

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL

**MOLECULAR GENETIC ASSESSMENT OF IMMUNE MODULATORS IN
EOSINOPHILS AND T CELLS BOTH *IN SILICO* AND *IN VITRO* UTILIZING
PHOTOACOUSTIC IMAGING APPROACHES**



M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

JULY 2025

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

**FOTOAKUSTİK GÖRÜNTÜLEME YAKLAŞIMLARINI KULLANARAK
EOZİNOFİLLER VE T HÜCRELERİNDE BAĞIŞIKLIK
MODÜLATÖRLERİNİN İN SİLİKO VE İN VİTRO MOLEKÜLER GENETİK
DEĞERLENDİRMESİ**

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To every heart where I have a place,



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ABBREVIATIONS

µg	:Microgram
µM	:Micromolar
mM	:Milimolar
7-AAD	:7-Aminoactinomycin D
AKT	: Protein Kinase B
ASC	:Apoptosis-associated Speck-like Protein Containing a CARD
BCR	: B cell receptor
C.A.	:Cell activator
BCA	:Bicinchoninic Acid
BSA	:Bovine serum albumin
CARD	:Caspase activation and recruitment domain
CD	:Cluster of differentiation
CLR	:C type lectin receptor
CYSLTR	: Cysteinyl Leukotriene Receptor
DAMP	:Damage-associated molecular pattern
DC	:Dendritic cell
DNA	:Deoxyribonucleic acid
EDTA	:Ethylenediaminetetraacetic acid
ELISA	:Enzyme-linked immunosorbent assay
ERK	: Extracellular signal-regulated kinase
FACS	: Fluorescent Activated Cell Sorting
FBS	:Fetal bovine serum
g	:Gram
h	:Hour
HRP	:Horseradish Peroxidase
ICC	: Immunocytochemistry
MNT	:Montelukast
NLR	:Nod-like receptor
PA	: Photoacoustic
PAMP	:Pathogen-associated molecular pattern
PRR	:Pattern recognition receptor
RT	:Room temperature
TCR	: T cell receptor
TLR	:Toll-like receptor



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SUMMARY

The immune system is a network of specialized cells and chemicals with distinct functions that have different roles in fighting infection. It protects the host from pathogenic invaders and provides memory of the infection to defend the host against future encounters. The ability of immune cells may extend beyond host defense to include growth, tissue homeostasis, and repair mechanisms. In recent decades, it has been demonstrated that the origin of inflammation determines the immune response and that the immune system plays a role in maintenance, renewal, and sterile inflammation. There are two kinds of responses to non-self: innate responses that can be developed regardless of exposure and adaptive responses that can develop more rapidly with repeated exposures. Anatomical and physiological barriers such as lysozyme in tears, mucosal secretions, gastric pH, and skin constitute the primary defense. When these barriers are compromised, innate immunity is activated to maintain tissue homeostasis and defend against infections, cancer, and toxins. The cells of the innate immunity are eosinophils, basophils, mast cells, monocytes, dendritic cells, neutrophils, and macrophages. They release chemokines and cytokines and remove pathogens by phagocytosis. Eosinophils are multifunctional granulocytes responding mostly to parasitic infections and allergies. They originate in the bone marrow from hematopoietic stem cells in response to IL-5 and GM-CSF. After maturation, they enter bloodstream and tissues via IL-5 and eotaxin signaling. On the other hand, the adaptive immune system includes B and T cells. T cells mature in the thymus and B cells in the bone marrow and produce antibodies. Upon maturation, they migrate to secondary lymphoid tissues to capture antigens and initiate responses primed by innate immune signals. T cells are categorized into naive, memory, and regulatory subsets. Upon activation, they produce IL-2, and they differentiate into effector cells, enhancing the clearance via cytokines and cytotoxic mediators.

Pattern recognition receptors (PRRs) sense molecular patterns associated with pathogens, apoptotic cells, and senescent cells. They link innate and adaptive immunity by producing non-specific protective mediators. PRRs are grouped by domain homology into TLRs, NLRs, RLRs, and CLRs. They recognize PAMPs and DAMPs, distinguish sterile and non-sterile inflammation. Although associated with innate immunity, PRRs are also expressed by T and B cells, suggesting a functional overlap. NLRP3 is the most studied inflammasome-related PRR, first identified via mutations causing cryopyrin-associated syndromes. It contains NACHT, LRR, and PYD domains, enabling ASC recruitment and caspase-1 activation, leading to pro-IL-1 β processing. NLRP3 is unique in being activated by diverse agonists including pathogens, toxins, environmental factors, and DAMPs.

The use of advanced imaging techniques to visualize biological processes has become a key focus in this field. Photoacoustic (PA) imaging is a hybrid biomedical imaging technique based on the thermo-acoustic effect generated by laser stimulation. It integrates the benefits of optical and ultrasonic imaging. Consequently, PA imaging is applicable to both pre-clinical research and clinical medicine for the diagnosis of cancer, microcirculation irregularities, and evaluation of therapy efficacy.

This thesis presents a two-part exploration of innate and adaptive immunity. It begins with a closer look at eosinophil-like cells in the context of PRRs and continues with the functional impact of montelukast on T cells using both molecular and PA imaging approaches. In the first chapter, the goal was to compare the basal expression of pattern recognition receptors (PRRs)—including both membrane-bound and cytosolic types—in human eosinophil-like cell lines (EoL-1 and HL-60) to human primary eosinophils (HPEs), using publicly available *in silico* databases. Given the limitations of working with primary eosinophils, determining cell lines that are most closely mimicking primary cells' receptor profiles was a necessary first step toward adopting the most suitable *in vitro* models. These findings were intended to support future mechanistic studies on eosinophil-driven immunity. The second chapter of this thesis focuses on how montelukast (MNT), a leukotriene receptor antagonist commonly prescribed for asthma, influences inflammatory signaling in Jurkat T cells. To serve this purpose, T cells, like Jurkat cells, were treated with MNT for 48 hours, either alone or in combination with cell activator (C.A.). Expression levels of several key immune-related genes and proteins, including CYSLTR1, NLRP3, ASC, IL-1 β , IL-10, and signaling molecules such as Akt and ERK, were measured using molecular assays including RT-qPCR, immunoblotting, ELISA, and immunocytochemistry (ICC). In addition to molecular approaches, photoacoustic (PA) imaging was used to examine how MNT treatment affected Jurkat cells changed optical signal characteristics. This combined strategy widened our view on the molecular changes induced by MNT in T cells while also testing the potential of PA imaging as a non-invasive approach for detecting drug-driven immune modulation.

In the first chapter, basal expression of PRRs' mRNA levels of human eosinophils were analyzed *in silico* and comparative profiles of EoL-1 and HL-60 cell lines with HPEs were determined. The analysis showed that levels of TLR2 and NLRP3 mRNA were significantly higher in both cell lines than in HPE ($p < 0.01$), whereas NLRP12, LGP2 and Dectin-1 receptors mRNA levels were higher in HPE than in both cell lines ($p < 0.01$). In addition, TLR6, MDA5, NOD2, NLRP1, CLEC4A were detected to be expressed at higher basal levels in EoL-1 and/or HL-60 than in PE. Although TLR3, TLR8 and TLR9 expressions were not detected in PE, these receptors were expressed in cell lines. TLR5 expression was unique to HPE. MDL-1 (CLEC5A) levels were significantly higher in HL-60 cells as compared to HPE, while CLEC4A expression was higher in EoL-1 cells than in HPE. These data suggested that EoL-1 and HL-60 cells are suitable *in vitro* models for certain PRRs, however, studying these receptors in cell lines may be challenging, particularly for those such as LGP2, NLRP12 and Dectin-1, which have high expression levels in HPE.

In the second chapter, effects of MNT on molecular inflammatory responses in Jurkat T cell line were evaluated at 48-hour post-stimulation. CYSLTR1 mRNA levels were

significantly upregulated after 10^{-5} M MNT treatment when compared to control group (~2.5-fold, $p < 0.0001$). NLRP3 expression was also upregulated by C.A. treatment ($p < 0.01$), and this response was further elevated in the MNT+ C.A. group as compared to control and only-C.A. ($p < 0.01$). These results showed that MNT may induce inflammation via NLRP3. When ASC adaptor protein was measured by immunoblotting, it was significantly downregulated in both C.A. and MNT+ C.A. groups ($p < 0.05$). We then measured IL-1 β release by ELISA and showed that there was no significant difference between the treatment and control groups (ns). However, intracellular IL-1 β expression was detected to be increased in the C.A. group ($p < 0.001$) and reached the highest level in the MNT+ C.A. group ($p < 0.01$) by immunocytochemical analysis. We also determine the signal transduction pathways that are potentially involved in the MNT responses. Initially, we investigated AKT protein, which was decreased in the C.A. group ($p < 0.05$) and no significant difference was observed in p-AKT level. However, the p-AKT/AKT ratio increased in the C.A. group ($p < 0.01$) and this increase was maintained in the MNT+ C.A. group (ns). Phosphorylated ERK 1/2 (p-ERK1/2) level increased in the C.A. group ($p < 0.05$) and reached the highest levels in the MNT+ C.A. group ($p < 0.01$). In addition, we examine the anti-inflammatory response. For this, IL-10 production was analyzed and expectedly, IL-10 was not significantly differentially expressed after treatment. As for the photoacoustic imaging results, PA signal amplitude was significantly decreased in the MNT-treated groups- both single treatment and in combination with C.A. when compared to the control and C.A. groups. The mean amplitude value was 2523.22 in the control group, 2283.59 in the C.A. group, 755.66 in the MNT group, and 1088.19 in the MNT+ C.A. group. This suggests that MNT effects on the cells was measured as a reduced photoacoustic signal.

In conclusion, the first chapter provided an *in silico* comparison of PRR expression profiles between eosinophil-like cell lines and HPEs, presenting practical insights into selecting more reliable and suitable models for future *in vitro* studies on eosinophil-driven immune mechanisms. The second chapter investigated the effects of MNT on inflammatory signaling in Jurkat T cells, revealing changes in cytokine expression, inflammasome-related proteins, and intracellular signaling pathways. The inclusion of photoacoustic imaging enabled the detection of biophysical changes at the cellular level during treatment. By integrating computational, molecular, and imaging-based approaches, this study presents a multi-way exploration of immune responses. Future research may be extended to primary human T cells and other drugs that require the determination of side effects for different therapeutic interventions.



FOTOAKUSTİK GÖRÜNTÜLEME YAKLAŞIMLARINI KULLANARAK EOZİNOFİLLER VE T HÜCRELERİNDE BAĞIŞIKLIK MODÜLATÖRLERİNİN IN SILIKO VE IN VITRO MOLEKÜLER GENETİK DEĞERLENDİRMESİ

ÖZET

Bağışıklık sistemi, enfeksiyonlarla kendine özgü şekilde mücadele edebilmek için farklı işlevlere sahip özelleşmiş hücreler ve kimyasallardan oluşan bir ağdır. İnsan vücudunu patojenik istilalardan koruyan ve konağı gelecekteki karşılaşmalara karşı savunmak için enfeksiyonlara ait özgül bir hafıza oluşturan, oldukça gelişmiş ve düzenlenmiş bir sistemdir. Bununla birlikte, bağışıklık hücrelerinin işlev ve kapasiteleri yalnızca konak savunmasıyla sınırlı kalmayıp; büyüme, doku homeostazı ve onarım mekanizmalarını da kapsayabilir. Son yıllarda, enflamasyonun kaynağının herhangi bir antijene karşı immün yanıtın şekillenmesinde belirleyici olduğu ve bağışıklık sisteminin yalnızca enfeksiyöz koşullarda değil, steril doku hasarlarında da önemli bir rol üstlendiği gösterilmiştir. İstilacı mikroorganizmalara karşı esasen iki tür bağışıklık yanıtı bulunmaktadır. Doğuştan gelen bağışıklık, enfeksiyon etkenine maruz kalma sıklığından bağımsız olarak tutarlı bir şekilde ortaya çıkarken, adaptif bağışıklık tepkileri belirli patojenlerle tekrar eden karşılaşmalarla gelişir. Gelişmiş immün yanıtlar öncesinde, anatomik ve fizyolojik bariyerler enfeksiyonlara karşı temel birincil savunmayı oluşturur. Bu bariyerler; gözyaşındaki bakteriyolitik lizozim, mukozal salgılar, gastrik asit ve deri gibi yapılar aracılığıyla sağlanır.

Fiziksel bariyerlerin aşılması durumunda, konak savunmasının bir sonraki basamağı olan doğuştan gelen bağışıklık sistemi aktive olur. Doğuştan gelen bağışıklık süreçleri, hücresel bütünlüğün, organizmal denge durumunun ve tüm canlı türlerinin hayatta kalmasının sürdürülmesi için kritik öneme sahiptir. Ayrıca, adaptif bağışıklık aktivasyonundan önce, enfeksiyonlar, kanser hücreleri ve toksik maddelere karşı birincil savunma mekanizmasını oluşturur. Bu sistem genel olarak hızlı, non-spesifik, adaptif olmayan ve immün hafızadan yoksun yanıtlarla karakterizedir. Bu yanıtı gerçekleştiren hücreler arasında eozinofiller, bazofiller, mast hücreleri, monositler, dendritik hücreler, nötrofiller ve makrofajlar yer alır. Bu hücreler, çeşitli kemokin ve sitokinler salgılayarak bağışıklık yanıtını düzenler ve ardından fagositoz yoluyla patojenleri ortadan kaldırır.

Doğuştan gelen bağışıklık hücrelerinden biri olan eozinofiller, çok işlevli granülositik lökositlerdir ve viral, bakteriyel, paraziter enfeksiyonların yanı sıra alerjik reaksiyonlara karşı da yanıt oluşturabilirler. İnterlökin-5 (IL-5) ve granülosit-makrofaj koloni uyarıcı faktör (GM-CSF) gibi spesifik sitokinlerin etkisiyle kemik iliğindeki pluripotent progenitörlerden türevlenirler. Olgunlaştıktan sonra, IL-5 ile eotaksin ailesi kemokinleri arasındaki sinerjik sinyalleşme sayesinde periferik dolaşıma geçerek dokulara göç ederler.

Adaptif bağışıklık sistemi, timusta olgunlaşan T lenfositleri ile kemik iliğinden köken alan ve farklılaştıktan sonra antikor üretebilen B lenfositlerinin çeşitli alt popülasyonlarından oluşur. Bu hücreler primer lenfoid organlarda olgunlaştıktan sonra, antijenleri tanıma görevini üstlendikleri sekonder lenfoid organlara göç ederler. Adaptif bağışıklık yanıtları bu bölgelerde başlar ve sıklıkla doğuştan gelen sistemden gelen sinyallerle yönlendirilir. Bu sinyaller, dolaşımdaki patojenlerden doğrudan ya da ikincil lenfoid organlara göç eden antijen sunan hücreler (APC'ler) aracılığıyla dolaylı biçimde iletebilir. T lenfositleri, bağışıklık yanıtının düzenlenmesinde kilit rol oynar ve naif T hücreleri, hafıza T hücreleri ve düzenleyici T hücrelerini içeren periferik alt popülasyonlara ayrılır. Bu hücreler, antijenler ve kostimülatör sinyallerle karşılaştıklarında IL-2 üretir, çoğalır ve efektör hücrelere farklılaşırlar. Ardından sitokin ve sitotoksik araçların salınımıyla patojen temizliğine katkı sağlarlar. T hücresi toleransının bozulması, alerjen spesifik Th hücrelerinin IL-2 üretimi ile karakterize alerjik tablolarla sonuçlanabilir.

Kalıp tanıma reseptörleri (PRR'ler), patojenlerin, apoptotik konak hücrelerin ve yaşlanmış hasarlı hücrelerin yüzeylerinde bulunan özgül moleküler yapıları tanıyabilir. PRR'ler, dendritik hücrelerin ardından, doğuştan gelen bağışıklık ile adaptif bağışıklık arasında moleküler bağlantıyı oluşturan önemli yapılardır. Antienfeksiyöz, antitümöral ve diğer immün koruyucu mediatörlerin üretimini başlatabilirler. PRR'ler çoğunlukla protein alanı homolojilerine göre şu alt gruplara ayrılır: 1) Toll-benzeri reseptörler (TLR'ler), 2) NOD-benzeri reseptörler (NLR'ler), 3) Retinoik asitle indüklenebilir gen-I benzeri reseptörler (RLR'ler), 4) C-tipi lektin reseptörleri (CLR'ler). Bu reseptörler, patojen ilişkili moleküler kalıplar (PAMP'ler) ve hasar ilişkili moleküler kalıplar (DAMP'ler) gibi özgül örüntüleri tanıır ve bu sayede hem steril hem de steril olmayan inflamasyonu algılar. PRR'lerin doğuştan gelen bağışıklıkla ilişkili olduğu bilinmesine rağmen, daha sonra T ve B hücrelerinin de PRR ekspresyonu yapabildiği ortaya konmuş ve bu durum doğuştan gelen ve adaptif bağışıklık arasında işlevsel bir örtüşmenin varlığını göstermiştir. NLRP3, bilinen inflamozom kompleksleri arasında en kapsamlı şekilde çalışılmış PRR'dir. Buna rağmen, işleyişine dair bazı önemli mekanizmalar hâlâ netlik kazanmamıştır. NLRP3 ilk olarak, otoinflamatuvar nitelikteki kriyopirin ilişkili periyodik sendromlarla ilişkili bir gen mutasyonunun yol açtığı fonksiyon kazanımı sonucu tanımlanmıştır. NLRP3, merkezi NACHT alanı, C-terminal LRR ve N-terminal PYD alanlarına sahiptir. Bu yapılar, adaptör protein ASC'nin PYD-PYD etkileşimi aracılığıyla komplekse katılımını ve böylece prokaspaz-1'in aktive olarak pro-IL-1 β 'yı olgunlaştırmasını sağlar.

Diğer inflamozomların aksine, NLRP3 inflamozomu patojenler, toksinler, çevresel faktörler ve endojen DAMP'ler gibi yapısal olarak oldukça farklı uyarıcılar tarafından aktive edilebilir. Moleküler düzeydeki bu biyolojik süreçlerin görselleştirilmesi amacıyla yenilikçi görüntüleme tekniklerinin kullanımı da son yıllarda araştırma alanında önem kazanmıştır. Bu bağlamda, fotoakustik (PA) görüntüleme, lazerle indüklenen termo-akustik etkiye dayalı, optik ve ultrasonik görüntüleme avantajlarını bir araya getiren hibrit bir biyomedikal görüntüleme yöntemidir. Klinik öncesi çalışmalardan klinik tıbbi uygulamalara kadar kanser, mikrosirkülasyon bozuklukları ve tedavi yanıtlarının değerlendirilmesinde kullanılmaktadır.

Bu tez çalışması, doğuştan gelen bağışıklık yanıtından başlayarak Montelukast'ın T hücrelerinde oluşturduğu etkilerle sonlanan iki ana bölümlü bir araştırma sürecini kapsamaktadır. İlk bölümde amaç, kamuya açık *in silico* veri tabanları aracılığıyla insan eozinofil benzeri hücre hatları (EoL-1 ve HL-60) ile birincil eozinofillerde hem membran bağlı hem de sitozolik örüntü tanıma reseptörlerinin bazal ekspresyonlarını karşılaştırmaktır. Primer eozinofillerle çalışmanın teknik sınırlamaları nedeniyle, bu analiz hücre hattı seçiminde referans oluşturmayı amaçlamıştır. İkinci bölümde ise, yaygın olarak astım tedavisinde kullanılan lökotrien reseptör antagonisti Montelukast'ın, Jurkat T hücrelerinde inflamatuvar sinyalizasyon üzerindeki etkileri araştırılmıştır. Hücreler, Montelukast'a tek başına ya da hücre aktivatörü (C.A.) ile birlikte 48 saat maruz bırakılmıştır. CYSLTR1, NLRP3, ASC, IL-1 β , IL-10, Akt ve ERK gibi gen ve proteinlerin ekspresyon seviyeleri RT-qPCR, Western blot, ELISA ve immünohistokimya ile analiz edilmiştir. Hücresel düzeydeki değişimlerin optik sinyal karakteristiklerini değerlendirmek amacıyla PA görüntüleme de kullanılmıştır.

İlk bölümde, eozinofil PRR'lerinin mRNA seviyeleri *in silico* olarak analiz edilmiş ve EoL-1 ile HL-60 hücre hatlarının insan primer eozinofilleri (HPE) ile karşılaştırmalı profilleri sunulmuştur. TLR2 ve NLRP3'ün her iki hücre hattında HPE'ye göre anlamlı düzeyde yüksek olduğu ($p<0.01$), NLRP12, LGP2 ve Dectin-1'in ise PE'de daha yüksek olduğu belirlenmiştir ($p<0.01$). TLR6, MDA5, NOD2, NLRP1 ve CLEC4A gibi bazı PRR'lerin bazal düzeyde hücre hatlarında HPE'ye kıyasla daha yüksek ekspresyon gösterdiği gözlenmiştir.

İkinci bölümde, 10^{-5} M MNT uygulaması sonrası CYSLTR1 mRNA seviyesi kontrol grubuna kıyasla yaklaşık 2,5 kat artmıştır ($p<0.0001$). NLRP3 ekspresyonu C.A. ile artmış ($p<0.01$) ve C.A.+MNT grubunda en yüksek seviyeye ulaşmıştır. ASC seviyesi bu gruplarda azalmıştır ($p<0.05$). IL-1 β salınımı ELISA ile anlamlı fark göstermese de, hücre içi ekspresyon C.A. grubunda artmış ($p<0.001$) ve C.A.+MNT grubunda en yüksek düzeye ulaşmıştır ($p<0.01$). p-AKT/AKT oranı C.A. grubunda artmış ($p<0.01$), p-ERK1/2 ise MNT+C.A. grubunda en yüksek seviyeye çıkmıştır ($p<0.01$). PA görüntüleme sonuçlarında, MNT uygulanan gruplarda PA sinyal genliği anlamlı şekilde azalmıştır; ortalama amplitüd kontrol grubunda 2523,22 iken MNT+C.A grubunda 1088,19'dur.

Sonuç olarak, bu tez çalışmasının ilk bölümünde, *in silico* analizlerle PRR profilleri karşılaştırılarak eozinofil temelli bağışıklık araştırmaları için en uygun *in vitro* modeller belirlenmiştir. İkinci bölümde, Montelukast'ın aktive edilmiş Jurkat T hücrelerinde inflamatuvar sinyalizasyonu nasıl etkilediği moleküler ve görüntüleme düzeyinde ortaya konmuştur. PA görüntülemenin bu süreçteki kullanımı, bağışıklık modülasyonunu noninvazif biçimde izleme potansiyelini göstermiştir. Çalışma; hesaplamalı, moleküler ve görüntüleme tabanlı yaklaşımların bütünleştirilmesiyle bağışıklık araştırmaları için çok boyutlu bir platform sunmaktadır. Gelecekteki çalışmalar, hastalık modelleri altında PRR ilişkili mekanizmaları doğrulamak ve immün hücre yanıtlarını dinamik biçimde görüntülemek için bu stratejiyi daha da ileriye taşıyabilir.



1. INTRODUCTION

1.1 Immune System

The immune system protects the human body against harmful external factors in a rational system and logic. It prevents the host from attaching foreign patterns, such as damage or pathogen-associated patterns, to the body and becoming a host. Nevertheless, the capacity of immune cells and their functions can extend beyond host defense to development, tissue homeostasis, and repair mechanisms. In the last decades, it is also depicted that the external or internal source of the inflammation is important in defining the type of response to any antigen and that the immune system is essential in terms of maintenance and renewing, not only in infectious situations but also highly crucial for sterile damages. The immune system works categorized as innate and adaptive immunity. There are many points where they work together to protect the host against disease-causing agents to which we are constantly exposed (Sattler, 2017; Zoghi et al., 2023)

Many cells work together to coordinate the functions of the immune system. The majority of these cells are white blood cells that develop from pluripotent haematopoietic stem cells. Most cells act roles in the innate immune system, such as mast cells, dendritic cells (DCs), granulocytes, and macrophages, differentiate from common myeloid progenitor (CMP). B and T lymphocytes, for example, are specialized cells of the adaptive immune response that originate from common lymphoid progenitor (CLP). Immune responses to antigens occur in the lymphoid organs, but the majority of immune system cells are found in the blood (Figure 1.1) (Zoghi et al., 2023).

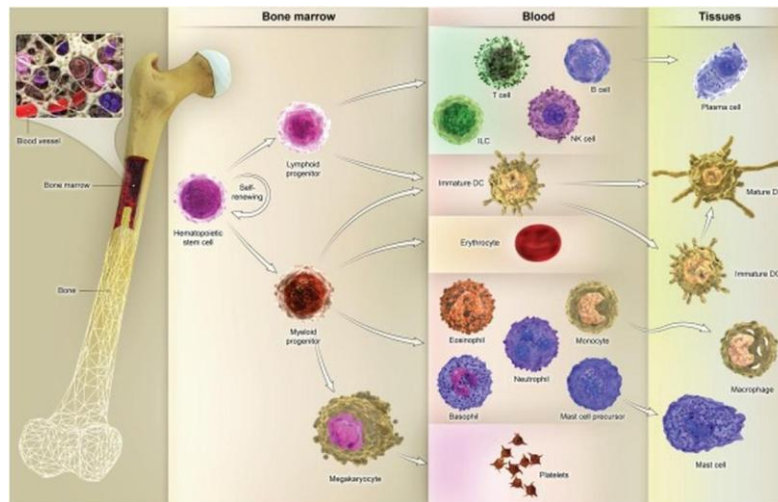


Figure 1. 1: Illustration of the process by which hematopoietic stem cells differentiate in the bone marrow to form mature immune cells that migrate into the blood and tissues. Cell types developing from lymphoid and myeloid progenitor cells and cells migrating to the tissue are indicated (Zoghi et al., 2023).

1.1.1 Innate immune system

The vital initial line of defense against infections is equipped by anatomical and physiological barriers. These barriers include bacteriolytic lysozyme in tears, saliva, mucosal secretions, low stomach pH, and, of course, intact skin. The high-level damages and risks of infection seen in individuals show that healthy innate and adaptive immune systems cannot make up for the breakdown of vital anatomical and physiological barriers. The defense provided by barriers is enhanced by innate immunity. Innate immunity targets sterile damages and microbial components. In spite of the fact that it has a restricted number of invariant receptors, it can compensate for this limitation and be used to detect invasive pathogens. One of the innate immune system's distinguishing features is its speed: minutes after being exposed to a pathogen, the immune system begins to produce a protective inflammatory response. Furthermore, the ensuing adaptive immune response is triggered via innate immune responses (Turvey & Broide, 2010).

The components of the innate immune system react differently to each germ; thus, the activation of the innate immune response itself can decide the reactivity of the adaptive

immune response. This means that the appropriate and efficient adaptive immune response is activated against each bacterium. Inflammation is one of the innate immune response's defense mechanisms, causing leukocytes and soluble chemicals to infiltrate the initial infection site or damaged tissues. Antiviral defense, which results in the destruction of virus-infected cells through the generation of anti-viral cytokines, type I interferons (IFNs) and the activation of natural killer (NK) cells (Zoghi et al., 2023).

Various kinds of white blood cells are responsible for combating infections. The innate immune system cells, eosinophils, basophils, mast cells, monocytes, dendritic cells, and especially neutrophils and macrophages, are the most crucial of them early in an immune response because they arrive at the location of an external breach. In their own right, these cell types are potent killers. They release extremely harmful chemicals, like various enzymes that break down the proteins. Then, in a process known as phagocytosis, they absorb and break down the damage (Nicholson, 2016).

1.1.1.1 Eosinophils

Eosinophils are the leukocytes essential for various vital functions such as maintaining immune homeostasis, contributing to effector and modulatory functions that shape innate and adaptive immune responses together (Celik et al., 2023). The activation of eosinophils through the connection of receptors for cytokines, immunoglobulins, and complementary mediators results in the release of various cytokines. Various types of granules, and secretory vesicles comprise distinct populations of secretory organelles found in eosinophils. The most of the secretory organelles are the crystalloid granules, which in eosinophils store most of the granule proteins (HOGAN et al., 2008). Eosinophils exhibit additional cell-surface proteins that facilitate cell-cell interactions, including adhesion proteins. Integrins facilitate the migration of eosinophils from the bloodstream to both normal and inflamed tissues. Eosinophils exhibit the leukocyte adhesion glycoproteins LFA-1 (CD11a), CR3 (CD11b), p150,95 (CD11c), and the common beta chain (CD18). In contrast to neutrophils, eosinophils can act like a leukocyte and migrate to inflammatory zone in individuals with leukocyte adhesion deficiency syndrome, indicating that eosinophils can utilize mechanisms both

dependent and independent of CD18 binding with intercellular adhesion molecule to adhere to vascular endothelial cells and infiltrate tissues (Weller., 1991).

1.1.1.2 Pattern recognition receptors

Microorganism detection is accomplished by pattern recognition receptors (PRRs) which are receptor family that germline-encoded. They execute the process via identifying structures known as two different motifs: 1) pathogen-associated molecular patterns (PAMPs), which are conserved across microbial species; 2) Damage-associated molecular patterns (DAMPs), which are endogenous chemicals generated by the damaged cells, are also recognized by PRRs. Four subfamilies of PRRs have been identified and classified thus far. Transmembrane proteins like Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) and cytoplasmic proteins like Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) are the subfamilies of PRRs. These are expressed by a variety of nonprofessional immune cells in addition to macrophages and DCs. Transcription implicated in inflammatory responses is upregulated when PRRs sense PAMPs or DAMPs, with the exception of certain NLRs. Proinflammatory cytokines such as IL-1 β , IL-6, IL-18, TNF- α , type I IFNs, various chemokines, some antimicrobial proteins, proteins that modulate receptor signalling, and other unidentified proteins are all encoded by these genes. Activated PRRs exhibit distinct patterns of inducible gene expression (Takeuchi & Akira, 2010).

1.1.1.3 NOD-like Receptors

Certain pathogens infiltrate the cytoplasm as part of their development cycle. For example, the cytoplasm is where virus particles are attached and where viral genes are frequently transcribed and translated. Furthermore, a number of escape approaches are available to some bacteria and parasites, including the ability to infiltrate the cytoplasm and create gaps in the phagosome membrane. Consequently, pathogens and their constituents, together with additional components generated by infection and damage, will be found in the cytoplasm, necessitating the body's detection of PRRs. NLRs are identified as intracellular PRRs and they are made up of three domains: 1) the N-

terminal effector domain, which is the protein interaction domain, such as the caspase activation and recruitment domain (CARD) or the pyrin domain (PYD); 2) LRRs at the C-terminus, which are used to identify ligands; and 3) the central nucleotide-binding domain (NBD), also referred to as the NACHT domain, which is shared by the NLR family and crucial for nucleic acid binding and oligomerization of NLRs. (Li & Wu, 2021).

The NLRC subfamily, which include CARD domain specifically; the NLRP subfamily, which includes PYD domain; the NLRB subfamily, which includes baculovirus inhibitors of apoptosis protein repeats; the NLRA subfamily, which includes acidic activation domains; and the NLRX subfamily, which includes additional NLR effector domains, are the five subfamilies into which the NLRs family can be separated based on the various N-terminal effector domains (Li & Wu, 2021).

1.1.2 NLRP3

The NLRP3 protein is the main mediator of the well-characterized immunological complex known as the NLRP3 inflammasome. One member of the NLR family is the NLRP3 protein. This complex also contains the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and procaspase-1. Although it is essential for the host immune system's defenses against different pathogens, its dysregulation has been connected to the etiology of a number of inflammatory conditions. Monocytes, dendritic cells, neutrophils, lymphocytes, osteoblasts, and epithelial cells are all express this 115 kDa cytosolic protein. It is made up of three domains: LRR domain, the NOD, and the PYD. By interacting with its pyrin domain, the PYD domain recruits ASC and initiates the inflammasome assembly process. While the LRR domain is in control of detecting danger signals, the NACHT domain possesses ATPase activity that is necessary for NLRP3 oligomerization after activation. After activation, NLRP3 monomers oligomerize via their NACHT domains, and then their PYD domains engage with ASC. (Kelley et al., 2019; Zahid et al., 2019). The physiologically inactive peptides pro-IL1 β and pro-IL-18 are converted into their active counterparts, IL-1 β and IL18, respectively, by activated caspase-1. Additionally, it cleaves and activates the membrane pore-forming

gasdermin D (GSDMD), a protein implicated in pyroptosis, a type of programmed cell death. Cardiolipin and phosphoinositides, two lipids found in plasma membranes, are highly affinized by GSDMDs N-terminal domain (GSDMD-NT). It oligomerizes after cleavage to create cell membrane holes. After the cell's osmotic potential is disrupted, pyroptosis occurs and internal materials, such as the inflammatory cytokines IL-1 β and IL-18, are released (Blevins et al., 2022).

Because the NLRP3 inflammasome responds to a variety of physically and chemically distinct stimuli, it cannot be able to bind directly to its activating stimuli. Instead, NLRP3 can detect a common cellular process triggered by different stimuli. Despite the fact that some of the details of this process are still under debate, a two-signal model has been proposed for NLRP3 inflammasome activation with all the known details and processes (Kelley et al., 2019). This model is illustrated in Figure 1.2.

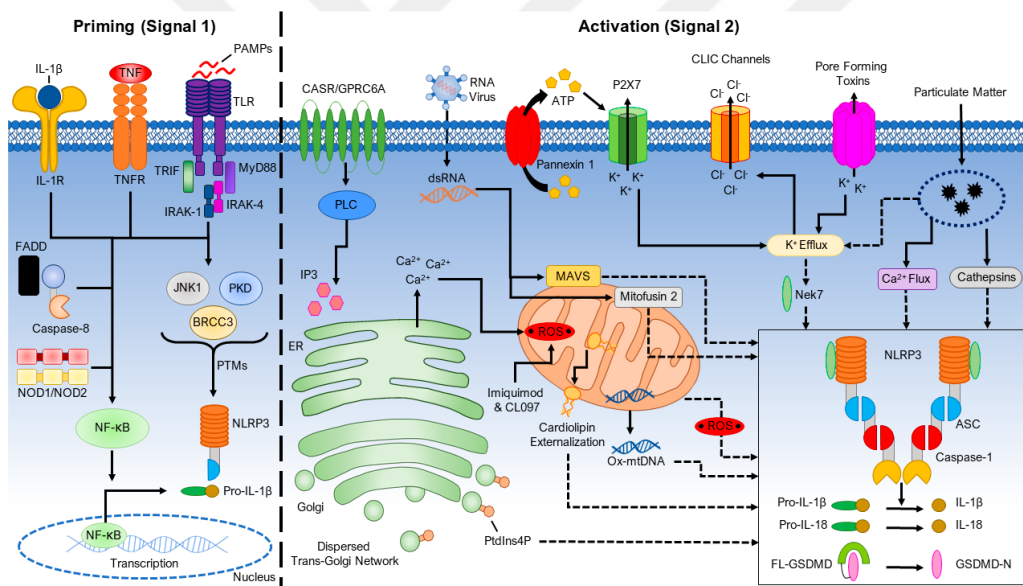


Figure 1. 2: The NLRP3 Inflammasome Activation Two-Signal Model. Microbial components or endogenous cytokines produce the signal 1, which initiates the signal 2 via activation of the transcription factor NF- κ B and causes NLRP3 and pro-interleukin-1 (pro-IL-1 β) to be upregulated Through their regulation of the NF- κ B pathway, caspase-8, FAS-mediated death domain protein (FADD), and NOD1/2 participate in the priming step. Post-translational changes to NLRP3 grant permission for its activation. (Kelley et al., 2019).

Currently, significant focus has been directed towards the involvement of the inflammasome in asthma and its association with asthma phenotypes. Besnard et al. demonstrated that the NLRP3 inflammasome plays a role in the progression of allergic airway inflammation (Besnard et al., 2011; Leszczyńska et al., 2022). The study demonstrated that the heightened infiltration of cells, predominantly eosinophils in bronchoalveolar lavage (BAL) and both eosinophils and lymphocytes in lung tissue, was observed in ovalbumin-challenged wild-type (WT) mice compared to NLRP3^{-/-} animals. Furthermore, the quantity of infiltrating granulocytes and lymphocytes was decreased in Nlrp3^{-/-} mice relative to OVA-challenged WT mice. Furthermore, the absence of NLRP3 influenced the recruitment of CD4⁺ T cells and the migration of dendritic cells (DC) in comparison to wild-type (WT) animals (Besnard et al., 2011; Leszczyńska et al., 2022). Based on this and several similar studies, the researchers concluded that NLRP3 inflammasome activity has a significant impact on the formation of allergic airway diseases through various triggers.

1.1.3 Pi3k-akt signalling pathway

Crucially activated downstream of the PI3 kinase signaling pathway is the molecule Akt. Different growth factors and cytokines boost the activity of the lipid enzyme PI3 kinase, so increasing PI(3,4)P₂ and PI(3,4,5)P₃ levels inside the cell. Akt and PDK1 (3-phosphoinositide-dependent protein kinase 1) are among the proteins with PH domains that find binding sites created by 3-phosphoinositides, so enabling their membrane recruitment. Usually in an inactive condition, Akt is maintained by an intramolecular interaction between the kinase domains. Akt's conformational change induced by the interaction between the PH domain and 3-phosphoinositides helps co-recruited PDK1 to reach the activation loop for the phosphorylation of Thr308 easier. Thr308's phosphorylation increases Akt activity almost 100-fold; but, optimal Akt activity also depends on phosphorylation of Ser473 inside the hydrophobic pattern. Activation of Akt affects the regulation of the cell proliferation, survival and metabolism (Hers et al., 2011).

In monocytes and macrophages, LPS stimulation activates AKT, which seems to exert an immunosuppressive effect by negatively regulating the immune response

downstream of LPS (Guha & Mackman, 2002; Zhao et al., 2020). In their study, Zhao et al. demonstrated that the immunosuppressive role of Akt, characterizing it as a dual regulator of the NLRP3 inflammasome. Additionally, it was noted that Akt phosphorylates NLRP3 at S5, which inhibits the oligomerization of NLRP3 and ASC, thereby reducing the production of inflammatory cytokines. Furthermore, Akt-mediated phosphorylation of S5 increases NLRP3 stability during the LPS priming phase by inhibiting TRIM31-mediated ubiquitination and degradation of NLRP3. Consequently, they established a physiological function for Akt in regulating NLRP3 (Zhao et al., 2020).

1.1.4 Mapk/erk signalling pathway

Extracellular signal-regulated kinase (ERK) is an effector kinase that has various responsibilities, such as cell cycle progression, differentiation, and survival. The malfunctions that will be done on this pathway are associated with various clinical conditions. Nowadays, several research studies have contributed to the understanding of the signaling of the mitogen-activated protein kinases (MAPK)-ERK pathway, which has facilitated the development of many possible therapeutic approaches aimed at this system. ERK1 and ERK2, generally called ERK1/2, are fundamental for cell development and activation because they have the basic components of the Raf/MEK/ERK signaling pathway. They can induce apoptosis and are responsible for the cell cycle arrest; besides these roles, their most well-known function is as cell-proliferative kinases. According to certain studies, the factors affecting pathway activity include spatial-temporal regulation and the molecular switches' non-canonical roles in this system (Park, 2023). The differentiation of CD4⁺ and CD8⁺ T cells during thymopoiesis also depends on the MAPK signaling pathways. While p38 and the c-Jun N-terminal kinase (JNK) promote negative selection, ERK1/2 is necessary for positive selection. The incapacity of CD4⁺ T cells to differentiate within the CD4 lineage is caused by the targeted deletion of ERK2. Pharmacological suppression of ERK1/2 promotes CD8⁺ T cell development. The maturation of T lymphocytes within the thymus is one mechanism whereby various cellular fates are connected to the activation of various families of MAPKs. T cell action in peripheral tissues depends on MAPKs. In a specific study that focused on ERK presence, they created ERK1^{-/-}

T cells which had a lower capacity to increase, a lower capacity to generate Th2 cytokines, and a higher tendency to develop into Th1 cells. ERK1^{-/-} mice develop more severe experimental autoimmune encephalitis. In the literature it is also showed that ERK1/2 induces Th2 differentiation by means of several pathways. A MEK1/2 inhibitor effectively reduces eosinophilic inflammation in the airway (Alam & Gorska, 2011) in a mouse model of asthma.

1.1.5 Adaptive Immune System

Highly specialized cells that can adjust to the threat of novel and diverse infectious agents make up the adaptive immune system. Thymus-derived T cells and bone marrow-derived B cells are the two main cell types that compose adaptive immunity. The adaptive immune system is sometimes titled "specific/acquired immune system" because of these cells' exceptional ability to precisely identify a wide variety of foreign antigens. The precise detection of foreign antigens by T cells and B cells is essential for the start and progression of an adaptive immune response. The expression of T cell receptors (TCRs) on T cells and B cell receptors (BCRs) on B cells mediates antigen recognition by T cells and B cells (Williams, 2011, p. 41). Mature B cells have ability to produce a high-affinity variant of their receptor, as an immunoglobulin (Ig) or antibody, which binds to its specific antigen for the purposes of microbial neutralization or clearance. In contrast to TCRs, which primarily identify short peptides produced from antigens, antibodies can recognize the epitopes (Vanguri, 2014).

1.1.5.1 Autoimmunity

Even though the adaptive immune system evolved during evolution, resulting in the acquisition of a response based on long-term memory, the intermediary cellular subsets of that evolution have persisted. They are unique in that they are highly prevalent in the early years of life, when the adaptive immune system is developing, but the high levels and overactivation later on might result in the context of allergies, autoimmunity, and also cancer (Hillion et al., 2020).

Environmental stimuli and genetic factors combine to cause autoimmune disease. People in the early stages of a disease are frequently subclinical and unaware of clinical symptoms. During the propagation phase, patients show signs of clinical disease, including tissue damage and self-sustaining inflammation brought on by the production of cytokines, the spread of epitopes, and an unbalanced effector T cell/Treg (Teff/Treg) ratio. Both cell-intrinsic inhibitory pathways and cell-extrinsic mechanisms involving regulatory T cells (Tregs) are activated to resolve autoimmune reactions. These mechanisms limit effector responses and restore equilibrium between Tregs and Teff. Because of a persistent conflict between regulatory mechanisms and pathogenic effector responses, patients in this phase often experience relapsing and remitting disease (Rosenblum et al., 2015).

Many autoinflammatory diseases have already been strongly linked to changes in inflammasome-forming NLRs. Excessive activation of the NLRP3 inflammasome by mutations increases IL-1 β secretion and causes cryopyrin-associated periodic syndromes (CAPSs). Different CAPS produced by NLRP3 gene mutations have been identified. A T cell-independent phase has been suggested for the start of rheumatoid arthritis (RA), a systemic autoimmune disease; the innate immune system influences the course of the autoimmune response. Given the variety of endogenous danger signals that activate NLRs and the effect of inflammasome products, such IL-1 β and IL-18, on adaptive immunity, the inflammasome most certainly contributes to some autoimmune diseases. By several pathways, IL-1 β affects lymphocytes: upregulation of IL-2 receptor expression, extension of T cell survival, increase of antibody production by B cells, and stimulation of B cell proliferation. Respectably, IL-1 β and IL-18 are essential for Th17 and Th1 cell development and proliferation to be promoted. IL-1 β and IL-18 thus improve T and B cell responses and might be a necessary link between NLR activation and adaptive immune responses (Shaw et al., 2011).

1.1.5.2 T Cells

T cells (also known as T lymphocytes) derive from bone marrow progenitors that migrate to the thymus for maturation and classification according to their

differentiation. Peripheral T cells consist of many subsets, including naive T cells, which are capable of responding to novel antigens; memory T cells, originating from prior antigen activation and facilitating long-term immunity; and regulatory T (Treg) cells, which are responsible for the modulation of immune responses. Immune responses initiate when naive T cells interact with antigens and costimulatory ligands presented by dendritic cells (DCs), leading to the production of interleukin 2 (IL-2), proliferation, and differentiation into effector cells that subsequently migrate to various locations to facilitate pathogen clearance via the secretion of cytokines and cytotoxic mediators (Kumar et al., 2018).

T cells are essential for sustaining tolerance to harmless antigens, and impaired T-cell tolerance contributes to the onset of various types of allergies. In healthy individuals, various T-cell populations exhibit responses to allergens. Allergen-specific Th cells in healthy individuals predominantly exist in a naïve state and express IL-2 upon allergen exposure. Additionally, a subset of cells exhibiting an antigen-experienced phenotype can produce cytokines such as IFN- γ and, infrequently, IL-10; however, they do not produce Th2-type cytokines, which are IL-4, -5, and -13, as assessed by short-term allergen restimulation (Suhkamp et al., 2023).

Development of the T cell can be briefly defined as they originated in the thymus from common lymphoid progenitors derived from the bone marrow. The seeding of the thymus is facilitated by the contact between platelet selectin glycoprotein 1 on progenitors and the adhesion molecule P-selectin on thymic epithelium. Recently arrived cells swiftly proliferate in response to IL-7, which receptor transmits signals via the common γ chain, encoded on the X chromosome, and is utilized by several other cytokine receptors (IL-2, -4, -9, -15, and -21) (Bonilla & Oettgen, 2010).

Two main signals and an extra signal are needed for T-cell activation. TCR recognition of these antigens gives the immune response specificity. The first signal consists of antigens that are presented by APCs as peptides linked to histocompatibility molecules. The second signal, known as the "costimulatory signal," functions in tandem with antigen stimulation and is crucial for T cell activation. APC molecules that interact with particular T cell costimulatory receptors deliver this signal. One

important factor affecting how a T cell interacts with an antigen is costimulation. The CD28 receptor is a key component of the most well-defined T-cell costimulatory pathway. It interacts with two costimulatory molecules, B7-1 (CD80) and B7-2 (CD86). Microbial presence and cytokines generated in response to microbial stimuli upregulate B7-1 and B7-2 expression on APCs. T cell responses are optimized by the controlled expression of B7 costimulators, which mainly activate T cells when pathogens are present (Sharpe & Abbas, 2006).

Upon receiving the essential signals, the T cell subsequently acquires additional instructions through cytokines. The factors dictate the differentiation of the cell type; for helper T cells, exposure to cytokines such as IL-12 leads to Th1 differentiation, IL-4 to Th2, and IL-6 and IL-23 to IL-17 production. Each cell executes a distinct function within the tissue and contributes to the advancement of immune responses. The resultant cell population migrates to the site of infection or inflammation to address the pathogen. Additional cells at the inflammation site, including neutrophils, mast cells, and epithelial cells, can release cytokines, chemokines, short peptides, and other molecules that promote further activation and proliferation of T cells (Figure 1.3) (Cavanagh & Gwyer Findlay, 2021).

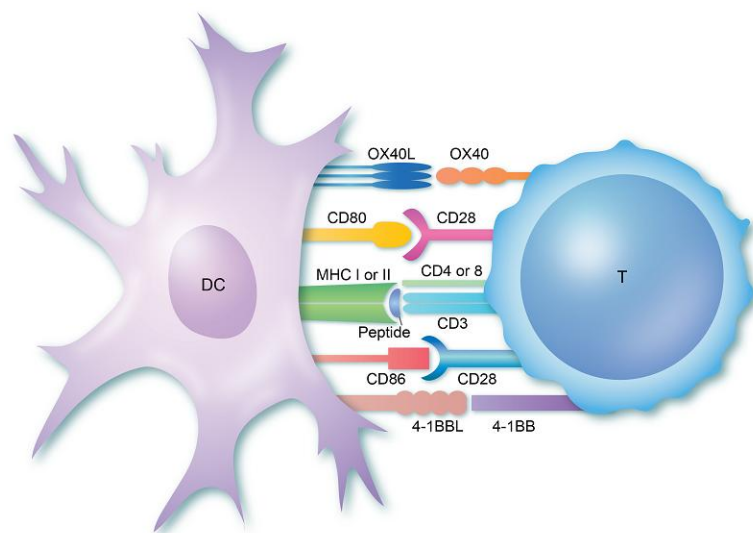


Figure 1. 3: Diagram illustrating the 3 signals of T cell activation. T cell interacts with a DC presenting its specific peptide in an MHC molecule, binding to the peptide-MHC complex via CD3 and either CD4 or CD8. Thereafter, co-stimulation transpires

via DC-associated CD86, CD80, OX40L (CD40L), and 4-1BBL. This triggers complete activation and effector functionality in the T cell (Cavanagh & Gwyer Findlay, 2021) .

1.1.5.3 T Cells in allergy

Th2 cells and type-2 cytokines control and enlarge the allergic inflammatory response. Th1 cells cross-regulate Th2 cells in some environments; it was hypothesised that Th1 cells downregulated Th2 cell effects and hence had a positive impact in allergic illness and asthma. Though Th1 cells were obviously pro-inflammatory, it became clear that the emergence of a Th1-associated inflammatory response would aggravate asthma and allergy condition. Often found at sites of allergic inflammation, IFN- γ is thought to be involved in the disorder. Furthermore, although the injection of allergen-specific Th1 cells into mouse lungs did not directly produce airway hyperreactivity (AHR), it did cause more rather than less inflammation in the airways. At last, it became quite clear that the increase in autoimmune, Th1-driven disease prevalence over the past 20 years coincided with the increase in atopic disease frequency. This demonstrated that the environmental changes causing increased asthma frequency cannot be ascribed just to a decrease in Th1 regulating elements. These results thus suggest that different forms of immunity than Th1 reactions have to be effective in control of the development of allergy diseases and asthma (Akbari et al., 2003) .

In a remarkable study by Bruchard and colleagues, the TCR in CD4⁺ T cells was revealed to activate NLRP3 expression via the signal transducer STAT5 and the IL-2 signaling pathway. Th2 cell differentiation was found to be completed in an inflammasome-independent fashion depending on NLRP3 expression on CD4⁺ T cells. By directly binding to DNA and so promoting DNA binding by the transcription factor IRF4, NLRP3 controlled the Th2 process. These led them to see a crucial function for NLRP3 as a Th2 differentiation transcriptional regulator. More importantly, NLRP3 expression in CD4⁺ T cells has physiological implications in allergic manner in asthma and also cancer by increasing TH2 polarization, asthma development, and Th2 cell-dependent tumor growth. Our findings gives the motivation to produce compounds that disturb the NLRP3-IRF4 connection and could

be regarded Tj2 response-modulating medicines. Such medications might be pertinent for the treatment of allergy diseases as well as Th2 cell–related disorders including malignancy (Bruchard et al., 2015).

1.1.6 Cysteinyl Leukotriene Receptors (cysltrs) & Montelukast (MNT)

Cysteinyl leukotriene receptors (CYSLTRs) are seven transmembrane spanning receptors that, when bound to their endogenous ligands which are leukotriene C4 (LTC₄), leukotriene D4 (LTD₄), and leukotriene E4 (LTE₄). They are able to interact with G proteins to initiate intracellular signaling pathways. These have been linked to a number of inflammatory conditions, including immune, gastrointestinal, skin, and cardiovascular diseases, and also they are important in respiratory conditions like asthma, allergic rhinitis, and other nasal allergies. Patients with atopic dermatitis, rheumatoid arthritis, Crohn's disease, or malignant astrocytoma have also been found to have increased urine excretion after episodes of unstable angina and acute myocardial infarction. Furthermore, this phenomenon is observed in people with a number of respiratory disorders, such as bronchial hyperreactivity, seasonal allergic rhinitis, virus-induced wheezing, and asthma (Capra, 2004).

US Food and Drug Administration (FDA) approved Montelukast (MNT) which presented as the selective antagonist of the LTD₄ receptor in 1998 for the treatment of asthma and for the treatment of allergic rhinitis after 4 years. In these times, due to their limited indications and lack of strong evidence regarding their safety or efficacy, LTRAs are currently classified as alternative controller in asthma treatment guidelines (Dykewicz et al., 2020), either as monotherapy or in combination with inhaled corticosteroids (ICS). The recommendation for allergic rhinitis limits the use of MNT as a treatment option to those whose condition cannot be adequately treated with other drugs (Markham & Faulds, 1998a; Mayoral et al., 2023).

Based on a case report review by Calapai et al., it is abundantly evident that several adverse drug reactions (ADRs) can burden MNT administration; of these, healthcare professionals should be aware of this situation since the combination of MNT with other drugs increases the risk of drug-drug interaction. Improved knowledge of the

processes generating adverse drug reactions connected to this anti-leukotriene will enable researchers to propose a therapeutic approach meant to lower MNT toxicity. Moreover, more precise epidemiological research on big populations is desirable to clearly identify risk variables favoring MNT-associated ADRs (Calapai et al., 2014) .

1.1.7 Jurkat t cell line

Jurkat T cell line (known as JM in the original study) was derived from a fourteen-year-old boy diagnosed with Acute Lymphoblastic Leukemia (ALL)(Gioia et al., 2018; Schneider et al., 1977). From the first day it was obtained, it enabled an outstanding number of discoveries and publications and was one of the first *in vitro* model organisms for studying T-cell biology. The Jurkat cell line has been especially used to model and clarify signaling mechanisms in T cell activation (TCA), a vital phase of the adaptive immune response, in T cell signaling. Surface signaling in the TCA model involves the T cell receptor (TCR) and auxiliary proteins CD3 and CD28 on T cells. This starts a sequence of molecular events that culminates in the transcriptional activation of several genes, including the important target gene for T cell activation, which is IL-2 (Montano, 2014).

1.1.8 Human Eosinophilic Leukemia Cell Line (eol-1) and HL-60 as PRR study model

The intricate functions of eosinophils render them both fascinating and tough to study; yet, numerous abilities have been clarified by research conducted over the past three decades (Celik et al., 2023). Therefore, in 1985, an eosinophilic leukemia cell line, termed EoL, was derived from the peripheral blood of a 33-year-old patient diagnosed with Philadelphia chromosome-negative eosinophilic leukemia (EL) (Saito et al., 1985). Under normal culture conditions, EoL-1 cells show cytological traits of myeloblasts; yet, several factors can induce their differentiation into eosinophilic granule-containing cells. Operating similarly to traditional eosinophils, the phenotypically unique EoL-1 cells Although they are leukemic and not always displaying the characteristics of normal eosinophils, EoL-1 cells are considered as a useful *in vitro* model for the research of human eosinophils (Mayumi, 1992).

Although HL-60 is a human promyelocytic leukemia cell line mostly used to model neutrophil and monocyte differentiation, several studies have shown its potential to acquire eosinophil-like features under particular conditions. Several mediators such as n-butyrate, or IL-5 can cause partial differentiation of HL-60 cells toward an eosinophilic phenotype, defined by restricted expression of eosinophil-associated markers (Ishihara et al., 2005; Plaetinck et al., 1990). This induced state does not, however, fully reflect the functional and molecular profile of real eosinophils or eosinophilic cell lines such as EoL-1. Thus, even if HL-60 is a surrogate model in the lack of more specialized cells, it is still an insufficient substitute for studies needing high-fidelity eosinophil biology.

1.2 Photoacoustic (PA) Imaging

Alexander Graham Bell first identified the PA effect in 1880 after observing sound being produced by the absorption of modulated sunlight. This marked the beginning of a long, if intermittent, history of investigating the basic physics behind photoacoustic (PA) techniques. After that, not much scientific study or technological advancement occurred until the laser was introduced in the 1960s. This device offered the high peak power, spectral purity, and directionality that were necessary for a variety of PA sensing applications (Beard, 2011). The advantages of optical and ultrasonic imaging are combined in PA imaging, a hybrid biomedical imaging technique that makes use of the thermo-acoustic effect produced by lasers. As a result, PA imaging has many uses in pre-clinical research and clinical medicine, such as diagnosing cancer, evaluating microcirculation abnormalities, and determining the effectiveness of therapy (Mutlu, et al., 2024).

In the PA imaging system, first, light energy converts into the sound energy. Optimal penetration depth is provided by the near-infrared (NIR) spectral region. In the context of biomedical way, PA imaging especially use effectively for visualizing vasculature *in vivo* or *in vitro*, with or without contrast agents, with high resolution even in microscopic scale. The creation of these sound waves within the tissue causes a volumetric expansion, resulting in the emission of acoustic waves. Detection was accomplished using ultrasonic transducers or specialized probes, and the image is

generated by investigating the arrival time of acoustic waves to the transducers, employing the established speed of sound. According to the data-of-interest, these signals can be processed as mean signal amplitudes (Figure 1.4) (Privitera et al., 2021).

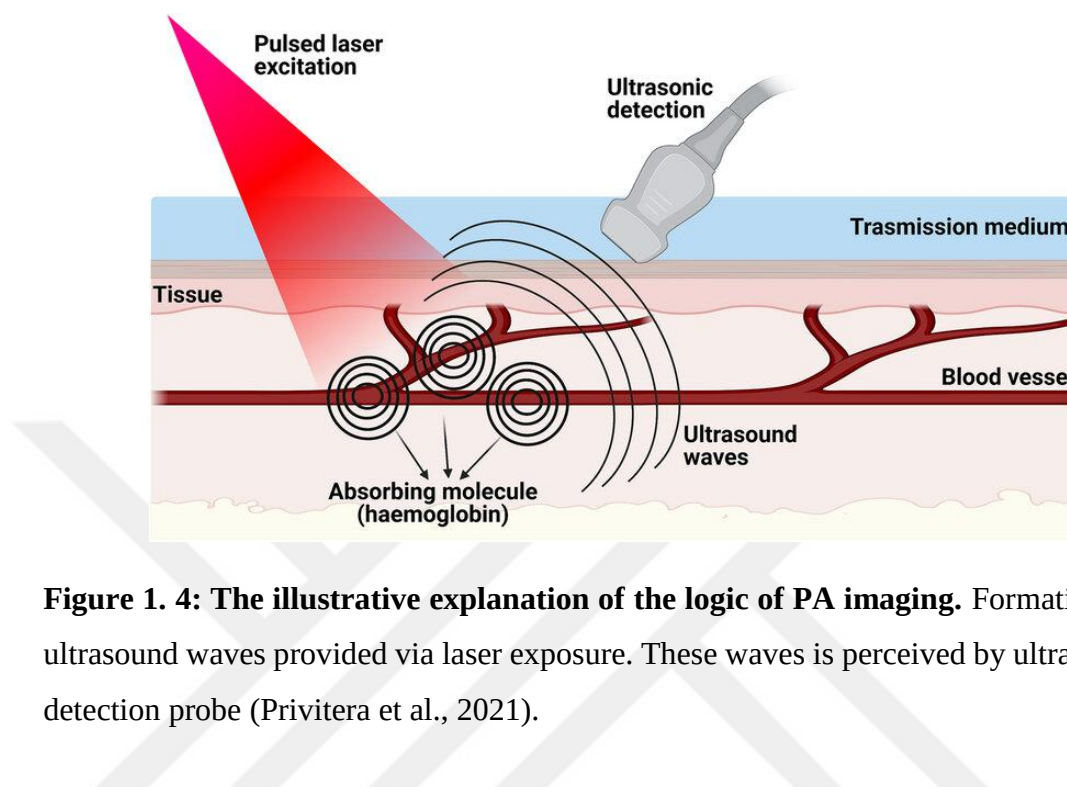


Figure 1. 4: The illustrative explanation of the logic of PA imaging. Formation of ultrasound waves provided via laser exposure. These waves is perceived by ultrasonic detection probe (Privitera et al., 2021).

1.3 Aim of the Study

In the first chapter of the thesis, the aim was to investigate and also compare the basal expression profiles of all subfamilies of PRRs, in EoL-1 and HL-60 human eosinophil-like cell lines and (HPEs) using *in silico* tools. Given the experimental limitations of working with primary eosinophils, identifying cell lines with comparable PRR expression patterns is critical for selecting reliable *in vitro* models. The findings are expected to provide a molecular basis for future mechanistic studies on eosinophil function and innate immune signaling.

The aim of the second chapter was to investigate and unveil the immunomodulatory effects of Montelukast, a cysteinyl-leukotriene receptor antagonist, in the manner of the expression of PRRs, particularly NLRP3, in the Jurkat T cell line. Additionally, the study seeks to elucidate whether Montelukast-induced molecular changes involve intracellular signaling pathways such as PI3K/Akt and MAPK/ERK1/2. Considering

the emerging role of photoacoustic (PA) imaging in the detection of intracellular biochemical alterations, this research also explores the potential of PAI as a non-invasive method to reflect immune activation-associated optical signatures in T cells. Ultimately, this study aims to provide mechanistic insight into how Montelukast may modulate innate immune responses and to assess the applicability of PA imaging in evaluating drug-induced immunological shifts.



2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Tools

Appendix A lists the tools utilized in this thesis project.

2.1.2 Commercial Kits

Appendix B lists the commercial kits used in this thesis project.

2.1.3 Commercial Chemicals

Appendix C lists the commercial chemicals and their brand names used in this thesis project.

2.1.4 Cell culture and stimulation

For culturing Jurkat T cell line, complete RPMI-1640 medium was used as growth media. Complete RPMI media was prepared using the components in Table 1. For activating Jurkat cells a pre-mixed cocktail with optimized PMA (phorbol 12-myristate-13-acetate) and ionomycin, Cell Activation Cocktail (without Brefeldin A, BioLegend), was used.

Table 2. 1: Ingredients of growth medium for Jurkat T cell culture.

Component	Quantity
RPMI 1640 media	500 mL
FBS (10%)	50 mL
L-glutamine (1%)	1 mL
Penicillin/Streptomycin (1%)	1 mL

2.1.5 BCA Quantification Assay

Pierce™ BCA Protein Assay Kit by Thermo Fisher Scientific (Catalog number: 23225) was used for protein assays and analysis during whole study to carry out BCA Assay. Bio-Rad Benchmark Plus Microplate Reader was also used for absorbance measuring processes. Standards and ingredients with finalized concentration are given in Table 2.2.

Table 2. 2: BSA standards' preparation protocol.

Standard	Diluent (μL)	BSA (μL)	Finalized BSA Concentration
A	0	300 μL from stock	2000
B	125	375 μL from stock	1500
C	325	325 μL from stock	1000
D	175	175 μL from B	750
E	325	325 μL from C	500
F	325	325 μL from E	250
G	325	325 μL from F	125
H	400	100 μL from G	25

2.1.6 Western Blot

2.1.6.1 Resolving Gel

Components of 10% resolving gel used in SDS-PAGE for western blotting are shown in Table 2.3 with their quantities.

Table 2. 3: Resolving gel components.

Constituent	Quantity (1 gel, 10%)
Distilled water (dH ₂ O)	2.375 mL
40% Acrylamide	1.25 mL
1.5 M Tris-HCl (pH 8.8)	1.25 mL
10% SDS	50 μL
10% APS	50 μL

TEMED	10 μ L
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2.1.6.2 Stacking Gel

Components of 5% stacking gel used in SDS-PAGE for western blot analyses are given in Table 2.4 with their quantities.

Table 2. 4: Ingredients of stacking gel and their quantities.

Component	Quantity
dH₂O	2.19 ml
40% Acrylamide	405 μ l
6.8 pH-0.5M Tris-HCl	375 μ l
10% SDS	30 μ l
10% APS	30 μ l
TEMED	3 μ l

2.1.6.3 10X Running Buffer

Constituents of 10X running buffer are illustrated in Table 2.5 with their quantities. 10X Running buffer was stored at +4 °C.

Table 2. 5: 10X Running buffer components.

Component	Quantity
Trizma-Base	15 g
Glycine	72 g
SDS	5 g
dH₂O	up to 1 L

2.1.6.4 Transfer Buffer (1X)

Components and quantities needed for transfer buffer preparation are shown in Table 2.6 with their quantities. Storage was done at +4°C for longer use.

Table 2. 6: Transfer buffer components.

Component	Quantity
10X Running Buffer	100 mL
Methanol	200mL
dH₂O	700mL (up to 1L)

2.1.6.5 10X TBS Stock

Components of 10X TBS are illustrated in Table 2.7 with their quantities. 10X TBS was stored at +4 °C.

Table 2. 7: 10X TBS components.

Component	Quantity
NaCl	87,66 g
Tris-base	24,22 g
KH₂PO₄	2,721 mL
dH₂O	up to 1 L
pH	7.6

2.1.6.6 TBS-Tween (TBS-T)

Constituents of TBS-T are shown in Table 2.8 with their quantities.

Table 2. 8: TBS-T components.

Component	Quantity
10X TBS	100 mL
dH₂O	900 mL

Tween-20	500 μL
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2.1.7 Antibody List

Antibodies used in the Western Blot, Flow Cytometry, Immunocytochemistry and ELISA are given in Table 2.9.

Table 2. 9: Antibody names together with their catalog numbers and usage dilution in the all experiments.

Component	Brand-Cat. No	Dilution (Technique)
Anti-NLRP3	CST #13158	1:500 (WB)
Anti-Akt	CST #9272	1:500 (WB)
Anti-phospho Akt (Ser473)	CST #4060	1:1000(WB)
Anti-p44/42 MAPK	CST #4695	1:1000(WB)
Anti-ASC	CST #67824	1:1000(WB)
Anti-phospho p44/42 MAPK	CST #9101S	1:1000(WB)
Anti-cleaved IL-1 β	CST #83186	1:800 (ICC)
Anti-rabbit IgG secondary	CST #7074	1:5000(WB)
HRP-linked Antibody		
Anti-mouse IgG secondary	CST #7076	1:5000(WB)
HRP-linked antibody		
Anti-Vinculin	CST #13901S	1:2000(WB)
Anti- β -actin	Biolegend #643802	1:1000 (WB)
PE- anti-human CD25	Biolegend #302606	5 μl for 10⁶ cells (FC)
APC-anti-human CD40L	Biolegend #310810	5 μl for 10⁶ cells (FC)
PerCP anti-human 7AAD	Biolegend #420404	5 μl for 10⁶ cells (FC)
Purified anti-human IL-1 β	Biolegend #508201	1:200 (ELISA)
Biotin anti-human IL-1 β	Biolegend #508301	1:200 (ELISA)

2.1.8 RT-qPCR

Component and their volumes and reaction steps for reaction of RT-qPCR per one chain are listed in Table 2.10 and in 2.11 respectively. All reactions were done in Roche LightCycler® 480 Real-Time PCR.

Table 2. 10: RT-qPCR reaction volumes.

Component	Volume
SYBR Green	10 µL
Primer (10 uM, forward)	1.25 µL
Primer (10uM, reverse)	1.25 µL
RNase-free dH ₂ O	5.8 µL
RT mix	0.2 µL
RNA sample (50 ng/ml)	1.5 µL
Total	20 µL

Table 2. 11: Reaction steps for RT-qPCR together with temperature and time.

Steps	Temperature	Time
Reverse Transcription	50 °C	30 min
Initial PCR Activation	95 °C	15 min
Denaturation	94 °C	12 sec (40 cycles)

Primers used for RT-qPCR process are given with their sequences and sizes in Table 2.12.

Table 2. 12: Primers' sequence specific for RT-qPCR.

Primer	Sequence (5' - 3')	Size
Human <i>HPRT1</i> forward	GACCAGTCAACAGGGGACAT	195 bp
Human <i>HPRT1</i> reverse	AACACTTCGTGGGGTCCTTTTC	
<i>CYSLTR1</i> forward	GCCGCCTCAGCACCTAT	407 bp

2.2 Methods

2.2.1 Cell Stimulation

Jurkat T cell lines were transferred from flask to a falcon and centrifuged at 1200 rpm for 5 min. Supernatant was discarded; 10 ml completed RPMI 1640 was added and pellet was solved. 900 µl completed RPMI 1640, 100 µl cell and 10 µl trypan blue dye were mixed in an eppendorf tube. 10 µl of it was poured to a hemocytometer. Cells were counted and cell number in stock was determined. How much volumes of cell stock and completed RPMI 1640, required to seed 1.5 million cells in each well, were calculated and prepared. 1 ml was added to each well of a 24-well plate. Later, Montelukast (M_w : 608.17 g/mol) was weighed and solved in 2 mL autocleaved distilled water (dH₂O) for each concentration in a range 10^{-9} to 10^{-5} M. Then, solution was filtered through a 0.22 nm pore sized filter in an Eppendorf tube. The volume was completed to 1 ml with autocleaved water and this new stock was used for subsequent preparations. For control sample, no MNT was added. Cells were incubated at 37 °C, 5% CO₂ for 48 hours.

2.2.2 Cell Lysis and Protein Extraction

Cells of each experiment group, were taken from 24-well plate, gathered in falcons and centrifuged at 2000 rpm for 6 min. Then, samples were placed in ice. Most of supernatant was collected and stored at -80°C; pellet was dissolved in remaining small amount of supernatant and transferred to eppendorf tubes. After centrifuging at 2000 rpm for 6 min once more, all of the supernatants were discarded. 140 µl 10X RIPA buffer, 200 µl 7X protease inhibitor and 1040 µl dH₂O were mixed as they would be diluted to 1X. Then, prepared 1X RIPA buffer with an amount of twice the volume of pellet was added to each tube. Pellets were solved in buffer and mixed harshly. Later, samples were mixed with vortexer for 30 sec and incubated for 10 min on ice. Mixing and incubating procedure was performed three times. Finally, lysates were stored at -

20 °C for 1 hour and then stored at -80 °C. In order to obtain precleared proteins, lysates were centrifuged at 13.000 rpm for 20 minutes at +4°C.

2.2.3 Protein Quantification with BCA

4 µl of each sample, which contained precleared proteins, were mixed with 36 µl 1X RIPA buffer-protease inhibitor in order to dilute samples with 1:10 ratio. 25 µl standard solutions: A, B, C, D, E, F, G, H, and I (1X RIPA buffer-protease inhibitor as blank) were added to 96-well plate in a duplicate, and 10 µl of samples were transferred to 96-well plate in a triplicate manner. 200 µl of reagent A-reagent B mixture with 50:1 ratio for all samples. Then, plate was incubated at 37 °C for 30 min. Finally, absorbance was measured using microplate reader.

2.2.4 Flow Cytometry

1.5 million cells were collected from each group - which included control group, 10^{-9} M to 10^{-5} M MNT stimulated cells - in eppendorf tubes separately and 100.000 cells was collected from control group (unstained group). The cells were centrifuged at 2000 rpm for 6 min. The supernatant was discarded and 200 µl Fluorescent Activated Cell Sorting (FACS) buffer (contains 2% FBS in TBS) were added to tubes to dissolve. Next, 0.5 µl 7-Aminoactinomycin D (7-AAD) and 49.5 µl FACS buffer per sample (+2) were added to each sample. Just for the unstained one only 50 µl FACS buffer was added. Then, they were incubated at room temperature, in dark for 20 min. 200 µl FACS buffer was added afterwards and centrifuged at 2000 rpm for 6 min at +4. FL-3 channel (for 7-AAD), FL-2 and FL-4 channels (for activation markers CD25 and CD40L, respectively) were used for detection at BD Accuri™ C6 Plus Flow Cytometer.

2.2.5 Western Blot

Resolving gel was prepared and poured between glasses and 0.1 % SDS was added to smooth the gel. After gel solidified, 0.1 % SDS was removed and washed with dH₂O

three times. Stacking gel was prepared and added between glasses onto resolving gel. Comb was placed and stilled for 40 min to let stacking gel solidify. Then, comb was removed. 10X running buffer was diluted to 1X by adding 100 ml 10X running buffer and completing total volume to 1000 ml with dH₂O. 4X sample buffer was prepared by adding 600 µl Tris-HCl, 400 µl glycerol, 0.1 g bromophenol blue, 0.1 g SDS and 50 µl β-mercaptaethanol. Samples were prepared by using 4X sample buffer, adding 30 µg sample and completing to total volume with dH₂O. Then, samples were incubated at 95 °C for 10 min and let sit at room temperature for 5 min to cool. Samples were loaded to gel and gel was run at 80 V for 40 min and then at 120 V for 60 min at room temperature.

PVDF membrane was placed in methanol, waited for 5 min. Membrane and gel was placed in transfer buffer and whatman papers were soaked in transfer buffer. Transfer sandwich was formed by placing the first sponge, two or one extra thick wet papers, gel, PVDF membrane, two or one extra thick whatman papers and again sponge on top of each other, respectively. Transfer was performed at 90 V for 1.5 hours at 4 °C.

After wet transfer process, membrane blocking was done using with 5% blocking buffer, which was prepared by solving 2 gr milk powder in 40 ml TBS-Tween solution and incubated for 1 hour at room temperature on shaker. Primary antibody was diluted in 3% blocking solution and membrane was incubated in primary antibody, overnight, on roller in cold chamber. Later, membrane was washed by incubating in TBS-Tween for 10 min and this procedure was repeated for three times. HRP-linked secondary antibody was diluted in 3% blocking solution and membrane was incubated in secondary antibody solution for 1 hour at room temperature on shaker. Membrane was washed with TBS-Tween solution for three times by incubating for 5 min on shaker. One unit of enhancer buffer was mixed with one unit of peroxide buffer in an eppendorf tube and mixture was added on membrane which was visualized by BioRad's Chemidoc System.

2.2.6 RNA Isolation

A commercial Total RNA isolation kit is used for the extraction of RNA. Jurkat cells are subjected to centrifugation at 2000 rpm for 6 minutes. The supernatant is discarded, and the pellet is then lysed with 150 µl of lysis buffer found in the kit. The samples are thoroughly vortexed, after which an equal volume of 64% ethanol (150 µl) is introduced into the same tube, and the samples are gently agitated. The complete mixture is extracted from the Eppendorf and deposited into the cartridge filter situated in the collecting tube. The samples underwent centrifugation at 13,000 rpm for 1 minute at 4°C. The liquid that traverses beneath the filter is extracted. 600 µl of Wash Solution 1 (named by brand) is introduced to the cartridge, and centrifugation is done at 13,000 rpm for 1 minute at 4°C once more. The liquid that traverses the filter is eliminated. 500 µL of Wash Buffer Solution 2/3 is introduced onto the filter. They were centrifuged at 13,000 rpm at 4°C once more for just one minute. Obtained liquid that flows beneath the filter is extracted. The identical step is reiterated. The filter is transferred to the new sterile tube. 25 µL of elution buffer (at 80 °C-preheated) is applied to the filter. The samples underwent centrifugation at 13,000 rpm for 1 minute at 4°C. The purity and concentration of RNA are assessed using NanoDrop. RNA specimens are preserved at -80°C.

2.2.7 RT- qPCR

CYSLTR1 mRNA levels were measured by quantitative (real-time) PCR from RNA isolated from Montelukast-stimulated Jurkat T cells. After 50ng/ml RNA was extracted from each sample, Qiagen One-step RNA Quantitech PCR kit was used for RT-qPCR technique. Each reaction was performed in a total volume of 20µl. Each cycle was performed with a Roche LightCycler® 480 Real-Time PCR device. Expression levels were calculated using the $\Delta\Delta C_t$ formula. HPRT reference gene was used for normalization. Statistical tests were performed with Graphpad Prism 8.0.

2.2.8 Enzyme-Linked Immunosorbent Assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) primarily quantifies cytokine levels released by cells. The sandwich ELISA method involves biochemical

examination through a spectrophotometer, where color change resulting from an enzyme response is monitored. On the first day, Nunc MaxiSorp flat-bottom plates are coated with purified anti-human antibodies specific to the target antigen, diluted in dPBS (1:200 or other dilution ratios). The plate was sealed and incubated for twelve hours at 4°C. On the second day, the plate is subjected to three washes with PBS-T, followed by the application of 250 µl of a 10% blocking solution (sterile or commercial PBS with 10% FBS) to each well for one hour at room temperature. After blocking solution is eliminated, 100 µl of each sample and standard is added into the wells. Standards are added to the wells through progressive dilution, commencing at a concentration suitable for the quantities of cytokines emitted from Jurkat cells as 1:2 serial dilution. Incubation is done overnight at 4°C. On last day, the plate undergoes four washes with PBS-T. Detection anti-human antibodies includes biotin targeting the specified antigen are generated by diluting them in a 10% blocking solution (1:200, or dilution of interest), with 100 µl aliquoted to each well. The plate is thereafter incubated for one hour at RT. Subsequent to incubation, the plate is rinsed five times with PBS-T. Avidin D conjugated with HRP, diluted in a 10% blocking solution (1:2000 generally), is prepared, and 100 µl is dispensed into each sample. The plate is again incubated for 30 minutes at ambient temperature, shielded from light. The plate undergoes six washes again. The TMB Peroxidase Substrate and TMB Peroxidase Substrate and Solution B are combined in a 1:1 ratio. 100 µl of the mixture is dispensed into each sample. Upon observing the color changes through the blueish in the wells, the reaction is terminated with 100 µl of 1 N HCl and color turns to yellow. The plate is measured at 450 nm using a spectrophotometer. The results are evaluated utilizing the GraphPad Prism 8 software (demo version).

2.2.9 Immunocytochemistry (ICC)

For the immunocytochemistry (ICC) evaluation of IL-1 β protein, experimental sets were prepared. For each group, 500.000 viable cells were count applied per round coverslip. Samples were fixed with methanol at -20°C for 5 minutes and air-dried. After washing with phosphate-buffered saline (PBS) for at least 5 minutes, staining procedures were carried out using aUltraVision Detection DAB System HRP Kit

(Thermo Scientific TP-060-HLHRP) kit. To minimize non-specific binding, blocking was performed with non-immune serum for 5 minutes.

Primary antibodies specific to human IL-1 β was diluted in dPBS at the desired dilution. Following the blocking step, samples were incubated overnight at 4°C with the respective primary antibodies. After incubation, the antibodies were removed, and the samples were washed three times with PBS for 5 minutes each. Subsequently, the samples were treated with a biotin-labeled secondary antibody up to 20 minutes. After another three PBS washes, incubation with streptavidin–peroxidase conjugate was performed for 20 minutes. Chromogen development was then achieved using a ready-to-use aminoethyl carbazole (AEC) substrate system (AEC Chromogen, Thermo Scientific, TA-125-HA). The reaction was terminated by removing the chromogen solution following microscopic evaluation of staining.

Following PBS washes, samples were mounted using Vision Mount Aqueous Mounting Medium (Thermo Scientific TA-125-UGand) for analysis. Immunohistochemical evaluations were performed under a light microscope at various magnifications. Immunoreactivity was assessed semi-quantitatively using the H-Score method. For each sample, five randomly selected fields were scored between 0 and 300, and the mean value was calculated.

2.2.10 Photoacoustic (PA) Imaging

Cell from each condition were fixed the slides as 500.000 viable cells for each slide and mounted with Entellan. They positioned within a photoacoustic tomography system, and photoacoustic data was obtained with the VERASONICS Vantage ultrasound system equipped with an L22-14vx ultrasound probe, comprising 128 transducer components. A pulsed laser with a wavelength of 532 nm and a pulse duration of 10 ns was employed for illumination. The cells were irregularly distributed within the circular area. The entire region was scanned incrementally, with each phase yielding data from a distinct fragment of the circular area. A step size of 1 mm was employed to scan the cells on the slide and obtained 3 different fragments for each sample. The photos acquired from each stage were digitally combined to create the

comprehensive image of the cells. At the end, data is processed in MATLAB 2024b to obtain mean amplitude values ($B_{\max}$).

2.2.11 Genevestigator and Human Protein Atlas Databases

PRRs' basal mRNA expression levels in HPEs and eosinophil-related cell lines were examined using two separate publicly accessible bioinformatics platforms: the Human Protein Atlas and Genevestigator (version 9.10.0, release date: 2023-06-16). Genevestigator incorporates carefully selected RNA-seq and microarray data from a range of biological sources and experimental setups. The Affymetrix Human Genome U133 Plus 2.0 array dataset was chosen for this study in order to obtain the mean expression values (log2 scale) of PRRs in HPEs and EoL-1 and HL-60 eosinophil-like cell lines. The formula $\log_2(MV_{\text{cell_line}}/MV_{\text{primary_eosinophils}})$, where mean value (MV) stands for mean value of expression, was used to perform fold-change analyses. To cross-check gene expression levels using normalized transcripts per million (nTPM) and validate the results, the Human Protein Atlas database was utilized as an extra in silico resource. The Atlas's Immune Cell section, which offers genome-wide RNA expression information derived from single-cell transcriptomic profiling, was chosen. $\log_2(nTPM_{\text{cell_line}}/nTPM_{\text{primary_eosinophils}})$ was used to compute fold changes in a similar manner. To evaluate the consistency and variation in PRR expression across cell types, data from both platforms were compared. The identification of candidate receptors and cell lines for subsequent experimental investigations with an eosinophil focus was made robust by the combined use of two separate datasets.



3. RESULTS

3.1 NLRP3 is Predominantly Expressed in EoL-1 and HL-60 Cells, While NOD2 Shows a Controversial Expression Profile Compared to HPEs

In the first chapter of the thesis, basal mRNA expression levels of PRRs were compared between EoL-1, HL-60 and HPEs. NLRs' expression levels for both databases are given in Figure 3.1.

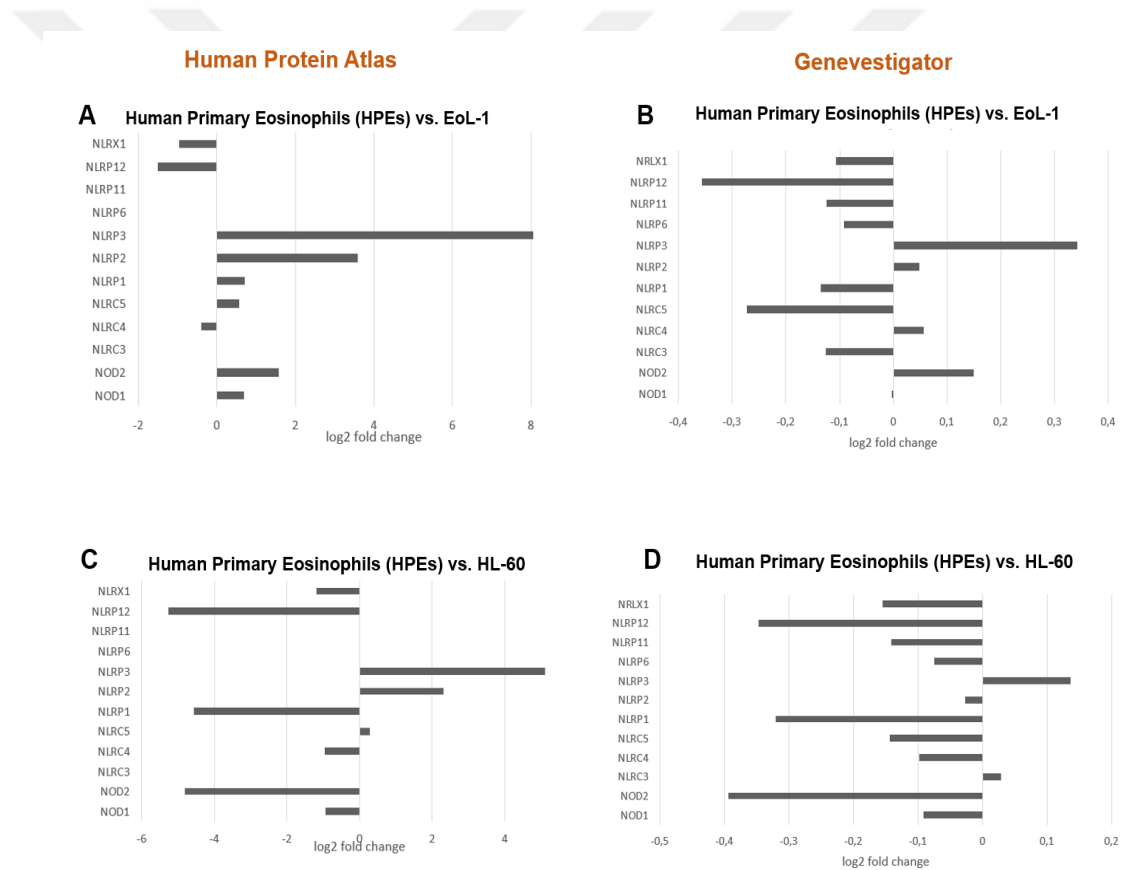


Figure 3. 1: Bar graphs represent the expression levels of NLRs based on their mRNA levels in EoL-1, HL-60, and human primary eosinophils (HPEs), obtained the data the Human Protein Atlas (A-C) and from Genevestigator (B-D). Expressions were given at the mRNA level and presented as MV for Genevestigator

and nTPM for the Human Protein Atlas and derived in log2 fold change. A-B) EoL-1 vs HPEs, C-D) HL-60 vs HPEs for both databases, respectively. Error bars represent the standard error of the mean (SEM). *NLRs: *Nod-like receptors*

As it can be seen both source demonstrated that mRNA levels of NLRP3 exhibited elevated mRNA expression for both EoL-1 and HL-60 relative to HPEs (Figure 3.1C, 3.1D, 3.1G, and 3.1H) ($p=0.03$). NLRP3 had the highest expression levels in both cell lines, succeeded by NLRP2, with the exception of HL-60 at the mRNA level (Figure 3.1D). Furthermore, the basal expression levels of NOD2 exhibited a consistent inverse relationship for both EoL-1 and HL-60 when their MVs and nTPMs were adjusted to those of HPEs, rendering this gene the most contentious among the other members of the NLR family (Figure 3.1C, 3.1D, 3.1G, and 3.1H). The nTPMs indicated protein levels; however, NLRP6 and NLRP11 (Figure 3.1G-H) were absent in both cell lines and HPEs. Indeed, EoL-1 cells were observed to lack baseline expression of the NLRP11 protein (data not shown), corroborating the *in silico* analysis. NLRP12 exhibited the greatest expression levels in HPEs according to both databases ($p=0.0001$).

3.2 TLR2 and TLR6 Are Highly Expressed in EoL-1 and HL-60, While TLR4 and TLR10 Are Lower

Analysis is continued with the comparison of basal mRNA expression levels of PRRs between EoL-1-HL-60, EoL-1-HPEs, and HL-60-HPEs, respectively (Figure 3.2).

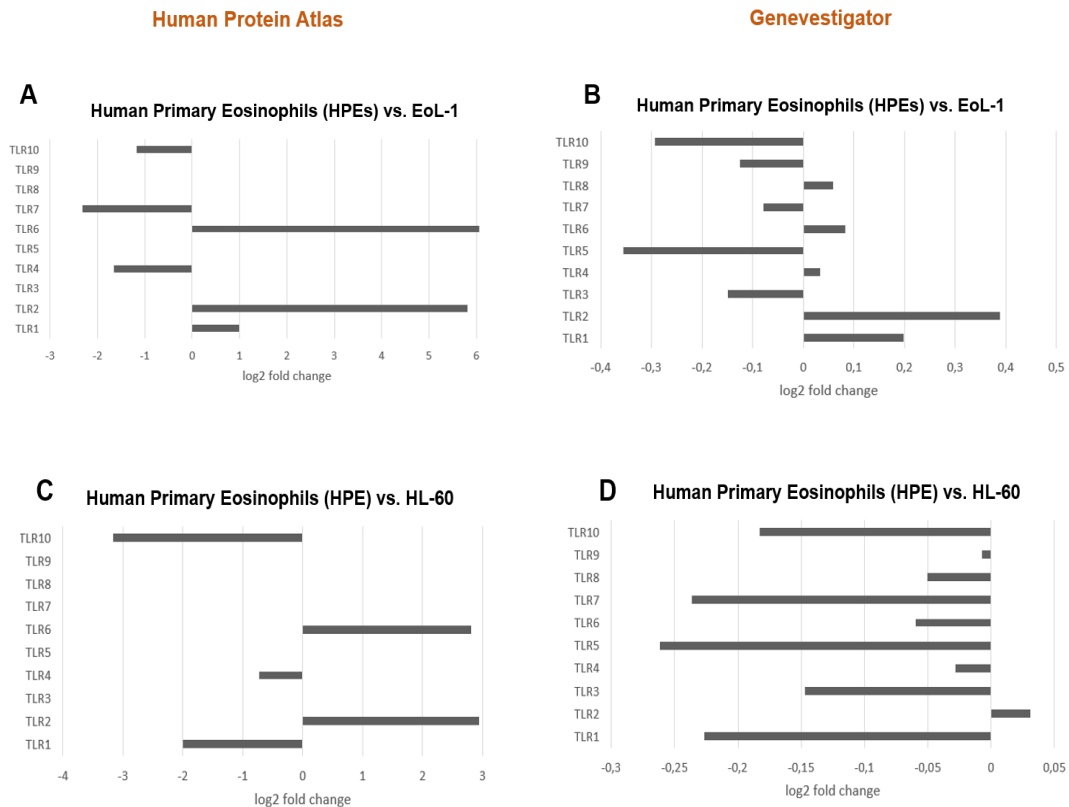


Figure 3. 2: Bar graphs represent the expression levels of TLRs based on their mRNA levels in EoL-1, HL-60, and human primary eosinophils (HPEs), obtained the data the Human Protein Atlas (A-C) and from Genevestigator (B-D). Expressions were given at the mRNA level and presented as MV for Genevestigator and nTPM for the Human Protein Atlas and derived in log2 fold change. A-B) EoL-1 vs HPEs, C-D) HL-60 vs HPEs for both databases, respectively. Error bars represent the standard error of the mean (SEM). *TLRs: Toll-like receptors

Genevestigator findings indicated that TLR2 exhibited elevated transcript levels in both cell lines compared to HPEs (Figure 3.2C, 3.2D) ($p=0.008$). Furthermore, The Human Protein Atlas findings indicated that the nTPM values for mRNA levels also correlated with protein levels, revealing that TLR2 and TLR6 were the most basally expressed TLRs in both cell lines, much surpassing those of HPEs (Figure 3.2E-3.2H). In contrast, the protein levels of TLR4 and TLR10 were decreased in both cell lines when they were compared to HPEs. Unexpectedly, mRNAs for TLR3, TLR8, and TLR9 were absent in HPEs, but TLR5 was also lacking in both cell lines.

3.3 MDA-5 is higher in EoL-1 and HL-60 Cells, LGP2 is lower in HPEs, While RIG-1 Expression Remains Inconclusive

After NLRs and TLRs, RLRs were also investigated according to the both databases. Bar graphs were shown in Figure 3.3.

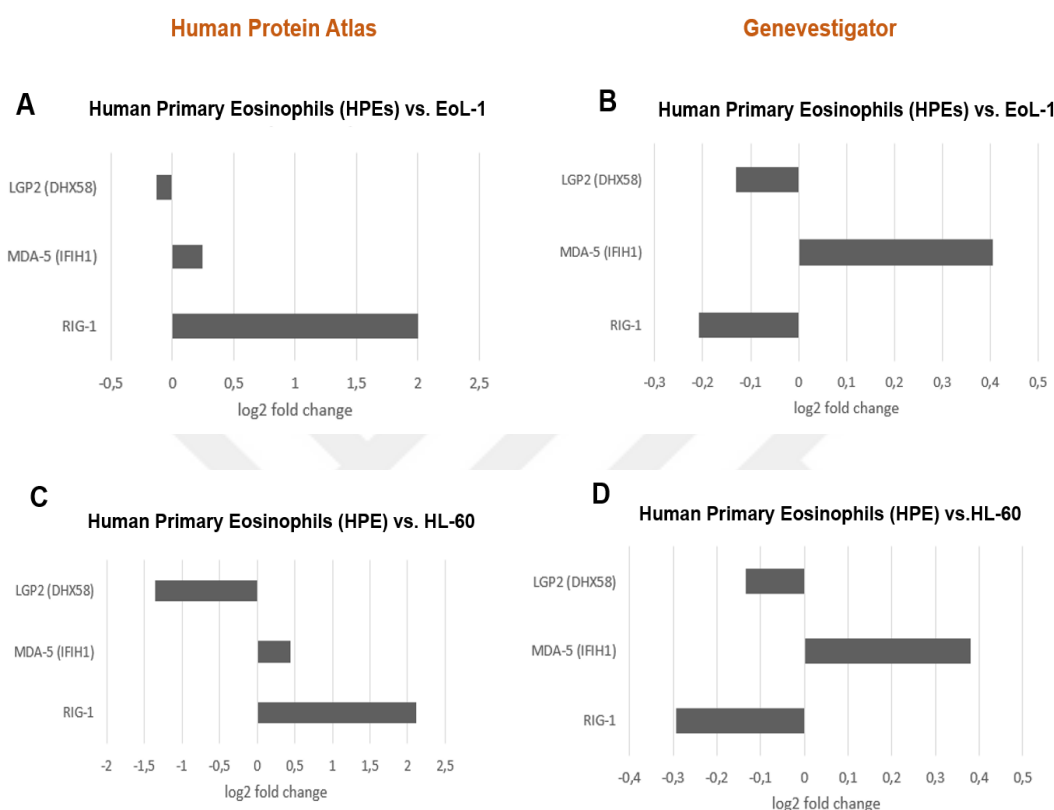


Figure 3. 3: Bar graphs represent the expression levels of RLRs based on their mRNA levels in EoL-1, HL-60, and human primary eosinophils (HPEs), obtained the data the Human Protein Atlas (A-C) and from Genevestigator (B-D). Expressions were given at the mRNA level and presented as MV for Genevestigator and nTPM for the Human Protein Atlas and derived in log2 fold change. A-B) EoL-1 vs HPEs, C-D) HL-60 vs HPEs for both databases, respectively. Error bars represent the standard error of the mean. *RLRs: Retinoic acid-inducible gene I-like receptors

Analysis indicated that mRNA levels of RLR family members were expressed at baseline levels in all three cell types examined in this study, according to both

databases (Figure 3.3). Upon comparing the mRNA expressions of cell lines to HPEs, we found that the gene, Laboratory of Genetics and Physiology 2 (LGP2), exhibited higher expression in HPEs compared to both cell lines ($p=0.004$), whereas MDA-5 demonstrated a lower mRNA expression in HPEs as compared to both cell lines (Figure 3.3C and D) ($p=0.0001$). Secondly, RIG-1 expression exhibited heterogeneity in its expression profile among cell lines and HPEs.

3.4 CLEC4A is Higher in EoL-1 Cells, While Dectin-1 is Predominantly Expressed in HPEs

As the last PRR family, CLRs, were also investigated according to the both databases results. Comparisons were given in Figure 3.4.

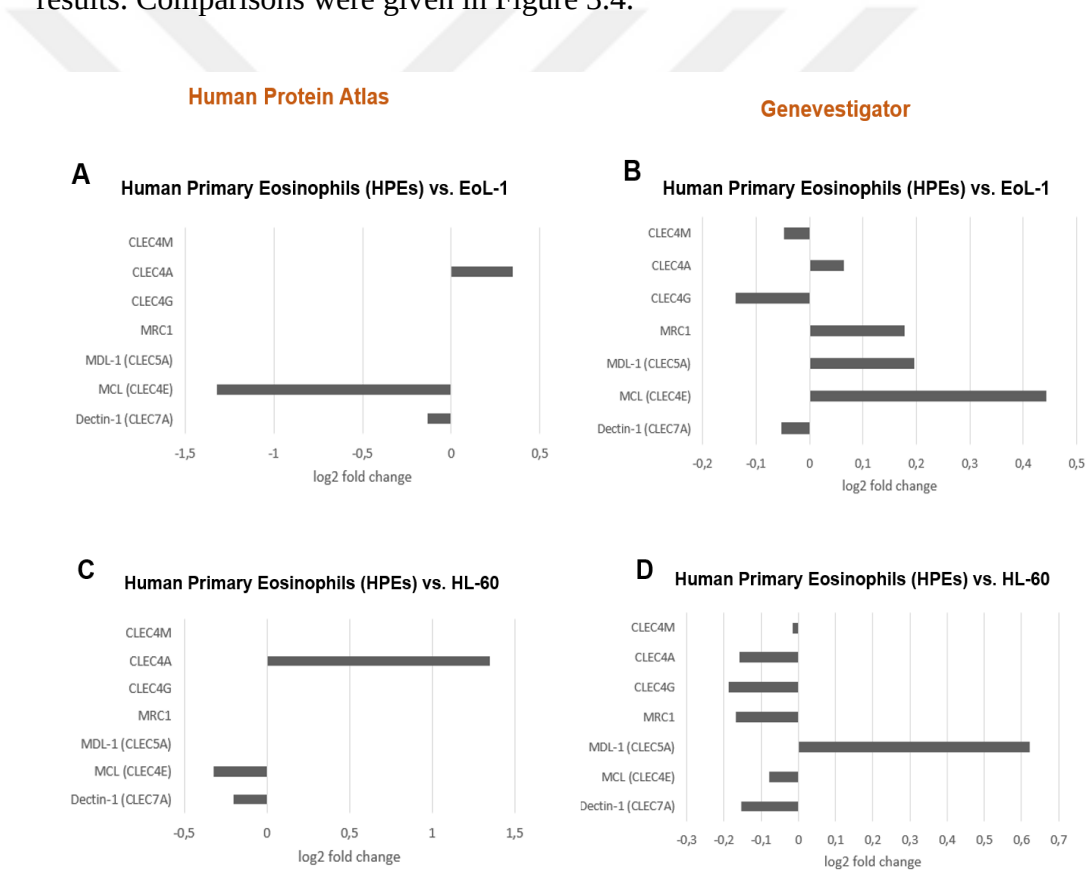


Figure 3. 4: Bar graphs represent the expression levels of CLRs based on their mRNA levels in EoL-1, HL-60, and human primary eosinophils (HPEs), obtained the data the Human Protein Atlas (A-C) and from Genevestigator (B-D). Expressions were given at the mRNA level and presented as MV for Genevestigator and nTPM for the Human Protein Atlas and derived in log2 fold change. A-B) EoL-1

vs HPEs, C-D) HL-60 vs HPEs for both databases, respectively. Error bars represent the standard error of the mean (SEM). *CLR: C-type lectin receptors

Upon examining the basal mRNA expressions of CLR family members in EoL-1, HL-60, and HPEs, it was evident that the cell lines had distinct basal mRNA expression profiles for CLEC4A, with EoL-1 cells demonstrating consistently higher levels than PE (Figure 4.3C). Dectin-1 exhibited elevated mRNA expression in HPEs compared to both cell lines throughout the databases employed in this investigation (p=0.04). These results may collectively indicate novel, unexamined roles for Dectin-1 in the functioning of HPEs.

3.5 NLRP12 and MDA5 Show the Most Significant Expression Differences Between Cell Lines and HPEs

After comparison of all PRR families, statistical comparisons were performed between cell lines and HPEs for members that showed significant changes. p values are shown in Table 3.1.

Table 3. 1: Statistical analysis of remarkably PRR members that showed differences in comparisons.

Receptor Name	HL-60 vs. HPEs (p-value)	EoL-1 vs. HPEs (p-value)
DECTIN-1	0.04	0.59
TLR2	0.6	0.0008
MDA5	<0.0001	0.0004
NLRP3	0.01	0.0001
NLRP12	<0.0001	0.0001
LGP2	<0.0001	0.004

*TLR: Toll-like receptor, MDA5: Melanoma differentiation-associated protein 5, NLR: Nod-like receptor, HPEs: Human primary eosinophils. Statistical analyses were performed using an unpaired Student's two-tailed t-test.

According to statistical analysis, there was a significant difference in the expression of NLRP3, NLRP12, MDA5, and LGP2 between HPEs and both eosinophilic cell lines. Notably, TLR2 and Dectin-1 only demonstrated selective significance in EoL-1 and HL-60, respectively, whereas NLRP12 and MDA5 showed highly significant changes in HL-60 cells ($p < 0.0001$).

The second part of the thesis aimed to explore the immunomodulatory effects of Montelukast, a cysteinyl-leukotriene receptor antagonist, on the expression NLRP3, in the Jurkat T cell line. In the light of this aim, optimization experiments were carried out first.

3.6 MNT did not change the viability of Jurkat Cells in a dose-dependent manner

Based on the information that the most commonly used MNT dose range in the literature, Jurkat cells were treated with 5 different doses (10^{-9} M, 10^{-8} M, 10^{-6} M, and 10^{-5} M) of MNT for 48 hours to control the viability via 7-AAD staining (Figure 3.5).

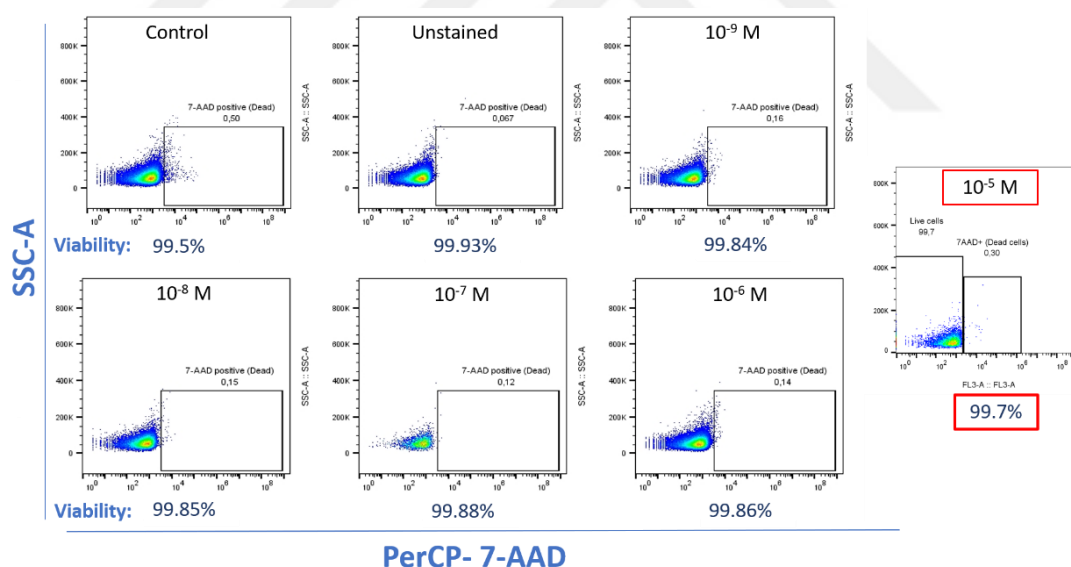


Figure 3. 5: Jurkat T cells were incubated with increasing concentrations of MNT (10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, and 10^{-5} M) for 48 hours. Cell viability was assessed using 7-AAD staining via flow cytometry. Representative dot plots indicate the percentage of viable (7-AAD⁻) and dead (7-AAD⁺) cells for each condition. Control (untreated) and unstained samples were included as references. *MNT: Montelukast, 7-

AAD: 7-Aminoactinomycin D, SSC-A: Side Scatter Area, **PerCP**: Peridinin-Chlorophyll-Protein complex, **FSC-A**: Forward Scatter-Area

When the percentages of 7-AAD staining dead cells were calculated, viability percentages were 99.5% in Control, 99.93% in Unstained, 99.84% in 10^{-9} M, 99.85% in 10^{-8} M, 99.88% in 10^{-7} M, 99.86% in 10^{-6} M and 99.7% in 10^{-5} M.

Clearly, no significant dose-related deaths were observed. It was therefore decided that the highest dose of 10^{-5} M was the optimal dose for the experiments.

3.7 24-Hour Combined Treatment with C.A. and MNT Enhances CD25 Expression and Slightly Modulates CD40L in Jurkat T Cells

After dose determination, the expression of CD25, a general activation marker, and CD40L, a late activation marker, were measured by flow cytometer to examine the time-dependent effect of another stimulant, C.A. First, control was achieved in cells treated for 24 hours. Cell conditions were untreated (control), 24h C.A., 24h MNT, and 24h MNT + C.A., respectively (Figure 3.6).

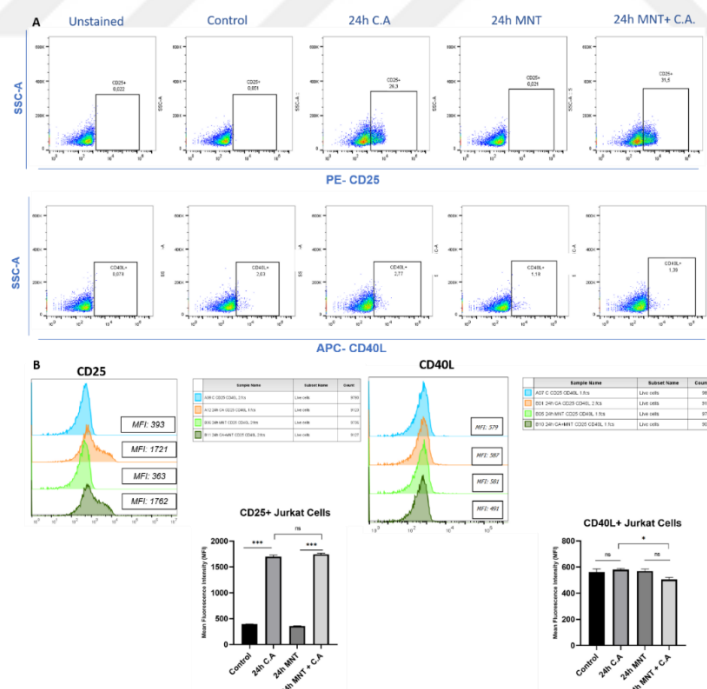


Figure 3. 6: Flow cytometry analysis showing the surface expression of activation markers CD25 and CD40L in Jurkat cells under different conditions: unstained,

control, 48-hour C.A., 48-hour MNT, and combined 24-hour MNT+C.A. treatment. A) CD25⁺ and CD40L⁺ cell percentages are displayed within each dot plot. B) Histogram overlays of CD25 and CD40L expression levels in Jurkat T cells, displaying mean fluorescence intensity (MFI) values for each condition. Quantification bar graphs (bottom row) show MFI values for CD25⁺ and CD40L⁺ populations. Statistical analysis was done using Unpaired student's T test. (*= $p < 0.05$, ***= $p < 0.001$) *C.A: Cell activator

The evaluation of CD25 and CD40L surface expression was based only on mean fluorescence intensity (MFI) values. For CD25, the MFI value was measured as 393 in the control group, and this value increased to 1721 after 24 hours of C.A. treatment. In the case of MNT alone, the MFI value of CD25 was 363 and remained at a similar level with the control. When MNT was administered together with C.A., the CD25 MFI value was determined as 1762 and this increase was shown to be statistically significant ($p < 0.001$). These results indicate that CD25 expression was significantly increased by C.A. alone, MNT alone was ineffective and the combined treatment maintained the effect of C.A.

In CD40L analysis, MFI values showed limited differences. CD40L MFI value, which was 519 in the control group, increased to 587 with C.A. treatment and this increase was found statistically significant ($p < 0.05$). In MNT treatment, the MFI value was measured as 501; this value decreased to 461 in the MNT + C.A group.

3.8 C.A. treatment significantly increased CD25 expression, whereas no significant effect of MNT alone, together with a limited increase in CD40L expression

After 24-hour determination, the expression of CD25 and CD40L were measured by flow cytometry to examine the effect of the MNT and C.A. in 48 hours. Cell conditions were untreated (control), 48h C.A., 48h MNT, and 48h MNT + C.A., respectively (Figure 3.7).

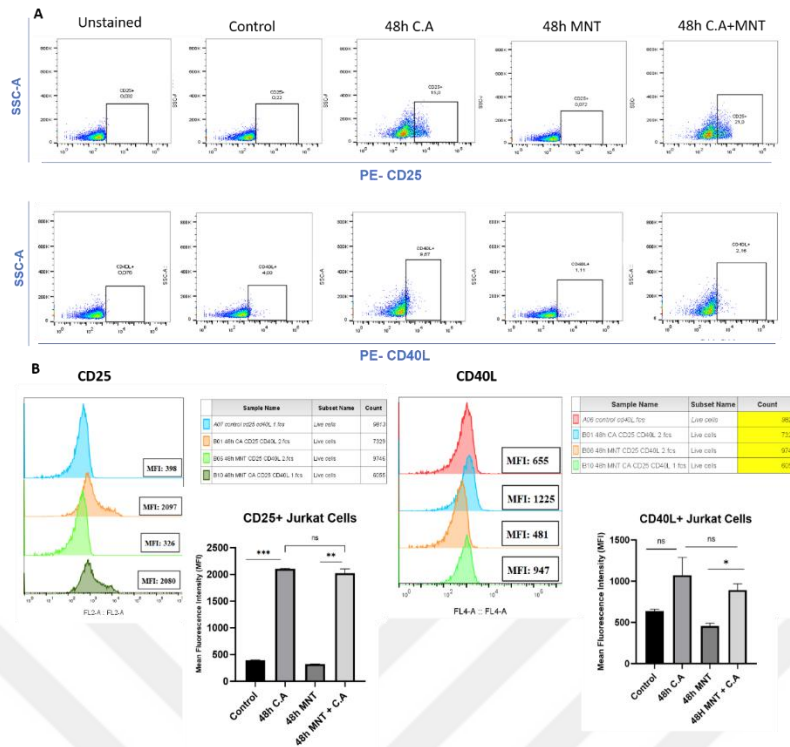


Figure 3. 7: Evaluation of CD25 and CD40L surface expression in Jurkat T cells by flow cytometry after 48 hours of C.A., MNT, and combined C.A.+MNT treatment. A) Contour plots of CD25 and CD40L expression are shown. The percentage of positive cells for each group is indicated in the corresponding region. B) histograms obtained for CD25 (left) and CD40L (right) and mean fluorescence intensity (MFI) values for each condition are presented. The bar graphs at the bottom show quantitative comparisons of the MFI values of the respective groups. Each group was analyzed with three independent samples. Statistical analysis was done using Unpaired student's T test. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

As a result of the analyses, it was shown that CD25 expression increased significantly with 48 hours of C.A. administration and this increase was maintained when administered together with MNT. While CD25 MFI value was 398 in the control group, this value increased to 2097 in the C.A. group and 2080 in the C.A.+MNT group. In the group in which MNT was administered alone, CD25 MFI value was 326 and no significant increase was observed.

In terms of CD40L expression, while the MFI value in the control group was 655, this

value increased to 1225 after 48 hours of C.A. administration. CD40L expression decreased with MNT treatment (MFI: 481), but a moderate increase was observed in the C.A.+MNT combination (MFI: 947). These findings suggest that C.A. has the potential to increase both CD25 and CD40L expression, but MNT may suppress this effect on CD40L.

3.9 CYSLTR1 mRNA levels were increased after MNT treatment

After stimulation of Jurkat T cells with 10^{-5} M Montelukast (MNT) for 48 hours, CYSLTR1 mRNA levels were analyzed by RT-qPCR. Results were given as a bar graph in Figure 3.8.

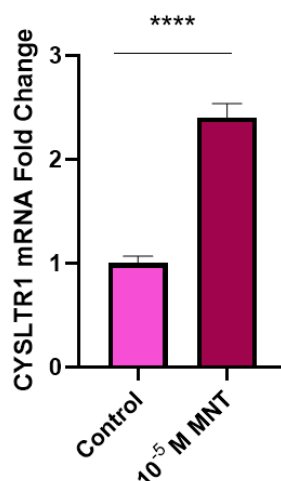


Figure 3. 8: Evaluation of mRNA levels of CYSLTR1 gene in Jurkat T cells after 10^{-5} M Montelukast (MNT) treatment by RT-qPCR. Results are shown as fold change, and data are presented as mean $\pm$ standard error of the mean (SEM). Statistical analysis was done using unpaired Student's T test. (****= $p < 0.0001$). Bar graph was illustrated via GraphPad Prism 8.0 software.

It was determined that CYSLTR1 expression increased approximately 2.5-fold in the MNT-treated group compared to the control group. The results are presented as fold change, and the increase was statistically significant (**** $p < 0.0001$).

3.10 MNT alone did not alter NLRP3 levels, but NLRP3 expression increased after co-treatment

After stimulation of Jurkat T cells for 48 hours, NLRP3 protein expression was evaluated by Western blotting. β -actin was used as loading control, band images and bar graph showing the fold change among the conditions were given in Figure 3.9.

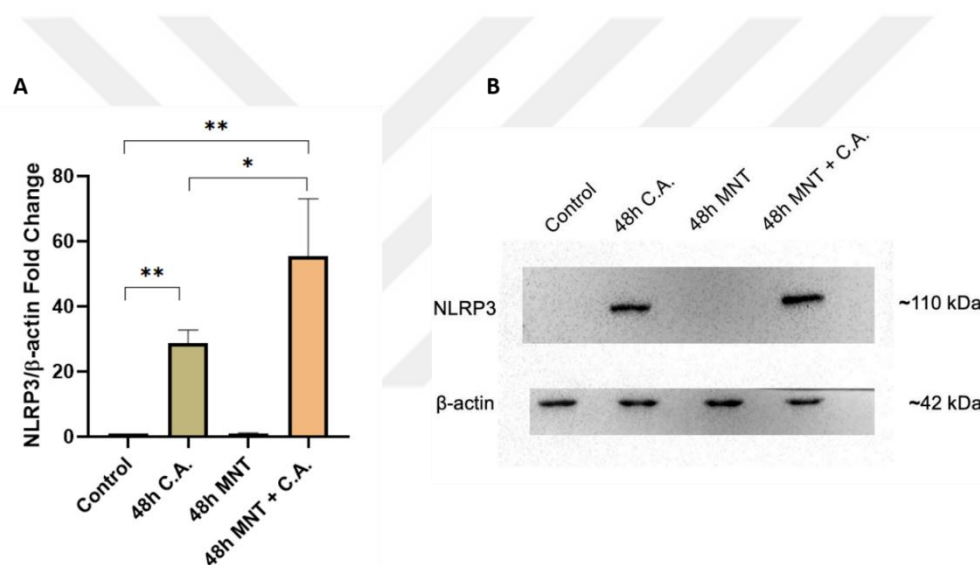


Figure 3. 9: Western blot results of NLRP3. A) the fold change plot of band intensities obtained according to NLRP3/ β -actin ratios. B) representative Western blot images of NLRP3 (~110 kDa) and β -actin (~42 kDa) proteins used as loading control. Data are expressed as mean $\pm$ standard error of the mean (SEM) and Statistical analysis was done using unpaired Student's T test. (* $p < 0.05$, ** $p < 0.01$). Bar graphs was illustrated via GraphPad Prism 8.0 demo version software.

According to the quantitative analysis results obtained by normalizing the Western blot band intensities, the NLRP3 protein level was significantly increased in the 48-hour C.A. treatment compared to the control group ($p < 0.01$). While no significant increase was observed in MNT treatment, NLRP3 protein level was significantly increased with C.A.+MNT treatment compared to both the control and MNT-alone groups ($*p < 0.05$, $**p < 0.01$).

3.11 ASC monomeric protein level decreased with 48h of C.A. and MNT+ C.A. treatments

After C.A., MNT and C.A.+MNT treatment of Jurkat T cells for 48 hours, ASC protein levels were evaluated by Western blot method. Quantitative analysis was performed by normalizing the band intensities to β -actin (Figure 3.10).

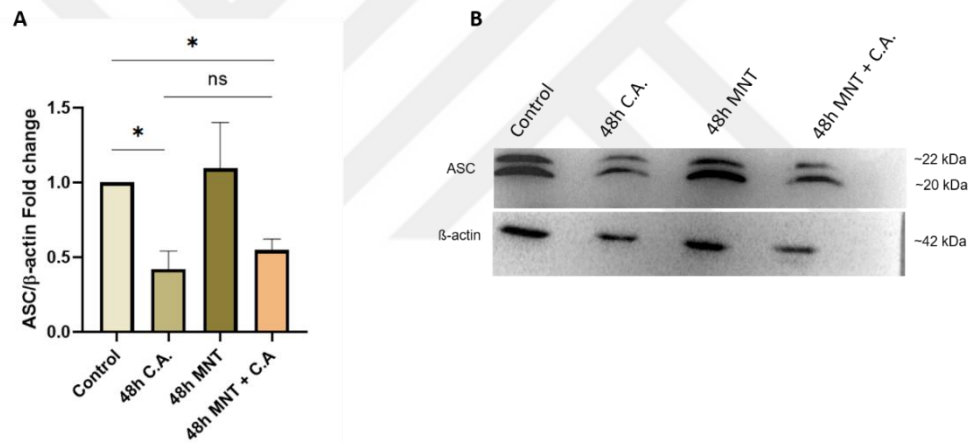


Figure 3. 10: Western blot evaluation of ASC protein levels in Jurkat T cells after 48 hours of C.A., MNT, and C.A.+MNT treatment. A) the fold change graph calculated according to ASC/ β -actin ratios is presented. B) representative Western blot bands of ASC (~20 & ~22 kDa) and β -actin (~42 kDa) proteins used as loading control are shown. Data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical significance was obtained with unpaired Student's T test and levels are indicated as $*p < 0.05$ and ns (not significant). Bar graph was illustrated via GraphPad Prism 8.0 demo version software.

A statistically significant decrease in ASC protein level was observed with 48 hours of C.A. treatment compared to the control group ($p < 0.05$). In the MNT group, ASC level remained at a similar level with the control group, Besides only C.A, in the C.A.+MNT groups, there were significant decreases compared to control ($p < 0.05$). There was no statistically significant difference between C.A. and MNT+ C.A. groups (ns).

3.12 Extracellular release of IL-1 β did not change among the groups after neither MNT nor C.A stimulation

After 48 hours of treatment of Jurkat T cells, IL-1 β levels in the culture supernatant were measured by the ELISA method. Results were given in Figure 3.11.

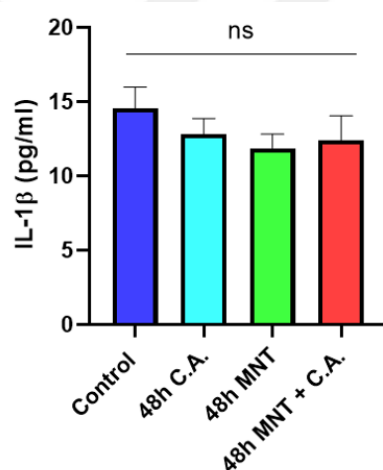


Figure 3. 11: Measurement of released IL-1 β levels after the treatment of MNT and C.A to Jurkat T cells via ELISA method. IL-1 β concentrations are presented in pg/ml in bar graphs, and data are expressed as mean $\pm$ standard error of the mean (SEM). No statistically significant difference was observed between the groups (ns, $p > 0.05$). Bar graph was illustrated via GraphPad Prism 8.0 demo version software.

According to the results obtained, no statistically significant difference was detected between the control and treatment groups in terms of IL-1 β release (ns). Extracellular IL-1 β levels remained at similar levels in all groups.

3.13 Intracellular expression of IL-1 β increased with 48 h of C.A. treatment; this increase reached the highest level in the combination of C.A. and MNT

IL-1 β protein levels in Jurkat T cells were evaluated by immunocytochemistry (ICC) and cellular positivity was semi-quantitatively analyzed by the H-score method. Results were shown in Figure 3.12.

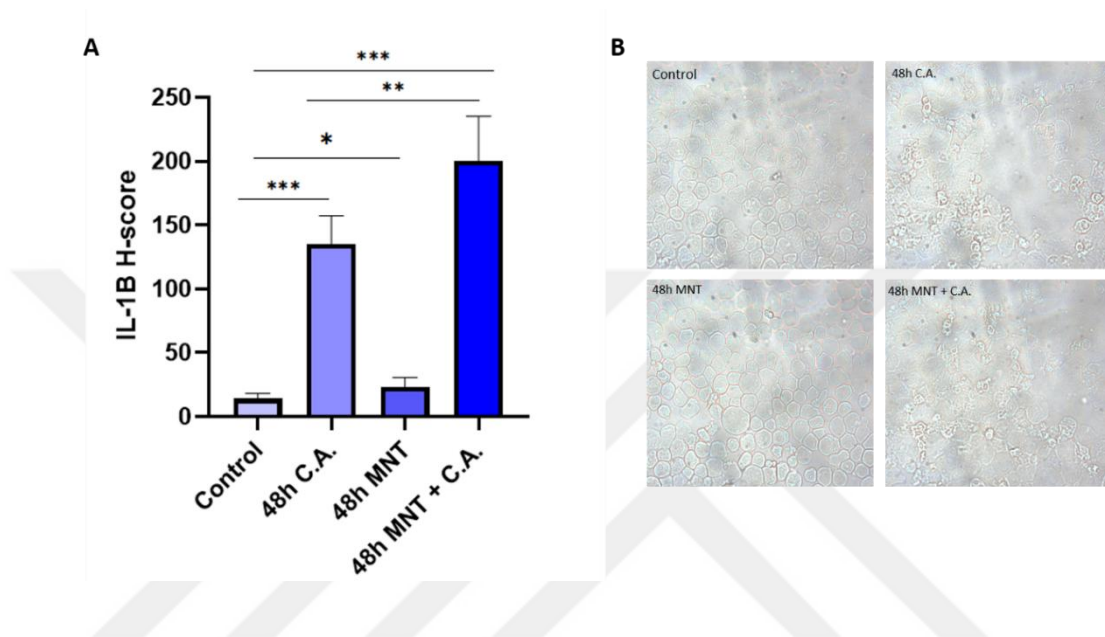


Figure 3. 12: Evaluation of IL-1 β protein expression by immunocytochemistry (ICC) and semi-quantitative analysis by H-score method after 48 hours of C.A., MNT and C.A.+MNT treatment of Jurkat T cells.A) H-score values are presented as mean $\pm$ standard error of the mean (SEM) in bar graphs. B) Representative microscopic images of each group are shown (x63). Statistical significance levels were determined by unpaired Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Bar graph was illustrated via GraphPad Prism 8.0 demo version software.

Compared to the control group, 48 h C.A. administration caused a significant increase in IL-1 β H-score values (*** $p < 0.001$). While the H-score value remained low in the MNT group, an even higher increase in IL-1 β level was observed when co-administered with C.A. ($p < 0.01$, $p < 0.001$) (Figure 3.13A). When representative microscopic images of each scoring group were analyzed, it can be said that IL-1 β remained inside the cell and these results represent intracellular IL-1 β production (Figure 3.13B).

3.14 p-AKT/AKT ratio increased with C.A. treatment, whereas MNT did not alter the increase

AKT and phospho-AKT (p-AKT) protein levels were analyzed by Western blot after 48 hours of C.A., MNT and MNT+ C.A. treatment of Jurkat T cells. Results were shown in Figure 3.13.

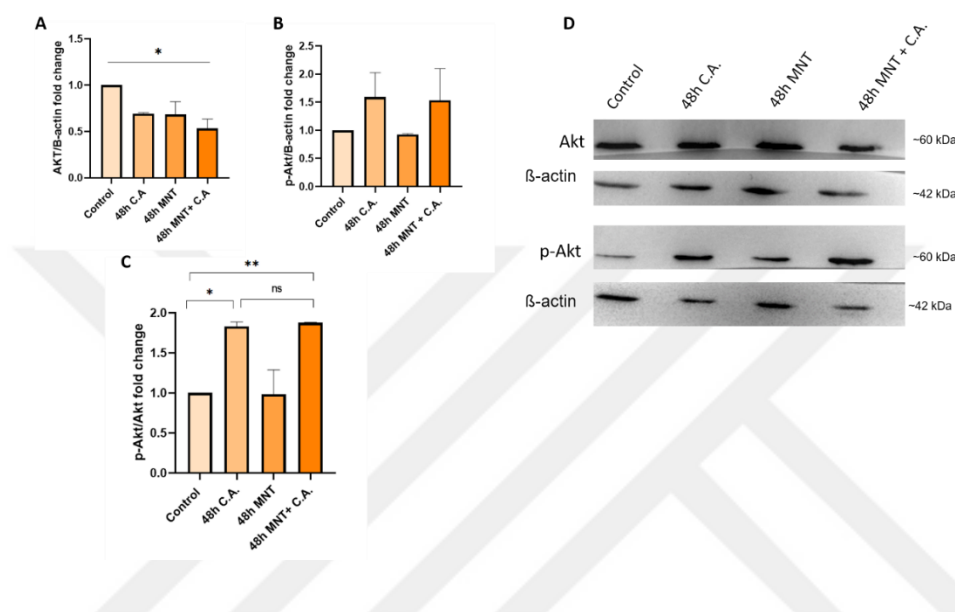


Figure 3. 13: Western blot evaluation of AKT and phospho-AKT (p-AKT) protein levels in Jurkat T cells after 48 hours of C.A., MNT and C.A.+MNT treatments. AKT/β-actin ratio is shown in (A) , p-AKT/β-actin ratio in (B) , and the p-AKT/AKT ratio in (C). Representative Western blot bands are presented. Band intensities were calculated by normalizing to β-actin (D). Data are presented as mean ± standard error of the mean (SEM); statistical significance was determined by unpaired Student's t test. (*p < 0.05, **p < 0.01, ns = not significant) Bar graphs were illustrated via GraphPad Prism 8.0 demo version software.

When the results were evaluated, the AKT protein level was significantly decreased in the C.A. group compared to the control group (*p < 0.05). p-AKT level did not show a significant difference between the treatment groups. The p-AKT/AKT ratio was significantly increased with C.A. administration (**p < 0.01); this increase was not observed in the MNT group. In the MNT+ C.A. group, the p-AKT/AKT ratio remained

similar to the C.A. group, and no statistical difference was found between the two groups (ns).

3.15 The p-ERK/ERK level, which was significantly different between all groups, was highest in the C.A.+MNT group

ERK1/2 (p42-44) and phospho-ERK (p-ERK-p42-44) protein levels were analyzed by Western blot after 48 hours of C.A., MNT and MNT+ C.A. treatment of Jurkat T cells. Results were shown in Figure 3.14.

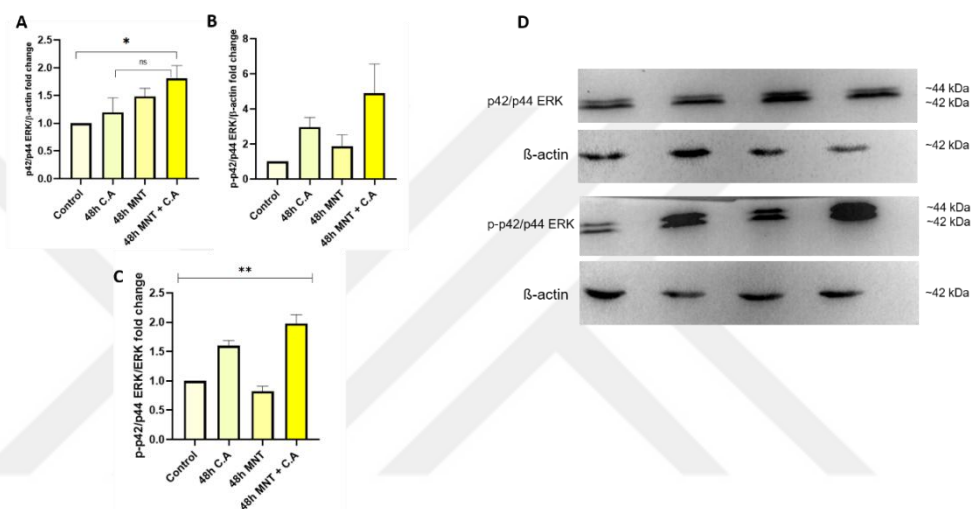


Figure 3. 14: Western blot evaluation of ERK1/2 and phospho-ERK1/2 (p-p42/p44) levels in Jurkat T cells after 48 hours of C.A., MNT and MNT+ C.A. treatments. A) phospho-p42/44 ERK/β-actin ratio, B) total ERK/β-actin ratio, and C) p-ERK/ERK ratio are presented as bar graphs. D) Representative Western blot bands of each group are shown. Data are presented as mean ± standard error of the mean (SEM) and statistical significance levels are identified via one-way ANOVA among whole groups and indicated as *p < 0.05, **p < 0.01 and ns (not significant). Bar graphs were illustrated via GraphPad Prism 8.0 demo version software.

The protein levels of p-p42/p44 ERK1/2 and total ERK1/2 were analyzed by Western blot after 48 hours of C.A., MNT and MNT+ C.A. treatment of Jurkat T cells. p-p42/p44 ERK1/2 levels were significantly increased in C.A. treatment compared to the control group (*p < 0.05). No significant increase was observed in the MNT group. In the

C.A.+MNT group, phospho-ERK level was measured at a higher level than in the C.A. group. The overall difference between the groups was evaluated by one-way ANOVA test and found to be statistically significant ($p < 0.01$). No significant difference was observed between the groups in ERK1/2 total protein level.

3.16 IL-10 level decreased in the group treated with 48h MNT+ C.A combination compared to the control

After 48 hours of treatment of Jurkat T cells, IL-10 levels in the culture supernatant were measured by the ELISA method. Results were given in Figure 3.15.

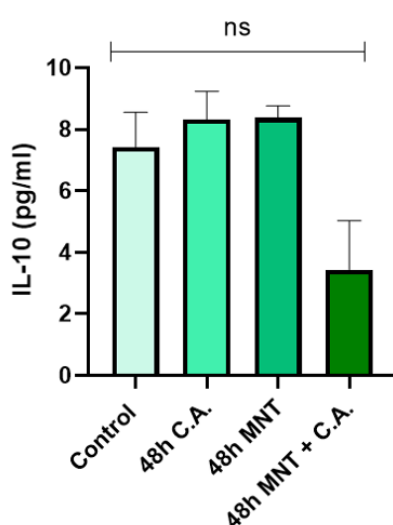


Figure 3. 15: After 48 hours of C.A., MNT, and C.A.+MNT treatment of Jurkat T cells, IL-10 levels in the supernatant were measured by the ELISA method. IL-10 concentrations are presented in pg/ml in the bar graph. Data are expressed as mean $\pm$ standard error of the mean (SEM). There was no statistically significant difference between the groups (ns). Bar graph was illustrated via GraphPad Prism 8.0 demo version software.

When the data obtained were interpreted, 48h C.A. and 48h MNT groups showed an expression profile close to the control. However, there was a significant decrease in IL-10 levels in the MNT+ C.A combination group compared to the control, but none of the inter-group changes were statistically significant ($p > 0.05$).

3.17 MNT caused a decrease in photoacoustic signal amplitude in Jurkat T cells

Jurkat T cells were prepared on slides after 48 hours of C.A., MNT and C.A.+MNT treatment, stained with eosin and examined in a photoacoustic imaging system. Imaging was performed using a pulse laser light source with a wavelength of 532 nm and an 18 MHz linear ultrasound probe. The raw signal data obtained from each sample were visualized with the system's built-in software and signal intensity distributions were obtained. Results were shown in Figure 3.16.

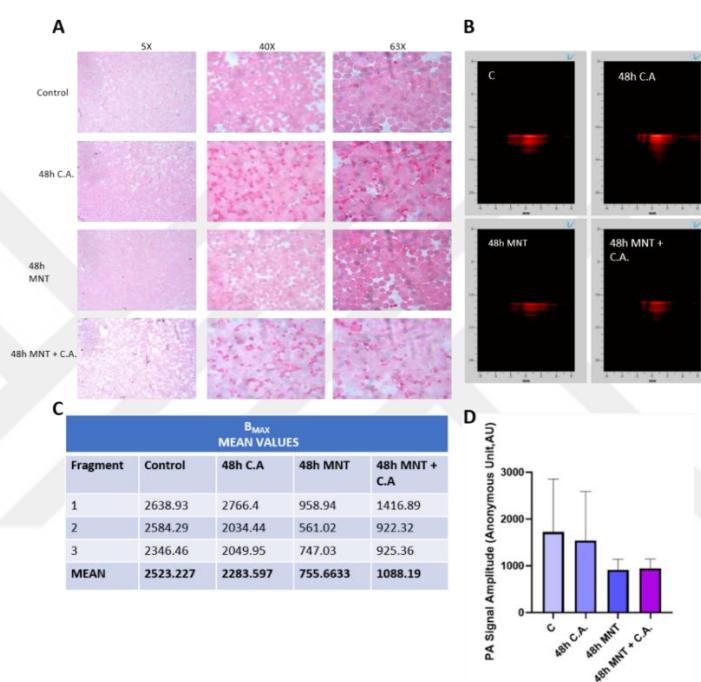


Figure 3. 16: Observation of Montelukast's field of action in photoacoustic imaging. A) Microscopic images of eosin-stained Jurkat T cells were presented at 5x, 40x, and 63x magnifications for each experimental group. B) Signal intensity obtained from the photoacoustic imaging system shows the characteristic signal distribution of each group. C) PA signals from three separate fragments from each slide surface were analyzed with MATLAB code, and the mean amplitude (B_{max}) values were presented in a table. D) The mean PA signal amplitudes were plotted and compared between groups. Data are expressed as mean $\pm$ standard deviation (SD). Microscopic images were obtained via Zeiss Blue Edition 3.0. Bar graph was illustrated via GraphPad Prism 8.0 demo version software.

According to the findings, the photoacoustic signal amplitude decreased in the MNT-treated groups. This decrease was similarly observed both in the group in which MNT was administered alone and in the combination group in which it was administered together with C.A. In both groups, the average signal amplitude was measured at lower levels compared to both the control group and the C.A.-only group. While the photoacoustic signal amplitude in the C.A.-treated group was close to the control group, these values were significantly lower in the groups containing MNT. Numerical data on signal amplitude were obtained from three different areas, averaged, and compared graphically. The average values presented together with the visual data show that the level of photoacoustic response is lower in the groups treated with MNT.



4. DISCUSSION

The diverse roles of eosinophils in the immune system have always been and continue to be a focus of study. One of the primary challenges in the investigation of human eosinophils is the procurement of sufficient purified cells, as they constitute a modest subset of leukocytes, and their isolation from other cells. Their too short lifespan is also one of the major problems (Mayumi, 1992). In this manner, researchers focused on these problems and created eosinophil-like cell line models to be able to investigate and study on them. Although cell lines are designed to be similar, they can have many morphological and functional differences both among themselves and relative to primary cells (Mayumi, 1992; Saito et al., 1985).

In this manner, the first part of the thesis aimed to investigate and compare the basal expression profiles of PRRs in EoL-1 and HL-60 human eosinophil-like cell lines and HPEs using *in silico* tools and it was conducted an original article (Celik et al., 2023). This is critical in order to determine which of these cell lines is more ideal for which PRR family. The findings are intended to serve as a guide for studies focused on eosinophil function and innate immune signaling.

When examining the mRNA expression obtained for the TLR family found on the cell surface, the most significant difference is in TLR2 expression. In both EoL-1 and HL-60 cells, TLR2 is significantly higher than in HPEs. This situation indicates that these cell lines can generate a strong inflammatory response to TLR2 ligands, such as bacterial, viral, fungal and parasitic lipoproteins. It also allows for more accurate in vitro study (Shen et al., 2013). However, the fact that receptors sensitive to viral PAMPs such as TLR3, TLR8, and TLR9 are absent or present at very low levels in HPEs suggests that these cells suppress these pathways in their basal state. One reason for this suppression is that eosinophils also play a role in tissue homeostasis (Akuthota et al., 2008; Gigon et al., 2023). Therefore, they may be regulated under a mechanism that keeps basal levels low in response to potential unnecessary inflammatory

responses. The fact that TLR10 is found only in HPEs suggests that this receptor may have immune regulatory roles specific to these cells, which is consistent with the literature (Fore et al., 2022).

Within the NLR family, NLRP3 was significantly overexpressed in both cell lines compared to primary eosinophils. This situation may make these cell lines advantageous for inflammasome activation and IL-1 β production, but it may also indicate that they exhibit an inflammatory profile that exceeds physiological and optimal levels (Sutterwala et al., 2006). On the other hand, NLRP12 was found at high levels only in primary eosinophils. This receptor is known to have anti-inflammatory effects on innate immunity in some certain cases such as bacterial infections, also highly expressed in primary eosinophils due to their regulatory roles (Zaki et al., 2014). This difference suggests that the cell lines may not adequately represent NLRP12-mediated regulatory signals and maybe causes misleading.

According to the comparisons to key family of viral immunity, RLRs, we found that RIG-I expression was lower in EoL-1 and HL-60 cells compared to HPEs, whereas MDA5 levels were higher in cell lines. Similarly, it was observed that the RLR signaling regulator LGP2 (DHX58) mRNA was higher in HPEs than in both cell lines. The literature reports that HL-60 cells express RIG-I and MDA5 at low levels in their basal state, but these receptors can be upregulated when the cells are differentiated into eosinophilic precursors (Berger et al., 2012). Therefore, the maturation status of the cell line and culture conditions can significantly affect RLR levels and the responses elicited. Importantly it can be stated that, low levels of several crucial proteins such as RIG-I in cell lines may lead to false negative results in *in vitro* assays or underestimation of the importance of these receptors (Celik et al., 2023).

Although there are relatively few studies in the literature on CLR expression levels and functions in eosinophils, existing evidence suggests that these cells may rely on CLR signals when interacting with fungi and similar pathogens (Kvarnhammar & Cardell, 2012). In our study, we found that some of the CLRs examined showed significant differences between HPEs and cell lines (Celik et al., 2023). For example, the mRNA level of Dectin-1 (CLEC7A), was found to be higher in HPEs than in EoL-

1 and HL-60. Dectin-1 is a well-characterized CLR that recognizes β -glucans and zymosan, the main components of the fungal cell wall (Kvarnhammar & Cardell, 2012). Mincle (Macrophage inducible C-type lectin) is a CLR member that recognizes microbial glycolipids and certain structures released after cell death (Linnemann et al., 2025). Interestingly, Mincle mRNA levels were found to be significantly higher in EoL-1 cell lines than in primary eosinophils. In HL-60 cells, Mincle expression is roughly similar to or slightly lower than that in HPEs (Celik et al., 2023). This finding may be related to the abnormal overproduction of Mincle by the malignant eosinophilic leukemia clone from which EoL-1 originates.

In conclusion, Even though PRR expression profiling shows differences in cell lines and human primary eosinophils, these cells still offer good models for mechanistic studies that require high cellular yield such as protein and RNA. EoL-1 cells, relatively serves as a better model to study PRRs than HL-60 due to discrepancies in different open sources.

In the second chapter of the thesis, it was demonstrated that the effects of MNT on cell signaling pathways and activation markers in Jurkat T cells were generally limited. The levels of T cell activation markers CD25 (IL-2 receptor α chain) and CD40L (CD154) increased significantly after stimulation with C.A., as expected. Montelukast alone did not trigger these activation markers. However, in a 24-hour co-treatment, MNT did not inhibit the increase in CD25 but rather maintained it; it also slightly reduced CD40L expression in the early phase. In the 48-hour treatment, MNT again sustained the CD25 increase triggered by activation but did not cause a significant change in CD40L levels. These data suggest that MNT does not completely suppress T cell activation but only causes small time-dependent changes in the expression of a few selected surface molecules. In the literature, it has been shown that they play regulatory roles in the Th1/Th2 balance and can increase or stabilize the expression of CD4⁺CD25⁺ regulatory T cells (Qu et al., 2018).

The significant increase in *CYSLTR1* mRNA levels after 48 hours of MNT stimulation is an unexpected result. This is because MNT is an FDA-approved drug that has been reported to inhibit increased CysLT₁ receptor expression under inflammatory stimuli

(Markham & Faulds, 1998). In our *in vitro* conditions, however, since there was no external leukotriene stimulus, instead of MNT suppressing receptor signaling, a possible compensatory increase in receptor levels occurred within the cell. This finding suggests that receptor antagonism may trigger feedback mechanisms in the cell, thereby increasing the target receptor level at the mRNA level, which requires validation at the protein level (Lynch et al., 1999).

MNT had different effects on T cell intracellular signaling in the AKT and ERK kinase pathways. MNT alone did not cause a change in AKT phosphorylation (p-AKT/AKT ratio), and its presence did not increase the number of cells stimulated by a cell activator. In other words, AKT activation was primarily due to TCR stimulation and was considered an expected condition, independent of montelukast. Interestingly, an effect of montelukast was observed in the ERK pathway. In cells stimulated with C.A. for 48 hours, the p-ERK/ERK ratio increased slightly with the addition of MNT. This unexpected result suggests that MNT may indirectly enhance the MAPK/ERK pathway under certain conditions. However, most studies have reported that MNT reduces ERK phosphorylation, particularly in cells activated by pro-inflammatory stimuli, thereby exerting an anti-inflammatory effect (Mansour et al., 2018; Woo et al., 2004). The increase observed in our study may be related to secondary effects associated with different stimulatory signals, cell type (Jurkat T cell), or long-term culture conditions. This suggests that MNT's effect on ERK signaling is binding-specific.

The role of MNT in inflammatory pathways has been investigated through NLRP3 inflammasome and related molecules. In our study, montelukast alone did not alter NLRP3 protein levels. However, NLRP3 levels increased after 48 hours of T cell activation (with C.A.), and this increase was also observed in the presence of montelukast and was even slightly higher. Thus, MNT demonstrated an NLRP3 expression-enhancing effect in activated T cells. In contrast, while ASC adaptor protein levels decreased significantly after 48 hours of activation, the addition of MNT did not cause any additional changes in ASC. When these results are considered together, it is understood that MNT increases NLRP3, a component of the inflammasome, at the primary level in T cells, but does not provide the other signals

necessary for full inflammasome activation. In addition, MNT treatment, either alone or in combination with C.A., did not alter the production of pro-inflammatory (IL-1 β) or anti-inflammatory (IL-10) cytokines released by T cells. However, an important finding emerged in intracellular cytokine staining which is intracellular IL-1 β production increased in Jurkat cells after 48 hours of activation, and this increase reached its highest level in the presence of MNT. Thus, MNT increased pro-IL-1 β accumulation within T cells but did not lead to the maturation and release of the cytokine. This might indicate that MNT enhances the primary signal of the inflammasome but cannot complete the inflammasome response in the absence of the second signal (caspase-1 activation and secretion). Additionally, it has been found that MNT might not fully trigger the NLRP3 inflammasome and might not induce IL-1 β release. This was unexpected when we compared to the literature. Some studies have reported that MNT suppresses NLRP3 inflammasome activity, thereby reducing inflammatory cytokines such as IL-1 β , IL-6, and TNF- α (El-Kashef & Zaghloul, 2022).

Another aspect of the study is the evaluation of Montelukast's effects using photoacoustic (PA) imaging. Interestingly, while no significant changes were observed in molecular analyses, PA imaging data revealed a decrease in signal intensity in cells treated with MNT for 48h. This finding suggests that MNT, which does not cause significant differences in surface markers and secreted cytokines, may have caused a measurable change in the optical-acoustic properties of cells. One possible explanation for these results is that MNT may cause changes in the chromophore content of cell morphology or composition. A study conducted by Beard et al. revealed that biophysical properties such as intracellular content density, organelle distribution, and cell size affect optical absorption properties and therefore have a decisive influence on PA imaging signals (Beard, 2011). For example, MNT and/or C.A. may cause slight shrinkage or a decrease in cell volume. If eosin stain retention by cells is reduced or cell density/preparation thickness differs in the MNT-treated groups, this will be reflected in the PA signal. It is also possible that MNT does not kill the cells (as shown in the 7-AAD viability test) but may have reduced the potential proliferation rate; although the measurements were performed with equal cell numbers, the metabolic

activities and macromolecular contents of the cells may affect the signal. At this point, in another study, we showed that an increase in the size of several immune cells, including the Jurkat T cell line, caused an increase in the signal (Mutlu et al., 2024). In another study we conducted, we reported that morphological differences observed by light microscopy and viability assays in EoL-1 eosinophilic cell line and Jurkat T cells treated with valproic acid (VPA) were reflected in the PA signal (Koksal et al., 2024). This finding is consistent with the results of our study, as even the subtle effects of montelukast may be detectable by PA. However, the statistical significance and reproducibility of the PA signal decrease in our data are limited. Therefore, this possibility should also be considered when interpreting these results. The need for optimization, repetition, and validation of the experiment remains important.

In conclusion, this project presents an interdisciplinary approach aimed at the multi-level characterization of immune cells through molecular profiling and functional imaging methods. The identification of best suitable model for eosinophilic cells through *in silico* analyses is an important preliminary step toward enhancing the biological validity of experimental systems. Following this, the evaluation of the effects of immunomodulatory agents such as MNT on T cells using both classical molecular techniques and new-generation, non-invasive biophysical approaches such as PA imaging provides a framework for analyzing drug-immune system interactions at the systems level. The findings indicate that MNT induces binding-specific modulation in signaling networks rather than directly triggering an inflammatory response, although these effects may not always be reflected at the phenotypic level. In this context, the integration of optical-acoustic technologies such as PA imaging with molecular biology represents a powerful tool that will enable more comprehensive, multi-parameter, and time-dependent monitoring of immune responses.

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APPENDICES

APPENDIX A: Tools used in this study are given with supplier brand names.

APPENDIX B: Commercial kits used in this study are given with supplier brand names.

APPENDIX C: Chemicals used in this study are given with supplier brand names.

APPENDIX A

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

Equipment	Supplier Company
Light microscope	Olympus CH30
Incubator	BINDER
Hemocytometer	Marienfield
Vortex	VWR
Micropipettes	Thermo Fisher 10,20,200,1000 μ L Eppendorf 10,200,1000 μ L
Serological pipettes	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.

Equipment	Supplier Company
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.

Equipment	Supplier Company
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	Sigma
Tween20	Fisher Scientific

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Celik, Z., Barutcu, E., Ozcelebi, Y., Sarac, E. Y., & Ciraci, C. (2023) *Turkish Journal of Immunology*, 11(3). DOI: 10.4274/tji.galenos.2023.28199

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