, they become dimerized and recruit downstream adaptor molecules, such as myeloid differentiation primary-response protein 88 (MyD88), MyD88-adaptor-like (MAL), Toll/interleukin (IL)-1 receptor (TIR)-domain-containing adaptor-inducing interferon- $\beta$  (TRIF), TRIF-related adaptor molecule (TRAM). MyD88 mediates the TLR signaling pathway that activates IRAKs (IL-1-receptor-associated kinases) and TRAF6 (tumour-necrosis-factor receptor-associated factor 6), and leads to the activation of the IKK complex (inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B)- kinase complex) consisting of IKK- $\alpha$ , IKK- $\beta$  and IKK- $\gamma$ . TLR1, TLR2, TLR4, TLR5, TLR6, TLR7 and TLR9 uses this pathway which releases NF- $\kappa$ B from its inhibitor so that NF- $\kappa$ B translocate into nucleus where it induces the expression of pro-inflammatory cytokines including interleukin (IL)-1, IL-6, IL-8, IL-12 and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) which their secretion foresees the threat posed by pathogens and enables the activity of other immune components (Anwar et al., 2013). Another TIR-domain containing adaptor, TRIF is essential for the MyD88-independent pathway. The non-typical IKKs IKK- $\epsilon$  and TBK1 (TRAF-

family-member-associated NF- $\kappa$ B activator (TANK)-binding kinase 1) mediate activation of IRF3 downstream of TRIF upon recognition of TLR3 and TLR4 ligands. TRAM is a TIR- domain containing adaptor which is specific to TLR4-mediated, MyD88-independent/TRIF-dependent pathway (Akira & Takeda, 2004)

### 1.4 Nod-Like Receptors (NLRs)

The NLRs are a cytosolic family of PAMP and DAMP sensors characterized by a C-terminal series of LRRs which recognize microbial molecules or danger signals, a central NACHT domain that facilitates NLR oligomerization and an N-terminal signaling domain (Werts et al., 2011). The NLR family have 22 members in humans classified in 5 subfamilies based on their N-terminal domain (Werts et al., 2011). These are, NLRA (CIITA) which contains acidic transactivation domain, NLRB (NAIP) subfamily having an N-terminal baculovirus inhibition of apoptosis repeat (BIR) domain, NLRC subfamily contains caspase activation and recruitment domain(CARD) and allows direct interaction of this NLR family members, NLRP subfamily contains pyrin domain (PYD) , NLRX subfamily contains a mitochondria-targeting sequence required for its trafficking (Werts et al., 2011).



**Figure 1.3:** Domain architecture of human NLRs(Werts et al., 2011).

Among them, a significant subset of NLRs including NLRP1, NLRP3, NLRC4, NLRP6 are reported to assemble large protein complex “Inflammasome” which

regulates the activation of caspases1 (Lamkanfi & Dixit, 2014)(Davis et al., 2011). The signaling pathway where the assembled inflammasome activates pro-caspase-1 into its catalytically active form is generally referred as the canonical inflammasome, whereas an undefined noncanonical inflammasome pathway regulates the activation of procaspase-11. Canonical inflammasome activation require two steps which are transcription and oligomerization. The first step is regulated by innate immune signaling, mainly by TLR signaling and/or cytokine receptors such as TNF which leads to production of biologically inactive pro IL-1 $\beta$ , IL-18 and NLR transcription via nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation. The second step leads to inflammasome oligomerization and eventually caspase-1 activation which, in turn, results in IL-1 $\beta$  and IL-18 processing and release (Pellegrini et al., 2017). Biologically active IL-1 $\beta$  and IL-18 promote inflammatory and antimicrobial response and activate different helper T cell subsets such as T<sub>H1</sub> and T<sub>H17</sub> cells (Bauernfeind & Hornung, 2013)(Ciraci et al., 2012). Furthermore, active caspase-1 results in a pyroptosis which is a conserved programmed of cell death. Pyroptosis is mediated by pore-forming effector protein gasdermin D, where its N-terminus is cleaved by active caspase-1 and oligomerizes to form pores on the host cell membrane. These pores allow water influx and leads to cell swelling and lysis. Physical rupture of the cell causes release of pro-inflammatory cytokines IL-1 $\beta$  and IL-18 and endogenous danger signals (Bergsbaken et al., 2009)(Man et al., 2017).

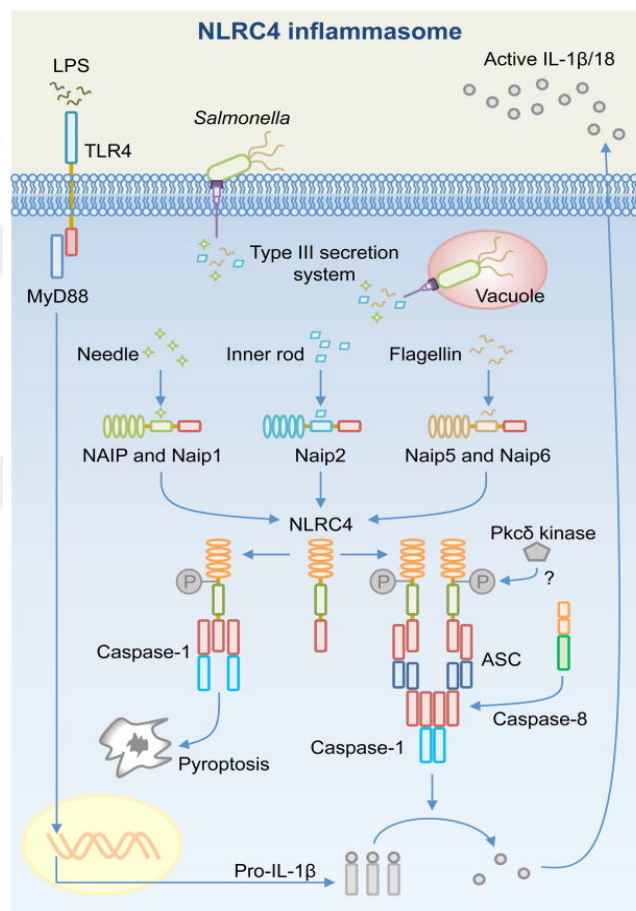
#### **1.4.1 NLRC4 Inflammasome**

NLRC4 or IPAF is expressed in myeloid cells where it has been shown to regulate caspase-1 activation and IL-1 $\beta$  processing via assembly of an inflammasome complex. NLRC4 contains an N-terminal CARD for signal transduction, a central NACHT domain and C-terminal LRRs (Man et al., 2017). NLRC4 binds to adaptor protein ASC which is required for cytokine processing and ASC directly and specifically associates with the pro-caspase-1 through CARD-CARD interactions, whereas pyroptosis initiated by NLRC4 signaling occurs ASC-independent manner (Man et al., 2017). Gram-negative bacteria such as *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Legionella pneumophila* has been shown to induce NLRC4 inflammasome assembly (Bauernfeind & Hornung, 2013)(Lage et al., 2014). and in addition to the conserved LRR domain, neuronal apoptosis inhibitory protein (NAIP) is required for recognition of bacterial proteins and inflammasome activation (Lage et al., 2014). The

NAIP-NLRC4 inflammasomes have been activated in macrophages infected by bacterial pathogens carrying components of type III secretion system(T3SS) or flagellin (X. Yang et al., 2018). The specificity of inflammasomes is consulted by NAIPs. In mice, NAIP2 has been shown to recognize T3SS bacterial rod and needle protein, whereas NAIP5 and NAIP6 recognize cytosolic flagellin (X. Yang et al., 2018)(Kofoed & Vance, 2011). Also, NAIP1 in mice and its human ortholog of NAIP have been served as receptors for T3SS needle proteins and like NAIP2/5, for activation of caspase-1 enzyme, they form ligand-induced NAIP-NLRC4 inflammasomes. Recognition of the ligand results in NLRC4-NAIP interaction and eventually NLRC4 activation. Once activated, NLRC4 forms a wheel-like structure of NAIP-NLRC4 inflammasome consisting of one NAIP and multiple NLRC4 molecules (X. Yang et al., 2018) which results in pro-IL-1 $\beta$  and pro-IL-18 processing via ASC-dependent mechanism by recruiting caspase-1.

Nonetheless, NLRC4 and pro-caspase-1 can interact with each other through their CARD domains and induces caspase-1-dependent pyroptosis in an ASC-independent mechanism (Man & Kanneganti, 2015). It is also reported that NLRC4 inflammasome recruits several effector proteins into the complex, including caspase-8 in macrophages infected with *S. Typhimurium*(Man et al., 2013). Caspase-8 is theorized to involved in delayed pro-IL-1 $\beta$  and pro-IL-18 processing and induces delayed cell death (Man et al., 2013). Furthermore, it has been reported that Pkc $\delta$  kinase-induced phosphorylation of an evolutionarily conserved residue, Ser533, found between the nucleotide-binding domain and the C-terminal LLR domain of NLRC4 is required for NLRC4 inflammasome assembly in response to *S. Typhimurium* infection (Qu et al., 2012). However, (Suzuki et al., 2014) demonstrated that phosphorylation of NLRC4 by Pkc $\delta$  kinase is dispensable for NLRC4 inflammasome activation in response to *Salmonella* or *Shigella* infection. The important role of NLRC4 inflammasome in the development of autoinflammation has been revealed by many studies. Two groups independently described gain of function mutations in NLRC4 which causes a syndrome of infantile enterocolitis and Macrophage activation syndrome (Duncan & Canna, 2018). Additionally, mutation in the NLRC4 gene was identified in patients with Familial Cold Autoinflammatory Syndrome (FCAS) which its mutant form promoted the formation of NLRC4 inflammasomes that cleave and activate pro-caspase-1 and enhance IL-1 $\beta$  production (Kitamura et al., 2014). Another study

demonstrating that NLRC4 inflammasome activation was sufficient to drive development of systemic inflammation in MRP8<sup>+</sup> cells which is a population consisting primary neutrophils (Nichols et al., 2017). Additionally, the involvement of NLRC4 in tumor development in various ways has been reported. It is reported that NLRC4 and IL-1 $\beta$  have been shown to promote breast tumor progression in diet-induced obese mice (Kolb et al., 2016). However, (Janowski et al., 2016) has demonstrated that in *Nlrc4*<sup>-/-</sup> mice, tumor growth increased in ASC and caspase-1 independent manner.

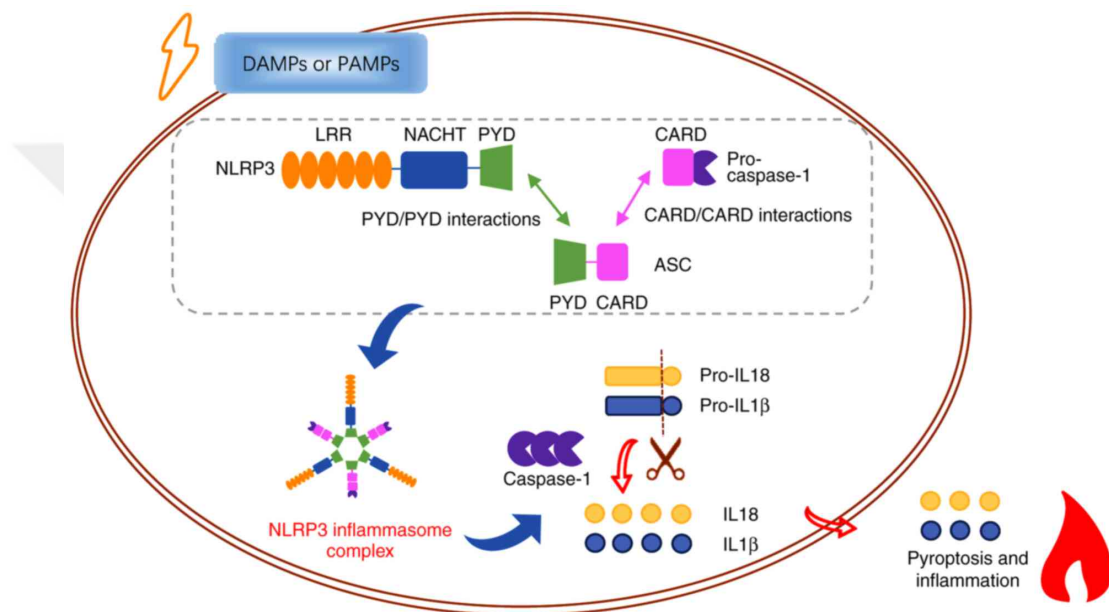


**Figure 1.4:** Regulation of NLRC4 inflammasome activation (Man & Kanneganti, 2015).

### 1.4.2 NLRP3 Inflammasome

The NLRP3 inflammasome is the well characterized and most studied inflammasome complex to date that is critical for host immune defenses against bacterial, viral and fungal infections. NLRP3 or NALP3 which is encoded by the cold induced auto inflammatory syndrome 1 (CIAS 1) gene, is expressed in cells of the immune and nervous systems (Ma et al. 2018). However, when dysregulated, it has been linked to

the pathogenesis of some inflammatory diseases such as cryopyrin-associated periodic syndromes (CAPS), diabetes, gout, Alzheimer’s disease, autoinflammatory diseases, and atherosclerosis (Kelley et al. 2019). The NLRP3 consists of three domains: A C-terminal leucine-rich repeats (LRRs) domain for ligand sensing, a central NACHT domain responsible for NLRP3 oligomerization and inflammasome assembly and N-terminal PYD domain which enable homotypic PYD/PYD interactions with apoptosis-associated speck-like protein containing CARD (ASC) consisting of PYD and CARD domains.



**Figure 1.5:** Structure of the NLRP3 inflammasome complex (Ma et al., 2018).

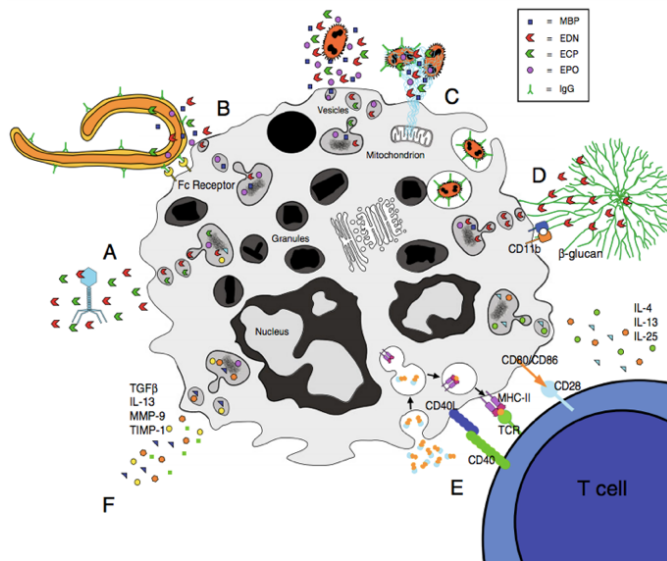
Upon recognition of PAMPs or DAMPs, activation of upstream signals and oligomerization of NLRP3 results in assembly of NLRP3 inflammasome. The NLRP3 inflammasome comprise of three cytoplasmic proteins: NLRP3, ASC and pro-caspase-1. Upon activation, the NLRP3 protein interacts with ASC via PYD and then recruits the pro-caspase-1 via CARD/CARD interactions to form NLRP3 inflammasome which subsequently cleaves and generates active caspase-1 enzyme targeting cleavage, maturation and secretion of proinflammatory cytokines IL-1 $\beta$  and IL-18, also inducing pyroptosis (Ma et al., 2018). A wide range of stimuli can be induced NLRP3 inflammasome activation including extracellular ATP, K<sup>+</sup> efflux, monosodium urate crystals, nigericin, pathogen associated RNA and bacterial and fungal toxins and components (Kelley et al., 2019).

## 1.5 Eosinophils and Their Functional Roles

Eosinophils are cells of innate immune system which constitute 1-6% of the circulating leukocytes (Long et al., 2016). Eosinophils are granulocytes that develop in the bone marrow from pluripotent progenitors and have critical roles in Th2 responses, including the IL-5 production that elevate eosinophilopoiesis and eosinophil activation (Long et al., 2016). The number of eosinophils in the blood and some tissues is known to increase during specific immune responses to parasitic infections and in allergic diseases including forms of asthma (Weller & Spencer, 2017). Studies in recent years have been identified many new roles of eosinophils in various pathological processes, including not only host protection against other microbes, but also anti-tumor immunity, allograft rejection and a number of idiopathic eosinophilic diseases (Long et al., 2016). Eosinophils differentiate in the bone marrow from an eosinophil lineage-committed progenitor (EoP) which are originated from the granulocyte/macrophage progenitor (GMP) in mice and common myeloid progenitor (CMP) in humans. The myeloid progenitors (GMP or CMP) must express C/EBP $\alpha$ , C/EBP $\epsilon$ , interferon regulatory factor 8 (IRF8), and PU.1 for eosinophil lineage commitment to occur. For EoP production from the myeloid progenitors, expression of friend of GATA-1 (FOG-1) declines which allows increased expression and activity of GATA TFs. XBP1 expression is increased to assist with the elevated granule protein synthesis and to promote survival in the EoP and eosinophil precursors during maturation process. Expression of C/EBP $\epsilon$  peaks together with ID2 during eosinophil maturation and declines during the final stages (Fulkerson, 2017). Differentiation of eosinophils from hematopoietic progenitors is also governed by cytokines or soluble mediators. IL-3, IL-5 and GM-CSF are important in the regulation of eosinophil development and survival. IL-3 and GM-CSF also induce differentiation of mast cells (Blanchard & Rothenberg, 2009). Of these three cytokines, IL-5 is the key cytokine specific to eosinophil lineage which has a profound roles in development, activation, survival of circulating and tissue eosinophils (Ramirez et al., 2018). IL-5 can be secreted by eosinophils in an auto/paracrine manner (Radonjic-Hösli & Simon, 2014) and Th2 lymphocytes, CD34<sup>+</sup> progenitor cells, group2 innate lymphoid cells (ILC-2), mast cells and invariant natural killer T cell are also major sources of IL-5. Likewise, chemokines such as CCL11, CCL24 and CCL26 (eotaxin1, 2, and 3, respectively) which are the ligand of CCR3 promote eosinophilia by cooperating with IL-5 and IL-

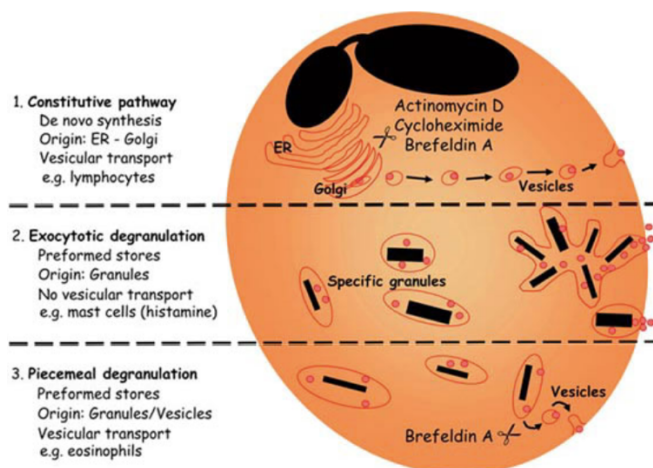
5-independent mechanism (Rosenberg et al., 2013). These chemokines have central role in eosinophil migration and recruitment into tissues and organs (Ramirez et al., 2018). Under physiological conditions, eosinophils can be detected in different organs where they strive various homeostatic tasks. Eosinophils are recruited into primary and secondary lymphoid organs such as the lymph nodes, the thymus, the spleen as well as Peyer's patches within the gut in order to assist the maturation and homing of other immune cells. Moreover, eosinophils promote survival of plasma cells within the bone marrow and the gut and provide a balance between T-regulatory and T-helper responses in the lungs and in the gut.

As intracellular organelles, eosinophils contain a plenty of secretory granules that are central to the functional responses of eosinophils (Neves et al., 2008). Human eosinophil granules comprise of four major cationic proteins: major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN). These cationic proteins are potent toxic against to human airway epithelium and parasitic helminths which damage cells by binding cell membranes thus disrupting the lipid bilayer or changing the activity odd enzymes within tissues (Long et al., 2016). The granules also contain various cytokines, chemokines, lipid mediators and immunomodulatory molecules including matrix metalloproteinases (Long et al., 2016). In addition to the tissue destructive properties of some mediators released from eosinophils, growth factors can also be released with tissue repair properties. Eosinophils may interact with tissue components and maintains tissue homeostasis or repair and remodeling under various physiological or pathological conditions. Unlike the cells of adaptive immunity which require hours or days to produce and release cytokines, eosinophils can secrete their preformed granule contents immediately by degranulation in response to certain stimuli (Long et al., 2016).



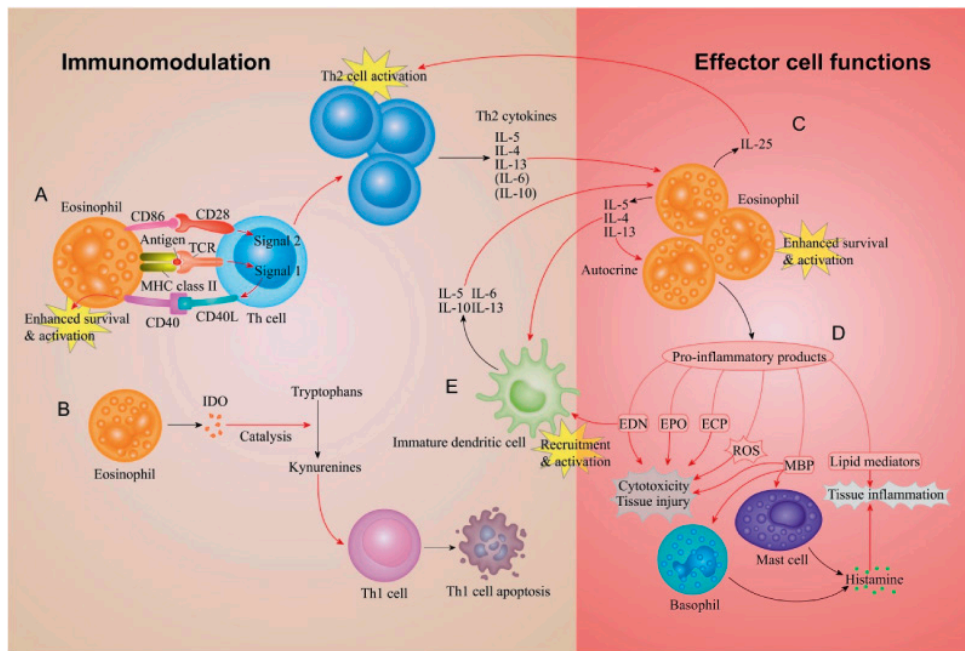
**Figure 1.6:** Innate immune functions of eosinophils and their contributions to host defense(Shamri et al., 2011).

In eosinophils, cytokine release may occur via different pathways including constitutive pathway, exocytotic degranulation and piecemeal degranulation (Bandeira-Melo & Weller, 2005). The constitute pathway can be blocked by protein synthesis inhibitor such as actinomycin D or cycloheximide which prevent release of mediators via vesicular transport. The constitutive pathway is a typical form of cytokine secretion in lymphocytes, not representing the main form of cytokine secretion in eosinophils. Eosinophils can store pre-formed cytokines and mediators within their specific granules and release those cytokines or mediators without *de novo* synthesis. Eosinophils uses exocytotic degranulation against to parasites and release their toxic proteins onto surface of helminths. However, exocytotic degranulation does not serve for granule-stored cytokines release. Furthermore, Dvorak et al. (1991) revealed a new secretory pathway in eosinophils named as piecemeal degranulation which is the major secretory pathway in eosinophils enabling the differential release of granule-stored cytokines.



**Figure 1.7:** Eosinophil pathways of cytokine release(Bandeira-Melo & Weller, 2005).

It is known that eosinophils have functional roles in both innate and adaptive immunity. In innate immunity, eosinophils have destructive and pro-inflammatory role in the Th2 immune response associated with parasite infection and allergic inflammation. Danger signals released by tissue injury or pathogen infections may recruit eosinophils which promote tissue remodeling and repair, and induce host defense against parasitic, bacterial, fungal or viral infection. Eosinophils also act as antigen presenting cells in response to parasitic infection or allergen challenge and in the meantime, they function as a versatile coordinator which regulates and interacts with various immune cells including T lymphocytes(Long et al., 2016). Effector functions of eosinophils are initiated by T helper 2 (TH2) cell-mediated immunity in response to allergens or pathogens through antigen presentation to TH cells and which promotes TH2 cell differentiation and activation, and TH2 cytokine release including IL-4, IL-5 and IL-13. Meanwhile, eosinophils are activated which favor the polarization of TH2 immunity through TH1- cell apoptosis by an IDO, an enzyme catalyzes the tryptophan catabolism in eosinophils. These TH2 cytokine promotes eosinophil differentiation, activation, survival and recruitment. The activated eosinophils successively produce and secrete cytokines including IL-4, IL-5, IL-13 and IL-25 thereby enhancing their own activity as an autocrine manner. Moreover, TLR ligands are capable of inducing eosinophil activation (Kvarnhammar & Cardell, 2012) and PRRs can also exert an anti- TH2 effect against to extracellular parasitic infections (Ditgen et al., 2014).



**Figure 1.8:** Mechanisms involved in the immunomodulatory functions and the effector cell functions of eosinophils in Th2 immunity (Long et al., 2016).

## 1.6 Extracellular Matrix and Its Components

The resulting immune response against to most infections are tissue-specific due to distinct extracellular matrix (ECM) signatures which consist of complex of secreted macromolecules such as carbohydrates and proteins which forms the ‘scaffolding’ of the airway and other tissues (Morwood & Nicholson, 2006) (Abdala-Valencia et al., 2018). As well as mechanical support, the ECM also plays important role in structural and functional maintenance of airway. Nevertheless, it is such a dynamic signaling network which has roles in migration, proliferation and differentiation of cells to maintain tissue homeostasis (Abdala-Valencia et al., 2018).

The major components of ECMs are collagens, elastins, laminins, fibronectins, hyaluronan (HA), proteoglycans (PGs). By interacting with each other, ECM molecules create meshwork structure that hosts for various cell types (Theoharis et al., 2019). Furthermore, ECM molecules interact with nonconventional receptors including toll-like receptors (TLRs) and growth factor receptors and regulate signaling (Theoharis et al., 2016). The components of ECM may also communicate with various cell types including immune cells, fibroblasts, epithelial cells and endothelial cells that reside within ECM to adjust their functions via several cell surface receptors including integrins, cell surface PGs and layilin (Theoharis et al., 2019). Since ECMs have a continued remodeling under normal circumstances such as wound healing or in

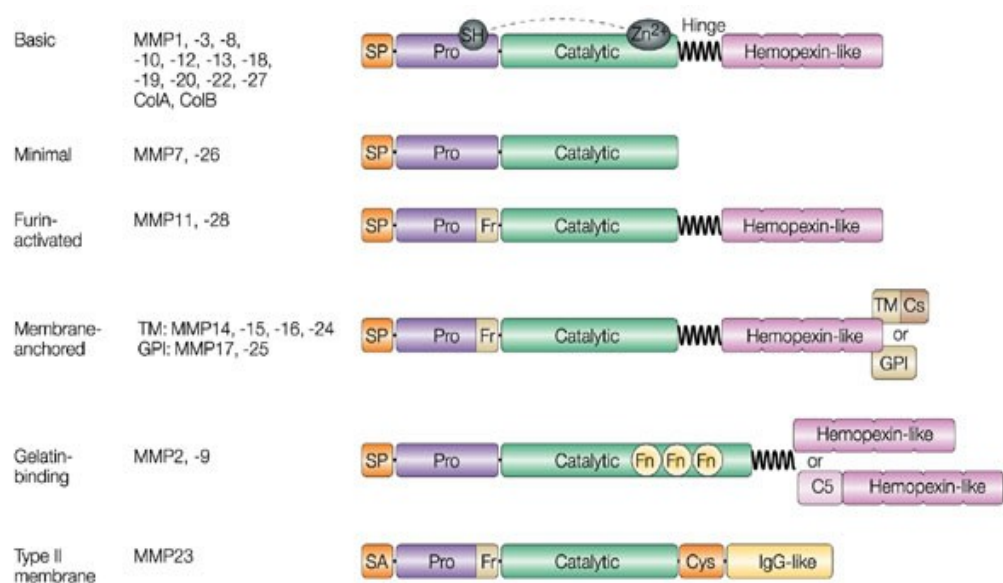
pathological conditions, maintaining ECM composition and structure properly is important for tissue integrity and functionality(Karamanos et al., 2019). Matrix remodeling is a well-programmed process which involves matrix degradation followed by production and accumulation of matrix components simultaneously (Theocharis et al., 2019) and thereby results in replacement of ECM. Matrix degrading enzymes including matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), ADAMs with thrombospondin motifs (ADAMTS), plasminogen activators, intracellular cathepsin and granzymes have been reported to involved in matrix remodeling(Theocharis et al., 2019). Even though fibroblasts constitute the main source of matrix molecules, all cells promote matrix formation during matrix remodeling.

### **1.6.1 Matrix Metalloproteinases (MMPs) family and their regulation**

The matrix metalloproteinases (MMPs) or matrixins are calcium dependent zinc-containing endopeptidases consisting of at least 24 members in vertebrates (Visse & Nagase, 2003) that are involved in ECM degradation(Verma & Hansch, 2007). Recent studies have been also revealed that MMPs are involved in the release or activation of cytokines, chemokines and growth factors that have roles in innate and adaptive immunity, inflammation, bone and airway remodeling and angiogenesis (Loffek et al., 2011). It has been also shown that each MMPs comprise of specific domain sequence and several domain motifs including the signal peptide(SP), the pro-peptide domain or pro-domain, the catalytic domain and the hemopexin-like domain at the C-terminus which exist in nearly all MMPs (Snoek-van Beurden & Von Den Hoff, 2005). MMPs contain a zinc ion ( $Zn^{2+}$ ) in the catalytic domain that is ligated to three conserved histidine residues in the HEXXHXXGXXH motif which is the signature of MMP family(Chen et al., 2013).

MMPs can be secreted or anchored to the cell membrane and are classified into collagenases, gelatinases, membrane- type MMPs, stromelysins, matrilysins and others based on the organization of MMP domains together with their structure and substrate specificity. MMP-1, MMP-8, MMP-13 and MMP-18 are collagenases which cleave interstitial collagen type I, II, and III and also some of the ECM and non-ECM proteins. Gelatinases degrade both gelatins and collagens and MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are the most studied MMPs that categorized as gelatinases. MMP-3, MMP-10 and MMP-11 are stromelysins and MMP-3 and MMP10 have

similar substrate specificities and involved in proteolysis of pro-MMPs, while MMP-11 have different structure and function. MMP-7 and MMP-26 are matrilysins which lack hemopexin-like domain. MMP-26 cleaves several ECM components, while MMP-7 process many cell surface molecules. Membrane-type (MT)-MMPs are expressed on the cell surface which have transmembrane or cytoplasmic domain at the C-terminus that consist of MMP-14, MMP-15, MMP-16 and MMP-24. Additionally, MMP-17 and MMP-25 are glycosyl-phosphatidylinositol-anchored MMPs which are also MT-MMPs. MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28 are other MMPs that are not assigned to any categories(Parks et al., 2004).



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**Figure 1.9:** Domain structure of the mammalian MMP family(Parks et al., 2004).

Since MMPs target degradation of ECM and non-ECM components and activation of other MMPs or growth factors release, catalytic activity of MMPs have been regulated at transcriptional and translational level, by zymogen (pro-enzyme) activation, by the endogenous and extracellular inhibitors, by subcellular or extracellular localization and internalization (Djuric & Zivkovic, 2017). The expression of MMPs is regulated by the activation of multiple signaling pathways including NF- $\kappa$ B, activating protein (AP)-1. There are also multiple factors which triggers different signaling cascade activation that modulates MMP gene expression that can be chemokines, cytokines or growth factors such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-  $\alpha$ ), epidermal growth factor(EGF) (Djuric & Zivkovic, 2017). Reactive

oxygen species (ROS), shear and tensile stresses or mechanical injury may also modulate the expression of MMPs. It has been also shown that MMPs could be down-regulated by the anti-inflammatory molecules such as transforming growth factor-  $\beta$  (TGF- $\beta$ ), nitric oxide (NO), interleukin-10 (IL-10). Integrins and adhesion molecules which have roles in cell-cell and cell-matrix interactions also regulates the MMPs expression(Djuric & Zivkovic, 2017).

One of the mechanisms that regulates MMP activation is zymogen activation. Most of the MMPs are synthesized and secreted as inactive form with a pro-domain which contains a cysteine switch sequence in close proximity to catalytic domain. These cysteine residue interacts with the catalytic zinc ion to prevent binding and cleavage of substrate and keep enzyme latent (Van Wart & Birkedal-Hansen, 1990). The activity of the MMP zymogen hinge on displacement of the pro-domain by conformational change or proteolysis which breaks apart cysteine-zinc pairing and thereby the enzyme can cleave and degrade its substrate(Loffek et al., 2011). Plasmin is a serine protease which is one of the most significant activators of MMPs in vivo which activates MMP-1,-3,-7,-8,-9,-10 and -13 (Lijnen, 2001). Other serine proteases including chymases and tryptases have potential to activate pro-MMPs. Furthermore, pro-MMPs have been also activated by active-MMPs. For instance, MMP-3 may activate pro-MMP-1,-7,-8,-9and -13. Active-MMP-7 then may active pro-MMP-1,-9 and -13. Additionally, thiol group modifying agents such as reactive oxygen species and oxidized glutathione could activate pro-MMPs. Therefore, multi-step and strict regulation and the balance between activation and inhibition of MMPs are critical to avoid from inflammation, uncontrolled ECM turnover, and dysregulation in cell growth and migration which eventually result in disease progression. For this, there are endogenous inhibitors of MMPs in tissues which are tissue inhibitors of metalloproteinases or TIMPs.

#### **1.6.1.1 Gelatinases**

Gelatinases or Type IV collagenases are one of the most studied and well characterized classes of matrix metalloproteinases which degrade collagen (e.g.gelatin) and contribute to the etiology of non-neoplastic and neoplastic pathologies. Gelatinases consist of two members which are gelatinase A or MMP-2 and gelatinase B or MMP-9(Ricci et al., 2016). MMP-2 is a non-glycosylated protein which is expressed in normal endothelial and epithelial cells, fibroblasts and in many cancer cells. In addition

to collagenases, MMP-2 also digests type I, II, and III collagens and target cleavage of various substrates including cytokines, receptor or growth factors. Unlike MMP-2, the pro-domain and the catalytic domain of MMP-9 contains two N-glycosylated sites(Ricci et al., 2016). MMP-9 is expressed in leukocytes, macrophages, endothelial cells and transformed cells which is involved in the remodeling of extracellular matrix by the cleavage of structural proteins including gelatin, elastin, laminin types IV, V, XI and XVI collagens. Nevertheless, MMP-9 is also involved in the regulation of numerous cellular functions including apoptosis, cell growth, invasion and metastasis, angiogenesis and immune response by the cleavage of growth factor precursors, cell surface receptors, cell adhesion molecules and other bioactive proteins(Ricci et al., 2016). It is also shown that MMP-9 expression is regulated by the extracellular matrix metalloproteinase inducer EMMPRIN or CD147 as well as cytokines and growth factors(Kim et al., 2009).

Several studies also show that as well as inflammatory mediators which induce leukocyte migration to sites of infection or injury, MMPs including gelatinases can provoke or repress inflammation by the proteolytic processing of cytokines and chemokines. One of the MMP substrates which are important in augmenting inflammatory response include cytokines such as Tumor necrosis factor-  $\alpha$  (TNF- $\alpha$ ) which is a pro-inflammatory cytokines seen in severe sepsis and in several autoimmune diseases including rheumatoid arthritis (RA) and multiple sclerosis(MS) (Kollias et al., 1999). Interleukin-  $1\beta$  (IL- $1\beta$ ) is another pro-inflammatory cytokine which requires proteolytic processing for activation. Caspase-1 or IL- $1\beta$ -converting enzyme (ICE) is required for IL- $1\beta$  processing but recent studies revealed the roles of other proteases for IL- $1\beta$  processing in ICE-null mice (Greten et al., 2007). It is shown that MMP-2, MMP-3 and MMP-9 can activate the IL- $1\beta$  precursor to the active 17-KDa form (Schönbeck et al., 1998) and mature IL- $1\beta$  can be degraded by MMP-1,-2,-3 and -9 which suggest that dual roles of MMPs in either promoting or suppressing IL- $1\beta$  (Ito et al., 1996). Therefore, further investigation into the IL- $1\beta$  activity through MMPs is required in normal and pathological processes.

Moreover, recent studies revealed the role of MMP-2 and MMP-9 in human diseases such as auto-immune and cardiovascular diseases, type 2 diabetes, neurodegenerative diseases and gelatinases is most extensively studied in cancer progression, invasion and metastasis (Ricci et al., 2016) (Klein & Bischoff, 2011). MMPs have also been

studied in asthma models and among them, MMP-9 has been the predominant one that is found in blood, bronchoalveolar lavage fluid (BALF) of patients with asthma(Dahlen et al., 1999).

### **1.6.2 Tissue Inhibitors of Metalloproteinases (TIMPs)**

Tissue inhibitors of metalloproteinases are endogenous inhibitors of MMPs that consist of four members which are TIMP-1, TIMP-2, TIMP-3 and TIMP-4 (Leco et al., 2006). TIMPs bind MMPs with a 1:1 molar ratio (Visse & Nagase, 2003). Normally, the ECM homeostasis is maintained by the balance between the active MMPs and TIMPs and disruption of this balance may lead to ECM degradation or ECM deposition that associated with various pathological conditions including asthma, pulmonary fibrosis, cancer invasion and metastasis (Leco et al., 2006). All mammalian TIMPs have similarities but they have different biochemical properties, Structural features and expression patterns. In general, the TIMPs are ranging from 21 to 28 kDa in size and have an N- and C-terminal domain and the N-terminal domain is able to inhibit MMPs. Among TIMPs, TIMP-3 is the only one that is bound to the ECM. TIMP-1, -2 and -4 can be found as soluble or cell surface associated. It has been defined that TIMP-1 or TIMP-3 can interact with pro-MMP-9 and TIMP-2, TIMP-3 or TIMP-4 with pro-MMP-2(Murphy & Nagase, 2009).

The TIMP1 is expressed prominently in the lungs, reproductive organs, bone and its expression can be regulated by various cytokines and growth factors. It has been reported that pro-inflammatory cytokines such as tumor necrosis factor-  $\alpha$  (TNF- $\alpha$ ), transforming growth factor  $\beta$  (TGF $\beta$ ), and interleukin 1 $\beta$  (IL-1 $\beta$ ) upregulated the expression of TIMP1 (Chirco et al., 2006). TIMP2 is also expressed in many different mammalian tissues and can be regulated by cytokines, growth factors and bacteria. For instance, TIMP2 expression had been upregulated by lipopolysaccharide (LPS) in macrophages while downregulated by TNF- $\alpha$  and TGF- $\beta$  (Masciantonio & Gill, 2017). Moreover, TIMP-3 which is expressed in the lung, heart, thymus and kidney, tightly regulated during proliferation and differentiation (WG, 2008). Several studied also revealed that pro-inflammatory conditions reduced TIMP3 expression in microvascular endothelia cells and increased inflammation has been observed in the multiple organs of injury models in mice lacking TIMP3(Khokha et al., 2013). Among all TIMPs, TIMP-4 is the least studied TIMP which is expressed in heart and

kidney and its expression is extremely reduced in many central nervous systems (CNS) diseases.

### **1.7 Eol-1 Cell Line**

Since eosinophils constitute %1-6 of circulating leukocytes and their life expectancy is very limited in vitro, Eol-1 cell line has been established from peripheral blood of a patient with Philadelphia chromosome-negative eosinophilic leukemia (Saito et al., 1985) and use as an in vitro eosinophilic model to investigate eosinophilic function (Jung, 2015). Studies revealed that stimulation with butyric acid differentiated Eol-1 cells into mature eosinophilic cells (Jung, 2015). Eol-1 cells have a FIP1L1 (fip1-like)-PDGFR $\alpha$  (platelet-derived growth factor receptor) fusion gene which is responsible for permanent activation of tyrosine kinase that then prompts to cell proliferation (Ishihara et al., 2008).

### **1.8 Aim of the Study**

The aim of this project to investigate the roles of NLRs and PRRs on regulation of matrix metalloproteinases and their tissue inhibitors in Eol-1 human eosinophilic cells by focusing on the effect of NLRC4 inflammasome activation. Recent studies reveal that PAM3CSK4 induces MMP-9 gene expression through the TLR-2/ MyD88-dependent mechanism that leads to activation of MEK1/2/ERK, p38 MAPK and NF- $\kappa$ B transcription factor in THP-1 monocytic cells (Al-Rashed et al., 2017) and LPS induces MMP-2 and MMP-9 gene expression in RAW264.7 murine monocytes (Y. H. Yang et al., 2015). Despite the relation between TLR signaling and MMP-9 gene expression in immune cells, the molecular effects of NLRC4, other NOD-like receptors and inflammasome complex are not studied either in eosinophils or other immune cells as well. Therefore, in this study, regulation of MMP-2, MMP-9 and their tissue inhibitors TIMP-1, TIMP-2 which have roles in remodeling of extracellular matrix and inflammation will be investigated through NLRC4 inflammasome in Eol-1 human eosinophilic cells. To serve this purpose we examine, 1) the effects of NLRC4 inflammasome activation on MMP-2, MMP-9, TIMP-1 and TIMP-2 expression and activation in Eol-1 cells, 2) the inflammasome independent IL-1 $\beta$  processing by MMP-9, 3) how protein synthesis inhibitor affects MMP-2 and MMP-9 production and release in Eol-1 cells, 4) the effects of NLRC4 inflammasome activation on

extracellular matrix metalloproteinase inducer (EMMPRIN or CD147), eosinophil degranulation and activation markers.





## 2. MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Equipment

Equipment used in this study are listed in Appendix A.

#### 2.1.2 Commercial Kits

Equipment used in this study are listed in Appendix B.

#### 2.1.3 Chemicals

Equipment used in this study are listed in Appendix C.

#### 2.1.4 Cell Culture

##### 2.1.4.1 Growth media

The components and their volumes for preparation of 10% FBS complete RPMI media were given in Table 2.1. These media were stored in 4°C refrigerator.

**Table 2.1:** The components of cell culture medium for EoL-1 cells.

Component	Amount	Final Concentration
RPMI 1640 Media	500 ml	
FBS	50 ml	10%(v/v)
Penicillin /Streptomycin	5 ml	1%(v/v)
L-glutamine	5 ml	1%(v/v)

##### 2.1.4.2 Eol-1 cell culture medium for transfection

The components for the preparation of EoL-1 cell culture medium for transfection are listed in Table 2.2. The medium was stored in 4°C.

**Table 2.2:** The components of the transfection medium.

Component	Amount	Final Concentration
RPMI 1640 Media	500 ml	
FBS	50 ml	10%(v/v)
Penicillin /Streptomycin	5 ml	1%(v/v)
L-glutamine	5 ml	1%(v/v)

**2.1.4.3 Freezing media for Eol-1 cells**

The components and their volumes for the preparation of freezing media were given in the Table 2.3. These media were stored in -20°C.

**Table 2.3:** The components of the freezing medium.

Component	Amount	Final Concentration
FBS	9 ml	90%(v/v)
DMSO	1ml	10%(v/v)

**2.1.5 Buffers used in SDS PAGE and western blotting****2.1.5.1 4X Sample Buffer**

The components for the preparation of the 4X Sample Buffer are listed in Table 2.4. The buffer was stored in -20°C.

**Table 2.4:** The components of the 4X sample buffer.

Component	Amount	Final Concentration
0.5 M Tris-HCl	0.6 ml	300 mM
Glycerol	0.4 ml	10%(v/v)
SDS	0.1 g	0.01%(v/v)
Bromophenol Blue	0.1 g	0.01%(v/v)
β-Mercaptoethanol (14.3 M)	50 μL	355mM

### 2.1.5.2 5X Running Buffer

The components for the preparation of the 5X Running Buffer are listed in Table 2.5. The buffer was stored in 4°C. 1X running buffer was prepared by diluting 5X running buffer with dH<sub>2</sub>O and used in SDS PAGE protein running step.

**Table 2.5:** The components of the 5X running buffer.

Component	Amount	Final Concentration
Trisma Base	15 g	1.5%(v/v)
Glycine	72 g	7.2%(v/v)
SDS	5 g	0.5%(v/v)
dH <sub>2</sub> O	1000 ml	

### 2.1.5.3 1X Transfer Buffer

1X transfer buffer was prepared before the transfer and used freshly. Components and their volumes of 1X transfer buffer were given in the Table 2.6. 1X transfer buffer was stored in 4°C.

**Table 2.6:** The components of the 1X transfer buffer.

Component	Amount	Final Concentration
Trisma Base	5.8 g	0.58%(w/v)
SDS	0.37 g	0.037% (w/v)
Glycine	2.9 g	0.29%(w/v)
Methanol	200 ml	20%(v/v)
dH <sub>2</sub> O	800 ml	

### 2.1.5.4 10X Phosphate-Buffered Saline pH 7.4 (PBS) and PBS-T

10X PBS was prepared as given in the Table 2.7 and pH is adjusted to 7.4. 1X PBS-T is prepared by diluting 10X PBS with dH<sub>2</sub>O and adding 0.5 mL Tween-20. 10X PBS and 1X PBS were stored in room temperature.

**Table 2.7:** The components of the 10X PBS.

Component	Amount	Final Concentration
NaCl (Mw: 58.44 g/mol)	80.06 g	8%(w/v)
KCl (Mw: 74.55 g/mol)	2.012 g	0.2%(w/v)
Na <sub>2</sub> HPO <sub>4</sub> (Mw: 141.96 g/mol)	11.365 g	1.1%(w/v)
KH <sub>2</sub> PO <sub>4</sub> (Mw: 136.0855 g/mol)	2.721 g	0.27%(w/v)
dH <sub>2</sub> O	1000	

#### **2.1.5.5 5% Blocking buffer (western blotting)**

Blocking buffer was used to block PVDF membrane after the transfer step to prevent non-specific binding of antibodies. 5% blocking buffer was prepared by dissolving 1 g skim milk in 20ml PBS-T.

#### **2.1.5.6 1.5M Tris-HCl pH 8.8**

90.75 g of Trisma-Base was dissolved in 400 mL dH<sub>2</sub>O and pH was adjusted to 8.8. Volume was completed to 500 mL. 1.5M Tris-HCl pH 8.8 solution was stored at room temperature.

#### **2.1.5.7 1.5M Tris-HCl pH 6.8**

90.75 g of Trisma-Base was dissolved in 400 mL dH<sub>2</sub>O and pH was adjusted to 6.8. Volume was completed to 500 mL. 1.5M Tris-HCl pH 6.8 solution was stored at room temperature.

#### **2.1.5.8 10%SDS**

10 g of SDS was dissolved in 100 mL dH<sub>2</sub>O. 10% SDS solution was stored at room temperature.

#### **2.1.5.9 10% Ammonium Persulfate (APS)**

10 g of APS was dissolved in 100 mL dH<sub>2</sub>O. 10% APS solution was prepared and used freshly.

### 2.1.5.10 SDS-PAGE Gels

10% resolving gel and 5% stacking gel were used in this study. Components and their volumes were given in Table 2.8.

**Table 2.8:** The components of the SDS PAGE gel.

Component	10% Resolving gel	5% Stacking gel
	Amount	Amount
dH <sub>2</sub> O	4.75 mL	4.38 mL
40% Acrylamide-Bisacrylamide (37.5:1)	2.5 mL	810 µL
1.5M Tris-HCl pH: 8.8	2.5 mL	-
1.5M Tris-HCl pH: 6.8	-	750 µL
10% SDS	100 µL	60 µL
10% APS	100 µL	60 µL
TEMED	10 µL	6 µL

### 2.1.5.11 Gel Staining and Destaining Solutions

For staining protein bands in SDS-PAGE gel, Coomassie blue solution was used and destaining solution was used to remove unbound stain. The components and their volumes of these solutions were listed in Table 2.

**Table 2.9:** The components of the staining and destaining solutions.

Component	Staining		Destaining	
	Amount	Concentrations	Amount	Concentrations
Acetic Acid	10 mL	10% (v/v)	10 mL	10% (v/v)
Methanol	50 mL	50% (v/v)	50 mL	50% (v/v)
Coomassie Brilliant Blue (G-250)	0.1 g	0.1 (w/v)	-	-
dH <sub>2</sub> O	Up to 100 mL		Up to 100 mL	

### 2.1.6 BCA Assay Standards

BCA assay standards were prepared as kit's instructions. 1X lysis buffer which contains protease inhibitor cocktail was used as diluent. The components and their volumes of BCA assay standards were listed in Table 2.10.

**Table 2.10:** BCA standards and their concentrations.

Standards	Diluent ( $\mu\text{L}$ )	BSA ( $\mu\text{L}$ )	Final BSA Concentrations
A	0	300 $\mu\text{l}$ from the stock	2000
B	125	375 $\mu\text{l}$ from the stock	1500
C	325	325 $\mu\text{l}$ from the stock	1000
D	175	175 $\mu\text{l}$ from B	750
E	325	325 $\mu\text{l}$ from C	500
F	325	325 $\mu\text{l}$ from E	250
G	325	325 $\mu\text{l}$ from F	125
H	400	100 $\mu\text{l}$ from G	25

### 2.1.7 Antibodies

Antibodies and their dilutions used in Western Blotting (WB), ELISA and flow cytometry (FC) were listed in Table 2.11.

**Table 2.11:** Antibodies used in this study and their dilutions.

Antibody	Catalog Number	Dilution
Anti-MMP2	CST 4022	1:1000 (WB)
Anti-MMP9	CST 3852	1:1000 (WB)
Anti-NLRC4	Biolegend 659702	1:1000 (WB)
Anti-Caspase-1 (p20-p22)	Ab207797	1:1000 (WB)
Cleaved-IL-1 $\beta$ (Asp116)	CST 83186	1:1000 (WB)
Anti- GAPDH	STJ97462	1:3000 (WB)
Anti- $\beta$ -Actin	CST 4970	1:5000 (WB)
Anti-rabbit IgG HRP-linked antibody	CST 7074	1:3000 (WB)
Anti-mouse IgG HRP-linked antibody	CST 7076	1:3000 (WB)
Purified anti-human IL-1 $\beta$	BioLegend 508201	1:250 (ELISA)
Biotin anti-human IL-1 $\beta$	BioLegend 508301	1:250 (ELISA)
Anti-CD147	Ab194401	0.5-1 $\mu$ l for 10 <sup>6</sup> cells(FC)
APC anti-human CD63	BioLegend 310909	5 $\mu$ l for 10 <sup>6</sup> cells(FC)
APC anti-human CD69	BioLegend 353007	5 $\mu$ l for 10 <sup>6</sup> cells(FC)
Goat Anti-Mouse IgG H&L (Alexa Fluor® 488) preadsorbed	Ab150117	1:2000 (FC)

### 2.1.8 Primers

Primers used in this study were listed in Table 2.12.

**Table 2.12:** Primers used in this study.

Primer	Sequence (5' – 3')	Size
Human HPRT1 forward	GACCAGTCAACAGGGGACAT	195 bp
Human HPRT1 reverse	AACACTTCGTGGGGTCCTTTTC	
Human MMP2 forward	CCGTGTTTGCCATCTGTTTTAG	157 bp
Human MMP2 reverse	AGGTTCTCTTGCTGTTTACTTTGGA	
Human MMP9 forward	TTCTCCAGAAGCAACTGTCC	151 bp
Human MMP9 reverse	TAGGTGATGTTGTGGTGGTG	
Human TIMP1 forward	AATTCCGACCTCGTCATCAG	230 bp
Human TIMP1 reverse	TGCAGTTTTCCAGCAATGAG	
Human TIMP2 forward	TTCATTCGTCTCCCGTCTTT	113 bp
Human TIMP2 reverse	ACCAACGTGTGTGGATCAAA	

### 2.1.9 RT-qPCR conditions

The component and their volumes and reaction steps for one reaction of RT-qPCR were listed in Table 2.13 and in Table 2.14 respectively.

**Table 2.13:** RT-qPCR reaction volumes.

Component	Volume
SYBR Green (2X)	10 $\mu$ L
Forward Primer (10 $\mu$ M)	1.25 $\mu$ L
Reverse Primer (10 $\mu$ M)	1.25 $\mu$ L
RNase free dH <sub>2</sub> O	5.8 $\mu$ L
Reverse Transcriptase Mix	0.2 $\mu$ L
Template (50 ng/ml)	1.5 $\mu$ L
TOTAL REACTION VOLUME	20 $\mu$ L

**Table 2.14:** RT-qPCR reaction steps.

Steps	Temperature	Time
Reverse Transcription	50°C	30 min
Initial PCR Activation	95°C	15 min
Denaturation	94°C	15 sec (40 cycles)
Annealing	59 °C	30 sec (40 cycles)
Amplification	72 °C	30 sec (40 cycles)
Melting Curve	From 60°C to 90°C	1 min – 15 sec (Measurements are taken every 1°C increase in temperature)

### 2.1.10 Buffer used in Gelatin Zymography and Reverse Zymography

#### 2.1.10.1 3mg/ml and 20 mg/ml Gelatin Solution

300 mg and 2g of gelatin and 0.02 g of sodium azide were dissolved into 100 ml of dH<sub>2</sub>O. Stock solution was stored in 4°C. Before using, stock solution was heated in a 37°C water bath.

#### 2.1.10.2 5X Sample Buffer

The components for the preparation of the 5X SDS Sample Buffer were listed in Table 2.15. The 5X sample buffer was stored in -20°C.

**Table 2.15:** The components of the 5X SDS sample buffer.

Component	Amount	Final Concentration
0.2 M Tris-HCl (pH:6.8)	0.6 ml	300 mM
Glycerol	0.8 ml	20%(v/v)
SDS	0.5 g	0.05%(v/v)
Bromophenol Blue	1 g	0.1%(v/v)

#### 2.1.10.3 5X Running Buffer

The components for the preparation of the 5X Running Buffer were listed in Table 2.15. The pH was adjusted to 8.3 and buffer was stored in 4°C. 1X running buffer was

prepared by diluting 5X running buffer with dH<sub>2</sub>O and used in gelatin zymography and reverse zymography conditioned medium running step.

**Table 2.16:** The components of the 5X running buffer.

Component	Amount	Final Concentration
Trisma Base	15 g	1.5%(v/v)
Glycine	92 g	9.2%(v/v)
SDS	5 g	0.5%(v/v)
dH <sub>2</sub> O	1000 ml	

#### 2.1.10.4 Renaturing Buffer

2.5% v/v Triton X-100 was prepared in dH<sub>2</sub>O and store in room temperature.

#### 2.1.10.5 Development (Incubation) Buffer

The components for the preparation of the development buffer were listed in Table 2.17. The pH was adjusted to 7.6 and volume was completed to 250 mL with dH<sub>2</sub>O and development buffer was stored in 4°C.

**Table 2.17:** The components of the development (incubation)buffer.

Gelatin Zymography	Reverse Zymography
50 mM Tris-HCl	50 mM Tris-HCl
0.2 M NaCl	0.15 M NaCl
5 mM CaCl <sub>2</sub>	10 mM CaCl <sub>2</sub>
1 μM ZnCl <sub>2</sub>	0.1μM ZnCl <sub>2</sub>
0.05% Brij35	0.05% Brij35
	TritonX-100 2.5%

#### 2.1.10.6 1.5M Tris-HCl pH 8.8

90.75 g of Trisma-Base was dissolved in 400 mL dH<sub>2</sub>O and pH was adjusted to 8.8. Volume was completed to 500 mL. 1.5M Tris-HCl pH 8.8 solution was stored at room temperature.

#### 2.1.10.7 0.5M Tris-HCl pH 6.8

30.25 g of Trisma-Base was dissolved in 400 mL dH<sub>2</sub>O and pH was adjusted to 6.8. Volume was completed to 500 mL. 1.5M Tris-HCl pH 6.8 solution was stored at room temperature.

#### 2.1.10.8 10%SDS

10 g of SDS was dissolved in 100 mL dH<sub>2</sub>O. 10% SDS solution was stored at room temperature.

#### 2.1.10.9 10% Ammonium persulfate (APS)

10 g of APS was dissolved in 100 mL dH<sub>2</sub>O. 10% APS solution was prepared and used freshly.

#### 2.1.10.10 Gelatin Zymography and Reverse Zymography gels

**Table 2.18:** The components of the gelatin zymography and reverse zymography gel.

Component	7.5% Resolving gel (GZ) Amount	10% Resolving gel (RZ) Amount	5% Stacking gel Amount
dH <sub>2</sub> O	2.07 mL	2.716 mL	3.05 mL
Gelatin (3 mg/ml)	3.33 mL	-	-
Gelatin (20 mg/ml)	-	1.25 mL	-
Recombinant MMP-2/MMP-9	-	0.16 µg/mL	-
30% Acrylamide-0.8% Bisacrylamide	2.5 mL	3.33mL	650 µL
1.5M Tris-HCl pH: 8.8	2 mL	2.5 mL	-
0.5M Tris-HCl pH: 6.8	-	-	1.25 mL
10% SDS	100 µL	100 µL	50 µL
10%APS	100 µL	100 µL	50µL
TEMED	10 µL	10 µL	5 µL

#### 2.1.10.11 Recombinant human MMP-2, MMP-9, TIMP-1

Sigma #M9070 (MMP-2), #8945 (MMP-9) and #T8947 (TIMP-1) were used as standards in gelatin zymography and reverse zymography assays.

## **2.2 Methods**

### **2.2.1 Cell culture**

EoL-1 cell line was used as a model for investigating the eosinophilic functions. The cells were revived in 20% complete RPMI medium. After recovery, EoL-1 cells were cultured in 10% complete RPMI medium. The medium of the cells was renewed every 2 days.

### **2.2.2 PAM3CSK4 stimulation**

The cells were plated in 24 well plates as  $1.6 \times 10^6$  cells/ml in growth medium (without penicillin/streptomycin). EoL-1 cells were stimulated with TLR2 agonist PAM3CSK4 (1ug/ml).

### **2.2.3 ATP stimulation**

After 4h of PAM3CSK4 stimulation, EoL-1 cells were stimulated with 5 mM of ATP. For stock solution, ATP was dissolved in PBS (pH:7.2). After 30 min stimulation with ATP, cell medium was renewed, and cells were lysed 10h after PAM3CSK4 stimulation.

### **2.2.4 Flagella transfection**

EoL-1 cells were transfected with flagella at 4h later with PAM3CSK4 stimulation using transfection reagent lipofectamine 2000. Flagella (100ng) and lipofectamine (1.5ug) were diluted in Opti-MEM separately and were incubated for 5 min at room temperature. After incubation, diluted flagella and lipofectamine solutions were mixed and incubated for additional 20 min at room temperature. After incubation, the mixture was added to each well as 100ng/ml flagella and 1.5ug lipofectamine concentrations. Cells were lysed 24h after PAM3CSK4 stimulation.

### **2.2.5 Cycloheximide stimulation**

10 mg of cycloheximide was dissolved in 1ml of dH<sub>2</sub>O and filtered in a new eppendorf tube.  $1.6 \times 10^6$  cells in 1ml growth medium (without penicillin/streptomycin) were plated in 24 well plates and cells were pretreated with 5,10 and 20ug/ml of cyclohexamide and 1 hour later stimulation with 5 ug/ml of cycloheximide, the cells were stimulated with PAM3CSK4. After 4 hours of TLR2 stimulation with

PAM3CSK4, Eol-1 cells were transfected with flagella. Cells were lysed 24h after PAM3CSK4 stimulation. Cell-free mediums were collected for performing gelatin zymography.

#### **2.2.6 Pan-Caspase inhibitor (Z-VAD-FMK) stimulation**

Pan-caspase inhibitor (Z-VAD-FMK) was reconstituted in DMSO. The final stock concentration was 20 mM. Eol-1 cells were seeded  $1.6 \times 10^6$  cells/ml as in 24-well plate and stimulated with PAM3CSK. PAM3CSK4-primed Eol-1 cells were treated with 20  $\mu$ M of Z-VAD-FMK 1 h before transfecting with flagella. Cells were lysed 24 h after PAM3CSK4 stimulation. Cell-free mediums were collected for performing gelatin zymography, reverse zymography and ELISA.

#### **2.2.7 MMP inhibitor (GM6001) stimulation**

GM6001 which is a broad-spectrum MMP inhibitor was reconstituted in DMSO to obtain 2.5 mM stock concentration. Eol-1 cells were seeded as  $1.6 \times 10^6$  cells/ml in 24-well plate and pretreated with 25  $\mu$ M of GM6001 1 h before PAM3CSK4 stimulation then transfected with flagella. Cells were lysed 24 h after PAM3CSK4 stimulation. Cell-free mediums were collected for performing gelatin zymography and ELISA.

#### **2.2.8 Recombinant IL-1 $\beta$ stimulation**

Recombinant Human IL-1 $\beta$  was reconstituted in sterile PBS containing at least 0.1% bovine serum albumin. Eol-1 cells were seeded as  $1.6 \times 10^6$  cells/ml in 24-well plate and treated with different concentrations of recombinant human IL-1 $\beta$  (100 pg to 10 ng). Cells were lysed 24 h after stimulation. Cell-free mediums were collected for performing gelatin zymography.

#### **2.2.9 BCA assay**

For determining the unknown protein concentration of the lysed cells, Thermo Scientific Pierce BCA Protein Assay Kit was used. For preclearance, lysed cells were centrifuged at 13000 rpm for 20 min at 4°C and supernatant was collected. Precleared protein samples were diluted at 1:10 ratio in 1X lysis buffer which contains 1X protease inhibitor cocktail. 25  $\mu$ l of standards and 10  $\mu$ l of protein samples were added to TPP 96 well tissue culture plate. Reagent A and reagent B were mixed at 50:1 ratio

to prepare working reagent. 200 ul of working reagent was added to each well. The plate was covered and incubated for 30 min at 37 °C. The absorbance value of unknown protein samples and standard were read at 562 nm by using a spectrophotometer.

#### **2.2.10 Western blotting**

After gel polymerization, glass plates containing gels were placed in electrode assembly in the electrophoresis tank and whole apparatus was filled with 1X running buffer. 30ug of protein samples were prepared in 4X sample buffer which contains SDS and denatured at 95 °C for 10 min. Denatured proteins were loaded into the wells and samples were run at 80 volts until they reach the resolving gel. After they reached the resolving gel, samples were run at 120 volts. Proteins were then transferred to PVDF membrane in transfer sandwich at 16 volts overnight at 4 °C. The PVDF membrane was blocked in blocking solution for 1h to prevent nonspecific binding and incubated in primary antibody overnight at 4°C which was diluted in blocking solution overnight at 4°C. After incubation, the membrane was rinsed with PBS-T 3 times for 10 minutes and immediately incubated in the secondary antibody for 1 hour at room temperature by gently shaking. Later on, the membrane incubated with secondary antibody was again rinsed with PBS-T 3times for 10 minutes. As HRP substrates, ECL solutions SuperSignal West Pico and SuperSignal West Femto PLUS were used to detect protein bands on the membrane by using ChemiDoc Imaging System.

#### **2.2.11 Gelatin Zymography**

After gel polymerization, glass plates containing gels were placed in electrode assembly in the electrophoresis tank and whole apparatus was filled with zymography 1X running buffer. Recombinant MMP-2 and MMP-9 (M9070 and M8945, respectively, Sigma) were used as controls. 25 ul of conditioned medium from each sample were prepare in 5X non-reducing sample buffer without denaturing. Samples were run at constant 90 volts at 4°C until the samples they the bottom of the gel. After electrophoresis, gels were washed in zymogram renaturing buffer two times for 30 min at room temperature with gentle agitation to allow proteins to renature. The gels were then incubated in zymogram development Buffer 2-4 days at 37°C. The zymogram gels were stained with coomassie blue solution for 2 h and destained in water and then

were visualized by using ChemiDoc Imaging system. The gelatinase enzyme activity was appeared as white bands against dark background.

#### **2.2.12 Reverse Zymography**

After gel polymerization, glass plates containing gels were placed in electrode assembly in the electrophoresis tank and whole apparatus was filled with zymography 1X running buffer. Recombinant TIMP-1 (T8947, Sigma) was used as control. 25 ul of conditioned medium from each sample were prepare in 5X non-reducing sample buffer without denaturing. Samples were run at constant 100 volts at 4°C until the samples they the bottom of the gel. After electrophoresis, gels were washed in zymogram renaturing buffer two times for 30 min at room temperature with gentle agitation to allow proteins to renature. The gels were then incubated in zymogram development buffer overnight at 37°C. The gels were stained with coomassie blue solution for 1 h and destained in water and then were visualized by using ChemiDoc Imaging system. The inhibitory activity of TIMP-1 appeared as dark blue bands against lighter background.

#### **2.2.13 RNA isolation**

For RNA isolation, RNAqueous Total RNA Isolation Kit was used. Cells which were collected from 24-well plate, were centrifuged at 2000 rpm for 6 min. The pellet was lysed with 150 ul Lysis/Binding solution and vortexed. Then, 150 ul of 64% ethanol was added and shaken gently. The mixture was added to filter cartridge inside the collection tube and centrifuged at 13000 rpm for 1 min at 4°C. After centrifugation, the supernatant was removed. The column was washed with Buffer Solution 1 and samples were centrifuged at 13000 rpm for 1 min at 4°C. The supernatant was removed, and Wash Buffer Solution 2/3 was added to filter cartridge and samples were washed two times. After centrifugation, supernatant was removed, and the filter cartridge was place into a new collection tube and elution buffer was added. The column was centrifuged at 13000 rpm for 1 min 4°C. The purity and concentration of RNA were measured by using NanoDrop.

#### **2.2.14 RT-qPCR**

The QuantiTect SYBR Green RT-qPCR kit was used for one-step RT-qPCR. The reaction mix and steps were prepared according to kit's instructions and Applied

Biosystem's StepOne System Cycler was used for performing cycling reactions. The results were analyzed by using GraphPad Prism 8.

### **2.2.15 ELISA**

The Sandwich ELISA method was used to measure the levels of cytokines secreted from cells. For this purpose, Nunc MaxiSorp 96-well plates were coated with antigen of interest specific purified capture anti-human antibodies which was diluted in 100 ul of PBS (1:250) and the plate was sealed and incubated overnight at 4°C. After coating, the plate was washed with PBST 3 times. Thereafter, wells were blocked with 250 ul of 10% Blocking buffer for 1 hour at room temperature. After blocking, 100 ul of samples and standards were added to wells and incubated overnight at 4°C. The standards were added to wells with serial dilution starting from an appropriate concentration. After incubation, samples were removed, and wells were washed with PBST 4 times. Next, antigen of interest specific biotin conjugated anti-human antibodies which was diluted 100 ul of 10% blocking solution (1:250) were added to the each well and incubated for 1 hour at room temperature. After incubation, the plates were wash with PBST 5 times and 100 ul of HRP Avidin D which was diluted in 10% blocking solution (1:2000) was added and incubated for 30 minutes at room temperature avoiding from the light. Then, the wells were washed with PBST 6 times and 100 ul mixture of TMB Peroxidase Substrate and TMB Peroxidase Substrate Solution B (1:1) was added to each well. After observing color changes in the wells, the reaction was stopped by adding 100 ul of 1 N HCl. The plate was read with spectrophotometer at 450 nm and data were analyzed by using GraphPad Prism 8.

### **2.2.16 Flow Cytometry**

FACS buffer was prepared which consist of 2% FBS containing PBS and 200 ul of FACS buffer was added to the cell pellet. After resuspending the pellet, the cell suspension was centrifuged at 1500 rpm for 8 minutes at 4°C. The supernatant was discarded. The cells were resuspended in 50 ul FACS buffer containing conjugated antibodies. For unstained sample, 200 ul FACS buffer was added to cells and samples were incubated at 4°C for 1 hour in dark. After incubation, 200 ul FACS buffer was added and samples were centrifuged at at 1500 rpm for 8 min at 4°C (For nonconjugated antibodies, Goat Anti-Mouse IgG H&L(Alexa Fluor® 488) was used).The supernatant was removed, and cell pellet was resuspended in 200 ul FACS

buffer and the cells were counted by using BD Accuri™ C6 flow cytometer. Data were analyzed using FlowJo software.





### **3. RESULTS**

#### **3.1 Matrix Metalloproteinases-2 And -9 Were Expressed And Inducible In Eol-1 Cells At mRNA Level.**

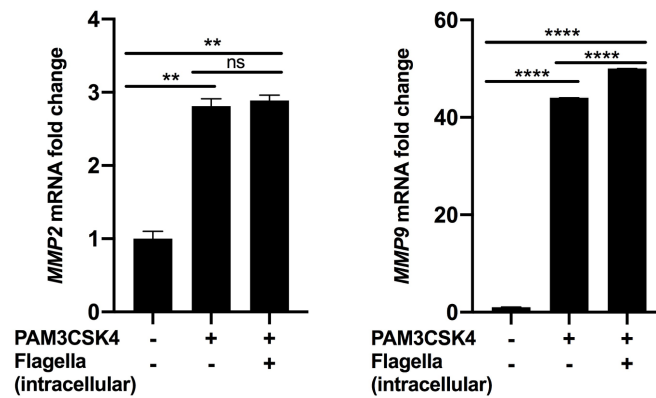
We previously showed that NLRC4 and NLRP3 inflammasomes are expressed and inducible in Eol-1 cells. To assess whether the mRNA expressions of MMP-2, MMP-9, TIMP-1 and TIMP-2 could be regulated through NLRC4 and NLRP3 inflammasomes, Eol-1 cells were stimulated with PAM3CSK4 and 4 h later transfected with flagella or stimulated with ATP and total RNA was isolated at 24 h and 10h post-stimulation with PAM3CSK4, respectively. The results from RT-qPCR analysis showed that the MMP-9 mRNA expression was significantly higher upon NLRC4 inflammasome activation. The expression of MMP-9 was significantly increased by 44-fold with TLR priming and 50 -fold by NLRC4 inflammasome activation at 24 h. The mRNA levels of MMP-2 increased by 2.81-fold with TLR priming and 2.88-fold by NLRC4 inflammasome activation which were not much as MMP-9. The results also showed that MMP-9 mRNA expression was significantly increased by 6.38-fold with TLR priming and 5.41-fold with NLRP3 inflammasome activation. The mRNA levels of MMP-2 increased by 1.4 -fold with TLR priming and 1.23-fold by NLRP3 inflammasome activation which were not much as MMP-9.

#### **3.2 Matrix Metalloproteinases-2 And -9 Were Expressed And Inducible In Eol-1 Cells At Protein Level.**

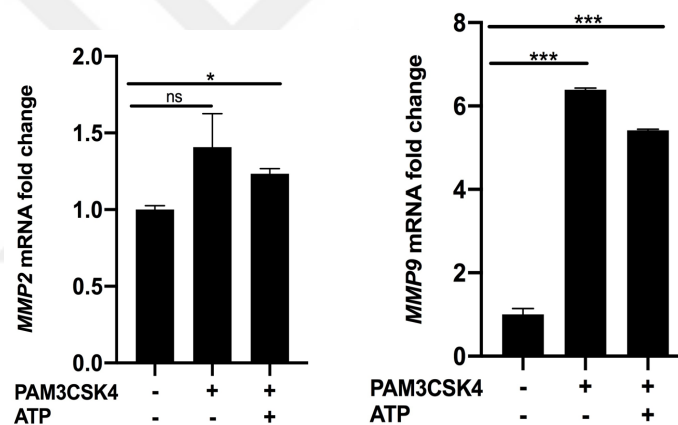
Next, we examined the protein expressions of MMP-9 and MMP-2 after NLRC4 and NLRP3 inflammasome activation by western blotting. The protein expression of MMP-9 increased by 2.1- fold with NLRC4 inflammasome activation while MMP-9 protein expression was increased by 1.25-fold with TLR priming step. However, NLRC4 inflammasome activation did not change the MMP-2 protein expression (Figure 3.2 A.). Moreover, the MMP-9 protein expression increased by 1.26-fold with

NLRP3 inflammasome activation and MMP-2 protein expression decreased by 0.84 - fold with NLRP3 inflammasome activation (Figure 3.2 B.).

A.

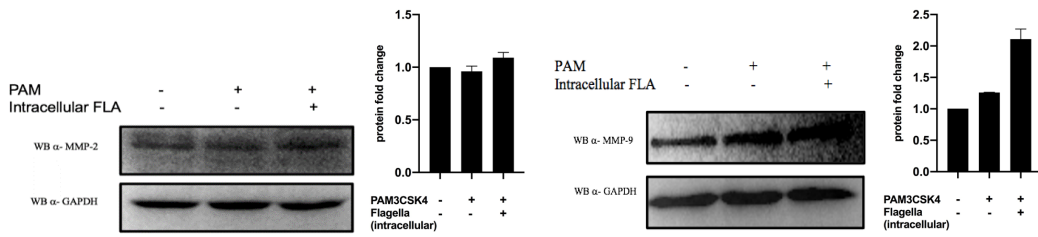


B.

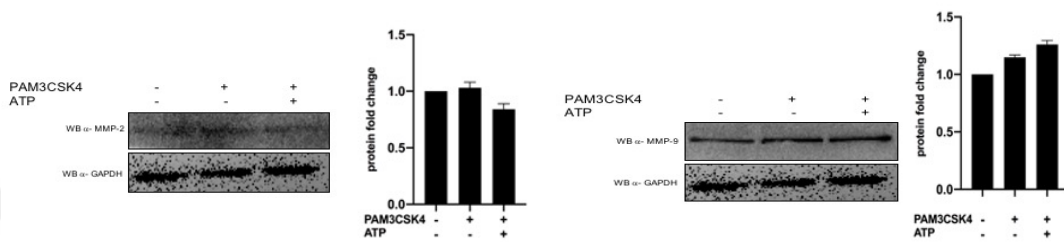


**Figure 3.1:** Matrix metalloproteinases-2 and -9 and their tissue inhibitors TIMP-1 and TIMP-2 is expressed and inducible in Eol-1 cells at mRNA level. (A-B) Eol-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later transfected with flagella (100 ng/ml) or stimulated with ATP (5 mM) and total mRNA was isolated from cells after 24- and 10-hours incubation, respectively. RT-qPCR was performed, and relative mRNA expression was normalized to HPRT1 mRNA expression. Graphs were prepared by using GraphPad Prism 8. Each value represents the mean  $\pm$  SEM of two replicates analyzed by Student *t* test. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001, ns: non-significant, PAM: PAM3CSK4, FLA: Flagella

A.



B.

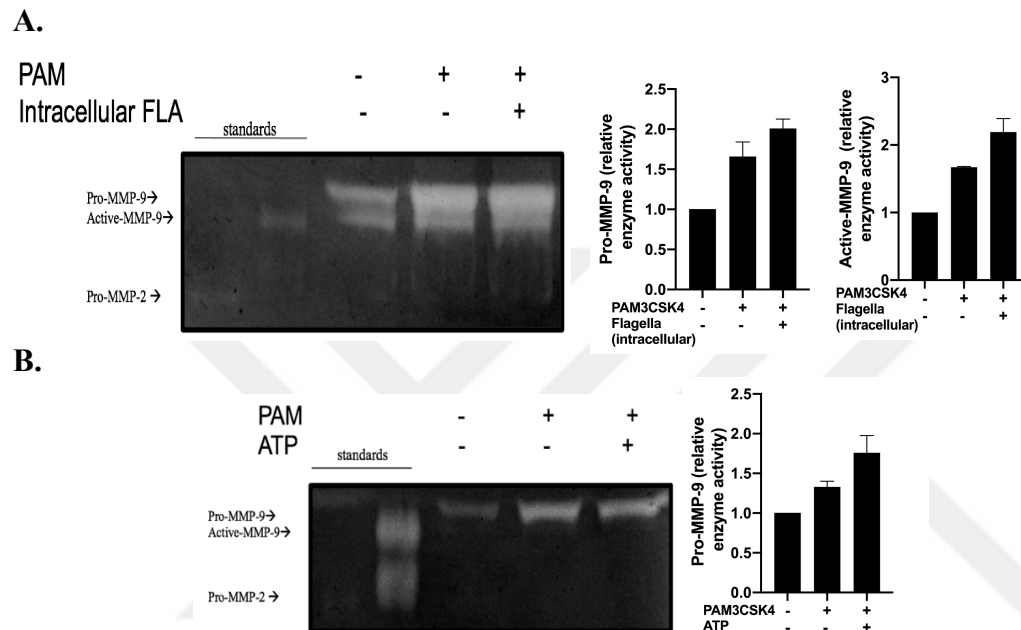


**Figure 3.2:** Matrix metalloproteinases-2 and -9 is expressed and inducible in EoL-1 cells at protein level. (A) EoL-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later transfected with flagella (100 ng/ml). Cells were lysed 24- hours after the stimulation with PAM3CSK4. (B) EoL-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later stimulated with ATP (5mM). Cells were lysed 10-hours after the stimulation with PAM3CSK4. The 30 ug of total protein samples were loaded into SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with indicated antibodies. MMP-9 is detected at 92 kDa. MMP-2 is detected at 72 kDa. GAPDH is detected at 36 kDa. Results were presented as relative optical density by using GraphPad Prism 8. PAM: PAM3CSK4, FLA: Flagella.

### 3.3 MMP-9 Activity Was Increased Upon NLRC4 And NLRP3 Inflammasome Induction In EoL-1 Cells, While MMP-2 Activity Was Not Inducible.

In order to evaluate the effect of NLRC4 and NLRP3 inflammasome activation on enzymatic activity of gelatinases secreted from EoL-1 cells, gelatin zymography was performed by using cell-free medium. The results showed that MMP-9 gelatinase activity was present and inducible through NLRC4 inflammasome activation in cell-free medium of EoL-1 cells as indicated with degradation of gelatin containing SDS-PAGE gel. The MMP-2 activity was not detected in cell-free medium of control and stimulated cells. Moreover, the MMP-9 activity was slightly induced upon NLRP3 inflammasome activation. According to gelatin zymography data, EoL-1 cells released MMP-9 as both pro- and active- forms and the pro-MMP-9 (~92 kDa) gelatinase activity induced by 2.27-fold through NLRC4 inflammasome activation and 2.23-fold with TLR-priming. The active-MMP-9 (~84 kDa) activity was also present in cell-free

medium and induced by 2.2- fold through NLRC4 inflammasome activation and 1.67- fold with TLR-priming (Figure 3.3 A.). The results also showed that NLRP3 inflammasome activation induced pro-MMP-9 gelatinase activity by 1.7- fold which was less than NLC4 inflammasome did. Additionally, there was less active-MMP-9 activity which was not inducible and observable in the gels upon NLRP3 inflammasome activation (Figure 3.3 B.).



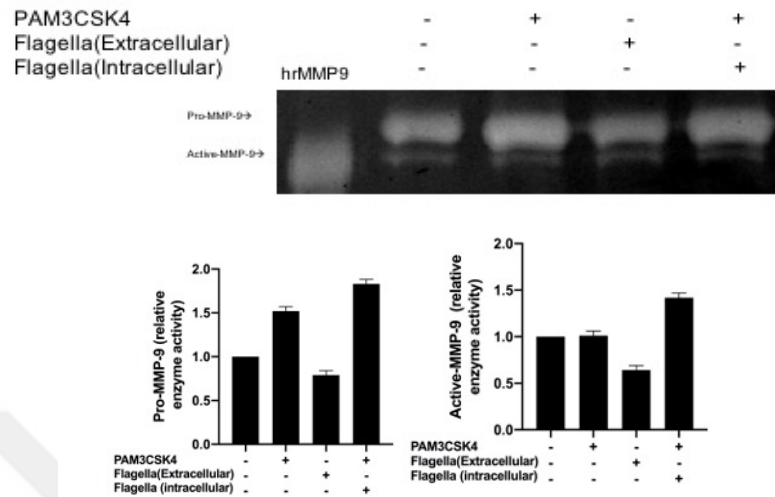
**Figure 3.3:** MMP-9 activity is increased upon NLRC4 and NLRP3 inflammasome activation in EoL-1 cells, while MMP-2 activity is not inducible. (A) EoL-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later transfected with flagella (100 ng/ml). Cell-free medium was collected 24-hours after stimulation with PAM3CSK4.

(B) EoL-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later stimulated with ATP (5mM). Cell-free medium was collected 10-hours after the stimulation with PAM3CSK4. Gelatin zymography was performed to measure MMP-2 and MMP-9 activities in the culture medium of control and stimulated EoL-1 cells. The gelatinolytic activities of purified MMP-2 and MMP-9 were shown on the two left lanes(10ng). The gelatinase enzyme activity was appeared as white bands against dark background. Pro- MMP-9 is represented at ~92 kDa. Active-MMP-9 is represented at ~84 kDa. MMP-2 is represented at ~72 kDa. Results were presented as relative optical density by using GraphPad Prism 8. PAM: PAM3CSK4, FLA: Flagella.

### 3.4 TLR5 Induction Had No Effect On MMP-9 Activity Released From EoL-1 Cells.

Bacterial flagella is a known ligand for TLR5. To further demonstrate that our observations are due to NLRC4 but not TLR5, we stimulated EoL-1 cells with flagella

without transfection. MMP-9 activity released from Eol-1 cells was analyzed by gelatin zymography. Expectedly, flagella treatment did not cause any induction on MMP-9 activity.



**Figure 3.4:** TLR5 induction had no effect on MMP-9 activity released from Eol-1 cell. (A) Eol-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later transfected with flagella (100 ng/ml) or just stimulated with flagella (100 ng/ml) . Cell-free medium was collected 24-hours after stimulation with PAM3CSK4. Gelatin zymography was performed to measure MMP-2 and MMP-9 activities in the culture medium of control and stimulated Eol-1 cells. The gelatinolytic activities of purified MMP-2 and MMP-9 were shown on the two left lanes(10ng). The gelatinase enzyme activity was appeared as white bands against dark background. Pro- MMP-9 is represented at ~92 kDa. Active-MMP-9 is represented at ~84 kDa. MMP-2 is represented at ~72 kDa. Results were presented as relative optical density by using GraphPad Prism 8. PAM: PAM3CSK4, FLA: Flagella.

### 3.5 TIMP-1 And TIMP-2 Are Regulated Through NLRC4 In Eol-1 Cells.

To evaluate the effect of NLRC4 and NLRP3 inflammasome activation and TLR2 on TIMP-1 and TIMP-2 mRNA expressions and activities secreted from Eol-1 cells, RT-qPCR and reverse zymography were performed. The mRNA expressions of TIMP1 and TIMP2 increased by 2.16-fold with TLR priming step during NLRC4 inflammasome activation (Figure 3.4 A.). TIMP-1 mRNA expression did not change while TIMP-2 mRNA expression increased with NLRC4 inflammasome activation. On the other hand, NLRP3 inflammasome activation led to reduction by 0.78- fold in TIMP-1 mRNA expression (Figure 3.4 A.). Since Eol-1 cells secrete both pro- and active-MMP9 but not MMP-2, only TIMP-1 activity was analyzed with reverse zymography approaches. As shown in Figure 3.B, while the area where TIMP-1

inhibits MMP-9 was seen as dark bands against bright background like as recombinant TIMP-1 did, there was no observed TIMP-1 activity secreted from Eol-1 supernatant.

### **3.6 Recombinant Human IL-1 $\beta$ Increased MMP-9 Protein Expression But Slightly Changed Gelatinase Activity Released From Eol-1 Cells.**

It has been reported that IL-1 $\beta$  induces MMP-9 production in many cell types and contribute to airway inflammation (C.-M. Yang & Yu, 2016). Since NLRC4 inflammasome activation induced MMP9 protein expression and activity released from Eol-1 cells, we assessed whether this induction is caused by NLRC4 inflammasome induced IL-1 $\beta$ . To address this question, Eol-1 cells were stimulated with different concentrations of recombinant human IL-1 $\beta$  (100 pg to 10 ng) for 24 hours. Western blot results showed that 10 ng of recombinant human IL-1 $\beta$  increased MMP-9 protein expression by 1.7-fold (Figure 3.5 A.). Gelatin zymography was also performed by using cell-free mediums of recombinant human IL-1 $\beta$  treated Eol-1 cells. Recombinant human IL-1 $\beta$  treatment slightly alter the pro- and active forms of MMP-9 activities secreted from Eol-1 cells.

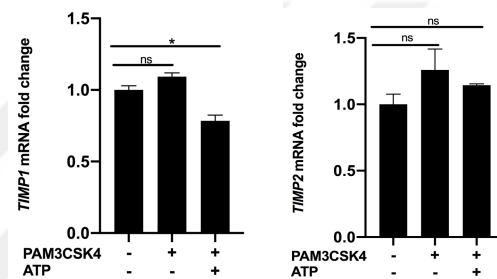
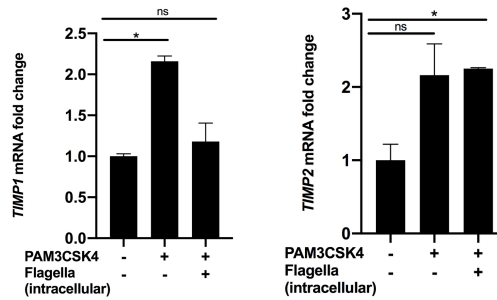
### **3.7 MMP And Caspase Inhibition Might Reduce NLRC4 Inflammasome Activation Induced MMP-9 Expression And Gelatinase Activity**

To confirm if the NLRC4 inflammasome formation in EoL-1 cells resulted in increased MMP-9 expression and activation, we pretreated Eol-1 cells with broad spectrum MMP inhibitor GM6001 or pan caspase inhibitor Z-VAD-FMK and then Eol-1 cells were stimulated with PAM3CSK4 and 4h later transfected with flagella. Then, cells were harvested for total protein extraction and western blot was performed. The results showed that both GM6001 and Z-VAD-FMK pretreatment augmented NLRC4 inflammasome activation induced MMP-9 protein expression in Eol-1 cells. GM6001 pretreatment reduced MMP-9 protein expression by 0.39- fold when compared with control group and pretreatment with GM6001 augmented NLRC4 inflammasome activation induced MMP-9 protein expression from 1.36-fold to 0.57-fold in Eol-1 cells (Figure 3.6 A.).

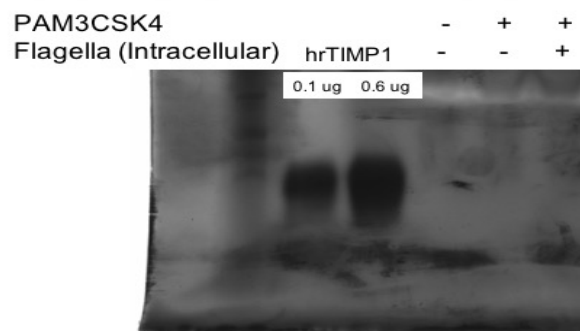
The western blot result also revealed that pretreatment with Z-VAD-FMK reduced MMP-9 protein expression by 0.36- fold when compared with control group and

augmented NLRC4 inflammasome activation induced MMP-9 protein expression from 1.36-fold to 0.17-fold in Eol-1 cells which demonstrated the correlation between MMP-9 expression and NLRC4 inflammasome activation (Figure 3.6 A.)

A.



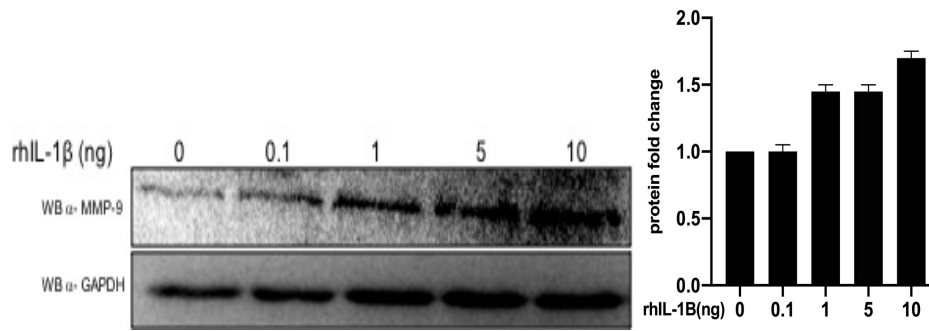
B.



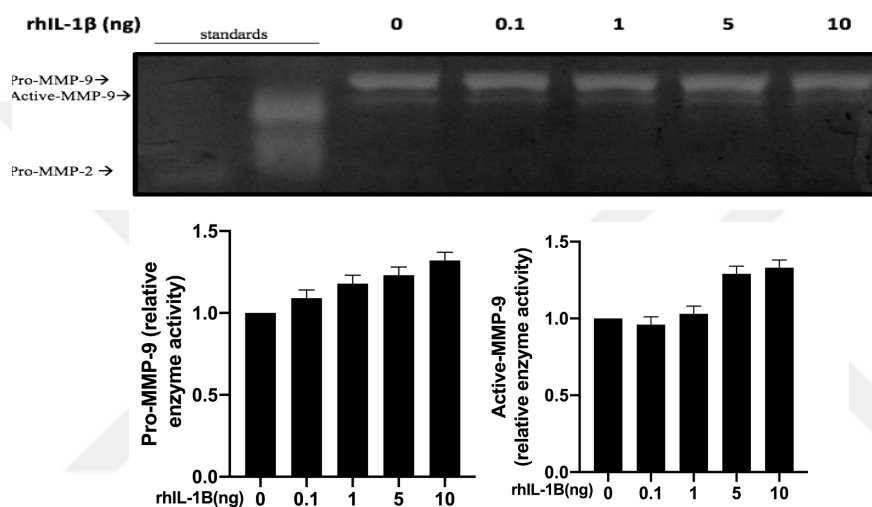
**Figure 3.5:** TIMP-1 and TIMP-2 are regulated through PRRs in Eol-1 cells. (A) Eol-1 cells were stimulated with PAM3CSK4 (1ug/ml) and 4 hours later transfected with flagella (100 ng/ml) or stimulated with ATP (5 mM). Total mRNA was isolated from cells after 24- and 10-hours incubation, respectively. RT-qPCR was performed, and relative mRNA expression was normalized to HPRT1 mRNA expression. Graphs were prepared by using GraphPad Prism 8. Each value represents the mean  $\pm$  SEM of two replicates analyzed by Student *t* test. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001, ns: non-significant, PAM: PAM3CSK4, FLA: Flagella. (B) Cell-free medium was collected 10-hours and 24-hours after the stimulation with PAM3CSK4.

Reverse zymography was performed to measure TIMP-1 activity in the culture medium of control and stimulated Eol-1 cells. The activity of recombinant human TIMP-1 was shown on the left two lanes (0.1 and 0.6 ug). The TIMP activity was appeared as dark bands against white background.

**A.**



**B.**



**Figure 3.6:** Recombinant human IL-1 $\beta$  increased MMP-9 protein expression but slightly changed gelatinase activity released from Eo1-1 cells. (A) Eo1-1 cells were stimulated with different concentrations of recombinant human IL-1 $\beta$  (100 pg to 20 ng). Cells were lysed 24 h after stimulation. The 30  $\mu$ g of total protein samples were loaded into SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with indicated antibodies. MMP-9 is detected at 92 kDa. MMP-2 is detected at 72 kDa. GAPDH is detected at 36 kDa. Results were presented as relative optical density by using GraphPad Prism 8. (B) Cell-free mediums were collected for performing gelatin zymography to measure MMP-2 and MMP-9 activities. The gelatinolytic activities of purified MMP-2 and MMP-9 were shown on the two left lanes (10 ng). The gelatinase enzyme activity was appeared as white bands against dark background. Pro- MMP-9 is represented at ~92 kDa. Active-MMP-9 is represented at ~84 kDa. MMP-2 is represented at ~72 kDa. Results were presented as relative optical density by using GraphPad Prism 8.

Next, gelatinase activity secreted from cells pretreated with GM6001 or Z-VAD-FMK was also analyzed with gelatin zymography. Pretreatment with GM6001 reduced NLRC4 inflammasome activation induced pro-MMP-9 and active-MMP-9 activity secreted from Eo1-1 cells from 1.78-fold to 0.86-fold and from 1.85-fold to 1.24-fold, respectively (Figure 3.6 B.). Pretreatment with Z-VAD-FMK reduced NLRC4

inflammasome activation induced pro-MMP-9 and active-MMP-9 activity secreted from Eo1-1 cells from 2,01-fold to 1,37 and from 1.48-fold to 1.03- fold, respectively. Interestingly, pro-MMP-2 activity was also induced by 1.6-fold upon NLRC4 inflammasome activation and TLR-priming and this induction was augmented by inhibition of caspase-1 with Z-VAD-FMK (Figure 3.6 C.).

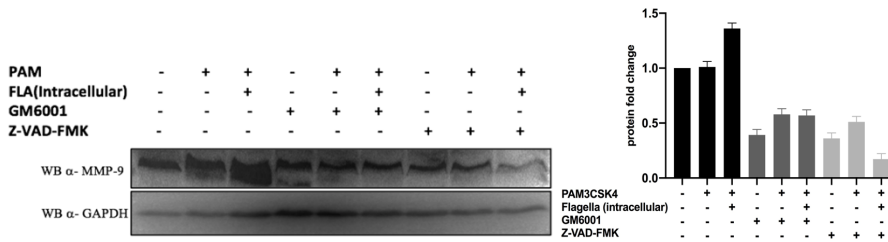
### **3.8 MMP And Caspase Inhibition Might Reduce NLRC4 Inflammasome Activation Induced IL-1 $\beta$ Cleavage And Secretion**

Since it is shown that in addition to inflammasome complexes, MMP-9 have ability to activate the IL-1 $\beta$  precursor to the active 17-KDa form. We next assessed whether NLRC4 inflammasome activation induced MMP-9 have role in IL-1  $\beta$  maturation or not. For this, we pretreated Eo1-1 cells with broad spectrum MMP inhibitor GM6001 or pan caspase inhibitor Z-VAD-FMK and then Eo1-1 cells were stimulated with PAM3CSK4 and 4h later transfected with flagella. Then, cells were harvested for total protein extraction. Western blotting and ELISA were performed for analyzed the cleaved IL-1 $\beta$  protein expression and IL-1 $\beta$  secretion from Eo1-1 cells, respectively. The results showed that, TLR2 and NLRC4 inflammasome augmented cleaved- IL-1  $\beta$  expression was reduced with MMP inhibition in Eo1-1 cells and cleaved- IL-1  $\beta$  expression was almost completely diminished (Figure 3.7 A.). These results were also confirmed by ELISA which showed that NLRC4 inflammasome activation induced IL-1  $\beta$  secretion was reduced with MMP and caspase inhibition as well, which revealed the role of MMP-9 in IL-1  $\beta$  maturation without caspase-1 in inflammasome complex.

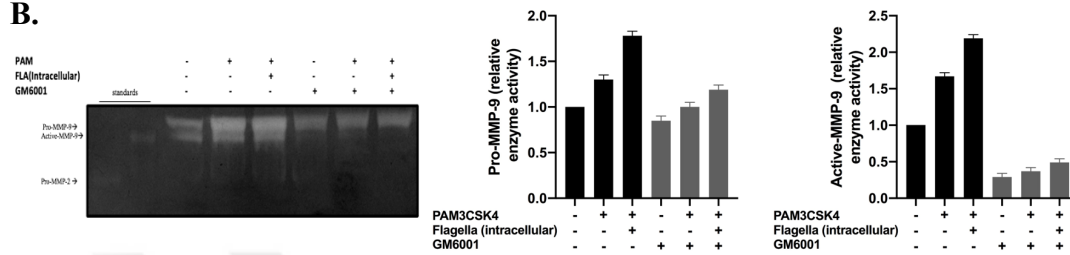
### **3.9 MMP-9 Protein Synthesis Was *De Novo*, But Enzyme Activity Originated From Pre-synthesized Enzyme**

Since eosinophil granules contain various cytokines, chemokines, lipid mediators and immunomodulatory molecules including matrix metalloproteinases(Long et al., 2016), Eo1-1 cells pretreated with protein synthesis inhibitor cycloheximide (CHX) to understand the involvement of protein synthesis in NLRC-4 inflammasome induced MMP-9 expression, and to reveal the mechanism of MMP-9 release (constitutive pathway or piecemeal degranulation) from Eo1-1 human eosinophils. CHX reduced the MMP-9 protein expression in Eo1-1 cells as a dose dependent manner.

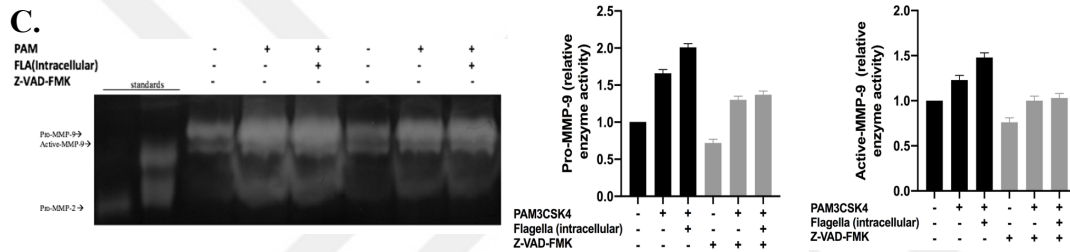
A.



B.



C.

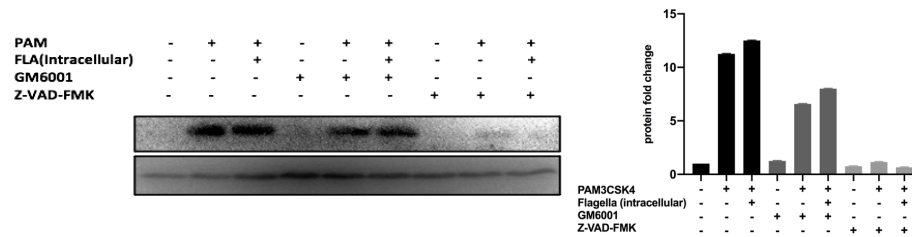


**Figure 3.7:** MMP and caspase inhibition might reduce NLRC4 inflammasome activation induced MMP-9 expression, gelatinase activity. (A) Eol-1 cells were pretreated with 25 uM of GM6001 1 h before PAM3CSK4 (1ug/ml) stimulation then transfected with flagella (100 ng/ml) or PAM3CSK4-primed Eol-1 cells were treated with 20 uM of Z-VAD-FMK 1 h before transfecting with flagella. Cells were lysed 24 h after stimulation with PAM3CSK4. The 30 ug of total protein samples were loaded into SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with indicated antibodies. MMP-9 is detected at 92 kDa. MMP-2 is detected at 72 kDa. GAPDH is detected at 36 kDa. Results were presented as relative optical density by using GraphPad Prism 8. (B, C) Cell-free mediums were collected for performing gelatin zymography to measure MMP-2 and MMP-9 activities. The gelatinolytic activities of purified MMP-2 and MMP-9 were shown on the two left lanes(10ng). The gelatinase enzyme activity was appeared as white bands against dark background. Pro- MMP-9 is represented at ~92 kDa. Active-MMP-9 is represented at ~84 kDa. MMP-2 is represented at ~72 kDa. Results were presented as relative optical density by using GraphPad Prism 8.

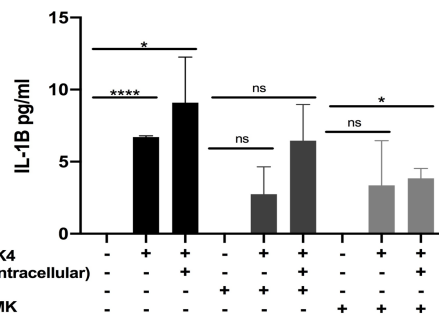
Interestingly, cycloheximide failed to block gelatinase activity released from Eol-1 cells (Figure 3.9 A.) which reveals that protein synthesis was not required for rapid MMP-9 release from Eol-1 cells. The results also showed that cycloheximide did not abolish NLRC4 inflammasome activation induced MMP-9 expression in Eol-1cells (Figure 3.9 B.). This result was confirmed by gelatin zymography which shows that pro-MMP-9 and active-MMP-9 activity secreted from Eol-1 cells upon NLRC4 inflammasome activation were reduced but not completely abolished by pretreatment

with cycloheximide which indicates that the effects are because of the release of pre-synthesized MMP-9 from granules (Figure 3.9 B.). This results also confirmed the importance of piecemeal degranulation of MMP-9 in Eol-1 human eosinophils.

A.



B.



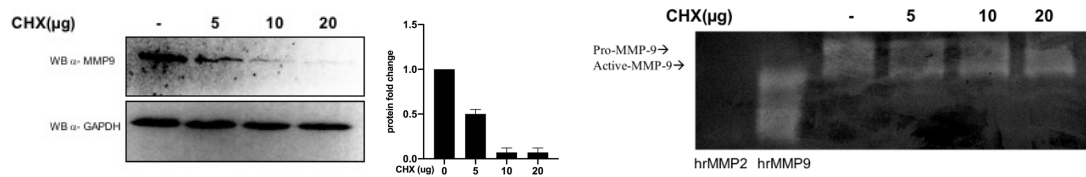
**Figure 3.8:** MMP and caspase inhibition might reduce NLRC4 inflammasome activation induced IL-1  $\beta$  cleavage and secretion. (A) Eol-1 cells were pretreated with 25  $\mu$ M of GM6001 1 h before PAM3CSK4 (1  $\mu$ g/ml) stimulation then transfected with flagella (100 ng/ml) or PAM3CSK4-primed Eol-1 cells were treated with 20  $\mu$ M of Z-VAD-FMK 1 h before transfecting with flagella. Cells were lysed 24 h after stimulation with PAM3CSK4. The 30  $\mu$ g of total protein samples were loaded into SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with indicated antibodies. MMP-9 is detected at 92 kDa. MMP-2 is detected at 72 kDa. GAPDH is detected at 36 kDa. Results were presented as relative optical density by using GraphPad Prism 8. (B) Cell-free mediums were collected for performing ELISA. GraphPad Prism 8 was used for the preparation of the graphs. Each value represents the mean  $\pm$  SEM of three biological replicates analyzed by t test. \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , ns: non-significant. PAM: PAM3CSK4, FLA: Flagella.

### 3.10 Induction Of NLRC4 Inflammasome Augmented The Expression Of CD63, CD69 And CD147 On Eol-1 Cells

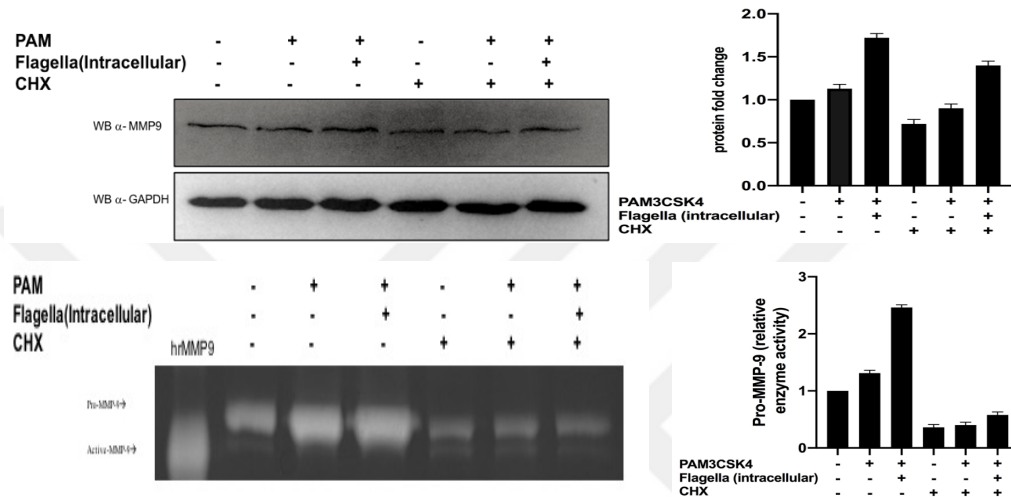
Since Eol-1 cells secrete MMP-9 at basal level and this MMP-9 release was induced through TLR-2 and NLRC4 inflammasome activation, we next assessed the effects of NLRC4 inflammasome activation on CD63 expression which is the member of transmembrane-4 glycoprotein superfamily and has been considered as a potential surface marker for eosinophil degranulation. The CD63 is present within granules and

undergoes secretion by piecemeal degranulation or exocytosis in response to stimuli. Hence, utilizing flow cytometry, we revealed that CD63 was expressed on the cell surface of Eol-1 cells. TLR2 stimulation and NLRC4 inflammasome activation increased CD63 expression (Figure 3.10). We also assessed the expression of CD69 which is an eosinophil activation marker. Eol-1 cells expressed CD69 scarcely at basal level and activation of NLRC4 inflammasome increased the expression of CD69 on Eol-1 cells which indicated that NLRC4 inflammasome activation induced Eol-1 cell activation as well. (Figure 3.10). We also investigate the effect of NLRC4 inflammasome activation on CD147 expression known as extracellular matrix metalloproteinase inducer (EMMPRIN) that have ability to induce MMP-9 expression in immune cells (Kim et al., 2009). NLRC4 inflammasome activation increased the expression of CD147 on Eol-1 cells (Figure 3.10).

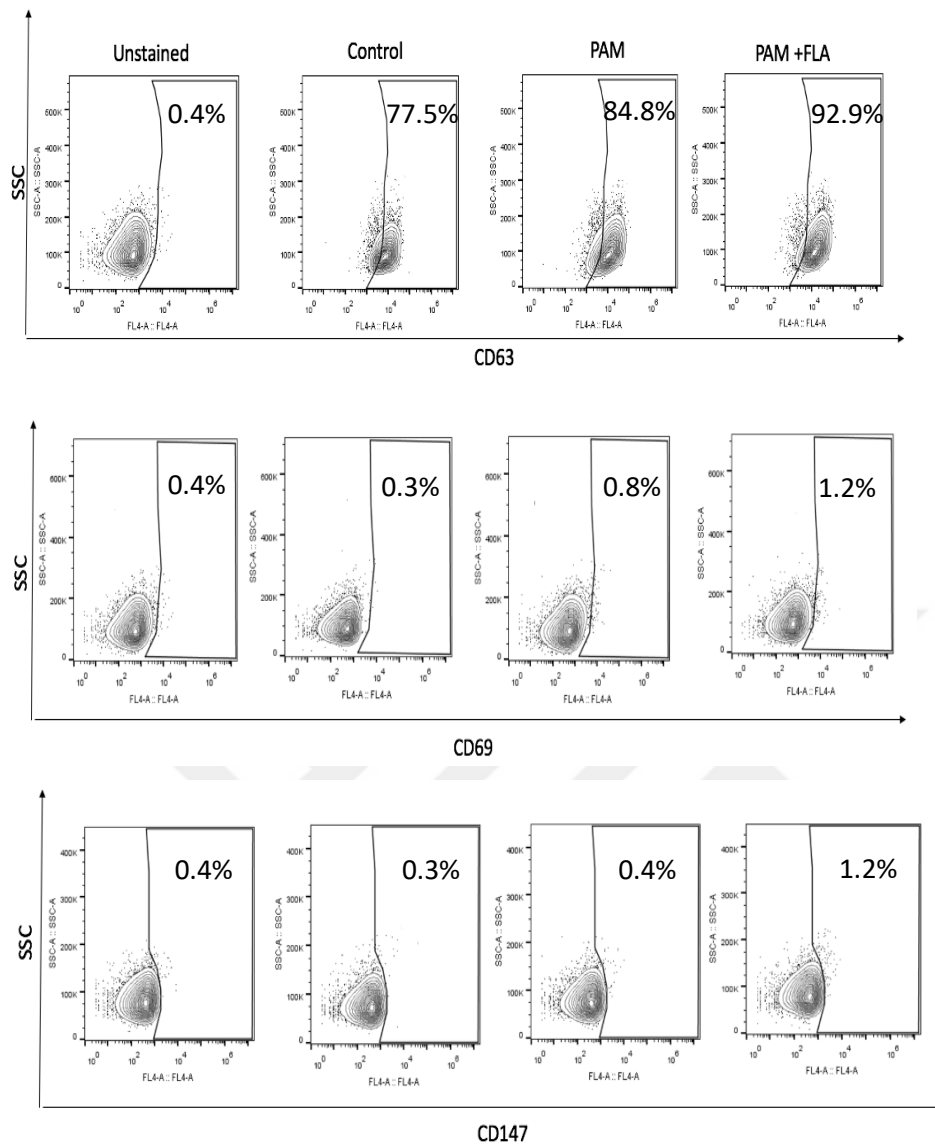
A.



B.



**Figure 3.9:** MMP-9 protein synthesis was *de novo*, but enzyme activity originated from pre-synthesized enzyme. (A-B) Eo1-1 cells were pretreated with 5μg/ml cycloheximide and 1 hour later stimulated with PAM3CSK4(1μg/ml) and 4 hours later transfected with flagella (100 ng/ml). Cells were lysed 24h after PAM3CSK4 stimulation. The 30 μg of total protein samples were loaded into SDS-PAGE gels and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with indicated antibodies. MMP-9 is detected at 92 kDa. MMP-2 is detected at 72 kDa. GAPDH is detected at 36 kDa. Results were presented as relative optical density by using GraphPad Prism 8. Cell-free mediums were collected for performing gelatin zymography to measure MMP-2 and MMP-9 activities. The gelatinolytic activities of purified MMP-2 and MMP-9 were shown on the two left lanes(10ng). The gelatinase enzyme activity was appeared as white bands against dark background. Pro- MMP-9 is represented at ~92 kDa. Active-MMP-9 is represented at ~84 kDa. Results were presented as relative optical density by using GraphPad Prism 8. PAM: PAM3CSK4, FLA: Flagella, CHX: Cycloheximide



**Figure 3.10:** Induction of NLRC4 inflammasome and TLR2 augmented the expression of CD63, CD69 and CD147. EoL-1 cells were stimulated with with PAM3CSK4 (1ug/ml) and 4 hours later, transfected with flagella (100 ng/ml). After 24 hours incubation, cells were collected and stained with anti-CD63, anti-CD69, anti-CD14 and Goat Anti-mouse IgG H&L- Alexa Fluor® 488 (for anti-CD147) antibodies for flow cytometry. Results were analyzed by FlowJo. PAM: PAM3CSK4, FLA: Flagella, SSC: Side scatter

#### 4. DISCUSSION

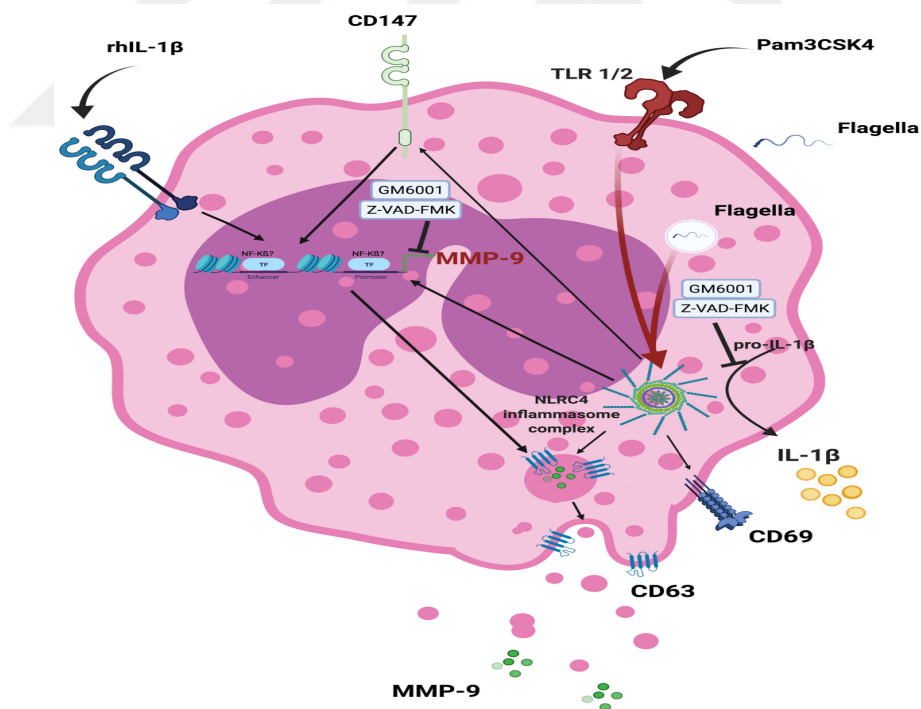
Eosinophils are granulocytes that have critical roles in Th2 immune responses, including the IL-5 production which elevate eosinophilopoiesis and eosinophil activation (Long et al., 2016). Since eosinophils are cells of innate immune system, they possess numerous TLRs and NLRs which recognize PAMPs or DAMPs and thereby mediating an immune response towards parasites and allergens. As intracellular organelles, eosinophils contain secretory granules which stores pre-formed cytokines, chemokines and immunomodulatory molecules such as matrix metalloproteinases (Long et al., 2016). Unlike the cells of adaptive immunity, eosinophils can release preformed granule contents by degranulation in response to certain stimuli (Long et al., 2016). The matrix metalloproteinases (MMPs) are calcium dependent zinc-containing endopeptidases that are involved in ECM degradation and tissue remodeling (Verma & Hansch, 2007). Among them, MMP-2 and MMP-9 (gelatinases) are one of the most studied and well characterized MMPs which their expressions and activations have been addressed in neoplastic pathologies and inflammatory disorders. Previous studies revealed the regulations of MMP-9 through TLR2 activation in human monocytes (Al-Rashed et al., 2017). Despite the relation between TLR signaling and MMP-9 expression in immune cells, the molecular effects of NLRs and inflammasome complex on MMPs have not been studied either in eosinophils or other immune cells. Even though human eosinophils are the source of MMP-2, MMP-9 and involved in immune response to parasitic infection and remodeling of airway, their regulation mechanisms through NLRs are rather unknown. Since NLRP3 and NLRC4 are two mostly studied NLRs known to assemble protein complexes called inflammasomes, we focus on regulations of MMPs through NLRC4 on this project. For this purposes, EoL-1 cell line was used to investigate eosinophilic function. Here, we report that EoL-1 cells expressed MMP-2 and MMP-9 at mRNA and protein levels. Treatment with PAM3CSK4 TLR2 ligand and NLRC4 inflammasome ligands (PAM3CSK4 stimulation and flagella transfection) significantly increased MMP-9 mRNA expression and augmented MMP-9 protein

expression while the MMP-2 mRNA expression increased with a minor change and protein expression did not change. We also revealed the effect of NLRP3 inflammasome activation on MMP-2 and MMP-9 mRNA and protein levels. NLRP3 ligands (PAM3CSK4 stimulation and ATP stimulation) also increased MMP-9 mRNA and protein expressions which were not much as NLRC4 ligands did. The MMP-2 mRNA and protein expression did not change with NLRP3 ligands. We also evaluated the enzymatic activity of gelatinases secreted from Eo1-1 cells upon NLRC4 and NLRP3 inflammasome activation. MMP-9 gelatinase activity was present in cell-free medium of Eo1-1 cells and augmented with NLRC4 inflammasome activation and TLR2 stimulation. Interestingly, MMP-2 activity was not detected. The NLRP3 inflammasome activation also induced MMP-9 gelatinase activity which was not much as NLRC4 ligands did. We also demonstrated that treatment with recombinant human IL-1 $\beta$  (10ng) increased MMP-9 protein expression in Eo1-1 cells. The MMP-9 gelatinase activity was induced slightly by the treatment with recombinant human IL-1 $\beta$ . The tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 were also regulated through NLRC4. TLR2 stimulation augmented the mRNA expression of TIMP-1, while NLRC4 inflammasome activation reduced TLR2 augmented TIMP-1 expression. NLRP3 inflammasome activation also reduced TIMP-1 mRNA expression.

The regulation of MMP-9 through NLRC4 inflammasome activation was also confirmed by pretreating Eo1-1 cells with pan caspase inhibitor Z-VAD-FMK or broad spectrum MMP inhibitor GM6001. Caspase-1 inhibition by Z-VAD-FMK reduced NLRC4 inflammasome activation induced MMP-9 protein expression like GM6001 did. Apart from the remodeling of extracellular matrix by degrading matrix component, MMP-9 have ability to activate IL-1 $\beta$  precursor to the active 17-KDa form in a caspase-1 independent manner. We revealed that TLR2 and NLRC4 inflammasome augmented cleaved- IL-1  $\beta$  expression and IL-1 $\beta$  secretion was reduced with MMP inhibition and almost completely diminished with caspase inhibition indicating the importance of MMP-9 in IL-1  $\beta$  maturation even if the majority of IL-1 $\beta$  secretion in Eo1-1 cells is caspase-1 dependent.

Eosinophils can store pre-formed mediators within their granules and release without *de novo* synthesis. We pretreated Eo1-1 cells with cycloheximide to understand the involvement of constitutive pathway in NLRC4 inflammasome induced MMP-9

expression and release. Cycloheximide did not reduce NLRC4 inflammasome induced MMP-9 expression, and not completely abolish MMP-9 activity released from Eo1-1 cell which indicates that the effects are because of the release of pre-synthesized MMP-9 from granules which also confirmed by increase in CD63 expression that is present within granules and undergoes secretion by piecemeal degranulation or exocytosis in response to stimuli. The increase in MMP-9 expression and activity upon NLRC4 inflammasome activation also correlates with increase in CD147 expression known as extracellular matrix metalloproteinase inducer (EMMPRIN) that have ability to induce MMP-9 expression in immune cells (Kim et al., 2009). Overall, we deduced that not only TLRs but also NLRs activates and regulates the expression of MMP-9 in Eo1-1 cells. It can be concluded that MMP-9 expression and activity may increase with PRR recognition in human eosinophils during parasitic infections or allergic responses. Further studies are required to investigate the relationship between immunomodulatory functions of MMPs and NLRC4 and other PRRs in primary human eosinophils.



**Figure 4.1:** Proposal model for regulation of MMP-9 in Eo1-1 cells through NLRC4.



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## **APPENDICES**

**APPENDIX A:** Equipments that used in this study are listed with supplier companies.

**APPENDIX B:** Commercial kits that used in this study are listed with supplier companies.

**APPENDIX C:** Chemicals that used in this study are listed with supplier companies.



## APPENDIX A

**Table A:** Equipments that used in this study are listed with supplier companies.

<b>Equipment</b>	<b>Supplier Company</b>
Light microscope	Olympus CH30
Incubator	BINDER
Hemocytometer	Marienfield
Vortex	VWR
Micropipettes	Thermo Fisher 10,20,200,1000 $\mu$ L
Serological pipettes	Eppendorf 10,200,1000 $\mu$ L Sarstedt (5ml, 10ml)
Falcon tubes	Sarstedt (15ml, 50ml)
Electronic pipette	Thermo Fisher
Centrifuge	VWR MicroStar 17R Thermo IEC CL10
Power supply	Biorad
Mini Protean Tetra Cell	Biorad
Step One Real Time PCR	Applied Biosystem
Flow Cytometry Accuri C6	BD Biosystems
Spectrophotometer Nanodrop	Thermo Fisher
Chemidoc	Biorad
Microplate reader	Biorad Benchmark Plus

## APPENDIX B

**Table B:** Commercial kits that used in this study are listed with supplier companies.

<b>Equipment</b>	<b>Supplier Company</b>
Pierce BCA Protein Assay	VWR
Pierce ECL Substrate	Thermo Fisher
Ambion RNA Isolation Kit	Thermo Fisher
Quantitech SYBR Green PCR Kit	Qiagen
SureBlue TMB Substrate Kit	VWR

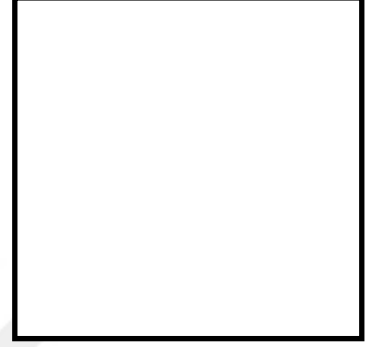
## APPENDIX C

**Table C:** Chemicals that used in this study are listed with supplier companies.

<b>Equipment</b>	<b>Supplier Company</b>
Acrylamide/Bis solution 30% (19:1)	Biorad
Acrylamide 40% (37.5:1)	Biorad
Ammonium persulfate	Sigma
APMA	Sigma
Bromophenol blue	Alfa aesar
B-mercapthoethanol	Sigma
Brij-35	Sigma
EDTA	Fisher Scientific
FBS	Gibco
Glycerol	MP
Glycine	MP
HCl 37%	Sigma
L-glutamine	Gibco
Methanol	Fisher Scientific
Penicilin/Streptomycin	Gibco
PBS	Lonza
SDS	MP
Trisma base	Sigma
Triton X-100	
Tween20	Fisher Scientific

## CURRICULUM VITAE

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### PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

- Akkaya I, Özel İ, **Oylumlu E**, Üzel G & Ciraci C. Bir Model Olarak İnsan Eozinofilik Hücre Hattının (Eol-1) Morfolojik ve Fonksiyonel Karşılaştırmalı Analizi. 25. Ulusal İmmünoloji Kongresi, Kasım 20-22, 2020 İstanbul, Türkiye.

### OTHER PUBLICATIONS, PRESENTATIONS AND PATENTS:

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