

T.C.
DOKUZ EYLÜL
ÜNİVERSİTESİ İZMİR
INTERNATIONAL
BIOMEDICINE AND
GENOME INSTITUTE

**ROLE OF INVARIANT NATURAL KILLER T
(NKT) CELLS IN NEUTROPHILIC (T_H1-
DRIVEN) ASTHMA**

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Molecular Biology and Genetics Ph.D. Program

Ph.D. THESIS

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ABBREVIATIONS

AAI	Allergic airway inflammation
ACK lysis	Ammonium-chloride-potassium lysis
AERD	Aspirin exacerbated respiratory disease
AF488	AlexaFluor 488
AF594	AlexaFluor 594
AF647	AlexaFluor 647
AF700	AlexaFluor 700
AHR	Airway hyper-responsiveness
ALI	Acute lung injury
AM	Alveolar macrophage
APC	Allophycocyanine
APCs	Antigen presenting cells
ARDS	Acute respiratory distress syndrome
B6	C57BL/6 strain mouse
BALF	Broncho-alveolar lavage fluid
BMI	Body-mass index
B_{reg}	Regulatory B cell
BSA	Bovine serum albumin
BUV395	Brilliant UV 395
BV421	Brilliant Violet 421
BV510	Brilliant Violet 510
BV570	Brilliant Violet 570
BV605	Brilliant Violet 605
BV650	Brilliant Violet 650
BV711	Brilliant Violet 711
BV785/6	Brilliant Violet 785/6
C	celsius
CCR-	Chemokine (C-C motif) receptor-
CF	Cystic fibrosis
CFA	Complete Freund's Adjuvant
COPD	Chronic obstructive pulmonary disease
CS	corticosteroid
CTL	Cytotoxic T lymphocyte
CXCR-	Chemokine (C-X-C motif) receptor-
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetat
EIA	Exercise-induced asthma
ELISA	Enzyme-linked immunosorbent assay
ExM	Exudate macrophage
F4/80	EGF-like module-containing mucin-like hormone receptor-

	like 1
FA	Formaldehyde
FBS	Fetal bovine serum
FEV	Forced expiratory volume
FITC	Fluorescein
FoxP3	Forkhead box P3
g	gram
Gata3	Gata binding protein 3
GFP	Green fluorescence protein
GL7	Ly77, T and B cell activation marker
h	Hour
H&E	Hematoxylin and Eosin stain
HDM	House dust mite
i.p.	intraperitoneal
i.v.	Intravenous
iBALT	Inducible bronchus-associated lymphoid tissues
ICCS	Intracellular cytokine staining
IF	Immunofluorescence
IFNγ	Interferon- γ
Ig	immunoglobulin
IgE	immunoglobulin E
IgG	immunoglobulin G
IgG1	immunoglobulin G1
IgG2b	immunoglobulin G2b
IgG2c	immunoglobulin G2c
IL-10	Interleukin-10
IL-13	Interleukin-13
IL-17	Interleukin-17
IL-21	Interleukin-21
IL-4	Interleukin-4
IL-5	Interleukin-5
ILC	Innate lymphoid cell
ILC2	Group 2 innate lymphoid cell
ILC_{reg}	Regulatory innate lymphoid cells
IM	Interstitial macrophage
ΔNKT	Invariant natural killer T cell
LD	Live Dead discrimination
LN	Lymph node
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6 complex locus C
Ly6G	Lymphocyte antigen 6 complex locus G6D
M	molar

MFI	mean fluorescence intensity
MHCI	Major histocompatibility complex class I
MHCII	Major histocompatibility complex class II
min	minutes
mL	milliliter
mLN	Mediastinal lymph node
mM	millimolar
MO	Monocyte
MPO	Myeloperoxidase
MΦ	Macrophage
NaN₃	Sodium azide
NET	Neutrophil extracellular traps
NK cell	Natural killer cell
NK1.1	Natural Killer 1.1
NKp46	Natural Killer cell protein 46
O.D	optical density
OVA	Ovalbumin
PacBlue	Pacific Blue
PAS	Periodic acid-Shiff stain
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline with Tween 20
pDC	Plasmacytoid DC
PE	Phycoerythrin
PE-CF594	Phycoerythrin cyanin based fluorophore 594
PE-Cy5	Phycoerythrin cyanine5
PE-Cy7	Phycoerythrin cyanine 7
PE-Dazzle 594	Phycoerythrin Dazzle 594
pen-strep	penicillin-streptomycin
PFA	Paraformaldehyde
PLZF	promyelocytic leukemia zinc finger protein ZBTB16
PMA/I	Phorbol 12-myristate 13-acetate/Ionomycin
PRRs	Pathogen recognition receptors
RBC	Red blood cell
RORγt	RAR-related orphan receptor gamma
ROS	Reactive oxygen species
RPMI medium	Roswell Park Memorial Institute medium
RT	room temperature
s.c.	Subcutaneous
<i>spp.</i>	species
SSC-A	Side scatter area

SSC-W	Side scatter width
Tbet	Tbox transcription factor TBX21
TCRβ	T cell receptor β chain
TGFβ	Tumor growth factor- β
T_h1	T helper 1
T_h17	T helper 17
T_h2	T helper 2
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
v/v	volume/volume
w/v	weight/volume
α-GalCer	α -galactosylceramide
μl	microliter



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ROLE OF INVARIANT NATURAL KILLER T (NKT) CELLS IN NEUTROPHILIC (T_h1-DRIVEN) ASTHMA

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Abstract

Asthma is a common chronic lung disease. It is categorized into two main endotypes called eosinophilic and neutrophilic asthma. The majority of asthma patients suffer from eosinophilic asthma and respond well to current treatments. However, 5-20% of patients with neutrophilic asthma respond poorly. Invariant Natural Killer T (NKT) cells have been described to enhance airway inflammation in an eosinophilic animal model of asthma. However, their role in neutrophilic asthma has not been clarified so far. We established mouse models to compare neutrophilic and eosinophilic asthma side-by-side. Using 19 parameter-flow cytometry, we analyzed cell frequencies, transcription factor expressions, and cytokine secretions of cell populations recruited to the inflamed lung and its draining lymph nodes. We compared lung inflammation in wild-type and NKT cell-deficient (J α 18^{-/-}) mice and found that NKT cells do not affect the disease severity of neutrophilic asthma. Furthermore, we discovered that the composition of macrophage and DC subsets in the airways allows the distinction of the two asthma endotypes. In particular, interstitial macrophages dominated the lungs of eosinophilic airway inflammation, whereas exudate macrophages and plasmacytoid DCs were more abundant in neutrophilic asthma. Moreover, the IL-10 secreting T cells, B cells, NKT cells, and innate lymphoid cells (ILCs) populations were comparable between the two endotypes. However, we found a unique subpopulation of neutrophils that expresses high levels of MHC class II molecule and could produce IL-10. These neutrophils were abundant in the lung during neutrophilic airway inflammation.

Keywords: Asthma, airway diseases, endotypes, neutrophils

NÖTROFİLİK (T_H1-KAYNAKLI) ASTIM ENDOTİPİNDE DOĞAL ÖLDÜRÜCÜ İNVARİYANT T (NKT) HÜCRELERİNİN ROLÜ

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ÖZET

Astım, eozinofilik ve nötrofilik olmak üzere iki ana endotipe kategorize edilen yaygın kronik bir akciğer hastalığıdır. Astım hastalarının büyük bir bölümü eozinofilik astıma sahiptir ve şu an sunulan tedavilere iyi yanıt verirler. Ancak, astım hastalarının %5-20'sini oluşturan nötrofilik astıma sahip hastalar bu tedavilere yanıt vermezler. İnvariant doğal öldürücü T (NKT) hücrelerinin, eozinofilik astım hayvan modelinde solunum yolu inflamasyonunu arttırdığı gösterilmiştir. Ancak, nötrofilik astımdaki rolleri henüz açığa kavuşmamıştır. Nötrofilik ve eozinofilik astımı yan yana karşılaştırmak için farelerde modelledik. 19-parametrelili akış sitometriyi kullanarak, inflamasyona uğramış akciğer ve mediastinal lenf düğümlerine toplanan hücrelerin, hücre frekanslarını, transkripsiyon faktör ekspresyonlarını ve sitokin salınımlarını analiz ettik. WT ve NKT hücreleri eksik (J α 18^{-/-}) fare suşlarını kullanarak, akciğer inflamasyonlarını karşılaştırdık ve NKT hücrelerinin nötrofilik astım şiddetini etkilemediğini bulduk. Ancak, solunum yolunda bulunan makrofaj ve dendritik hücre alttipleri kompozisyonunun astım endotiplerini tanımlayıcı özelliği olduğunu keşfettik. İntersititli makrofajlar, eozinofilik inflamasyonlu akciğerleri domine etmişken, ekzudat makrofajlar ve plazmasitoid dendritik hücreler nötrofilik astımda çokça bulunmaktaydı. Ayrıca, IL-10 salgılayan T, B, NKT veya doğal lenfoid hücre (ILC) popülasyonlarında iki astım endotipi arasında bir farklılık bulunmamaktaydı. Ancak, nötrofilik astımlı akciğerde MHC sınıf II molekülünü eksprese eden ve IL-10 üretebilen özel bir nötrofil alt popülasyonu saptadık. Bu nötrofil popülasyonunun özellikle, nötrofilik solunum yolu inflamasyonu sırasında akciğerde bol miktarda bulunduğu ortaya koyduk.

Anahtar Kelimeler: Astım, solunum yolu hastalıkları, endotipler, nötrofiller

1. Introduction and objectives

Asthma is a disease caused by chronic airway inflammation. As a result of the varying triggers and clinical symptoms, the severity of exacerbations, and the response to treatment, asthma is a heterogeneous disease, and many phenotypes exist. The endotype concept aims to define patient subgroups based on the underlying pathobiological mechanism identified by specific cellular and molecular biomarkers. Due to the significant variance in patient discomfort and the intensity of symptoms, the clinical characterization and pathogenesis of asthma are challenging. In the absence of crucial information on the molecular pathogenesis and the classification of asthma patients, the current treatment strategies follow a rather empirical approach. Our understanding of asthma to date is mainly based on the allergen-induced, eosinophilic asthma, characterized by airway eosinophilia, mast cell recruitment to the bronchia, and augmented production of antigen-specific IgE antibodies. Mouse models of eosinophilic asthma demonstrated remodeling of the airway mucosa via activated ILC2s, mast cells, basophils, and Th2 cells resulting in an intense type 2 immune response. Corticosteroids and anti-IgE therapies give successful results for eosinophilic asthma patients. However, a minority of asthma patients are treatment-resistant, as they suffer from neutrophilic asthma. These patients exhibit intense discomfort, leading to severe lung dysfunction and chronic obstructive pulmonary disorder (COPD). The situation requires an increased utilization of health care resources, causing an increased burden for public health.

NKT cells are innate-like lymphocytes involved in diverse disease pathologies ranging from autoimmune disease to lung infections and are crucial modulators of eosinophilic asthma. However, their role in neutrophilic asthma remains elusive. In this project, we combined sophisticated *in vivo* approaches with extensive multiparametric flow cytometry analysis. With the proposed experiments, we aimed:

(1) To establish adjuvant-induced and LPS-induced *in vivo* mouse models to

investigate eosinophilic and neutrophilic asthma;

(2) To determine the involvement of *NKT* cells in neutrophilic asthma in the mouse model;

(3) To analyze the frequency and phenotype of *NKT* cell subsets in neutrophilic asthma in the mouse model;

(4) To investigate in detail the myeloid cells and their subsets that are recruited to the lung during eosinophilic and neutrophilic airway inflammation.

We hypothesize that identifying the role of *NKT* cells in neutrophilic asthma and the detailed side-by-side comparison of neutrophilic and eosinophilic asthma **will provide valuable information on the differential pathogenesis of the two asthma endotypes**. As the invariant TCR α chain of *NKT* cells is identical in every human, the potential of *NKT* cell-based therapies is tremendous. This project will be the first to analyze side-by-side the role of *NKT* cells and *NKT* cell subsets in neutrophilic and eosinophilic asthma. It will decipher critical points in the differential pathogenesis and promise to develop brand-new approaches for diagnosis and therapy of these different asthma endotypes.

2. Literature background and scope

Asthma is a chronic disease of the airways affecting 5 – 20% of the population within various countries (Global initiative for Asthma et al. ,2020). It is associated with airway hyper-responsiveness and inflammation, which are induced or exacerbated by physical and environmental factors, including weather changes, allergens, pollution, respiratory infections, obesity, and smoking (I Agache et al. ,2012; HY Kim et al. ,2010; Global initiative for Asthma et al. ,2020). Clinically, asthma is diagnosed by wheezing, shortness of breath, chest tightness, and cough, together with restricted airflow. Statistics show that female gender, allergic rhinitis, a high body-mass index (BMI), and a family history of asthma are associated with a higher risk of developing asthma (Edwards et al. ,2017; Lefaudeux et al. ,2017; Postma et al. ,2007). However, the term 'asthma' does not define a single disease etiology but a multifarious disorder that encompasses an array of symptoms with varying persistence and intensity between individuals (Global initiative for Asthma et al. ,2020; Lambrecht et al. ,2015; Fahy et al. ,2015). Therefore, many phenotypes exist (IO Agache et al. ,2013; I Agache et al. ,2018). The characterization of this heterogeneous disease into phenotypes (clinical, physiological, and biological traits) and endotypes was introduced in 2006 (Simpson et al. ,2006) (cellular and pathophysiological traits) to overcome the challenging task of diagnosis (**Figure 1**).

Asthma is now commonly divided into two inflammatory pheno/endotypes, a Th2-high (Peters et al. ,2014; I Agache et al. ,2012; Hyde et al. ,2019; Haspeslagh et al. ,2017) and a non-Th2 or low-Th2 (Maltby et al. ,2017; Antolín-amérigo et al. ,2018; Sze et al. ,2020) endotype. The Th2-high asthma endotype represents the most common and best-studied asthma endotype, which is coupled with an allergic immune response, sputum eosinophilia, and which can be currently treated (Peters et al. ,2014; I Agache et al. ,2012; Hyde et al. ,2019; Haspeslagh et al. ,2017). In contrast, the non-Th2 type asthma endotype is a more severe form of disease

coupled with airway neutrophilia (Maltby et al. ,2017; Antolín-amérigo et al. ,2018; Sze et al. ,2020). This non-Th2 asthma responds poorly to the main treatment options currently available for asthma patients, severely impacting the quality of their life. Thus, a clearer understanding of the underlying immunopathology of the different asthma endotypes is essential to devise effective diagnosis and treatment approaches for the treatment-resistant non-Th2 endotype.



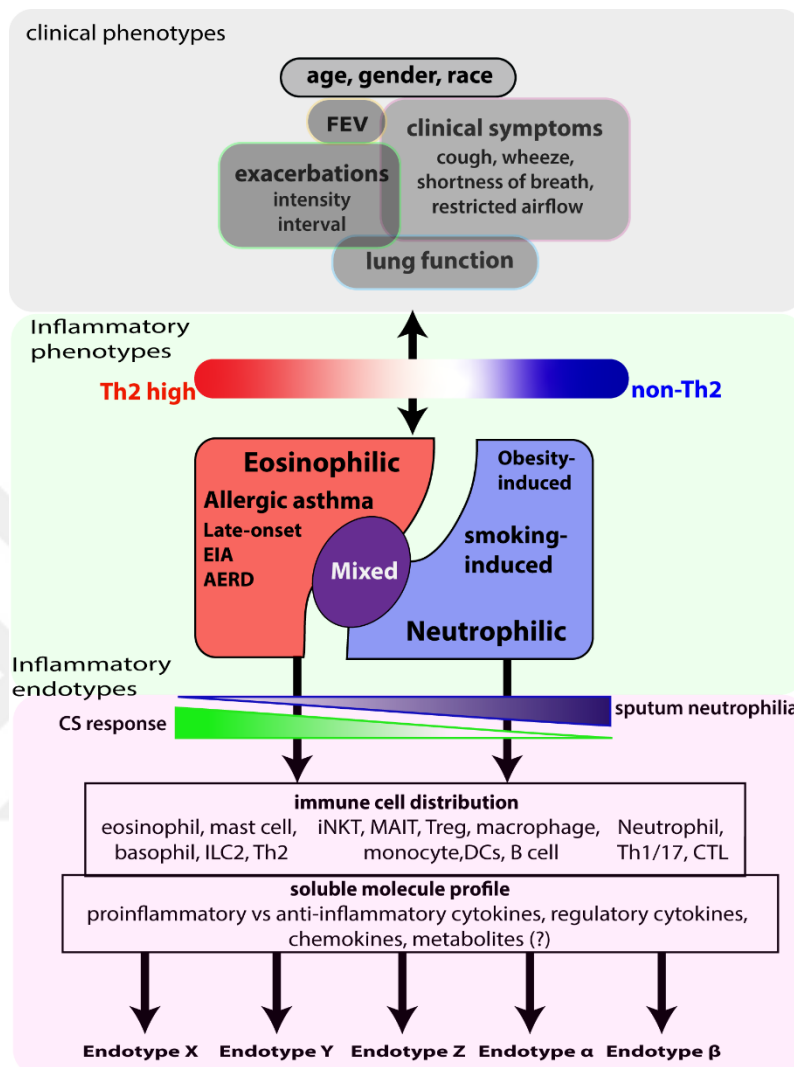


Figure 1. Defining endotypes for asthma. Patients are diagnosed according to their symptom history, severity, and pattern. The diagnosis was further verified by the lung function tests, allergy, and bronchial provocation test. The additional blood work and sputum content analyses provide information to determine the type of airway inflammation. Generally, the inflammatory pheno/endotypes are divided into two, according to the cell recruited to the airway. Sputum neutrophilia specifies neutrophilic asthma, where blood and sputum eosinophilia indicates eosinophilic asthma. Aspirin-exacerbated respiratory disease (AERD), exercise-induced asthma (EIA), late-onset asthma, and allergic asthma are examples of eosinophilic type airway inflammation. Patients who belong to the eosinophilic asthma group respond well to corticosteroid (CS) treatment. However, neutrophilic asthma, in other words, severe corticosteroid-resistant asthma, can be triggered by obesity, smoking. These clinical, physiological, and biological traits alone are not enough to help manage treatment-resistant asthmatics groups' symptoms. Thus, recently a more comprehensive analysis of immunological cell and soluble molecule distribution along with genetics analysis, open revenues to further characterize those into more specific smaller patients groups in asthma endotypes.

Asthma endotypes and mouse models

Asthma is a long-term persisted chronic airway disease induced by extreme airway hyper-responsiveness affecting millions of people worldwide (Global initiative for Asthma et al. ,2020). The underlying pathology associated with dysregulated immune responses and pulmonary inflammation is diverse (HY Kim et al. ,2010; I Agache et al. ,2012; Wenzel et al. ,2012). However, it is possible to broadly characterize asthma based on the overall pulmonary immune response into 'type-2 high' and 'type-2 low' asthma (Sugita et al. ,2019; Peters et al. ,2014). Traditionally, the term 'asthma' is attributed to allergic asthma, also called eosinophilic asthma, associated with a type-2-polarized immune response (Fallon et al. ,2020). It is often triggered by inhaled antigens, derived for example, from house dust mites (HDMs), ragweed pollen, mold, or cockroaches, which lead to the remodeling of the airway mucosa (HY Kim et al. ,2010; Lambrecht et al. ,2015; Edwards et al. ,2017). The immunopathology is aggravated by elevated levels of eosinophils (eosinophilia) and mast cells in the bronchia, the augmented production of antigen-specific IgE antibodies, as well as the production of the Th2 cytokines IL-4, IL-5, and IL-13 that leads to goblet cell hyperplasia and bronchial hyper-responsiveness (Fahy et al. ,2015; Fallon et al. ,2020; Ray et al. ,2017). Type-2 high pulmonary inflammations are treated with corticosteroids, and therefore, diagnosed asthmatics are usually prescribed corticosteroids to manage their asthma (Petsky et al. ,2017; Hopkin et al. ,2012). However, 5-20% of the patients do not respond to corticosteroids or other conventional medications used to relieve asthma exacerbations, including anti-IgE (Samitas et al. ,2015) and anti-IL-5 (Maltby et al. ,2017; Stokes et al. ,2016) treatments. The pulmonary inflammation in such patients exhibits a neutrophil-dominated low- or non-type-2 immune response (Ray et al. ,2017; Fallon et al. ,2020; Edwards et al. ,2017). Although some researchers argue that this severe corticosteroid-resistant asthma is a chronic and prolonged version of eosinophilic

asthma, the clinical data (Lefaudeux et al. ,2017) and the studies with mouse models demonstrate that a non-eosinophilic (McGrath et al. ,2012), neutrophil-dominated, and corticosteroid-resistant asthma phenotype exists (**Figure 2**) (Simpson et al. ,2007; Hodge et al. ,2016; I Agache et al. ,2012). This type of neutrophilic asthma carries a distinct inflammatory signature (Bogaert et al. ,2011; Dejager et al. ,2015; Mcalees et al. ,2015; Wilson et al. ,2009; Whitehead et al. ,2014; Loza et al. ,2016; Lefaudeux et al. ,2017). Neutrophilic asthma lacks the type-2 immune response biomarkers, like IL-4, IL-5, IL-13 cytokine secretion, IgE production, Th2 cells activation, and eosinophilia (Fallon et al. ,2020; Loza et al. ,2016; Ntontsi et al. ,2020). Instead, the corticosteroid-unresponsive neutrophilic inflammation is characterized by neutrophilia and augmented production of IFN γ and IL-17 (Ray et al. ,2016; Chambers et al. ,2015; Fallon et al. ,2020). Due to the limited therapy options that ease neutrophilic asthma, the disease progresses, leading to tissue remodeling and to an increased propensity for pulmonary infections, including viruses (Geerdink et al. ,2015; Cortjens et al. ,2017; Busse et al. ,2010) and bacteria (Ray et al. ,2017). These respiratory infections are implicated in triggering asthma onset and exacerbations (Teo et al. ,2015; An et al. ,2018); however, further investigation is needed to establish their role in the development of disease pathology.

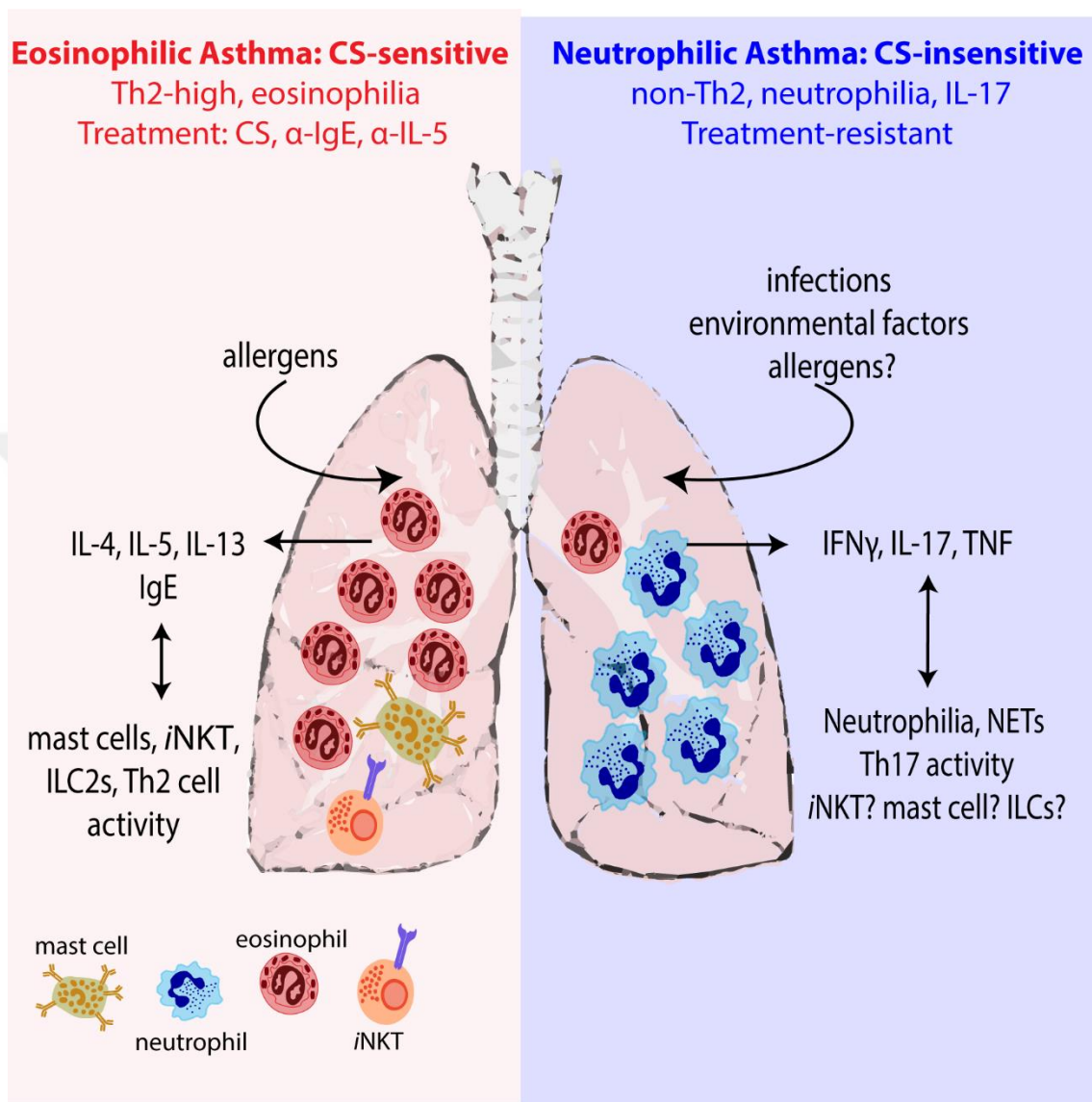


Figure 2. Immunology of asthma endotypes. Airway inflammation and asthma have long been modeled *in vivo* in mouse models. Treatment-responsive widely studied eosinophilic asthma, triggered majorly by allergens, is associated with Th2 high immune response, airway eosinophilia, production of IL-4, IL-5, IL-13 cytokines, IgE by the mast cells, *i*NKT cells, ILC2s. Th2 low, treatment-resistant, neutrophilic asthma provoked by infections, smoking, environmental irritants induce the release of IFN γ , IL-17, and TNF from neutrophils, Th1/17 cells, ILCs. However, many aspects of the neutrophilic airway inflammation and the role of particular cells, soluble molecule effect on the pathogenesis of such asthma endotypes, remain elusive. *i*NKT, invariant natural killer T cell; ILC2s, group 2 innate lymphoid cells; IFN γ , interferon-gamma; TNF, tumor necrosis factor.

Airway inflammation and asthma have long been modeled *in vivo* in mouse models to gain more insight into the progression, pathogenesis, and genetic

background of the disease (Barrios et al. ,2008). The modeling a human airway disease in the mouse has some drawbacks, as it does not mimics the whole human pathology. However, the main clinical features, like emphysema, airway hyper-responsiveness, high IgE levels, mucous hypersecretion, and eosinophilia, can be modeled in the mouse (Edwards et al. ,2017; Fallon et al. ,2020; Bonniaud et al. ,2018). Ovalbumin (OVA) is a commonly used antigen for mouse studies. Although OVA does not trigger an allergic immune response in humans, the use of OVA along with the adjuvant Alum (Aluminium hydroxide) is a well-described procedure to induce a type-2 immune response in airways of mice (Haspeslagh et al. ,2017; Bogaert et al. ,2011; Chuang et al. ,2011; Nakada et al. ,2014; Fallon et al. ,2020). However, the severe form of clinical asthma, which is unresponsive to inhaled corticosteroids, anti-IgE, and anti-IL-5 conventional treatment, is more diversified and more challenging to model in the mouse (Wadhwa et al. ,2019). This challenge can be overcome by tailoring the mouse models according to (i) the particular outcome, like airway neutrophilia or treatment-unresponsiveness, and (ii) the inducers of the severe airway inflammation/exacerbation; like infection-induced or ozone-induced (Fallon et al. ,2020; Maltby et al. ,2017; Gubernatorova et al. ,2019; Wadhwa et al. ,2019). Subcutaneous administration of either LPS (Wilson et al. ,2009; Whitehead et al. ,2014) or CFA (Bogaert et al. ,2011; Dejager et al. ,2015) along with the model antigen OVA, or pulmonary infections with *Aspergillus spp* or *Alternaria spp* (Bonniaud et al. ,2018; Albacker et al. ,2013), or administration of *ex vivo* differentiated T_H1 (Hayashi et al. ,2007) are all valid mouse models to induce neutrophilic, corticosteroid-resistant asthma.

To date, there is limited evidence that links disease-associated pathological features to a particular clinical outcome or treatment strategies. Hence, further research into the underlying molecular mechanisms is required to develop new endotype-specific asthma treatments. In particular, a side-by-side comparison of the

immune response in the inflamed lung during eosinophilic and neutrophilic asthma appears best suited to accomplish this aim.

The role of *NKT* cells in airway inflammations

Invariant natural killer T (*NKT*) cells are lipid-reactive, unconventional T cells that share features with innate Natural Killer (NK) cells and memory T cells (Shimizu et al. ,2014). The Va14-Ja18 semi-invariant T cell receptor on mouse *NKT* cells can recognize glycolipids presented on CD1d molecules, leading to the activation of the *NKT* cells. Upon activation, *NKT* cells rapidly produce large amounts of both type 1 (IFN γ) and type 2 (IL-4) cytokines, unlike conventional T cells, which usually secrete either type-1 or type-2 cytokines. The fast and copious release of both IFN γ and IL-4 from *NKT* cells promotes the recruitment and activation of a broad range of immune cells (Im et al. ,2009). Therefore, *NKT* cells are a unique immune cell type that shares innate and adaptive immune cell features. Recent studies suggest that similar to CD4⁺ T helper subsets, *NKT* cells can be divided into several subsets. *NKT*1 (Albacker et al. ,2013), *NKT*2 (Lynch et al. ,2015), *NKT*17 (Michel et al. ,2007; Coquet et al. ,2008), and *NKT*_{fh} (King et al. ,2012; Chang et al. ,2012; Tonti et al. ,2012) cells, and IL-10 releasing *NKT*10 cells (Wingender et al. ,2015; Sag et al. ,2014;Lynch et al. ,2015)

NKT cells are involved in the regulation of many diseases, ranging from autoimmune diseases, like rheumatoid arthritis and diabetes (Nel et al. ,2016), to bacterial and viral infections (Wingender et al. ,2011; Gorski et al. ,2013; Ishikawa et al. ,2010), as well as asthma and allergy (Akbari et al. ,2003; Hachem et al. ,2005; Lisbonne et al. ,2003; Matsuda et al. ,2010; Akbari et al. ,2010) (**Figure 3**). Although *NKT* cells are not frequent in the pulmonary niche, studies showed that *NKT* cells play a protective role against the pulmonary pathogens *S. pneumonia* (Thanabalasuriar et al. ,2016; Kinjo et al. ,2011; Kawakami et al. ,2003) and

influenza (Gorski et al. ,2013; Ho et al. ,2008; Ishikawa et al. ,2010; Kok et al. ,2012). Furthermore, studies on allergen-induced, eosinophilic asthma showed that *NKT* cells promote airway hyper-responsiveness and asthma (Lombardi et al. ,2010; Omid Akbari et al. ,2003; JO Kim et al. ,2004; Shasby et al. ,2002; Lisbonne et al. ,2003; Wingender et al. ,2011) (**Figure 3**). However, after activation of *NKT* cells with the strong agonist α GalCer, *NKT* cells were now protective against eosinophilic asthma (Okamoto et al. 2010; Hachem et al. 2005a). This diverse function of *NKT* cells in eosinophilic asthma has been attributed to the differential secretion of IFN γ (Hachem et al. ,2005; Okamoto et al. ,2010) and TNF (Dejager et al. ,2015), but the exact mechanisms underlying such different outcomes remain elusive (Hachem et al. ,2005; Okamoto et al. ,2010; Bogaert et al. ,2011; Dejager et al. ,2015).

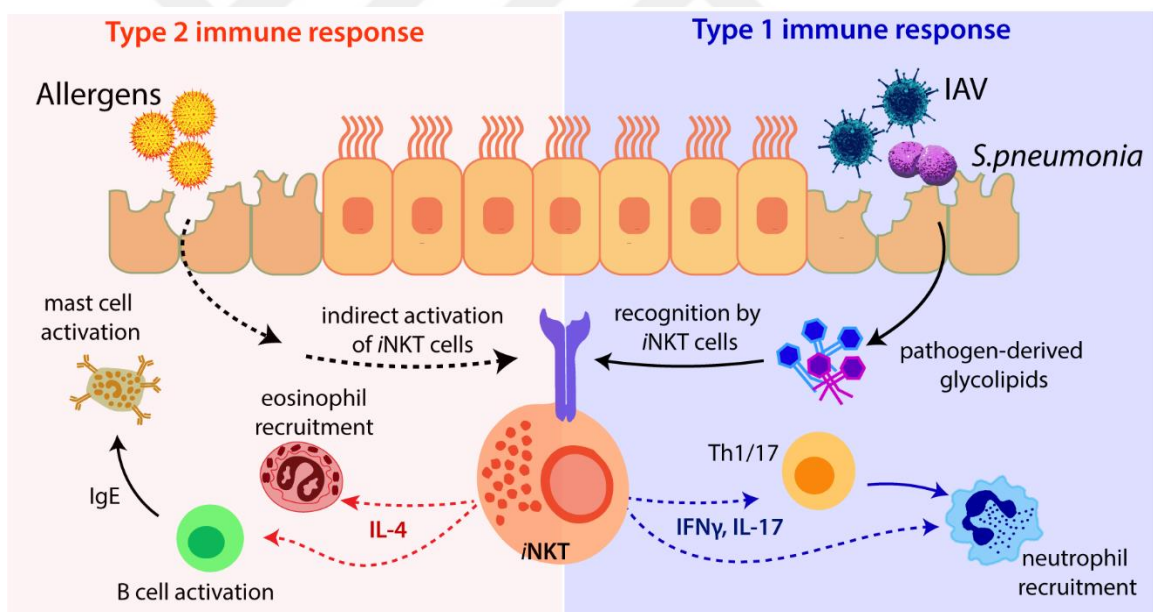


Figure 3. *NKT* cells in airway inflammations. *NKT* cells constitute a relatively small percentage of the pulmonary cell population—however, their ability to respond quickly to inflammation orchestrates the immune response initiation. Besides their rapid response interval, they have the ability to produce both type1 and type 2-specific cytokines. This ability of *NKT* cells, enable them to control or trigger two different immune response simultaneously and interact with an array of innate and adaptive immune cells. In the lung, *NKT* cells were previously described as protective against pulmonary infections (*S. pneumonia*, Influenza) by inducing type 1 immune response via IFN γ release. On the other hand, they are harmful in allergen-induced lung inflammation by triggering a pathogenic type 2 immune response.

The role of IL-10 in airway diseases

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that can limit immune responses during inflammation (Ouyang et al. ,2019). IL-10 reduces the activity of effector T cells, NK cells, and macrophages (Ouyang et al. ,2019; Branchett et al. ,2019). It limits tissue damage and restores immune homeostasis (Ouyang et al. ,2019). IL-10 can be produced by several immune cells, including B cells (Amu et al. ,2010; F Braza et al. ,2015), T cells (Jang et al. ,2017; Böhm et al. ,2015), macrophages/monocytes (Bracken et al. ,2015; Kawano et al. ,2016), dendritic cells (Han et al. ,2006; Branchett et al. ,2019), innate lymphoid cells (S Wang et al. ,2017), NKT cells (Sag et al. 2014), and epithelial cells (Branchett et al. ,2019).

In the airways, a role for IL-10 has been described for chronic obstructive pulmonary disease (COPD), asthma, and acute respiratory distress syndrome (ARDS) (Branchett et al. ,2019). For example, IL-10 release in adult (Raeiszadeh Jahromi et al. ,2014; Xystrakis et al. ,2006) and pediatric (Gupta et al. ,2014) asthma patients were defective, implicating the essential role of IL-10 to resolve airway inflammation (Ogawa et al. ,2008; Branchett et al. ,2019). In mouse models of asthma, IL-10 producing B cells have been shown to prevent HDM- (F Braza et al. ,2015) and casein-induced (A-R Kim et al. ,2016) allergic airway inflammation. Furthermore, the adoptive transfer of IL-10 treated DCs (Koya et al. ,2007) or of FoxP3⁺ regulatory T cells (Kearley et al. ,2005; Oh et al. ,2002) prevented allergic airway inflammation in mice by inhibiting Th2 activity. Although IL-10 secreting regulatory T cells are essential to halt allergic airway inflammation in HDM-induced mouse model of allergic asthma (Coomes et al. ,2017), the numbers of infiltrating FoxP3⁺ regulatory T cells were comparable between neutrophilic and eosinophilic asthma (Jang et al. ,2017). For myeloid cells, studies showed that alveolar inflammation could be ameliorated by IL-10-producing interstitial macrophages in neutrophilic asthma (Kawano et al. ,2016), by alveolar macrophages in HDM-induced allergic asthma

(Bracken et al. ,2015), and by neutrophils in pulmonary bacterial infection (Peñaloza et al. ,2019).

The role of neutrophils in airway inflammations

Neutrophils are innate immune cells that constitute up to 30% of the white blood cells in the blood of healthy humans (Ley et al. ,2018). They are produced in the bone marrow during steady-state granulopoiesis and released upon CD184 (CXCR4) mediated licensing to the circulation (Ley et al. ,2018; Nicolás-Ávila et al. ,2017; José M. Adrover et al. ,2016). Neutrophils are indispensable players in infection and autoimmunity. Upon inflammation, they quickly adhere to the inflammation site through either activation via TLR engagement or translocation via chemotaxis (Silvestre-Roig et al. ,2016; Nicolás-Ávila et al. ,2017). This translocation is achieved by the expression of an array of chemokine receptors; CD191/CCR1, CD192/CCR2, CD193/CCR3, CD195/CCR5, CD182/CXCR2, CD183/CXCR3, and CD184/CXCR4 that enables the ability of neutrophils to respond to the majority of chemokines (Sadik et al. ,2011; Ley et al. ,2018). Neutrophils shape immune responses during inflammation via (i) the formation of neutrophil extracellular traps (NETs) (Pham et al. ,2017; Sanz-Moreno et al. ,2018; Khandpur et al. ,2013), (ii) the secretion of soluble molecules like alarmins and cytokines (Persson et al. ,2018; Baines et al. ,2010; Ley et al. ,2018), (iii) the response to pathogen-associated molecular patterns (PAMPs) by pathogen recognizing receptor (PRRs) (Acorci-Valério et al. ,2010; Tsuda et al. ,2004), and (iv) the ability to act as antigen-presenting cells (APCs) to activate adaptive immune cells (Li et al. ,2019; Potter et al. ,2001; Takashima et al. ,2015) (**Figure 4**). Although they were considered a homogenous population of short-lived cells, recently, it was suggested that subsets of neutrophils could be recognized and that some of these can survive for weeks under pathological conditions (Deniset et al. ,2018; Silvestre-Roig et al. ,2016). Neutrophils have long been associated with respiratory diseases including, COPD (Bewley et al. ,2017),

ARDS (Williams et al. ,2014), cystic fibrosis (Robledo-Avila et al. ,2018), and corticosteroid-resistant asthma (Panettieri et al. ,2018; Gernez et al. ,2010).

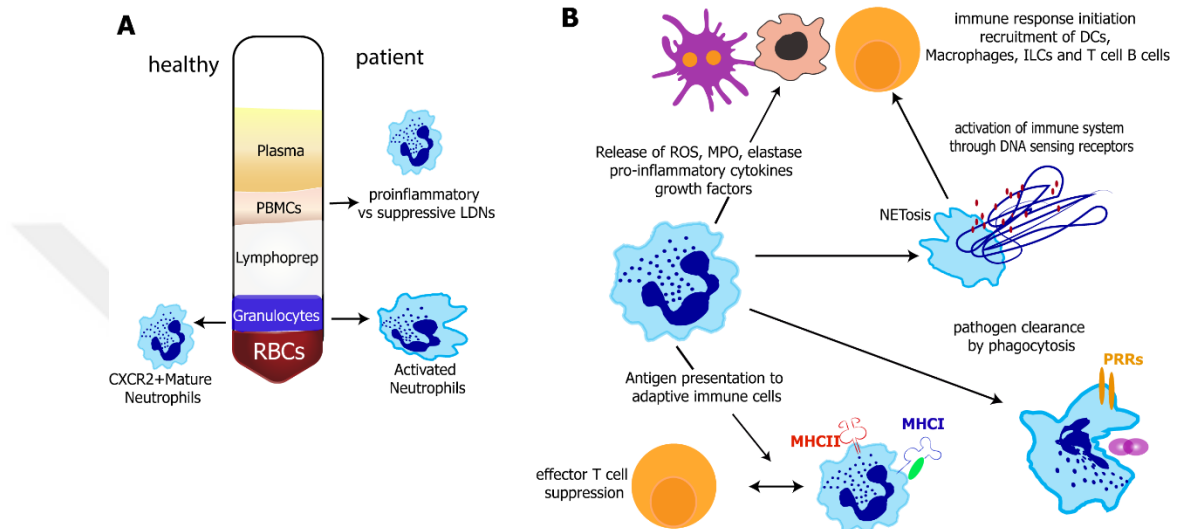


Figure 4. Neutrophils. Neutrophils are the most populated granulocytes in the circulation. They are the aggressive first responders to the inflammation. Once they 'sense' that the immune barrier has breached, they got activated and recruited to the inflammation site via a process called chemotaxis. Although they were considered a homogenous population of short-lived cells, subpopulations of neutrophils can be recognized, and that some of these can survive for weeks under chronic inflammatory conditions. Neutrophils are frequently isolated from the blood by gradient centrifugation for research purposes, and **(A)** their isolation from the blood is heavily affected by the inflammatory state of the sample. **(B)** Once they are in an active state, they shape the immune response by the release of alarmins and toxic compounds, e.g., ROS, NO; releasing their DNA content, a process that is known as NETosis to capture pathogens and trigger further DNA sensing related immune mechanisms; identifying and eliminating pathogens via pathogen-recognition receptors (PRRs) and present antigen to the adaptive immune cells.

Neutrophil subsets with differential functions were implicated in severe airway inflammations. The first description of neutrophil subsets characterized N1 and N2 neutrophils in the blood by their expression of CD49d and CD11b and by the release of IL-10 or IL-12 (Tsuda et al. ,2004). Later, further subsets were identified and called 'immature' or 'aged' neutrophils, or 'tissue-retained' neutrophils (Silvestre-Roig et al. ,2016; JH Kim et al. ,2018). The bronchoalveolar lavage (BAL) of pediatric patients with severe asthma showed an activated CD16^{hi} CD11b^{hi} CD62L^{hi} phenotype

with a high NET forming capacity (Grunwell et al. ,2019). The frequency of these neutrophils correlated with the disease severity, and they were resistant to apoptosis (Grunwell et al. ,2019). Similarly, a neutrophil phenotype, characterized as CD16^{hi} CD62L^{dim}, was associated with airway hyperreactivity due to their TNF release, which stimulated airway smooth muscles (Ekstedt et al. ,2019). Furthermore, these CD16^{hi} CD62L^{dim} neutrophils could prime T cells (Arebro et al. ,2017), contributed to eosinophil recruitment in allergic rhinitis (Arebro et al. ,2017), and were implicated in viral pulmonary infections in infants (Cortjens et al. ,2017).

The mouse models of eosinophilic and neutrophilic asthma showed that corticosteroids only induced apoptosis of eosinophils but not neutrophils (Tian et al. ,2017). Neutrophil recruitment to the inflamed lungs upon challenge with LPS (X Wang et al. ,2020) or ragweed pollen (Hosoki et al. ,2016) was CD182-dependent. Although neutrophils mount the initial immune response against the inflammation, persisting inflammation can cause extended tissue damage and secondary pathology due to elastase release and NET-formation (NETosis) from neutrophils (Margaroli et al. ,2019; Ley et al. ,2018) in a CD184-dependent fashion (Radermecker et al. ,2019). A CD182-dependent mechanism can also drive a spontaneous granule release by neutrophils and can prevent their NETosis and further cytotoxicity in LPS-induced lung injury (Jose M. Adrover et al. ,2020).

Neutrophils are highly abundant cells throughout the body. Besides the chemokine receptors mentioned above, CD54 (ICAM1) is associated with neutrophil transmigration to the inflamed gut (Sumagin et al. ,2014) and the brain, and with the acquisition of APC-like properties (Whittaker Hawkins et al. ,2017). Furthermore, CD54⁺ neutrophils showed enhanced phagocytosis and effector functions in a model of LPS-induced endotoxemia (Woodfin et al. ,2016).

Besides effector functions, neutrophils also possess regulatory functions to

shape innate and adaptive immune responses (Li et al. ,2019). A particular subset of neutrophils that expresses MHC class II and the costimulatory molecules CD80 or CD86 has an APC-like property (Geng et al. ,2013; Li et al. ,2019) and is able to present antigens to T cells (Abdallah et al. ,2011) during fungal (Fites et al. ,2018) or bacterial (Barquero-Calvo et al. ,2013) infections.

Under pathogenic conditions, neutrophils are known to produce pro-inflammatory cytokines; like IL1 α , IL1 β , IL-6, IL-17, and IFN γ , but also anti-inflammatory cytokines, like IL-4, IL-10, and TGF β (Cassatella et al. ,2019; Tamassia et al. ,2018). Although IL-10-producing neutrophils have been reported in mouse models (Bazzoni et al. ,2010; Tsuda et al. ,2004; Kasten et al. ,2010; Ocuin et al. ,2011; Perobelli et al. ,2016), their existence in humans is still controversial (Tamassia et al. ,2018; Cassatella et al. ,2019; De Santo et al. ,2011; Acorci-Valério et al. ,2010).

3. Materials and methods

3.1 Type of the research:

The research was an experimental research study in its entirety.

3.2 Location and the time frame of the research:

The research was conducted from August 2017 to January 2021 at the Izmir Biomedicine and Genome Center (IBG) in the laboratory of Dr. Wingender.

3.3 Research population, sampling, and experimental groups:

This study did not include human specimen(s).

3.4 Research materials:

Samples were collected from wild-type C57BL/6 mice and from IL-10GFP (B6(Cg)-Il10tm1.1Karp/J, also known as Vert-X) and J α 18-deficient (J α 18^{-/-}) mice on the C57BL/6 background that have been provided by the La Jolla Institute for Immunology (LJI, La Jolla, US). All mice were housed under SPF conditions in the Izmir Biomedicine and Genome Center (IBG, Izmir) animal facility in accordance with the Institutional Animal Care Committee guidelines. Experiments were performed according to experimental animal ethics committee guidelines (10/2016 and 05/2019). CD1d/PBS57-tetramer-BV421 and CD1d/PBS57-tetramer-PE were provided by the NIH Tetramer Core Facility (Atlanta, USA). Additional reagents, including chemicals, antibodies, kits, consumables, and devices are outlined in detail in section **3.6.1**.

3.5 Research variables:

The variables of the research were different asthma endotypes/phenotypes modeled either with different adjuvants or different concentrations of LPS. The efficiency of models was assessed by immune cell frequencies, the expression of surface markers, cytokines, transcription factors,

cytokine concentrations in the BALF, and OVA-specific antibody concentration in sera.

3.6 Methods:

3.6.1. Reagents, Devices, Tools:

3.6.1.1 Antibodies

Table 1: Antibodies

Marker	Conjugate	Clone	Catalog number	Company /Source	Purpose	Dilution
GL7	AF488	GL7	144612	BioLegend	Flow cytometry	1:400
IL-4	AF488	11B11	504109	BioLegend	Flow cytometry	1:400
Tbet	AF488	O4-46	561266	BD Biosciences	Flow cytometry	1:100
CD4	AF647	RM4-5	100530	BioLegend	Flow cytometry	1:1000
CD169 / Siglec-1	AF594	3D6.112	142416	BioLegend	IF	1:400
TCR β	AF594	H57-597	109238	BioLegend	IF	1:400
CD45R / B220	AF647	RA3-6B2	103226	BioLegend	Flow cytometry	1:400
CD54	AF647	YN1/1.7	116114	BioLegend	Flow	1:200

		.4			cytometry	
CD45R / B220	AF647	RA3-6B2	557683	BD Biosciences	Flow cytometry	1:400
CD11c	AF700	N418	56-0114-82	eBioscience	Flow cytometry	1:200
CD335 / NKp46	AF700	29A1.4	561169	BD Biosciences	Flow cytometry	1:200
CD4	AF700	RM4-5	100536	BioLegend	Flow cytometry	1:800
CD45	AF700	30-F11	103128	BioLegend	Flow cytometry	1:400
CD45.1	AF700	A20	110724	BioLegend	Flow cytometry	1:400
CD86	AF700	GL-1	105024	BioLegend	Flow cytometry	1:1000
FoxP3	AF700	FJK-16s	56-5773-82	ThermoFisher	Flow cytometry	1:1000
IL-17A	AF700	TC11-18H10.1	506914	BioLegend	Flow cytometry	1:100
TCR β	AF700	H57-597	109224	BioLegend	Flow cytometry	1:200
CD19	APC	MB19-1	17-0191-82	ThermoFisher	Flow cytometry	1:200

CD3ε	APC	145.2C11	20-0031-U100	Tonbo Biosciences	Flow cytometry	1:200
CD44	APC	IM7	17-0441-82	ThermoFisher	Flow cytometry	1:400
IFNγ	APC	XMG1.2	20-7311-U100	Tonbo Biosciences	Flow cytometry	1:200
IL-4	APC	11B11	20-7041-U100	Tonbo Biosciences	Flow cytometry	1:400
IL-10	APC	JES5-16E3	505010	BioLegend	Flow cytometry	1:1000
Ly6G	APC	1A8	20-1276-U100	Tonbo Biosciences	Flow cytometry	1:400
TCR β	APC	H57-597	J15845		Flow cytometry	1:400
CD11c	APC	N418	20-0114-U100	Tonbo Biosciences	Flow cytometry	1:200
CD127	APC-Cy7	A7R34	135040	BioLegend	Flow cytometry	1:800
CD45.2	APC-Cy7	104	109824	BioLegend	Flow cytometry	1:200
IL-17A	APC-Cy7	TC11-	560821	BD	Flow	1:200

		18H10		Biosciences	cytometry	
MHC II (I-A/I-E)	APC- Fire750	M5/114 .15.2	107652	BioLegend	Flow cytometry	1:1000
CD3ε	BUV395	145- 2C11	563565	BD Biosciences	Flow cytometry	1:100
Ly6G	BUV395	1A8	563978	BD Biosciences	Flow cytometry	1:400
CD1d/PB S57- Tetrame r	BV421	N/A		NIH core	Flow cytometry	1:400
CD64 / FcγRI	BV421	X54- 5/7.1	139309	BioLegend	Flow cytometry	1:400
Ly6G	BV421	1A8	127628	BioLegend	Flow cytometry	1:400
CD127	BV510	A7R34	135033	BioLegend	Flow cytometry	1:200
CD19	BV510	6D5	115546	BioLegend	Flow cytometry	1:200
CD19	BV570	6D5	115535	BioLegend	Flow cytometry	1:200
CD4	BV570	RM4-5	100542	BioLegend	Flow cytometry	1:200
CD8α	BV570	53-6.7	100740	BioLegend	Flow cytometry	1:200
CD90.2 / Thy1.2	BV570	30-H12	105329	BioLegend	Flow cytometry	1:200

CD103	BV605	2E7	121433	BioLegend	Flow cytometry	1:200
CD4	BV605	RM4-5	100548	BioLegend	Flow cytometry	1:200
CD45	BV605	30-F11	103139	BioLegend	Flow cytometry	1:200
CD95	BV605	SA367H8	152612	BioLegend	Flow cytometry	1:200
CD11b	BV650	M1/70	101259	BioLegend	Flow cytometry	1:200
CD127	BV650	A7R34	135043	BioLegend	Flow cytometry	1:200
CD45	BV650	30-F12	103151	BioLegend	Flow cytometry	1:100
CD45R / B220	BV650	RA3-6B2	563893	BD Biosciences	Flow cytometry	1:200
CD8a	BV650	53-6.7	100741	BioLegend	Flow cytometry	1:400
IFN γ	BV650	XMG1.2	505831	BioLegend	Flow cytometry	1:200
ROR γ t	BV650	Q31-378	564722	BD Biosciences	Flow cytometry	1:200
CD117 / cKit	BV711	2B8	105835	BioLegend	Flow cytometry	1:100
GATA3	BV711	L50-823	565449	BD Biosciences	Flow cytometry	1:100
TNF	BV711	MP6-	506349	BioLegend	Flow	1:100

		XT22			cytometry	
CD11b	BV711	M1/F70	101242	BioLegend	Flow cytometry	1:400
CD62L	BV711	MEL-14	104445	BioLegend	Flow cytometry	1:400
CD8α	BV711	53-6.7	100748	BioLegend	Flow cytometry	1:200
CD45.1	BV785	A20	110743	BioLegend	Flow cytometry	1:400
CD90.2 / Thy1.2	BV785	30-H12	105331	BioLegend	Flow cytometry	1:200
IFNγ	BV785	XMG1.2	505838	BioLegend	Flow cytometry	1:200
Ly6C	BV785	HK1.4	128041	BioLegend	Flow cytometry	1:200
CD103	BV786	M290	564322	BD Biosciences	Flow cytometry	1:100
Tbet	BV786	O4-46	564141	BD Biosciences	Flow cytometry	1:800
FoxP3	eF660	FJK-16s	50-5773-82	ThermoFisher	Flow cytometry	1:200
CD45R / B220	eFluor 660	RA3-6B2	50-0452-82	ThermoFisher	Flow cytometry	1:200
CD11c	FITC	N418	11-0114-	ThermoFisher	Flow cytometry	1:200

			85			
CD122	FITC	TM-b1	11-1222-82	ThermoFisher	Flow cytometry	1:200
CD170	FITC	S17007L	155504	BioLegend	Flow cytometry	1:200
CD24	FITC	M1/69	101806	BioLegend	Flow cytometry	1:200
CD49d	FITC	R1-2	103606	BioLegend	Flow cytometry	1:100
Ly6G	FITC	1A8	35-1276-U100	Tonbo Biosciences	IF	1:200
TCR β	FITC	H57-597	109206	BioLegend	IF	1:400
TNF	FITC	MP6-XT22	554418	BD Biosciences	Flow cytometry	1:200
CD19	Pacific Blue	6D5	115523	BioLegend	Flow cytometry	1:400
CD24	Pacific Blue	M1/69	101820	BioLegend	Flow cytometry	1:400
CD4	Pacific Blue	GK1.5	100428	BioLegend	Flow cytometry	1:400
Ly6A/E (Sca-1)	Pacific Blue	D7	108120	BioLegend	Flow cytometry	1:400
MHC II (I-A/I-E)	Pacific Blue	M5/114 .15.2	107620	BioLegend	Flow cytometry	1:400

MHC II (I-A/I-E)	Pacific Blue	M5/114 .15.2	107620	BioLegend	Flow cytometry	1:400
CD11b	PE	ICRF44	12- 0118- 42	ThermoFis her	Flow cytometry	1:800
CD11c	PE	N418	117308	BioLegend	Flow cytometry	1:800
CD19	PE	MB19-1	12- 0191- 82	ThermoFis her/	Flow cytometry	1:800
CD1d/PB S57- Tetrame r	PE	N/A		NIH core	Flow cytometry	1:600
CD4	PE	RM4-5	100512	BioLegend	Flow cytometry	1:800
CD44	PE	IM7	12- 0441- 83	ThermoFis her	Flow cytometry	1:800
F4/80	PE	BM8	123110	BioLegend	Flow cytometry	1:800
PLZF	PE	9E12	145804	BioLegend	Flow cytometry	1:400
TCR β	PE	H57- 597	12- 5961- 83	ThermoFis her	Flow cytometry	1:400
CD170 /	PE-CF594	E50-	562757	BD	Flow	1:400

Siglec F		2440		Biosciences	cytometry	
IL-17A	PE-CF594	TC11-18H10	562542	BD Biosciences	Flow cytometry	1:800
ROR γ t	PE-CF594	Q31-378	562684	BD Biosciences	Flow cytometry	1:800
CD11b	PE-Cy7	M1/70	101216	BioLegend	Flow cytometry	1:400
CD45R / B220	PE-Cy7	RA3-6B2	25-0452-82	ThermoFisher	Flow cytometry	1:400
CD49d	PE-Cy7	R1-2	103618	BioLegend	Flow cytometry	1:400
CD86	PE-Cy7	GL-1	105014	BioLegend	Flow cytometry	1:400
CD8 α	PE-Cy7	53-6.7	25-0081-82	ThermoFisher	Flow cytometry	1:400
Fc ϵ RI α	PE-Cy7	MAR-1	134318	BioLegend	Flow cytometry	1:400
GATA3	PE-Cy7	L50-823	560405	BD Biosciences	Flow cytometry	1:200
GL7	PE-Cy7	GL7	144620	BioLegend	Flow cytometry	1:200
IL-4	PE-Cy7	11B11	504118	BioLegend	Flow cytometry	1:400
Ly6G	PE-Cy7	1A8	127618	BioLegend	Flow cytometry	1:400

PLZF	PE-Cy7	9E12	145806	BioLegend	Flow cytometry	1:400
IL-10	PE-Dazzle 594	JES5-16E3	505034	BioLegend	Flow cytometry	1:100
CD11b	PerCP-Cy5.5	M1/70	101228	BioLegend	Flow cytometry	1:600
CD45R / B220	PerCP-Cy5.5	RA3-6B2	45-0452-82	ThermoFisher	Flow cytometry	1:800
CD90.2 / Thy1.2	PerCP-Cy5.5	53-2.1	140322	BioLegend	Flow cytometry	1:800
TCR β	PerCP-Cy5.5	H57-597	65-5961-U100	Tonbo Biosciences	Flow cytometry	1:800
IL-13	PerCP-eF710	eBio13A	46-7133-82	ThermoFisher	Flow cytometry	1:400
MHC I (H-2Kb)	PerCP-eF710	AF6-88.5.5.3	46-5958-80	ThermoFisher	Flow cytometry	1:400
CD44	V500	IM7	560780	BD Biosciences	Flow cytometry	1:200
CD45	V500	30-F11	561487	BD Biosciences	Flow cytometry	1:200
goat anti-mouse	HRP	N.A	1030-05	Southern biotech	ELISA	1:2500

total IgG-HRP						
goat anti- mouse IgG1- HRP	HRP	N.A	1071- 05	Southern biotech	ELISA	1:2500
goat anti- mouse IgG2a- HRP	HRP	N.A	1081- 05	Southern biotech	ELISA	1:2500
goat anti- mouse IgG2b- HRP	HRP	N.A	1091- 05	Southern biotech	ELISA	1:2500
goat anti- mouse IgG2c- HRP	HRP	N.A	1078- 05	Southern biotech	ELISA	1:2500
ChromP ure Mouse IgG	Purified		015- 000- 003	Jackson Immuno research	Flow cytometry	
ChromP	Purified		012-	Jackson	Flow	

ure Rat IgG			000- 003	Immunores earch	cytometry	
anti- mouse CD16/C D32	Purified	Ly-17	101302	BioLegend	Flow cytometry	

3.6.1.2. Chemicals

Table 2: Chemicals

Name	Catalog number	Company	Purpose
Complete Freund's Adjuvant	F5881	Sigma	Immunization
Imject Alum	77165	ThermoSci entific	Immunization
Endograde Ovalbumin	321001	Hyglos	Immunization, ELISA
LPS, C ₁₇₅ H ₃₁₇ N ₅ O ₁₀₁ P ₆	L6386	Sigma	Immunization
Fetal Bovine Serum (FBS)	S 0115	Biochrom	Cell culture, Flow cytometry
Phosphate buffer saline	L 1825	Biochrom	
ACK Lysis Buffer	10-548E	Lonza	Tissue processing
Calcium hydroxide, Ca(OH) ₂	102047	Millipore	Cell Culture
Hydrochloric Acid, HCl 1 mol/l (1N) volumetric solution	A1434,1 000	Applichem	ELISA
Sodium Azide, NaN ₃	S2002- 500G	Sigma	Flow cytometry

Ficoll™ paque PLUS	GE17-1440-03	GE Health care	Cell isolation
Lymphoprep™	07851	Stem Cell	Cell isolation
EDTA, C ₁₀ H ₁₆ N ₂ O ₈	EDT001.500	BioShop Canada	Coagulant
DNase I	10104159001	Roche	Tissue processing
Collagenase D	11088866001	Roche	Tissue processing
Phorbol 12-myristate 13-acetate. C ₃₆ H ₅₆ O ₈	P8139-1MG	Sigma	Cell culture
Ionomycin from Streptomyces conglobatus, ≥98% (HPLC)	I9657-1MG	Sigma	Cell culture
RPMI medium		Lonza	Cell culture
BSA, C ₁₂₃ H ₁₉₃ N ₃₅ O ₃₇	A1391,0250	Applichem	Histology
Triton X-100, C ₁₄ H ₂₂ O(C ₂ H ₄ O) _n)	A4975,0100	Applichem	Histology
Tween-20, C ₅₈ H ₁₁₄ O ₂₆	A4974,0500	Applichem	Elisa
4% Histofix	256462.0931	Applichem	Flow cytometry
Prolong Diamond antifade mountant	P36970	Thermo Scientific	Histology
DAPI	D1306	Thermo Scientific	Histology

Mouse IFN γ ELISA	DY485	R&D	ELISA
Mouse IL-5 ELISA	DY405	R&D	ELISA
Mouse IL-13 ELISA	DY413	R&D	ELISA
Mouse IL-17 ELISA	DY421	R&D	ELISA
Cytofix/Cytoperm™ Fixation and Permeabilization Solution, 125 mL	554722	BD Biosciences	Flow cytometry
Cytofix™ Fixation Buffer, 100 mL	554655	BD Biosciences	Flow cytometry
Perm/Wash™ Perm/Wash Buffer, 100 mL	554723	BD Biosciences	Flow cytometry
Transcription factor buffer set	562574	BD Biosciences	Flow cytometry

3.6.1.3. Consumables

Table 3: Consumables

Name	Catalog number	Company	Purpose
FACS Tubes	352052	Corning	Flow cytometry
70 μ m mesh	542070	Greiner	Tissue processing
V-bottom 96 well plates		Greiner	Flow cytometry
50 mL falcon tubes	227261	Greiner	Sample processing
15 mL falcon tubes	188271	Greiner	Sample processing
6-well plates	657160	Greiner	Tissue

			processing
24-well plates	662160	Greiner	Cell culture
48-well plates	677180	Greiner	Cell culture
96-well plates Flat bottom	655180	Greiner	Cell culture
Serological pipette 10mL, sterile	607180	Greiner	Cell culture
Serological pipette 25mL, sterile	760180	Greiner	Cell culture
Serological pipette 5mL, sterile	606180	Greiner	Cell culture
Reservoirs, sterile	006.13.055	Interlab	Cell culture
Reservoirs	006.03.055	Interlab	Lab
Parafilm	058.01.002	Interlab	
Weighing paper	037.33.001	Interlab	
Sterile PES Syringe Filters Pore:0.22(µm)	S04-PS22-T	CHROMFILT R	Immunization
RC-L250 multichannel tips	17001118	Rainin	
Cryostat Blades C35	FE2075000 03	Feather	Histology
Superfrost Plus Microscope Slides	J1800AMNZ	Thermo Scientific	Histology
Cryomold	4557	Tissue-Tek	Histology
O.C.T Compound	4583	Tissue-Tek	Histology
4% PFA	30525-89-4	Santa Cruz	Histology
Multichannel pipet-lite L8 - 200XLS+	17013805	Rainin	Pipette
Pipette, single channel, PIPETMAN G starter kit, P20G, P200G, P1000G	F167900	Gilson	Pipette

Pipette pump, electrical	010.01.006	Isolab	Pipette
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3.6.1.4. Mouse Dissection Tools

Table 4: Mice dissection tools

	Catalog number	Company
Delicate operating scissors 4.75in str s/s	RS-6702	Roboz Surgical Instrument
Microdissection forceps 4inches serrated slt crv .8mm tip	RS-5135 Graefe Forceps	Roboz Surgical Instrument
Semken forceps 5inches serrated	RS-5240 Semken Forceps	Roboz Surgical Instrument
Light operating scissors 5inches crv s/s	RS-6753	Roboz Surgical Instrument
Moloney forceps 4.5in slt crv serr	RS-8254 Moloney Forceps	Roboz Surgical Instrument
Instrument tip protectors; assortment pack of 20	IT-1000	Roboz Surgical Instrument
Intramedic Polyethylene Tubing, 0.58mm x 30.5m [for BALF]	598326	Harvard Apparatus

3.6.1.5 Devices

- Autoclave (Systec, VX-150, 166L, SN:8373)

- Biological safety cabinet (ThermoFisher Scientific, class II type A2- SN:41806507)
- Cell analyser, LSR FortessaX20 (BD Biosciences, # 647780S1, SN:H647780S1001, model no:N/A)
- Centrifuge (IKA, model: mini G, SN: 100046858)
- Centrifuge 5702 (Eppendorf, SKU: Z606936EU, SN: 5702DR5336846)
- Centrifuge microCL 17R (ThermoFisher Scientific, # 75002455, SN: 41793777)
- CO2 incubator (MEMMERT, INCOmed 246 0°C/+50°C, SN: 0215-0125)
- Confocal Microscope (Zeiss LSM 880)
- Cryostat (Leica SM 1950)
- Freezer, -20°C (Bosch, 161x70 cm, SN: GSN51AW30)
- Ice maker (HOSHIZAKI, model number: FM-300AKE-N, SN:E11212)
- Microscope, inverted (Carl Zeiss, model: Axio Vert.A1, SN: 3849001095)
- Pipette pump, electrical (Isolab, 0.1-200 ml, # 010.01.006)
- Pipette stand, carrousel (GILSON, SKU: F161401)
- Refrigerator, +4°C (Bosch, model: KSV36AI31/09)
- Spectrophotometer (Thermo Scientific, Multiskan™ FC Microplate Photometer)
- Vortex (ThermoFisher Scientific, LP vortex mixer, SN: EAKT18071, # 88880018)
- Water bath (Nüve laboratory & sterilization technology, model no: NB 9, SN: 02-2531)
- Anesthesia machine (E-Z systems, model: classic, # EZ-150C)
- Biological safety cabinet (LABGARD, class II, model no: NU-S677-400E, SN:182313100617)
- Charcoal filter canister (E-Z systems, ReFresh, # EZ-258)
- Induction chamber (E-Z systems, sure-seal mouse chamber 10.16 cm long x 10.16 cm width x 10.16 cm height, # EZ-177)

3.6.2. Methods

3.6.2.1 Mice

3.6.2.1.1. Common procedures: Most of the research caused no unusual pain, distress, or discomfort to the animals involved, as they were subjected only to simple injections (s.c., i.p.), blood sampling, or terminal euthanasia for organ removal. For painful procedures, animals received anesthesia using inhaled isoflurane. In general, we closely monitored mice during all experiments and humanely euthanized any mouse that appears in distress, based on general appearance, including hunched posture and piloerection of the fur, or that appears otherwise moribund. Any effort made to minimize discomfort, pain, and injury of experimental animals.

3.6.2.1.2. Airway allergen sensitization and challenge model(s):

Adjuvant-induced asthma models: Complete Freund's Adjuvant (CFA) (Sigma, USA), Imject Alum (ThermoScientific, USA), Ovalbumin (OVA) (Hyglos, Germany) were purchased from commercial vendors. Each of the adjuvants is mixed vigorously to homogenize the suspension and yield homogenous solutions. Neutrophilic asthma was induced by one subcutaneous injection (d0) per mouse with 0.5 mg/mL CFA (Complete Freund's Adjuvant, Sigma-Aldrich, St. Louis, MO) mixed with 20 µg OVA (Ovalbumin, Hyglos, Germany) (Bogaert et al. ,2011; Dejager et al. ,2015). Eosinophilic asthma was induced by weekly intraperitoneal injections (d0, d7, d14) per mouse of 1 mg Alum ('Imject Alum', ThermoFisher Scientific) mixed with 20 µg OVA (Bogaert et al. ,2011; Dejager et al. ,2015). Seven days after the last Alum/OVA injection, all mice were challenged with 50 µg OVA/mouse by pharyngeal/laryngeal installation once on two consecutive days (d21, d22). 16-18 hours later, the mice were sacrificed, and the blood, BALFs, and lungs were collected to assess immune responses by flow cytometry, ELISA, and immunohistochemistry or immunofluorescence. The total volume for i.p and s.c injections were 200 µl/mouse where the total volume for the challenge was 50 µl/mouse.

LPS-induced asthma models:

Neutrophilic asthma was induced by weekly administration (d0, d7) of 50 μ l PBS per mouse, containing 10 μ g LPS (Lipopolysaccharide, #L6386, Sigma-Aldrich, St. Louis, MO) and 50 μ g OVA (Ovalbumin, Hyglos, Germany), via pharyngeal/laryngeal aspiration (Mcalees et al. ,2015; Whitehead et al. ,2014). Eosinophilic asthma was induced by weekly administration (d0, d7) of 50 μ l PBS per mouse, containing 0.1 μ g LPS (Lipopolysaccharide, #L6386, Sigma-Aldrich, St. Louis, MO) and 50 μ g OVA (Ovalbumin, Hyglos, Germany) via pharyngeal/laryngeal aspiration (Mcalees et al. ,2015; Whitehead et al. ,2014). Seven days after the last administration, all mice were challenged with 100 μ g OVA/mouse by pharyngeal/laryngeal installation once (d14). 48 hours later, the mice were sacrificed and the blood, BALFs, and lungs were collected to assess immune responses by flow cytometry, ELISA, and immunohistochemistry or immunofluorescence.

LPS-induced lung injury model:

Lung inflammation was induced by three daily administrations (d0, d1, d2) of 50 μ l PBS per mouse, containing 10 μ g LPS (#L6386, Sigma-Aldrich, St. Louis, USA) via pharyngeal/laryngeal aspiration. 16-18 hours after the last challenge, the mice were sacrificed, and lungs, spleens, and mediastinal lymph nodes were collected (Katsura et al. ,2015; Donovan et al. ,2015).

3.6.2.2. Mouse cell preparation

- Blood: Blood was collected from the mice either via retro-orbital collection at prime or via cardiac puncture at the end of the experiment. To obtain sera, the whole blood samples were incubated at 37°C for 30 minutes and centrifuged at 7000 rpm for 20 minutes at 4°C. The upper layer, the clear sera, was collected and stored at -80°C for further ELISA analysis (H Zhao et al. ,2014). To collect PMNs (polymorphonuclear leukocytes), the blood was collected into EDTA-treated tubes. After 1:1 dilution of the blood with PBS, the sample was layered on top of Ficoll

paque PLUS or Lymphoprep solutions to perform density-gradient centrifugation following the manufacturer's instructions. After the centrifugation, the middle layer containing the PBMCs and the layer just above the RBCs that contained the PMNs were collected (Silvestre-Roig et al. ,2019; Marchi et al. ,2014; Ubags et al. ,2018).

- Lung: Single-cell suspensions from mouse lungs were prepared as described (Moro et al. ,2015). Lungs were removed and minced into smaller pieces in a 6-well plate (Greiner, Germany). The digestion mixture, composed of 1 mg/mL collagenase D and 0.1 mg/mL DNase I (both from Roche, Switzerland) in complete RPMI medium (Gibco, USA) supplemented with 5% (v/v) fetal calf serum (FCS) (Gibco, USA), 1% (v/v) Pen-Strep-Glutamine (10.000 U/ml penicillin, 10.000 µg/ml streptomycin, 29.2 mg/ml L-glutamine, Life Technologies, USA) was added to the samples and incubated for 30 min at 37°C on a lateral shaker. The lung samples were filtered through a 100 µm mesh, washed twice with PBS, and the red blood cells were eliminated with ACK lysis buffer (Lonza, USA) and resuspended in complete RPMI medium.

- Broncho-alveolar lavage fluid (BALF): BALF was collected by inflating the murine lungs with 1 mL of ice-cold PBS, which was repeated twice with fresh PBS. The three washes were centrifuged separately at 400 *g* for 7 min at 4°C. The supernatant of the first wash was collected for the ELISA analysis, the cells of all three washes were pooled for the flow cytometric analysis (Moro et al. ,2015).

3.6.2.3. *In vitro* stimulation

In case of intracellular staining the homogenized cells were cultured with complete medium (RPMI 1640, Life Technologies, USA) supplemented with 5% (v/v) fetal calf serum (FCS) (Gibco, USA), 1% (v/v) Pen-Strep-Glutamine (10.000 U/ml penicillin, 10.000 µg/ml streptomycin, 29.2 mg/ml L-glutamine, Life Technologies, USA) and 1.08mM Ca⁺⁺ (Millipore, USA). Lymphocytes were stimulated *in vitro* with

PMA (50 ng/mL) and ionomycin (500 ng/mL) (both Sigma-Aldrich, St. Louis, MO) for four hours at 37°C in the presence of both Brefeldin A (GolgiPlug) and Monensin (GolgiStop). As GolgiPlug and GolgiStop (both BD Biosciences, San Diego, CA) were used together, half the amount recommended by the manufacturer was used, as suggested previously (Siebert et al. ,2008; Sag et al. ,2017).

3.6.2.4. Flow Cytometry

For staining of cell surface molecules, dead cells were labeled with Zombie Dye UV in PBS (1:500, BioLegend) according to the manufacturer's suggestions and washed once with ice-cold PBS. Then, cells were resuspended in staining buffer (PBS, 1% BSA (w/v), 0.01% NaN₃ (w/v) containing FcγR-blocking αCD16/32 (2.4G2) antibody and unconjugated rat IgG (Jackson ImmunoResearch) to prevent non-specific binding and were then stained with fluorochrome-conjugated antibodies according to the manufacturer's recommendations or at empirically determined concentrations for 30 minutes in a total volume of 50 μl. Samples are washed twice with PBS and fixed with 150 μl of Cytofix/Cytoperm buffer (BD Biosciences) on ice for 10 minutes. The cells were washed once with PBS and once with staining buffer. Samples were resuspended in staining buffer and kept at 4°C, protected from light until the samples were analyzed with the flow cytometer. The staining of lymphocytes with CD1d tetramers loaded with PBS57, a α-galactosylceramide homolog, was performed according to NIH Tetramer Facility's suggestion. In brief, cells were stained with the tetramers together with other surface antibodies in a staining buffer at 4°C for 30 minutes. Murine NKT cells were defined throughout as live CD8α⁻ CD19/CD45R⁻ CD44⁺ TCR/CD3ε⁺ CD1d/PBS57-tetramer⁺ cells. For the measurement of transcription factors, the cells were fixed using the fixative within the Transcription Factor Buffer Set (BD Biosciences) for 60 minutes at 4°C. In case the samples were collected from IL10^{GFP} mice, they were fixed instead with freshly prepared 2% formaldehyde (FA) for 40 minutes at 4°C (Heinen et al. ,2014). Later,

cells are permeabilized with the Perm/Wash buffer (BD Biosciences) and incubated overnight with Perm/Wash solution (BD Biosciences) containing fluorochrome-conjugated antibodies and unconjugated rat and mouse IgG. The following day, the samples were washed with Perm/Wash buffer once, followed by additional 5 min incubation on ice in order to decrease unbound antibodies. The cells were measured with an LSR-Fortessa (BD Bioscience) at IBG's Flow Cytometry and Cell Sorting Core Facility as described (G. Wingender et al. ,2015; Sag et al. ,2017). Data were analyzed with FlowJo (Tree Star) software. Graphs derived from digital data are displayed using a 'bi-exponential display' (Roederer et al. ,2004; Tighe et al. ,2019).

CD1d-tetramer-BV421 and CD1d-tetramer-PE were provided by the NIH Tetramer core facility (Atlanta, USA). Zombie Dye UV (BioLegend), fluorochrome-conjugated CD3 ϵ , CD4, CD8 α , CD11b, CD11c, CD19, CD45, CD45.1, CD45R, CD64, CD90.1/2, CD103, CD117, CD127, CD170, Fc ϵ R1, FoxP3, Gata3, IFN γ , IL-4, IL-13, IL-2, IL-17A, Ly6C, Ly6G, MHCII, PLZF, Ror γ t, Sca-1, T-bet and TNF antibodies are purchased from BioLegend, Tonbo, eBioscience, or BD Biosciences.

3.6.2.5. ELISA

The IFN γ , IL-5, and IL-13 cytokine levels in the BALF were measured with the respective Sandwich-ELISA kits (R&D Systems, MN, US) according to the manufacturer's instructions. OVA-specific antibodies were measured in the sera, collected from mice via cardiac puncture, as described (Kuroda et al. ,2016). For detecting total IgG, IgG1, IgG2, and IgA antibodies, the sera were serially diluted and loaded onto 10 μ g OVA-coated plates. Horse-radish peroxidase (HRP)-conjugated anti-mouse IgG, IgG1, IgG2, IgA antibodies (Southern Biotech, USA) were used as detection antibodies. To detect OVA-specific IgE antibodies, the sera were diluted 1/10 with PBS containing 1% (w/v) BSA and loaded onto anti-mouse IgE coated (BD biosciences) plates. anti-OVA-HRP antibodies (AbD Serotec, BioRad) were used as

detection antibody (Kuroda et al. ,2016). The colorimetric change, resulting from the enzymatic reaction between the HRP portion of detection antibody and the substrate TMB, was measured as absorbance at 450 nm (OD450) with a Spectrophotometer (Thermo Scientific, Multiskan™ FC Microplate Photometer). The titers were defined from the reciprocal value of the absorbance at OD450.

3.6.2.6. Tissue Sectioning, Hematoxylin & Eosin (H&E) and Alcian Blue/PAS staining, Immunofluorescence (IF)

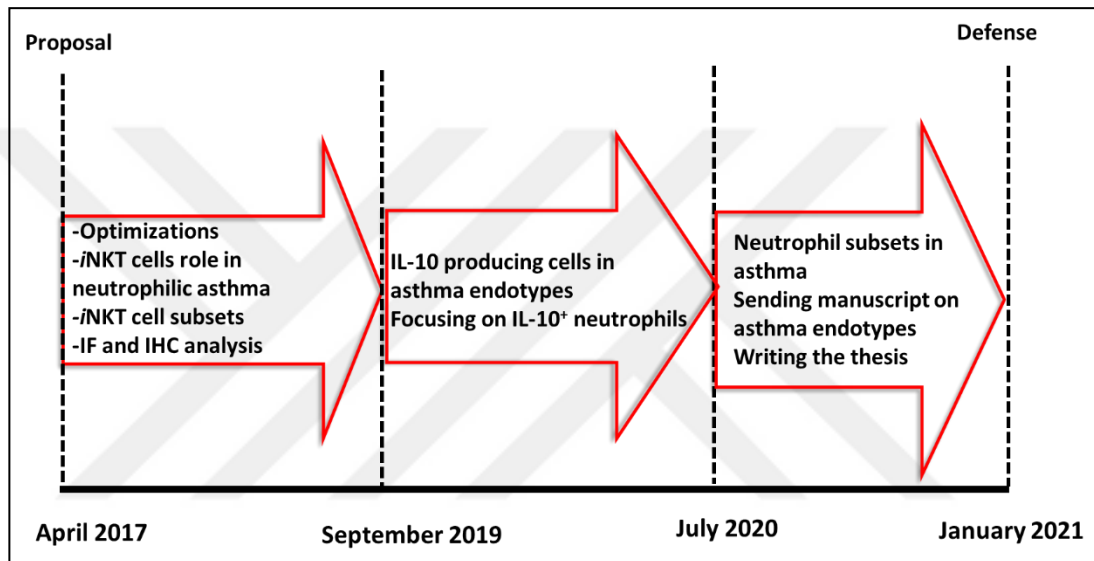
Lungs were inflated with 4% PFA (Cell Signaling, San Diego, US) and fixed for four hours on a lateral shaker at 4°C. After dehydration with 30% sucrose overnight on a lateral shaker at 4°C, samples were embedded in O.C.T, (Tissue Tek, Sakura, US) and snap-frozen. 7-10 µm thick sections were prepared with a cryostat (Leica CM 1950) (H Zhao et al. ,2014).

H&E Staining: The tissue sections were stained with hematoxylin (Sigma, USA) and eosin (Sigma, USA) for two minutes each. Slide contrast was increased by a brief HCl/ethanol treatment (1/1000, v/v) (Sigma, USA). Slides were fixed by ascending concentration of ethanol (70%, 80%, 90%, 95%, 100%) (Sigma, USA) and a final 5 second xylene (Sigma, USA) treatment. Once the slides dried. The tissue sections were stained with an Alcian Blue/PAS staining kit according to the manufacturer's recommendations (Bio optica, Italy). Once the slides dried, they were mounted with entellan and examined with a light microscope (Olympus IX71).

Immunofluorescence (IF): Sample slides were blocked with 3% BSA (v/v) (Applichem, Germany), 0.01% Triton X-100 (v/v) (Applichem, Germany) solution, and incubated overnight with the primary antibody at 4°C. For staining with biotin-conjugated antibodies, after the overnight incubation with the primary antibodies at 4°C, the slides are washed with PBS, and the samples were incubated with the appropriate secondary antibody for 30 min at RT (H Zhao et al. ,2014). After brief

washing, the slides were mounted with ProLong Diamond Antifade reagent (Invitrogen, USA) and visualized by the confocal microscope, Zeiss LSM880 (IBG's Optical Imaging Core Facility).

3.7. Research plan and timeline:



3.8. Data analysis:

Flow cytometric data were collected as either population frequencies (%) or MFI (mean fluorescence intensity). The concentrations of the cytokines IL-5, IL-13, and IFN γ were determined directly via a standard curve calculated with computer-based curve-fitting software using a 4-parameter logistics curve-fitting algorithm. OVA-specific antibody concentrations were calculated as the titers defined from the reciprocal value of the absorbance at OD450 (Zhou et al 2015).

All data are presented as mean and standard error mean (SEM). All statistical analysis was performed with Graphpad Prism 7.0 software. Mann-Whitney U test or one-way ANOVA followed by Holm-Sidak posthoc test are used to compare p values regarded as * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. The tests that are performed to define statistical significance according to (a) number of experimental groups, (b) the size of sample pool in each group and (c) the data distribution Gaussian vs non-Gaussian. Except the data in Figure 12B and Figure 14B, the data are analysed by one-way ANOVA followed by Holm-Sidak posthoc test (experimental groups larger than two, sample pool size $n=10-17/\text{group}$, non-gaussian distribution).

3.9. Limitations of the research:

In this research, asthma endotypes are modeled *in vivo* by using different adjuvants. Due to the use of adjuvants, different pathways involved in the pathology of the airway inflammation might be missed.

The number of mice within experimental groups were calculated using GPower, to determine the baseline number mouse population needed in each experimental group. The mouse number/groups divided among different repetitions (a) to show the reproducibility, (b) to facilitate handling and experiment analyses. Although the distribution of some of the myeloid cell population shows the statistical significance between two asthma endotypes, a larger pool of mice might be needed to assess smaller changes among less frequent myeloid cell subsets.

IBG vivarium encountered an infection among colonies. Taking into account the *in vivo* data cleared according to the level of inflammation observed in the naïve mice by neutrophils, eosinophils and macrophage population levels as well as splenomegaly upon sample collection. The experiments with a 'suspected' level of unexpected inflammation is repeated and those analysis included in the thesis. However we would like to make a note here and would like our result to be evaluated accordingly.

3.10. Ethical committee approval:



DOKUZ EYLÜL ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU

35340, Inciraltı, İzmir-232 4122234

Gündem No/ Toplantı No/Yıl : 03/05/2016

Toplantı Tarihi : 01 Mart 2016

İzmir Uluslararası Biyotıp ve Genom Enstitüsü Müdürlüğü'ne

10/2016 Protokol No'lu; Müdürlüğünüz Öğretim Üyelerinden Yard.Doç.Dr.Gerhard WINGENDER yürütücüsü olduğu "iNKT Hücrelerinin Solunum Yolu Aşırı Duyarlılığı Tepkileri Sırasında Hücre-Hücre Etkileşimleri (Cell-Cell Interactions of iNKT Cells During Airway Hypersensitivity)" isimli; DVM MSc Aslı ÇELİK'in araştırmacı olduğu, projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.



**İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(İBG-HADYEK)
KARARI**

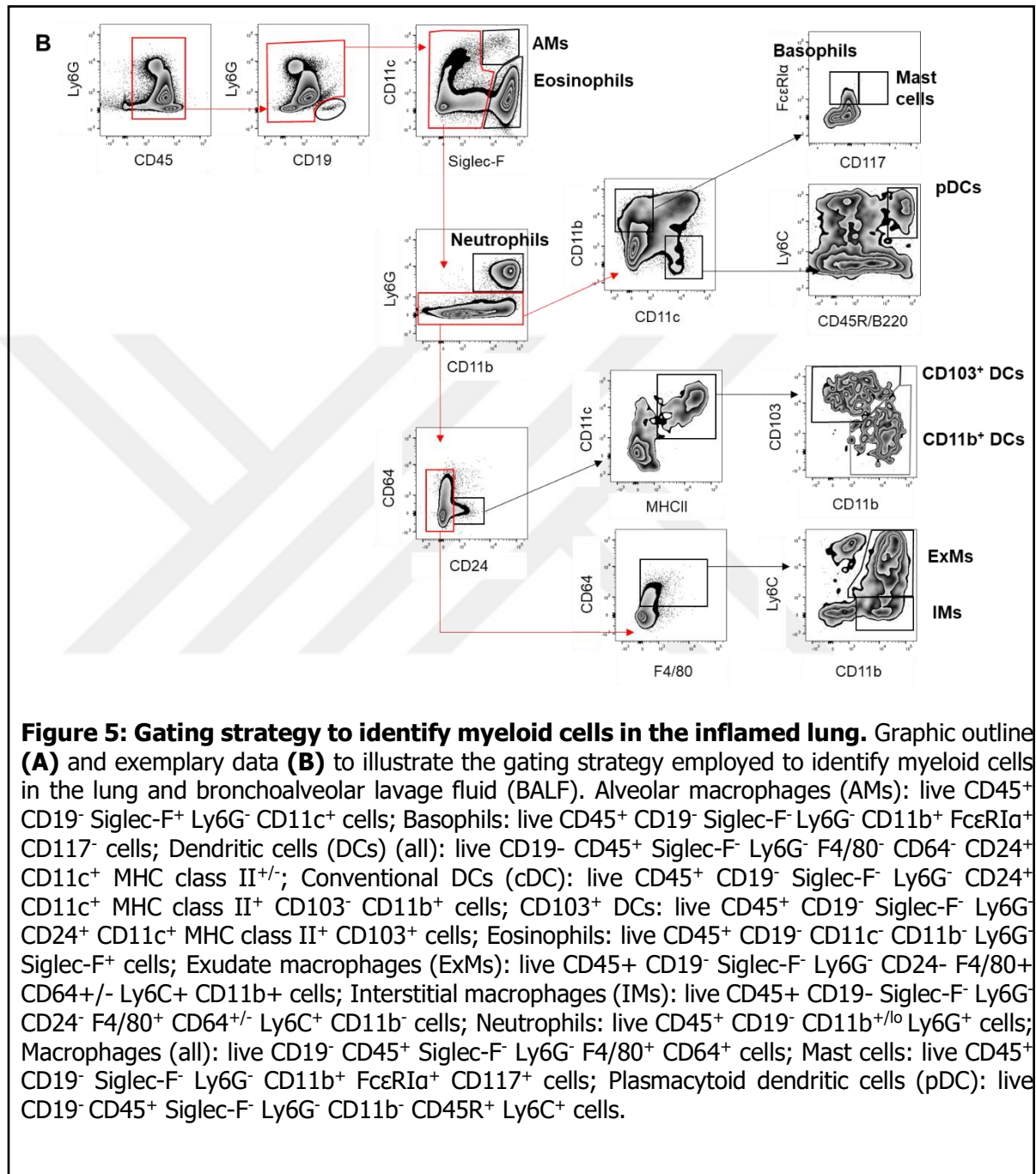
TOPLANTI TARİHİ	13/02/2019	TOPLANTI GÜNÜ	Çarşamba
TOPLANTI SAYISI	2	TOPLANTI SAATİ	15.30

Assoc. Prof. Gerhard Windenger,

05/2019 Protokol No'lu; yürütücüsü olduğunuz “The role of invariant natural killer T (iNK) cells in neutrophilic, Th1-driven, asthma ” başlıklı projenin uygulanmasında etik açıdan sakıncı olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.





We adopted the protocols for two adjuvant-induced mouse models to compare the immune responses in the inflamed lungs during eosinophilic and

neutrophilic asthma side-by-side (Bogaert et al. ,2011; Dejager et al. ,2015; Jang et al. ,2017) (**Figure 6A**). To induce neutrophilic asthma, we administrated the adjuvant Complete Freund's Adjuvant (CFA) subcutaneously mixed with the model antigen ovalbumin (OVA) once, without subsequent boost. To induce eosinophilic asthma, we injected the adjuvant alum mixed with OVA intraperitoneally three times in weekly intervals (d0, d7, 14). Based on the literature (Bogaert et al. ,2011; Dejager et al. ,2015) and our preliminary experiments (data not shown), these two adjuvant-induced mouse models allow the reliable side-by-side comparison of the two different asthma endotypes *in vivo*. To illustrate how well the models reflected the eosinophilic and neutrophilic asthma, we analyzed (i) the eosinophilia and neutrophilia in the inflamed lung, (ii) the IL-5, IL-13, and IFN γ release in the bronchoalveolar lavage fluid (BALF), and (iii) the OVA-specific antibody concentration in the sera (**Figure 6 and Figure 7**). As expected, the cell analysis from the lung showed elevated levels of eosinophils (**Figure 6B**) in the eosinophilic (Alum/OVA) and augmented levels of neutrophils (**Figure 6C**) in the neutrophilic (CFA/OVA) asthma groups. This was mirrored in the BALF (**Figure 6B, C**). Hematoxylin-eosin (H&E) stains lymphocytes and red blood cells and is graded through a red-blue color scale: cell nuclei appear in blue-purple color, basophils and eosinophils are defined by a reddish color scheme, muscles are dark red, red blood cells are cherry red, while a light pinkish color can identify collagen and mitochondria. H&E staining of inflamed lungs showed severe inflammation coupled with extreme bleeding in the neutrophilic (CFA/OVA) group (**Figure 6D**, upper panel). Alcian Blue/PAS stains polysaccharides and mucin-producing cells characterized by a blue-magenta color scale: dark blue color indicates cell nuclei, blue color shows acidic mucins, magenta color for neutral mucins, and blue-purple color indicates acidic-neutral mucins. However, the hyper-mucus secretion demonstrated by Alcian Blue/PAS staining, which is an essential driver of airway

obstruction in asthma (Global initiative for Asthma et al. ,2020), was comparable for both neutrophilic and eosinophilic asthma endotypes (**Figure 6D**, lower panel).

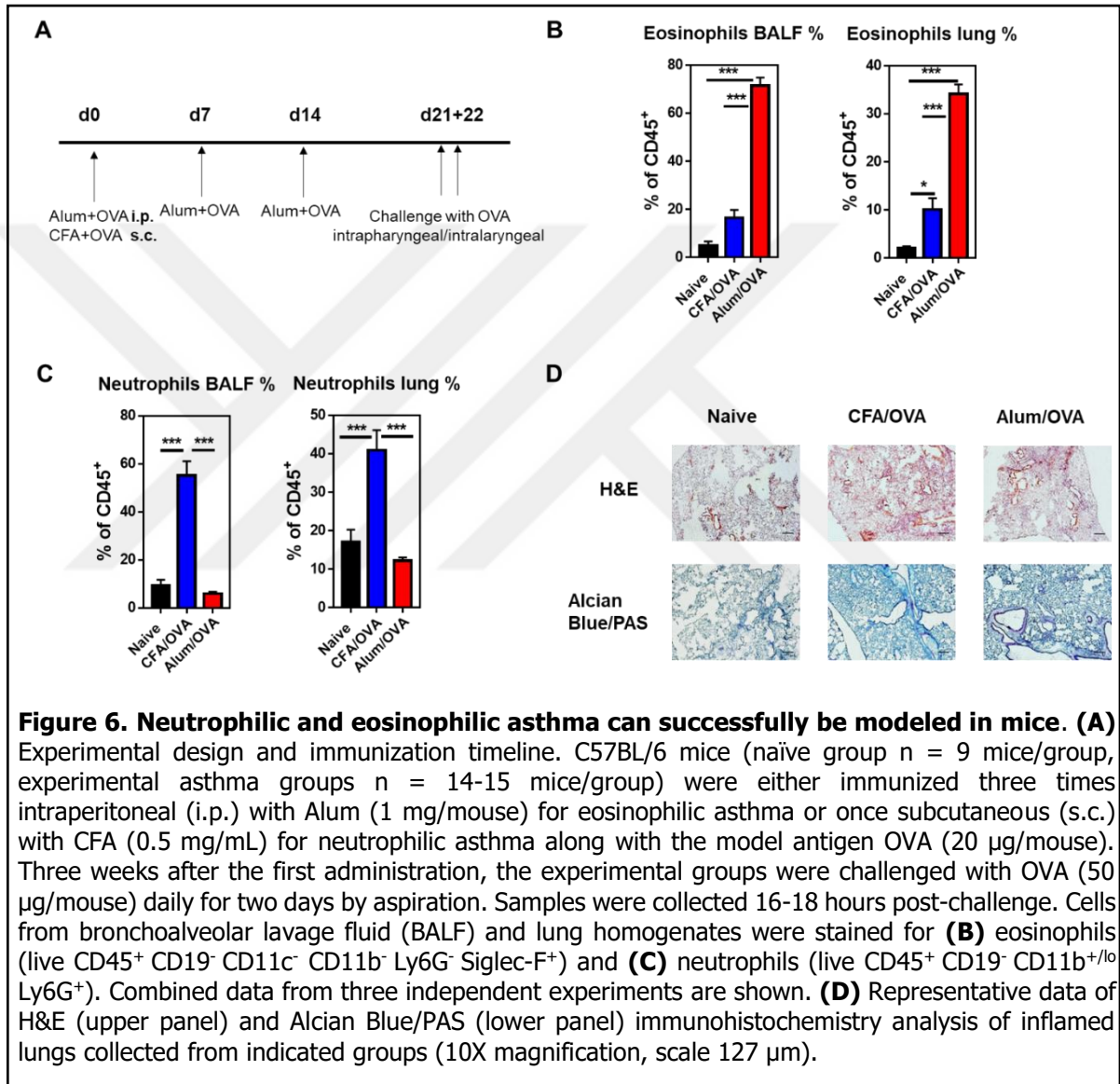
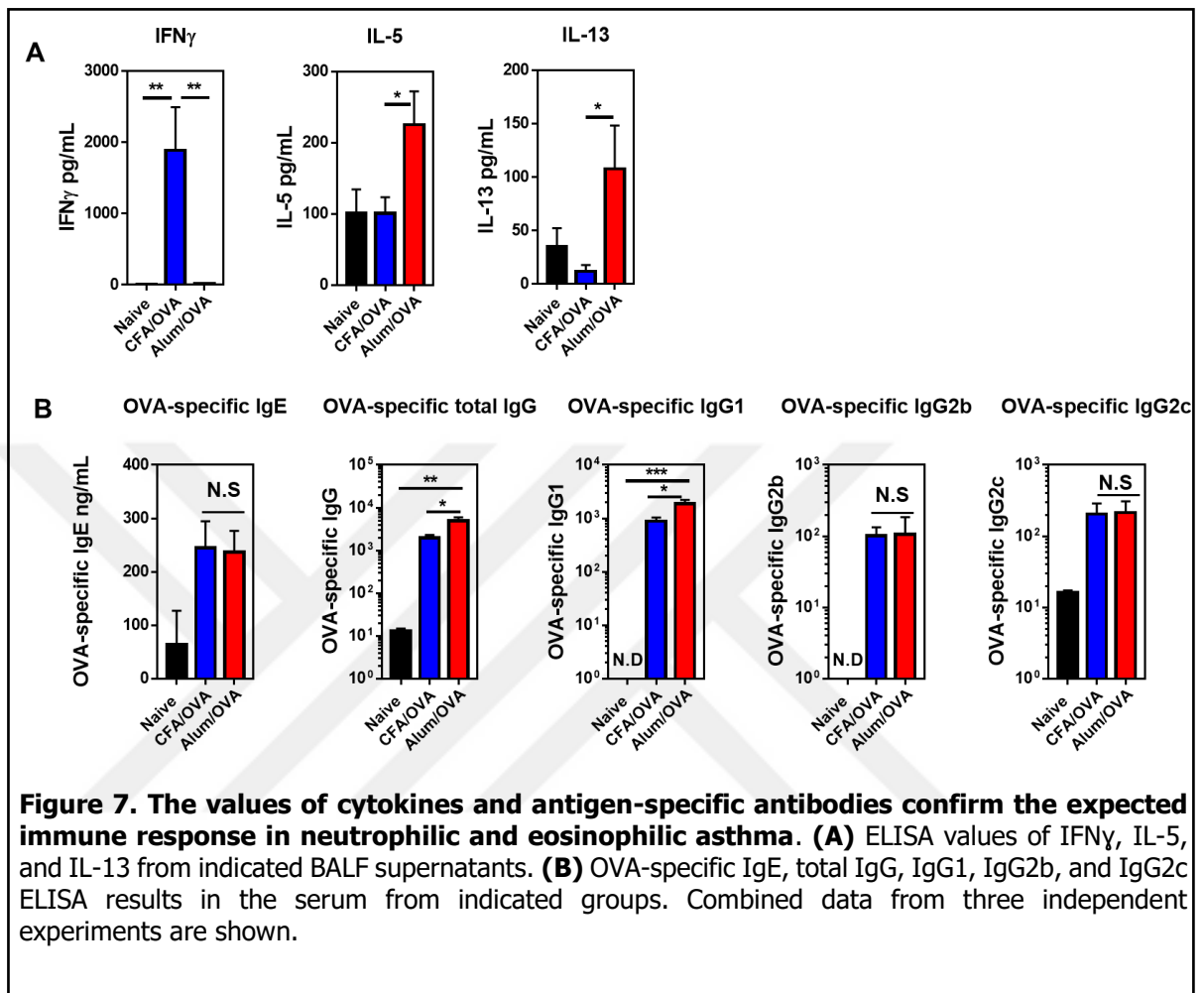


Figure 6. Neutrophilic and eosinophilic asthma can successfully be modeled in mice. (A) Experimental design and immunization timeline. C57BL/6 mice (naïve group n = 9 mice/group, experimental asthma groups n = 14-15 mice/group) were either immunized three times intraperitoneal (i.p.) with Alum (1 mg/mouse) for eosinophilic asthma or once subcutaneous (s.c.) with CFA (0.5 mg/mL) for neutrophilic asthma along with the model antigen OVA (20 μg/mouse). Three weeks after the first administration, the experimental groups were challenged with OVA (50 μg/mouse) daily for two days by aspiration. Samples were collected 16-18 hours post-challenge. Cells from bronchoalveolar lavage fluid (BALF) and lung homogenates were stained for **(B)** eosinophils (live CD45⁺ CD19⁻ CD11c⁻ CD11b⁻ Ly6G⁻ Siglec-F⁺) and **(C)** neutrophils (live CD45⁺ CD19⁻ CD11b^{+/lo} Ly6G⁺). Combined data from three independent experiments are shown. **(D)** Representative data of H&E (upper panel) and Alcian Blue/PAS (lower panel) immunohistochemistry analysis of inflamed lungs collected from indicated groups (10X magnification, scale 127 μm).

Furthermore, in line with the literature (Edwards et al. ,2017; Loza et al. ,2016; I Agache et al. ,2018), the concentration of the Th2 cytokines IL-5 and IL-13 were significantly elevated in eosinophilic asthma, whereas the Th1 cytokine IFN γ predominated in neutrophilic asthma (**Figure 7A**). Next, we determined the systemic immune response via quantification of the antigen-specific antibody concentration in the serum. For serum IgE, higher levels were reported in eosinophilic asthma patients than in neutrophilic asthma patients (W Wu et al. ,2014; Ray et al. ,2017), and α IgE therapy was effective for eosinophilic asthma (W Wu et al. ,2014; Huang et al. ,2019). However, in our mouse models, the OVA-specific serum IgE antibody levels were comparable in the eosinophilic (Alum/OVA) and neutrophilic (CFA/OVA) asthma groups (**Figure 7B**). Furthermore, and in line with clinical data (Ray et al. ,2017; Verduyn et al. ,2019), the antigen-specific serum IgG and IgG1 antibody levels were significantly higher in the eosinophilic than the neutrophilic asthma endotype (**Figure 7B**). No difference was observed for OVA-specific IgG2b and IgG2c antibody levels (**Figure 7B**).

Together, these data demonstrate that the mouse models used here successfully replicate key features of human eosinophilic and neutrophilic asthma and are suitable for investigating asthma endotypes.



4.1.1 Neutrophilic asthma is characterized by high numbers of plasmacytoid dendritic cells in the BALF

We observed a more robust influx of leukocytes into the BALF during neutrophilic (CFA/OVA) than eosinophilic (Alum/OVA) asthma, with up to 4×10^5 total cells/mL (**Figure 8A**). The frequency of myeloid cell populations recovered from the BALF, in descending order, were neutrophils/eosinophils (**Figure 6B, 6C**), monocytes, macrophages, dendritic cells (DCs) (**Figure 8A**), and few basophils (**Figure 8B**), and mast cells (**Figure 8C**). In contrast to the BALF, basophils (**Figure 8B**) and mast cells (**Figure 8C**) were enriched in the lung tissue of mice with eosinophilic (Alum/OVA) asthma. These data suggest that basophils and mast cells do not migrate to the BALF during lung inflammation. Although DCs constituted less than 1% of the BALF cells (**Figure 8A**), they were previously associated with the induction of Th2 immune responses in patients with allergic asthma (Upham et al. ,2002). To better understand the role of DCs in eosinophilic and neutrophilic asthma, we determined the frequency of DC subsets in the inflamed lung as CD11b⁺ conventional (myeloid) DC (cDCs), CD103⁺ lung resident DCs (CD103⁺ DCs), and CD45R⁺ plasmacytoid dendritic cells (pDCs). Both CD11b⁺ cDC (**Figure 8D**) and lung resident CD103⁺ DCs (**Figure 8E**) were more frequent in the lung tissue of mice with eosinophilic (Alum/OVA) than neutrophilic (CFA/OVA) asthma. In contrast, the frequency of pDCs did not change in the lung during the inflammation (**Figure 8F**). However, pDCs appeared to be the only DC subtype that populated the alveolar space, which was more prominent during neutrophilic (CFA/OVA) than eosinophilic (Alum/OVA) asthma (**Figure 8F**).

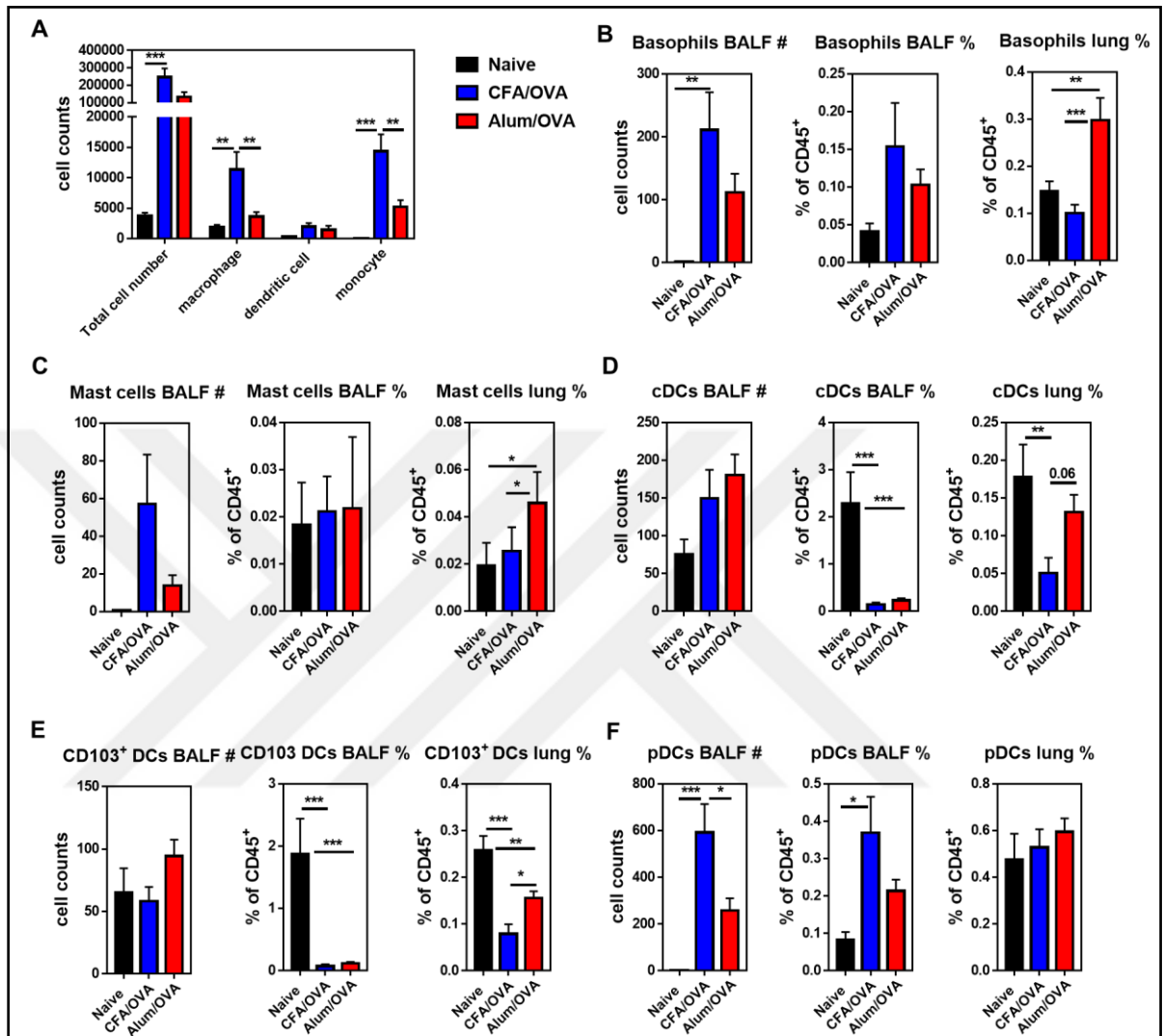
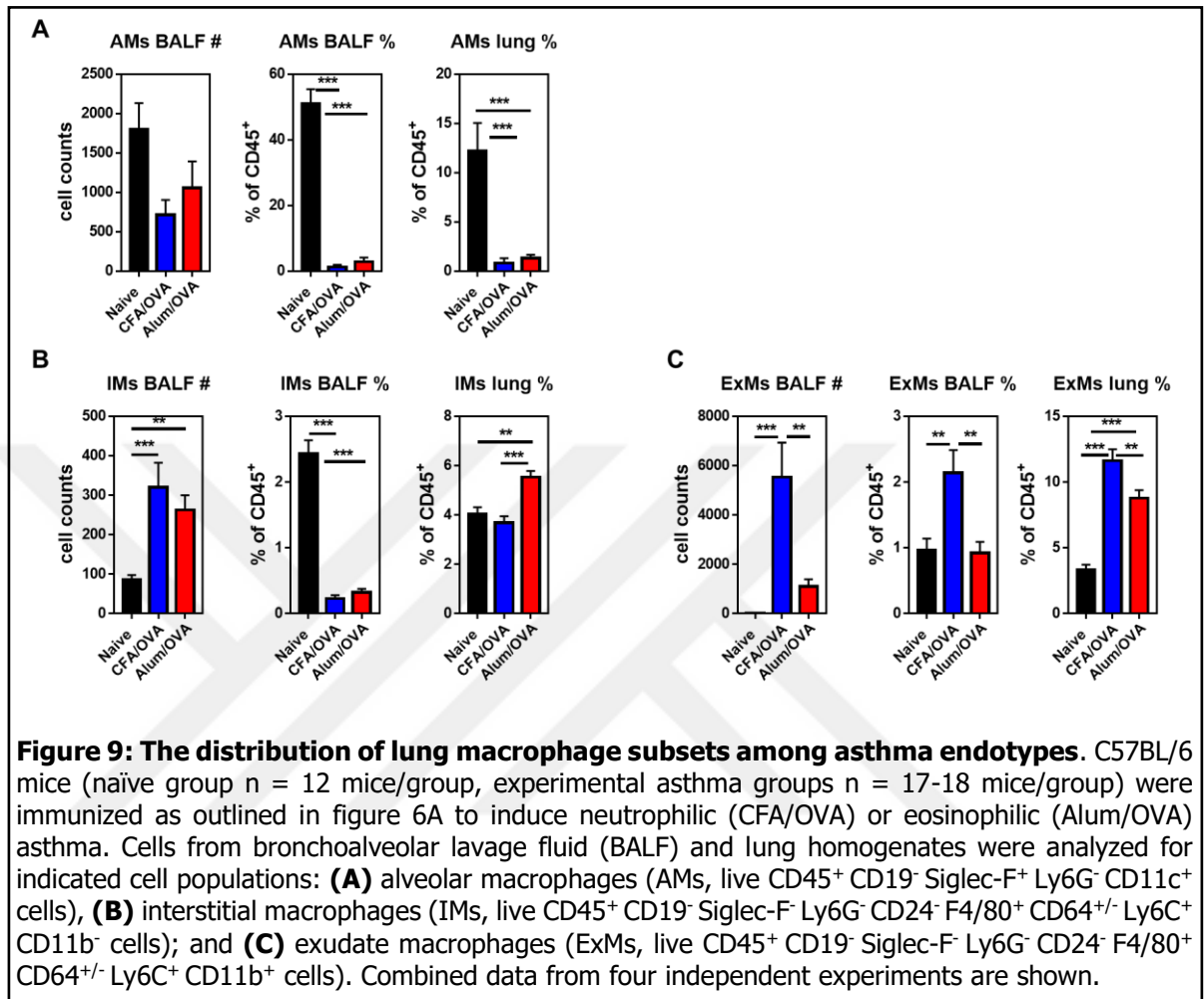


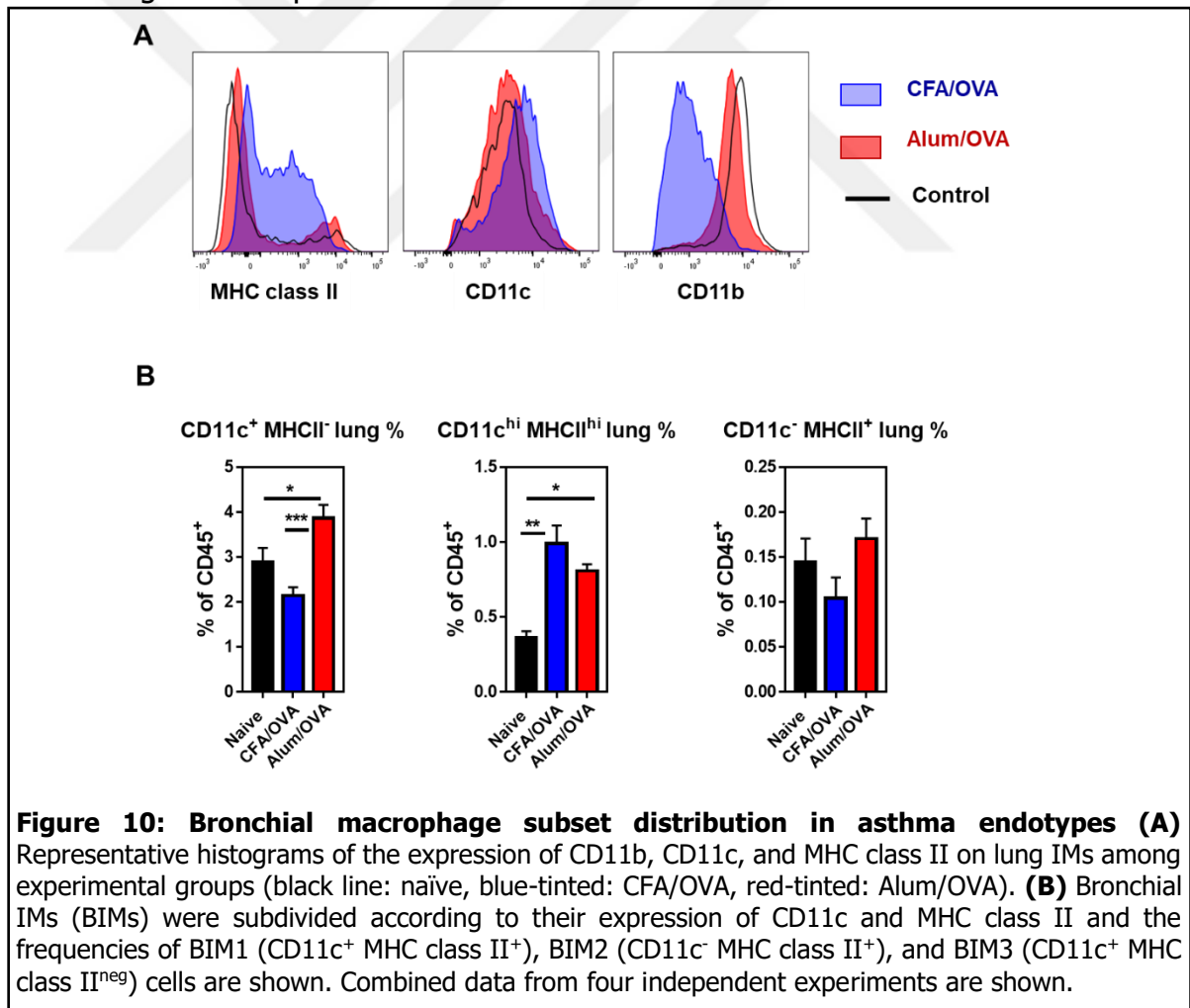
Figure 8. DC subsets, mast cells, and basophils are differentially recruited to the inflamed lungs in neutrophilic and eosinophilic asthma. C57BL/6 mice (naïve group $n = 12$ mice/group, experimental asthma groups $n = 17-18$ mice/group) were immunized as outlined in Figure 6A to induce neutrophilic (CFA/OVA) or eosinophilic (Alum/OVA) asthma. Cells from bronchoalveolar lavage fluid (BALF) and lung homogenates were analyzed for indicated cell populations. **(A)** Total cell counts of all leukocytes (live CD45⁺ cells), macrophages (live CD19⁻ CD45⁺ Siglec-F⁻ Ly6G⁻ F4/80⁺ CD64⁺), dendritic cells (live CD19⁻ CD45⁺ Siglec-F⁻ Ly6G⁻ F4/80⁻ CD64⁻ CD24⁻ CD11c⁺ MHC class II⁺), and monocytes (live CD19⁻ CD45⁺ Siglec-F⁻ Ly6G⁻ F4/80⁺ CD64⁻ CD24⁻ CD11c⁻ MHC class II⁺ Ly6C⁺) in the BALF. Total cell counts and/or relative cell frequencies of **(B)** basophils (live CD45⁺ CD19⁻ Siglec-F⁻ Ly6G⁻ CD11b⁺ FcεRIα⁺ CD117⁻ cells); **(C)** mast cells (live CD45⁺ CD19⁻ Siglec-F⁻ Ly6G⁻ CD11b⁺ FcεRIα⁺ CD117⁺ cells); **(D)** conventional DCs (cDC, live CD45⁺ CD19⁻ Siglec-F⁻ Ly6G⁻ CD24⁺ CD11c⁺ MHC class II⁺ CD103⁻ CD11b⁺ cells); **(E)** CD103⁺ DCs (live CD45⁺ CD19⁻ Siglec-F⁻ Ly6G⁻ CD24⁺ CD11c⁺ MHC class II⁺ CD103⁺ cells); and **(F)** plasmacytoid dendritic cells (pDC, live CD19⁻ CD45⁺ Siglec-F⁻ Ly6G⁻ CD11b⁻ CD45R⁺ Ly6C⁺) in indicated organs. Combined data from four (A, D) or three (B, C) independent experiments are shown.

4.1.2. The frequency of exudate and interstitial macrophages markedly differs between asthma endotypes

In the steady-state, the lung houses two main subsets of macrophages, alveolar macrophages (AMs) and interstitial macrophages (IMs) (Byrne et al. ,2016). AMs, which represent the major portion of lung resident macrophages, are tissue-resident macrophages of embryonic origin that self-renew in the lung (Ginhoux et al. ,2016; Evren et al. ,2020). In contrast, IMs are derived from blood monocytes infiltrated into the lung parenchyma (Gibbings et al. ,2017; Evren et al. ,2020). Another blood monocyte-derived macrophage population migrate into the lung during lung inflammations and are named non-resident Ly6C^{hi} or exudate macrophages (ExMs) (Taut et al. ,2008; Osterholzer et al. ,2011; ,2013; Herbold et al. ,2010). Since pulmonary macrophages have pivotal roles in resolving lung inflammations (Gibbings et al. ,2017; McQuattie-Pimentel et al. ,2018), we measured the frequency of AMs, IMs, and ExMs in the BALF and lungs of the two asthma endotypes. In line with the massive increase of macrophages in the lung (**Figure 9A**), the frequency of AMs considerably declined in the BALF and the lungs of both asthma groups compared to the control group (**Figure 9A**). As IMs are located mainly in the lung parenchyma (Evren et al. ,2020), it was expected that their numbers would not significantly increase in the BALF, indicated by a sharp decline in their relative frequency in the BALF (**Figure 9B**). Interestingly, however, we observed an increase in the frequency of IMs in the lungs of the eosinophilic (Alum/OVA) inflammation group (**Figure 9B**). In contrast, the influx of ExMs was more prominent in the neutrophilic (CFA/OVA) than the eosinophilic (Alum/OVA) asthma group, which was particularly striking in the BALF (**Figure 9C**).

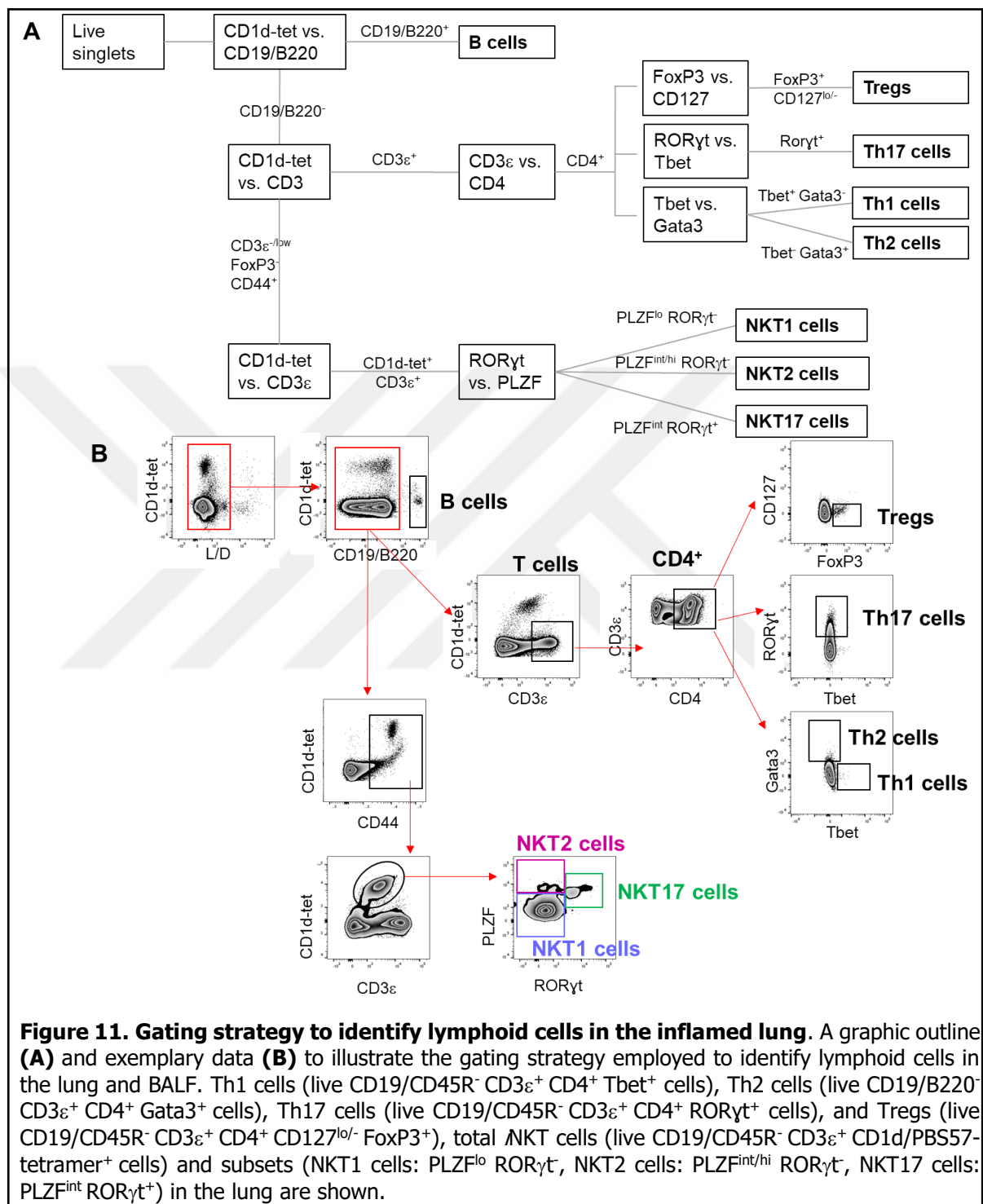


It was recently suggested that the IMs themselves could be classified into three subsets, based on their expression of CD11b, CD11c, and MHC class II (Gibbins et al. ,2017). When these subsets were analyzed (**Figure 10A, B**), we noticed that the IM population in the eosinophilic (Alum/OVA) inflammation mainly consisted of the CD11c^{lo} MHC class II^{neg} population (**Figure 10C**), which was described to have the highest phagocytic activity and the lowest turnover rate among the IM subsets (Gibbins et al. ,2017). Together, these data indicate that eosinophilic and neutrophilic asthma involve significantly different macrophage populations, with IMs increasing in the lungs with eosinophilic asthma and ExMs increasing in neutrophilic asthma.



4.2. Adaptive immune response in adjuvant-induced asthma models

The adaptive immune response is essential to resolve chronic inflammations (Iwasaki et al. ,2015). The type 1 immune response is mainly associated with the clearance of bacterial/viral infections by inducing Th1 and cytotoxic T cells, and by the release of the pro-inflammatory cytokines, IFN γ , TNF, and IL-1 β (Iwasaki et al. ,2015; ,2017). In contrast, the type 2 immune response is characterized by Th2 cells and group 2 innate lymphoid cells (ILC2s), and the production of the cytokines IL-4, IL-5, and IL-13 (Gause et al. ,2013). To analyze the adaptive immune cells, we designed another flow cytometry panel to identify T cell and NKT cell subsets, as well as B cells according to their expression of Tbet, Gata3, ROR γ t, PLZF, and FoxP3 (**Figure 11**).



4.2.1. Th17 cells are significantly expanded in neutrophilic asthma

Both conventional CD4⁺ T cells (Robinson et al. ,2010; Gelfand et al. ,2017) and innate-like invariant Natural Killer T (NKT) cells (Omid Akbari et al. ,2003; O Akbari et al. ,2006; Lisbonne et al. ,2003; Wingender et al. ,2011) are involved in asthma in humans and in mouse models. Therefore, we measured the subset distributions of CD4⁺ T cells (Th1, Th2, Th17, Treg) in mice with eosinophilic or neutrophilic asthma. Surprisingly, we did not observe a significant difference for the frequency of Th1 and Th2 cells and of Tregs in the lungs of mice with eosinophilic (Alum/OVA) or neutrophilic (CFA/OVA) asthma (**Figure 12A**). Similarly, the production of the cytokines IFN γ , IL-4, IL-13, and TNF by CD4⁺ T cells did not differ between the eosinophilic (Alum/OVA) and neutrophilic (CFA/OVA) asthma groups (**Figure 12B**). The activity of Th17 cells and elevated levels of IL-17 are indicators of neutrophilic asthma (J Zhao et al. ,2012; Chesné et al. ,2014; Hinks et al. ,2016). Our analysis showed that the frequency of Th17 cells in the lung (**Figure 12A**), as well as the production of IL-17 by CD4⁺ T cells (**Figure 12B**), was significantly higher in the lungs of mice with neutrophilic (CFA/OVA) than with eosinophilic (Alum/OVA) asthma.

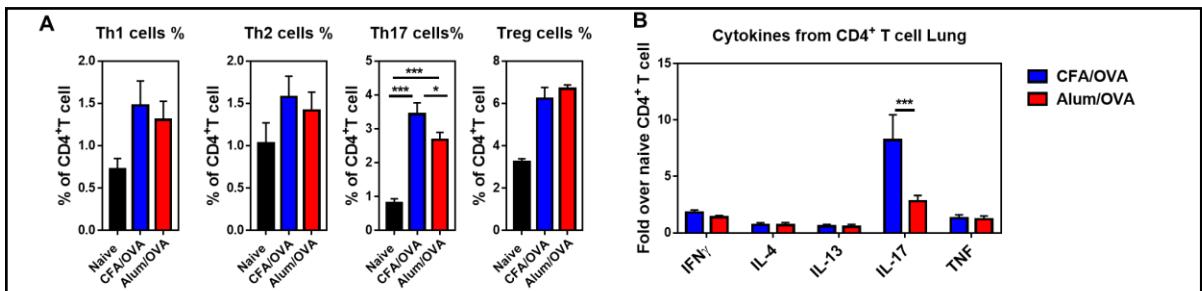
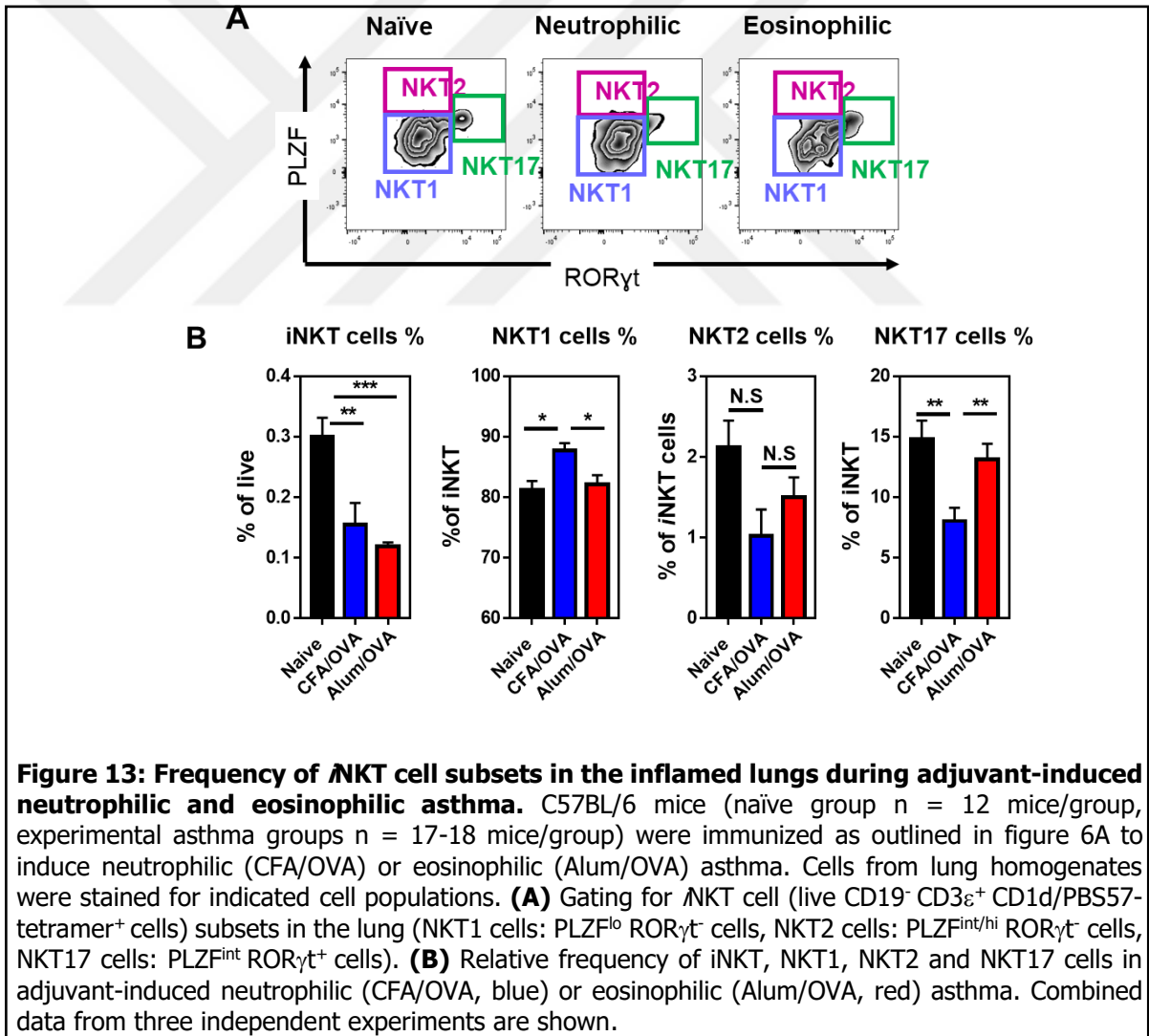


Figure 12. Frequency of CD4⁺ T cell subsets in the inflamed lungs during neutrophilic and eosinophilic asthma. C57BL/6 mice (naïve group n = 12 mice/group, experimental asthma groups n = 17-18 mice/group) were immunized as outlined in figure 6A to induce neutrophilic (CFA/OVA) or eosinophilic (Alum/OVA) asthma. Cells from lung homogenates were stained for indicated cell populations. **(A)** The relative cell frequency of lung Th1 cells (live CD19⁻ CD3 ϵ ⁺ CD4⁺ FoxP3⁻ Tbet⁺ cells), Th2 cells (live CD19⁻ CD3 ϵ ⁺ CD4⁺ FoxP3⁻ Gata3⁺ cells), Th17 cells (live CD19⁻ CD3 ϵ ⁺ CD4⁺ FoxP3⁻ ROR γ t⁺ cells), and Tregs (live CD19⁻ CD3 ϵ ⁺ CD4⁺ CD127^{lo/-} FoxP3⁺ cells) is shown. **(B)** Production of the indicated cytokines by lung CD4⁺ T cells following *in vitro* stimulation with PMA and ionomycin. Combined data from four (A) or two (B) independent experiments are shown.

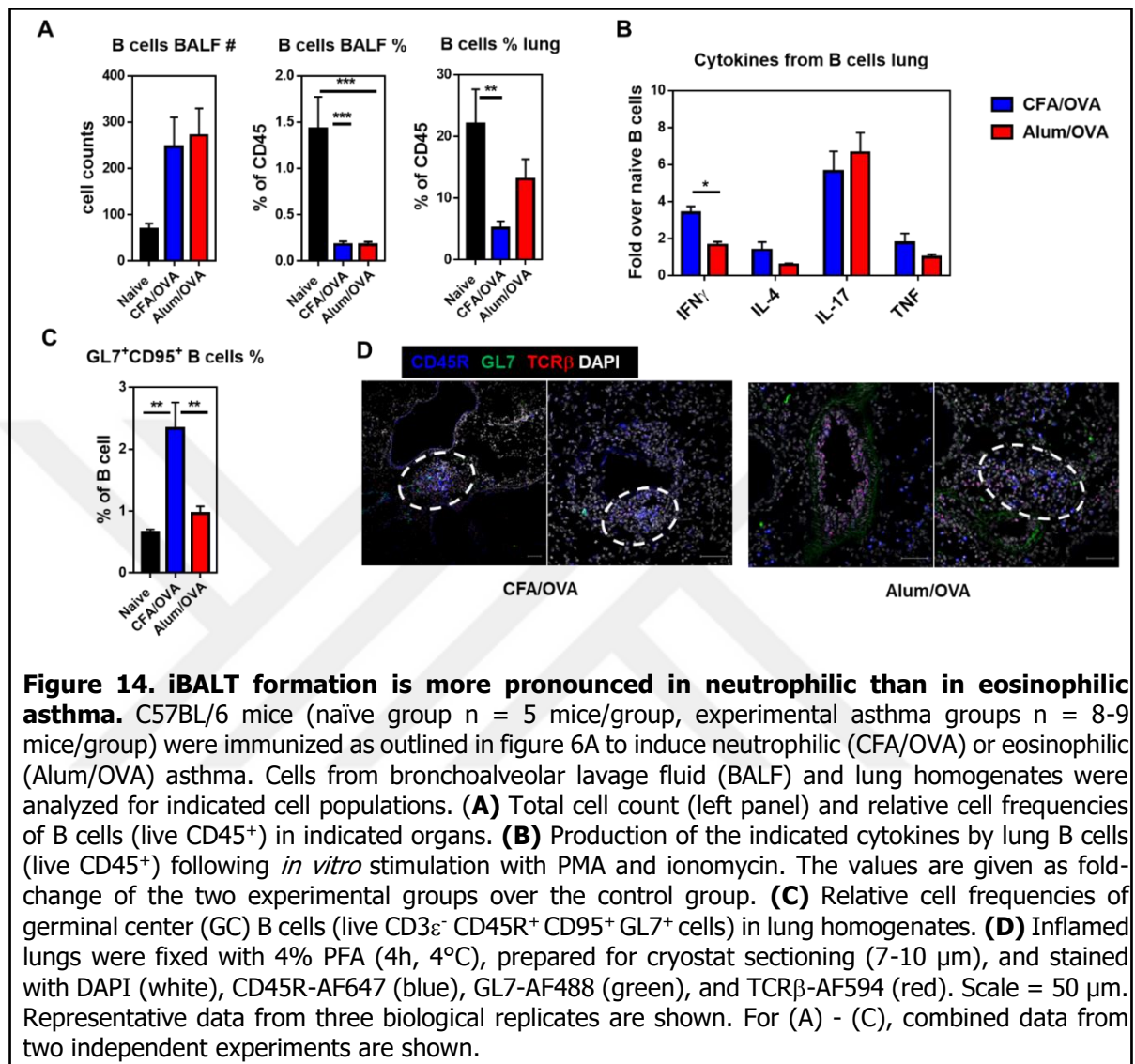
4.2.2. Analysis of NKT cell subsets in asthma endotypes

We determined the frequency of NKT1 , NKT2 , and NKT17 cells in the inflamed lungs (**Figure 13A**). In the adjuvant-induced asthma models, the lung NKT cells (**Figure 13B**) were mostly NKT1 cells (**Figure 13B**), and their frequency increased during neutrophilic (CFA/OVA) asthma (**Figure 13B**). We did not detect any changes in the lungs for NKT2 cells (**Figure 13B**). However, the frequency of NKT17 cells decreased during neutrophilic (CFA/OVA) asthma (**Figure 13B**), which was, interestingly, the opposite of the changes observed for Th17 cells (Figure 8B).



4.2.3. Neutrophilic asthma is characterized by an increased frequency of activated B cells

Besides their ability to produce allergen-specific antibodies (Lambrecht et al. ,2015), B cells are involved in the pathogenesis of chronic lung inflammation either by presenting antigens to T cells (Wypych et al. ,2018) or by the formation of 'inducible bronchus-associated lymphoid tissue' (iBALTs) (Foo et al. ,2010). iBALTs are tertiary lymphoid structures that are rapidly induced and provide an effective local site to drive lymphocyte activation and immune response in the lung (Marin et al. ,2019). To assess the B cell response during eosinophilic (Alum/OVA) and neutrophilic (CFA/OVA) asthma, we measured the frequency of B cells and their cytokine production, as well as the frequency of germinal center (GC) B cells in the lungs. Although the overall frequency of B cell was comparable between the BALFs and lungs with eosinophilic (Alum/OVA) or neutrophilic (CFA/OVA) asthma (**Figure 14A**), the IFN γ production of B cells from lungs with neutrophilic asthma was significantly higher than in the neutrophilic asthma group (**Figure 14B**). We did not observe a difference for IL-4, IL-17, or TNF production by the B cells (**Figure 14B**). Importantly, we noticed a significant increase of CD45R/B220⁺ GL7⁺ CD95⁺ germinal center (GC) B cells in the lungs of mice with neutrophilic (CFA/OVA) asthma, which was not observed in the lungs of mice with eosinophilic (Alum/OVA) asthma (**Figure 14C**). These data indicate that iBALT formation was only supported during neutrophilic asthma in our mouse models, a finding that was supported by the immunofluorescence of the inflamed lungs (**Figure 14D**).



4.3. The role of *NKT* cell in the pathogenesis of neutrophilic asthma

NKT cells were shown to modulate the induction of airway hyperreactivity and airway inflammation in the mouse model of eosinophilic asthma. Research with *NKT* cell-deficient *CD1d*^{-/-} or *Jα18*^{-/-} mice demonstrated that *NKT* cells could exacerbate airway hypersensitivity and asthma pathogenesis (Hachem et al. ,2005; O Akbari et al. ,2010). However, their role in neutrophilic asthma has not been investigated.

4.3.1. *NKT* cell are not involved in CFA-induced neutrophilic asthma

To determine whether *NKT* cells are involved in the pathogenesis of neutrophilic asthma, we immunized *NKT* cell-deficient *Jα18*^{-/-} mice along with their C57BL/6 controls with the previously adopted CFA-driven model for neutrophilic asthma (Figure 6A). We found that *NKT* cell deficiency did not affect the neutrophil influx to the inflamed lungs and BALF (**Figure 15A**). Although the pDCs and ExMs were predominant in neutrophilic asthma, pDC levels were not altered by *NKT* cell deficiency (Figure 8F, Figure 9C). Interestingly, the ExM population was reduced due to a lack of *NKT* cells (**Figure 15B, C**). Further assessment is needed to decipher the relation of *NKT* cells and ExMs. Additionally, neither IFN γ production (**Figure 15D**) nor Th17 activity (**Figure 15E**) was affected by *NKT* cell deficiency.

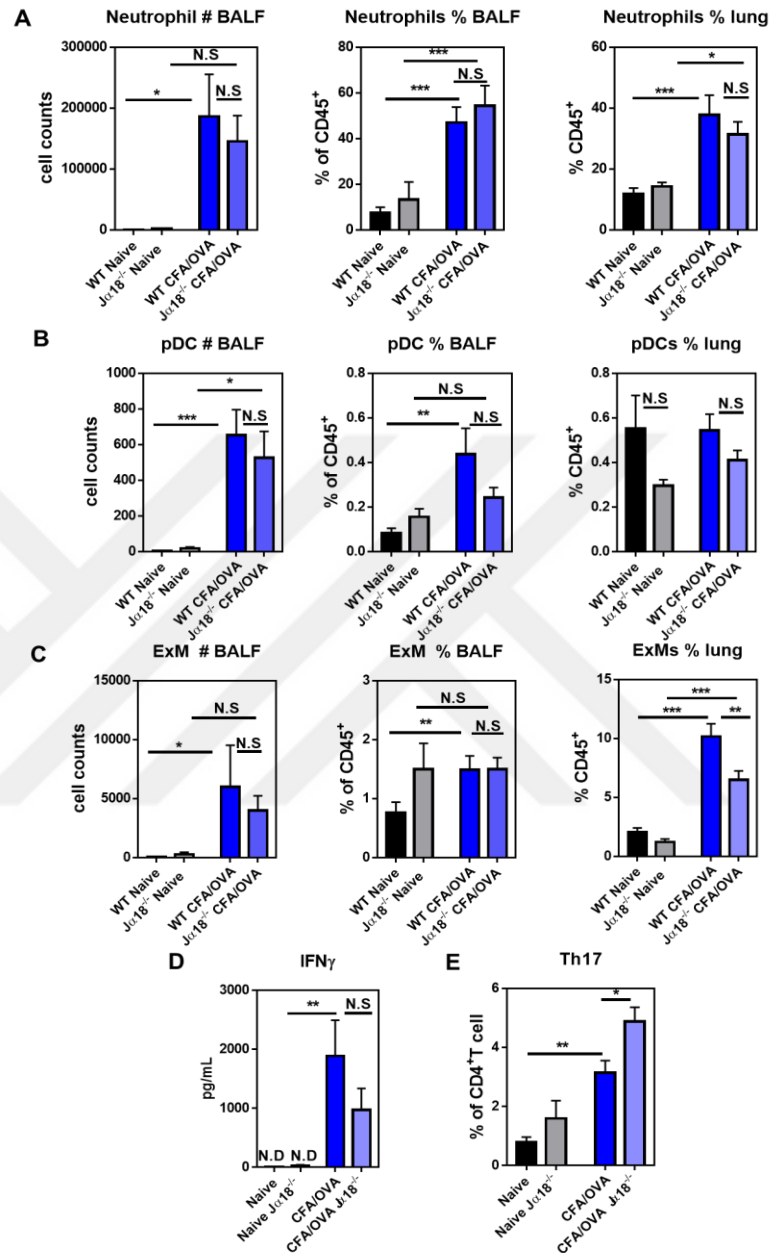
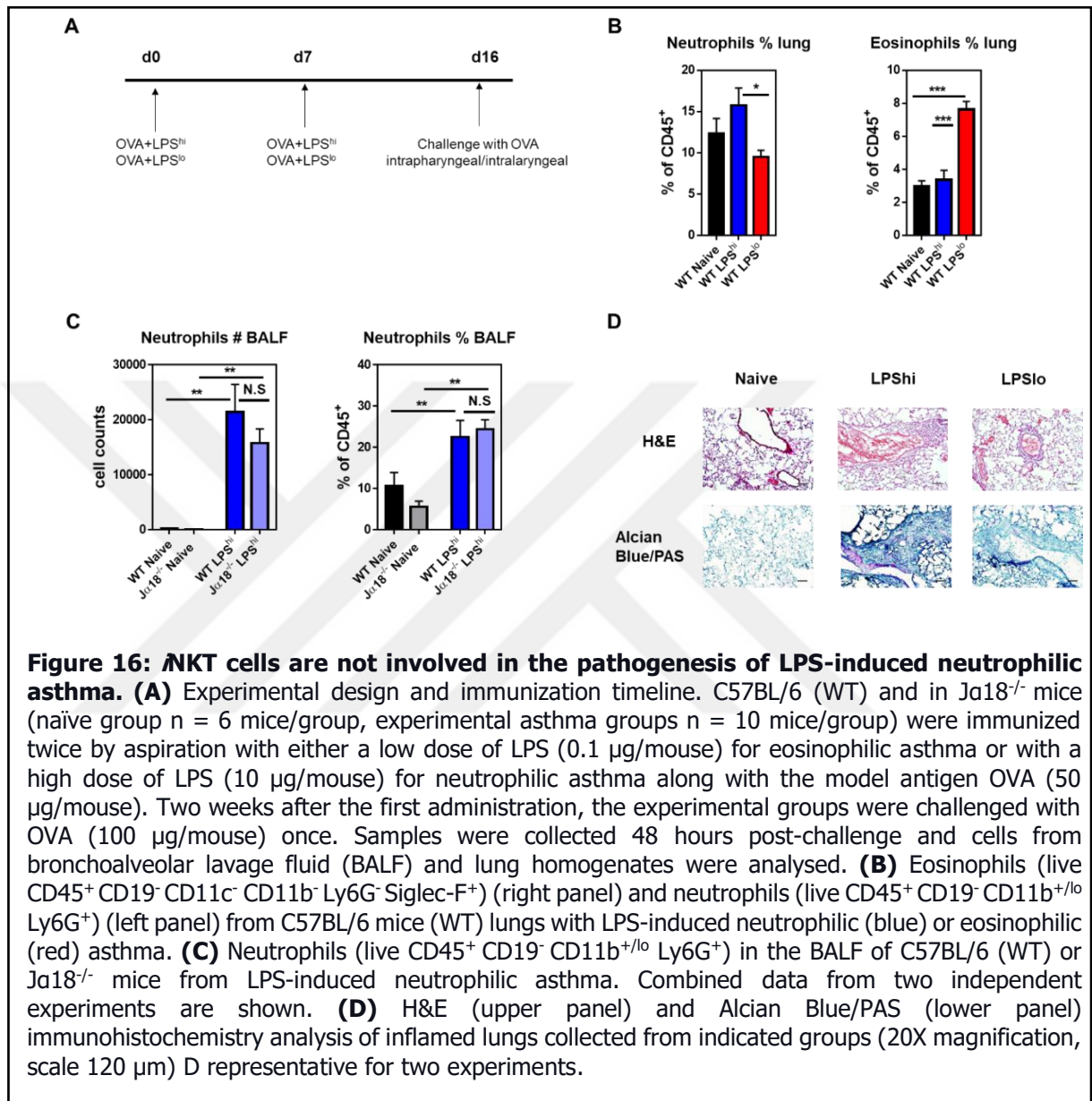


Figure 15. *NKT* cells are not involved in the pathogenesis of neutrophilic asthma. C57BL/6 or *NKT* cell-deficient *Jα18^{-/-}* mice (naïve group $n = 12$ mice/group, experimental asthma groups $n = 17-18$ mice/group) were either immunized three times intraperitoneal (i.p.) with Alum (1 mg/mouse) for eosinophilic asthma or once subcutaneous (s.c.) with CFA (0.5 mg/mL) for neutrophilic asthma along with the model antigen OVA (20 μ g/mouse). Three weeks after the first administration, the experimental groups were challenged with OVA (50 μ g/mouse) daily for two days by aspiration. Samples were collected 16-18 hours post-challenge. Cells from bronchoalveolar lavage fluid (BALF) and lung homogenates were stained for **(A)** neutrophils (live CD45⁺ CD19⁻ CD11b^{+/lo} Ly6G⁺), **(B)** plasmacytoid dendritic cells (pDC, live CD19⁻ CD45⁺ Siglec-F⁻ Ly6G⁻ CD11b⁻ CD45R⁺ Ly6C⁺), and **(C)**

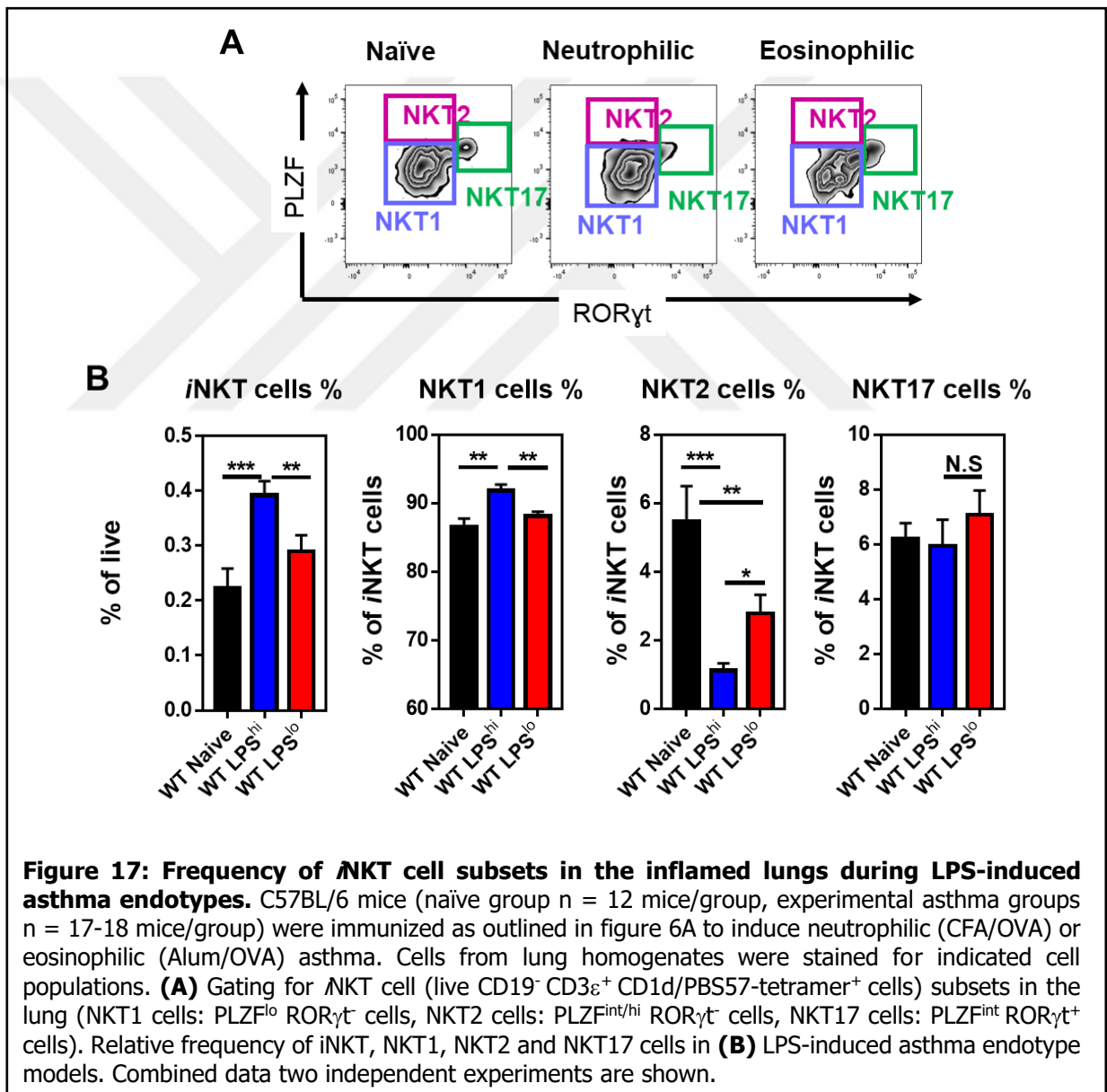
exudate macrophages (ExMs, live CD45⁺ CD19⁻ Siglec-F⁻ Ly6G⁻ CD24⁻ F4/80⁺ CD64^{+/-} Ly6C⁺ CD11b⁺ cells). **(D)** ELISA values of IFN γ from the BALF supernatants of indicated groups. **(E)** The relative cell frequency of lung Th17 cells (live CD19⁻ CD3 ϵ ⁺ CD4⁺ FoxP3⁻ ROR γ t⁺ cells) is shown. Combined data from three (A-D) or two (E) independent experiments are shown.

4.3.2 $\mathcal{N}$ KT cells are not involved in the pathogenesis of LPS-induced neutrophilic asthma

To further verify that $\mathcal{N}$ KT cells are redundant in the pathogenesis of neutrophilic asthma, we used another model to induce neutrophilic asthma. It is possible to induce eosinophilic or neutrophilic asthma by administering low or high concentration of LPS, respectively (Whitehead et al. 2015) (**Figure 16A**). After establishing that the LPS-induced asthma endotype models work in our hands (**Figure 16B**), we compared the high-dose LPS model in J α 18^{-/-} and C57BL/6 mice. Similar to the data we obtained from the CFA-induced model (Figure 15), the neutrophilia persisted in the $\mathcal{N}$ KT cell-deficient mice (**Figure 16C**). Although immunohistochemical analysis showed lung inflammation upon LPS inhalation (aspiration described at Materials and Methods), there was no significant difference in the severity between the two endotypes (**Figure 16D**).

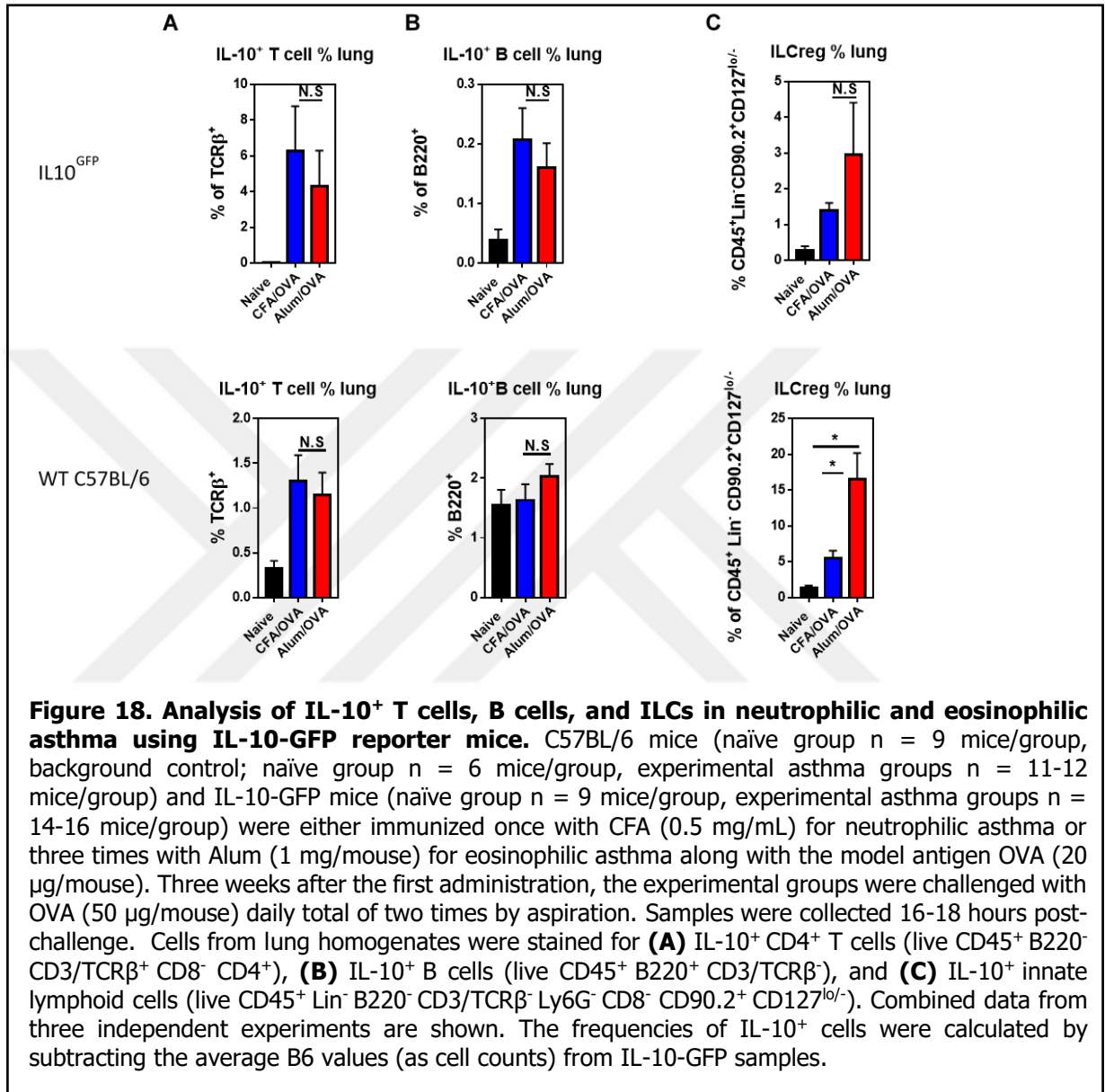


In the LPS-induced asthma models, NKT1 cells were more prevalent in neutrophilic (LPS^{hi}) asthma, whereas NKT2 cells were more abundant in eosinophilic (LPS^{lo}) asthma (**Figure 17B**). Interestingly, similar to alum-induced eosinophilic asthma, the NKT17 cell population expanded slightly more in the LPS-induced eosinophilic than neutrophilic asthma group, but this increase was not statistically significant (**Figure 17B**).



4.4. IL-10 secreting cells in adjuvant-induced asthma endotypes

IL-10 is an anti-inflammatory cytokine responsible for the modulation of local immune responses via subsiding immune cell activity. Many cell types secrete IL-10, which is often produced upon pro-inflammatory cytokine release. The dysregulated release of IL-10 is involved in many respiratory diseases including, asthma, allergic airway disease (AAD), chronic obstructive pulmonary disease (COPD), and pulmonary infections (Branchett 2019). In the lung, FoxP3⁺ and FoxP3⁻ regulatory T cells (Branchett 2019), CD8⁺ T cells (Sun 2015), B cells (A-R Kim et al. ,2016; F Braza et al. ,2015), alveolar macrophages (AM) (Bracken 2015), interstitial macrophages (IM) (Kawano 2016), airway and alveolar epithelial cells (Branchett 2019), as well as airway-associated dendritic cells (DCs) (Branchett 2019) were shown to limit lung inflammation via IL-10 secretion. However, due to the low percentage of IL-10⁺ cells within most cell populations and due to the low intensity of the flow cytometric staining of intracellular IL-10, the clear identification of IL-10 producing cells is challenging (Sag 2017). Therefore, various reporter mouse lines were developed to avoid the need for intracellular cytokine staining to identify IL-10 producing cells (Bouba 2016). Here, we used the IL-10^{GFP} (VeRT-X) mouse strain, where an IRES-enhanced green fluorescence fusion protein (GFP) is inserted downstream of the *Il10* locus (Madan 2007). We analyzed the IL-10 production via the IL10^{GFP} signal in T cells, B cells, myeloid cells, and ILCs, from immunized IL-10^{GFP} and C57BL/6 controls mice. We found that the frequency of IL-10⁺ T and B cells were comparable between both asthma endotypes (**Figure 18A, B**), whereas ILC_{regs} increased during eosinophilic asthma (**Figure 18C**). We also confirmed that the IL-10-signal derived from the GFP reporter in these lymphoid cells adequately represented actual IL-10 production by comparing the values with the ICCS staining of C57BL/6 control cells (**Figure 18A, B, C lower panels**).



However, when we compared the IL10^{GFP} and IL-10 ICCS signals of myeloid cells, we noticed that an increased background signal masked the true GFP signal, especially in neutrophils (**Figure 19**). The frequency of IL-10^{GFP} - positive neutrophils in the BALF was significantly higher in mice with neutrophilic asthma

than eosinophilic asthma (**Figure 19A, upper panel**). In contrast, the frequency of IL10^{GFP} neutrophils was comparable between endotypes in the inflamed lungs (**Figure 19B, upper panel**). However, when we quantified the IL-10 production via ICCS in similar treated C57BL/6 WT mice, no difference for IL-10⁺ neutrophils in the BALF was observed (**Figure 19A, lower panel**) and in the lungs, the IL-10⁺ neutrophils expanded in eosinophilic asthma (**Figure 19B, lower panel**).

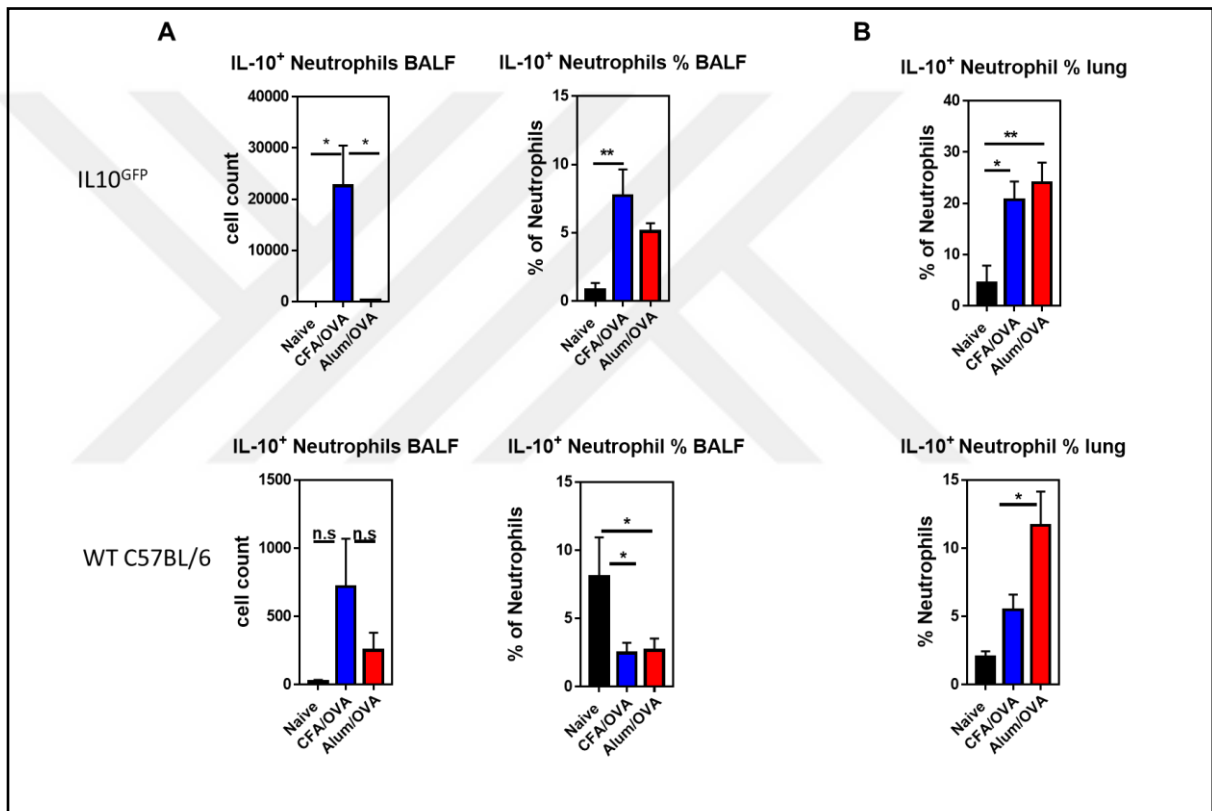
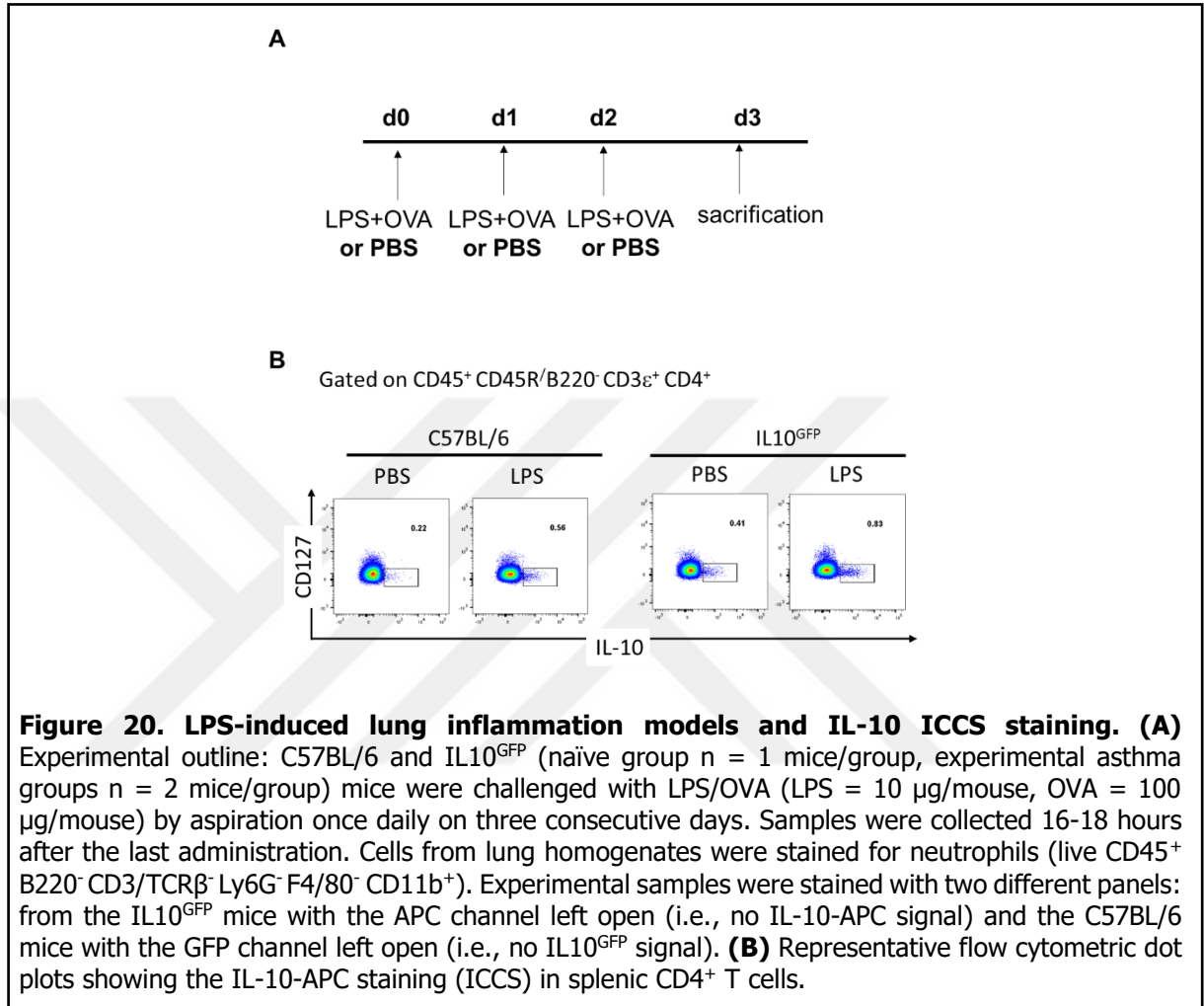
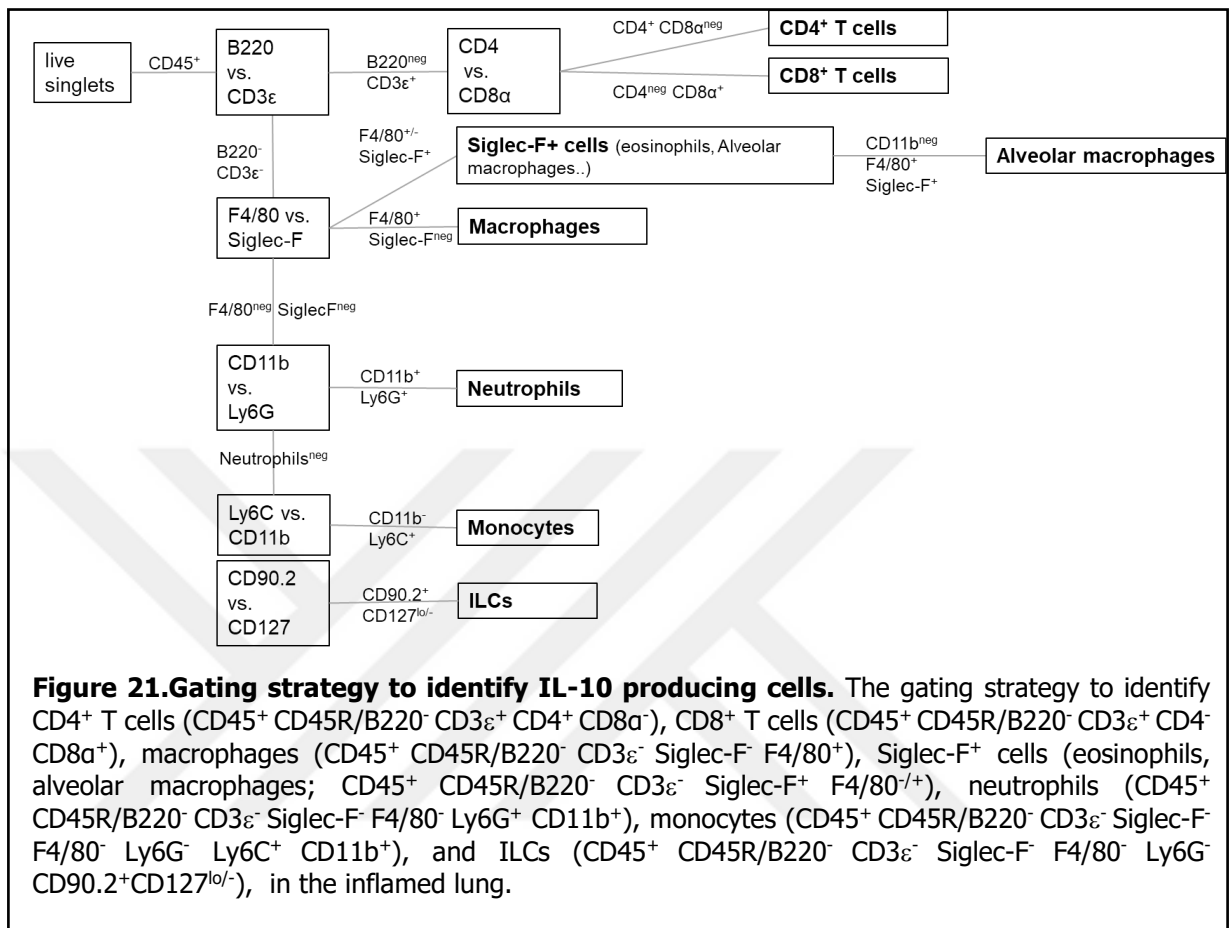


Figure 19: The frequencies of IL-10⁺ neutrophils measured with IL10^{GFP} reporter mice or with ICCS do not match in the inflamed lung. C57BL/6 mice (naïve group n = 9 mice/group, background control; naïve group n = 6 mice/group, experimental asthma groups n = 11-12 mice/group) and IL-10^{GFP} mice (naïve group n = 9 mice/group, experimental asthma groups n = 14-16 mice/group) were either immunized once with CFA (0.5 mg/mL) for neutrophilic asthma or three times with Alum (1 mg/mouse) for eosinophilic asthma along with the model antigen OVA (20 µg/mouse). Three weeks after the first administration, the experimental groups were challenged with OVA (50 µg/mouse) daily total of two times by aspiration. Samples were collected 16-18 hours post-challenge. Cells from broncho-alveolar lavage fluid (BALF) (**A**) and lung homogenates (**B**) were analysed for IL-10 production of neutrophils (live CD45⁺ B220⁻ CD3/TCRβ⁻ Ly6G⁺ F4/80⁻ CD11b⁺) via IL10^{GFP} (top panel) or via ICCS (bottom panel). Combined data from two (lower panel) or three (upper panel) independent experiments are shown. For IL10^{GFP} mice experiments, the frequencies of IL-10⁺ cells were calculated by subtracting the average B6 values (as cell counts) from IL10^{GFP} samples.

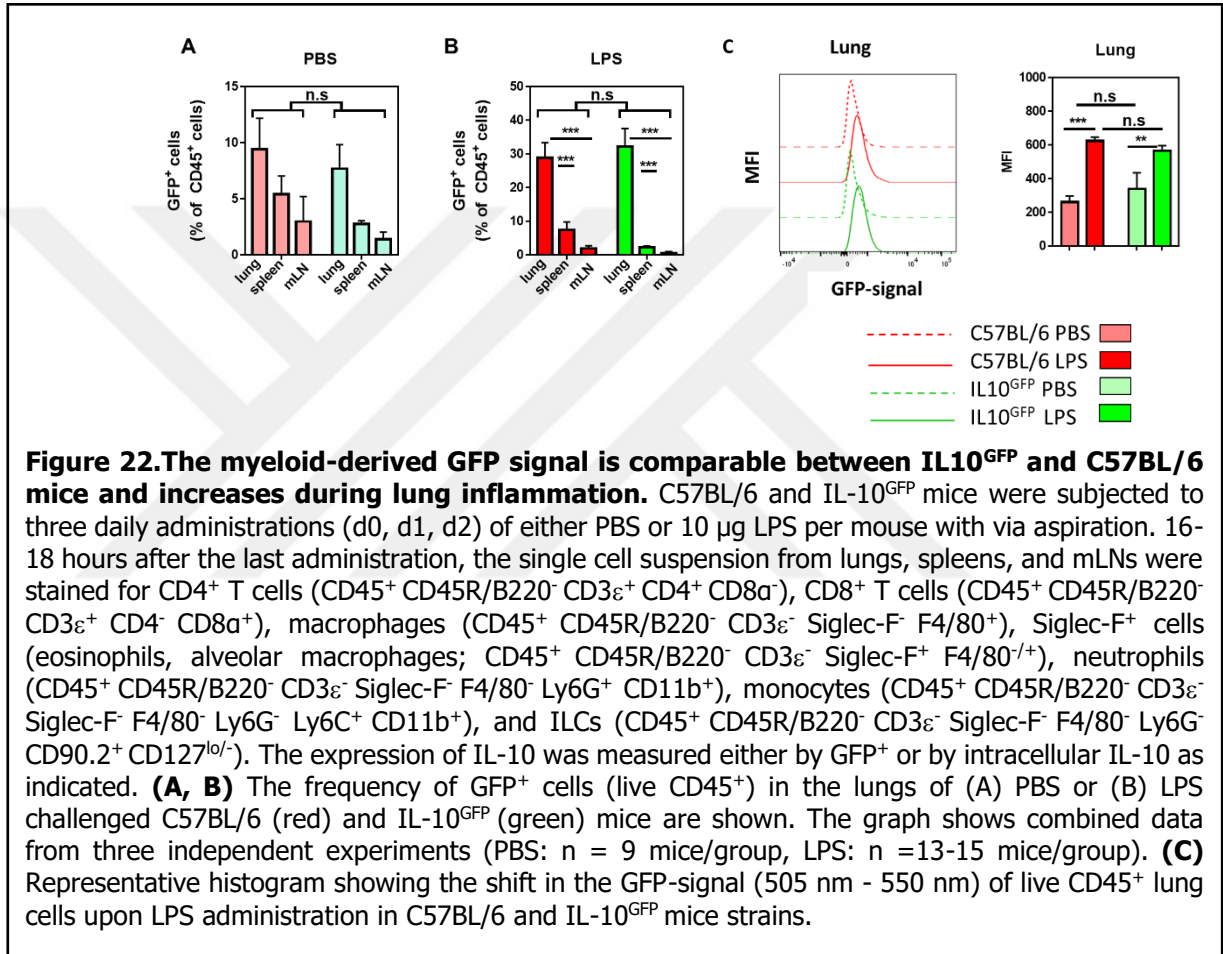


To investigate this background issue further, we turned to the LPS-induced lung inflammation models (**Figure 20A**), due to its shorter experimental timeline, allowing us to progress faster. After verifying that we can successfully stain IL-10 production via ICCS (**Figure 20B**), we analyzed IL-10 production by CD4⁺ T cells, CD8⁺ T cells, alveolar macrophages, macrophages, neutrophils, monocytes, and ILCs, as identified as outlined in figure 21.



We aimed to analyze the reliability of the GFP-derived vs. ICCS-derived IL-10 signal in different tissues, including lung, mLN, and spleen. During our analysis, we noticed that the background signal in the GFP-channel (505 nm - 550 nm) of leukocytes from lung, spleen, or mediastinal lymph node (mLN) was comparable in non-inflamed controls of either C57BL/6 (WT) or IL-10^{GFP} mice (**Figure 22A**). Upon LPS-induced lung inflammation, the GFP-signal increased, particularly in the lungs (**Figure 22B**). Nevertheless, the GFP-signal from WT and IL-10^{GFP} mouse-derived cells remained comparable for the percentage (**Figure 22A, B**) or the mean fluorescence intensity (MFI, **Figure 22C**), irrespective of the organ analyzed and the inflammatory condition. These data suggest that the GFP-signal detected in the

IL-10^{GFP} - derived cells was a background signal. To further define which specific cell types produce IL-10, we directly compared the IL-10 signal derived from the GFP-signal or ICCS for CD4⁺ and CD8⁺ T cells, neutrophils, and macrophages, Siglec-F⁺ cells (eosinophils, alveolar macrophages), monocytes, and ILCs.



We determined that GFP-derived IL-10 signal from CD4⁺ and CD8⁺ T cells well correlate with ICCS-derived IL-10 signals (**Figure 23A, B**). However, the frequency of IL-10⁺ neutrophils (**Figure 23C**), macrophages (**Figure 23D**), or Siglec F⁺ cells (**Figure 23E**) was much higher when based on the GFP-signal than when based on the ICCS data. Additionally, the GFP-signals derived from monocytes (**Figure 23F**) and ILCs (**Figure 23G**) were substantially higher only in the lung tissue, suggesting a tissue-specific component to the GFP-channel autofluorescence.

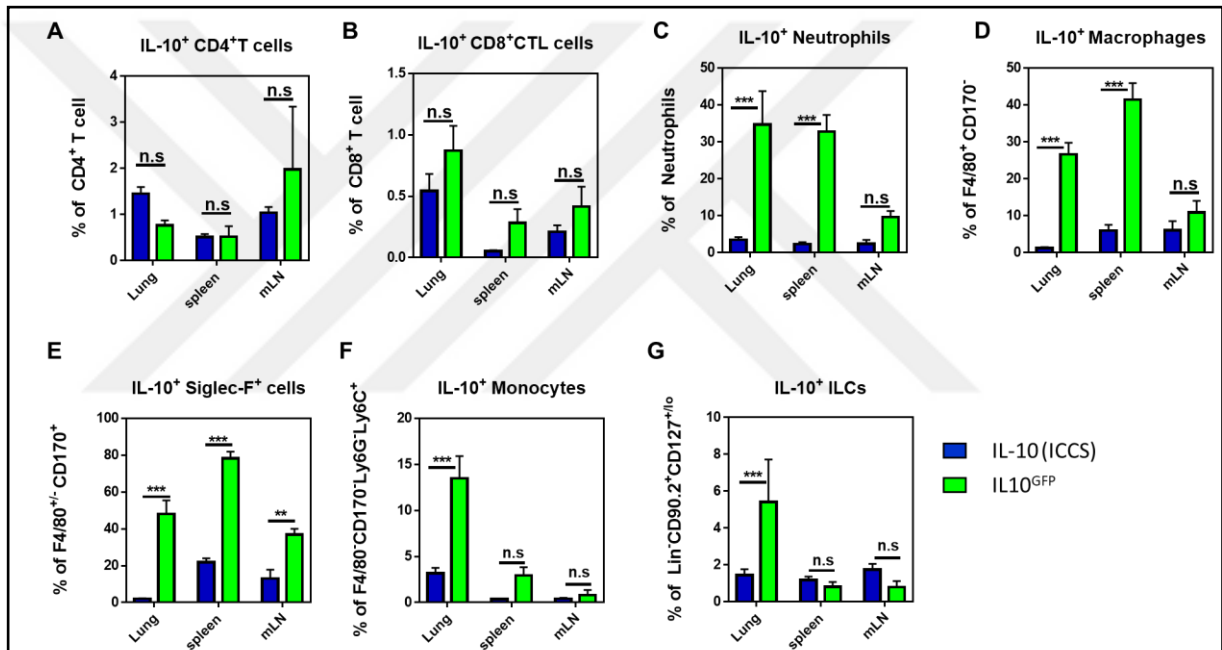
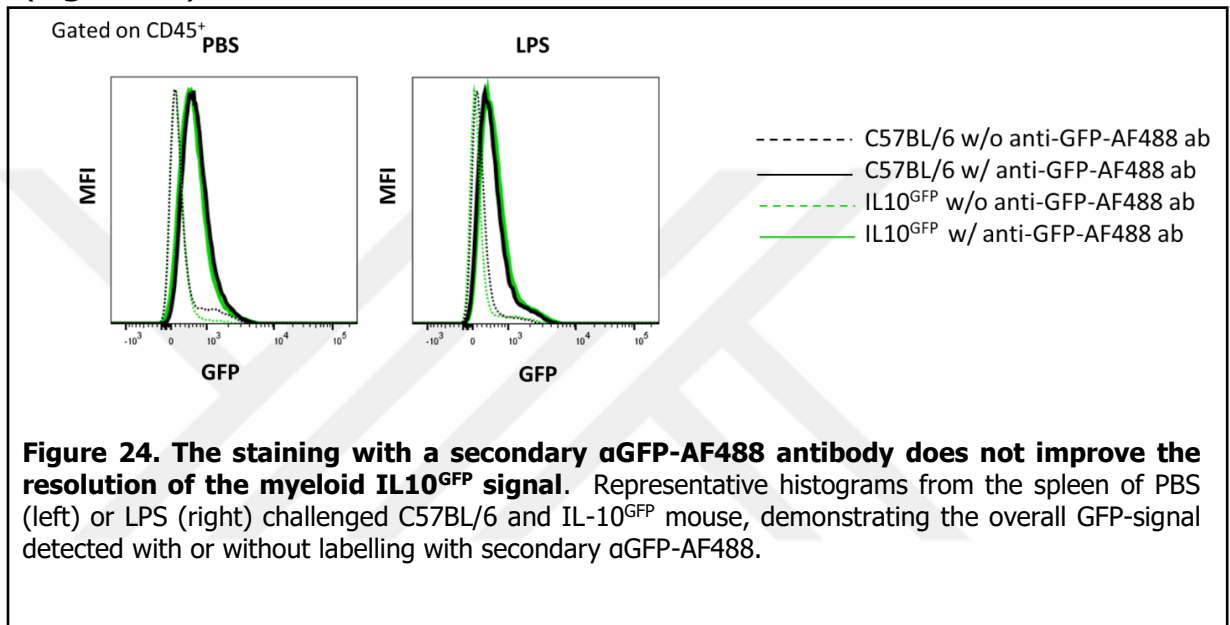
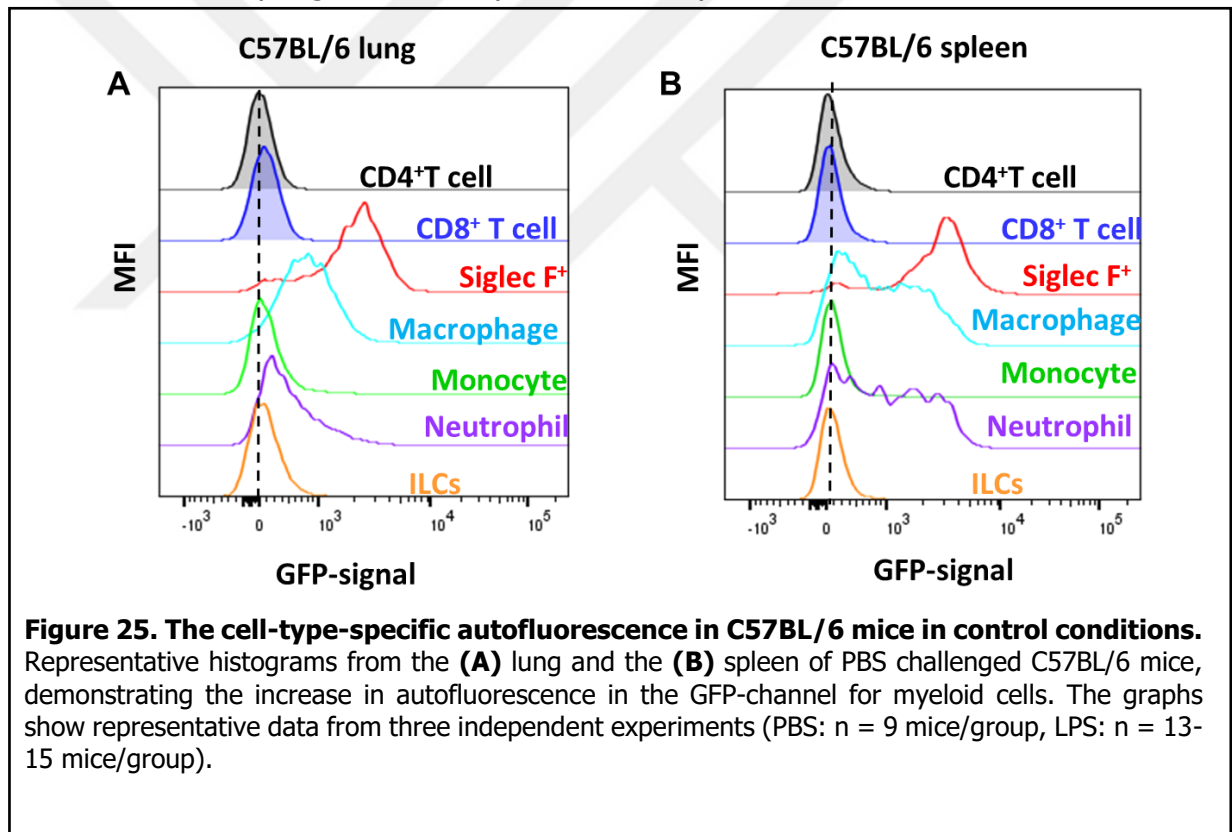


Figure 23: The myeloid-derived IL-10^{GFP} signal is masked by autofluorescence in granulocytes. C57BL/6 and IL-10^{GFP} mice were subjected to three daily administrations (d0, d1, d2) of 10 µg LPS per mouse with via aspiration. 16-18 hours after the last administration, the single cell suspension from lungs, spleens, and mLNs were stained for CD4⁺ T cells (CD45⁺ CD45R/B220⁻ CD3ε⁺ CD4⁺ CD8α⁻), CD8⁺ T cells (CD45⁺ CD45R/B220⁻ CD3ε⁺ CD4⁻ CD8α⁺), macrophages (CD45⁺ CD45R/B220⁻ CD3ε⁻ Siglec-F⁻ F4/80⁺), Siglec-F⁺ cells (eosinophils, alveolar macrophages; CD45⁺ CD45R/B220⁻ CD3ε⁻ Siglec-F⁺ F4/80^{+/+}), neutrophils (CD45⁺ CD45R/B220⁻ CD3ε⁻ Siglec-F⁻ F4/80⁻ Ly6G⁺ CD11b⁺), monocytes (CD45⁺ CD45R/B220⁻ CD3ε⁻ Siglec-F⁻ F4/80⁻ Ly6G⁻ Ly6C⁺ CD11b⁺), and ILCs (CD45⁺ CD45R/B220⁻ CD3ε⁻ Siglec-F⁻ F4/80⁻ Ly6G⁻ CD90.2⁺ CD127^{lo/-}). The expression of IL-10 was measured either by GFP⁺ or by intracellular IL-10 as indicated. **(A-G)** Comparison of the IL-10 signal derived from intracellular cytokine staining (ICCS) or from the GFP-signal (IL10^{GFP}) of IL10^{GFP} mice in (D) CD4⁺ T cells, (E) CD8⁺ T cells, (F) neutrophils, (G) macrophages, (H) Siglec-F⁺ cells (eosinophils, alveolar macrophages), (I) monocytes, and (J) ILCs from indicated organs. The graph shows combined data from two independent experiments (PBS: n = 6 mice/group, LPS: n = 7-9 mice/group).

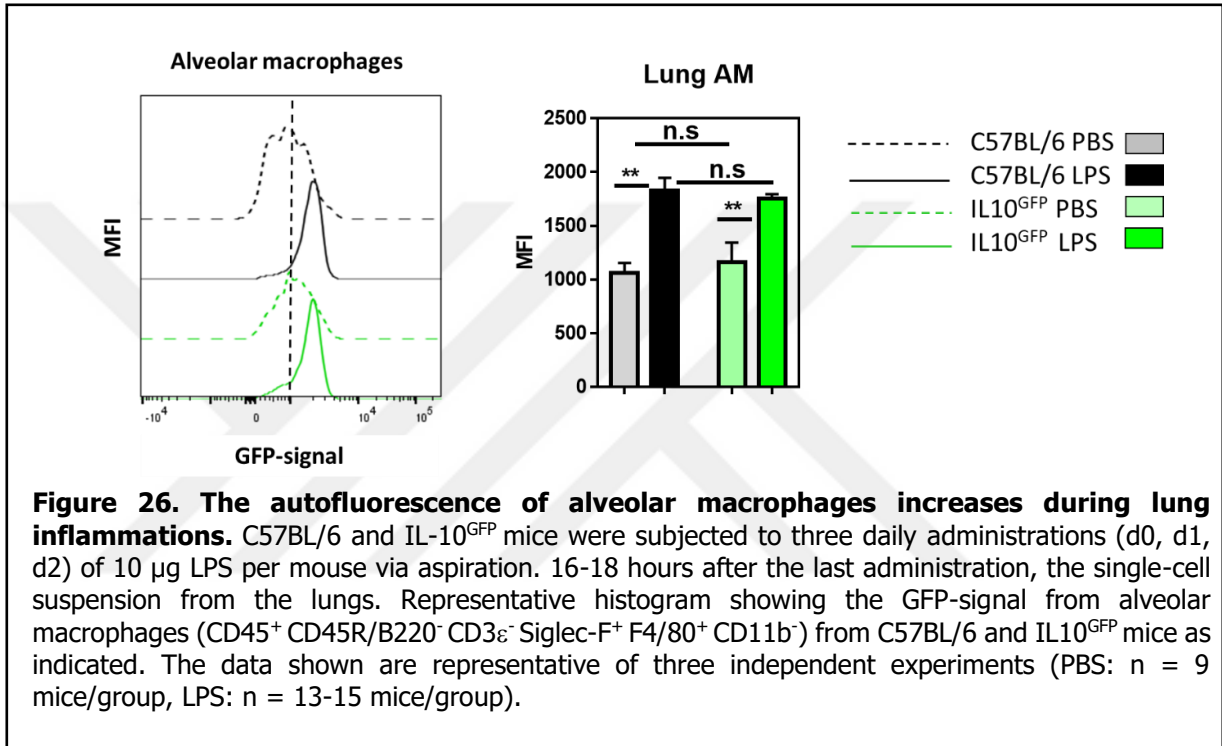
Furthermore, we were not able to improve the specificity of the IL10^{GFP}-signal either by the arithmetic subtraction of the GFP-signals of control (non-stimulated) C57BL/6 samples (data not shown) or by the staining with a secondary α GFP-AF488-antibody (**Figure 24**).



Several myeloid cells possess an autofluorescence around 525 nm (Mitchell et al. ,2010), which coincides with the emission maxima of GFP around 530 nm (Mitchell et al. ,2010; Kretschmer et al. ,2016). To further decipher the autofluorescent cell types, we overlaid the GFP-channel derived signals of CD4⁺ and CD8⁺ T cells, neutrophils, macrophages, Siglec-F⁺ cells (eosinophils, alveolar macrophages), monocytes, and ILCs from C57BL/6 control mouse lungs (**Figure 25A**) and spleens (**Figure 25B**). The histograms show that the intensity of the autofluorescence differs between immune cell in a cell type-specific manner: Siglec-F⁺ cells > macrophages > neutrophils > monocytes/ILCs/T cells.



In a similar fashion, we also observed a significantly increased GFP-channel autofluorescence when alveolar macrophages from inflamed lungs were analyzed (**Figure 26**).

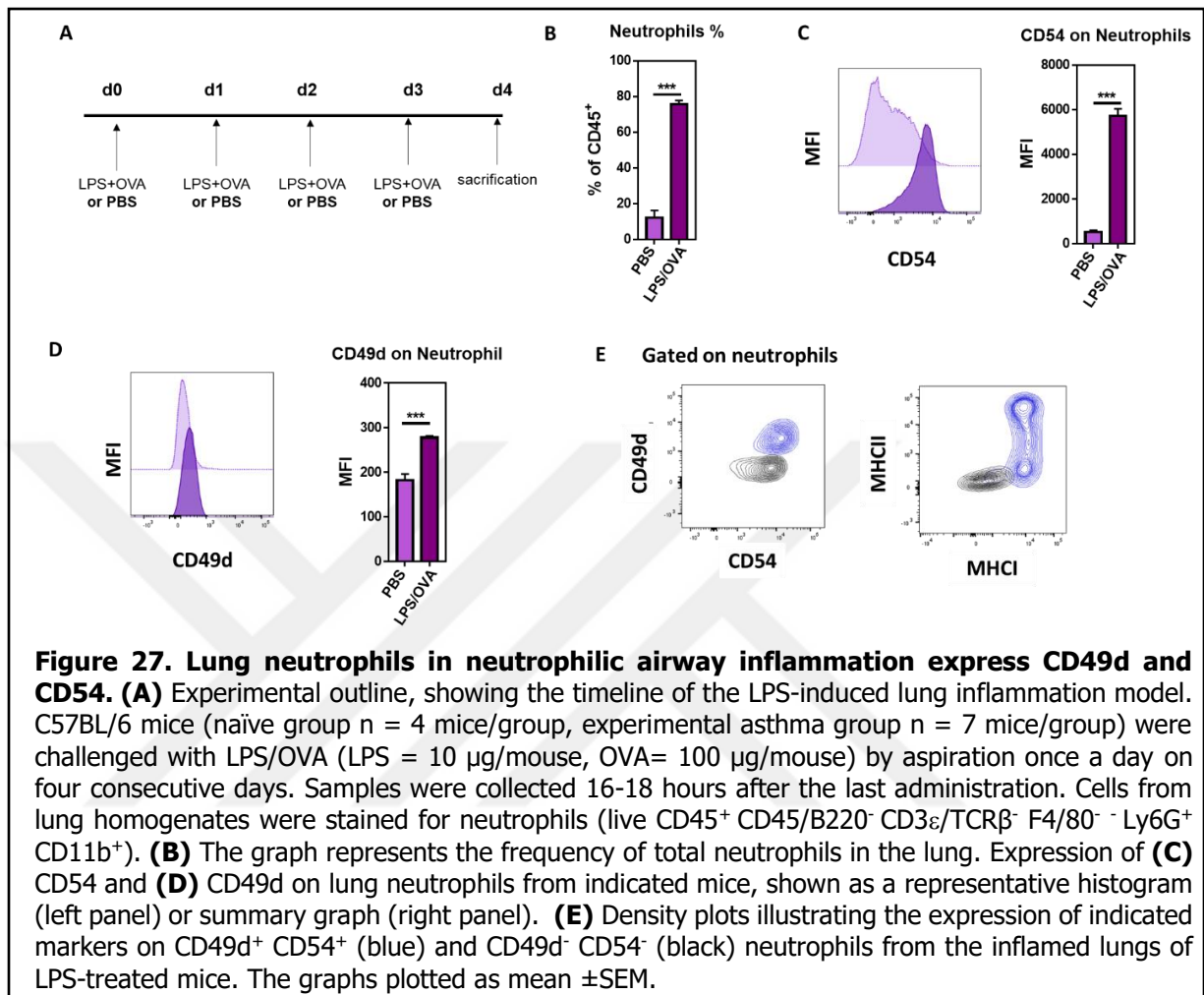


4.5. Neutrophils in asthma endotypes

Neutrophils are an essential part of the innate immune system and they respond quickly and aggressively to local inflammations (Nicolás-Ávila et al. ,2017). Although neutrophils are usually described as short-lived innate effector cells, recent publications indicate that neutrophils are not homogenous. Several different neutrophil subsets were defined in the circulation (Tsuda et al. ,2004; Silvestre-Roig et al. ,2019) and tissues (Geng et al. ,2013; Hampton et al. ,2015; Fites et al. ,2018; Silvestre-Roig et al. ,2019; Abdallah et al. ,2011). Neutrophils are also known to have the ability to secrete copious amounts of soluble factors; including IFN γ , TNF, IL-1 β , IL-12, IL-13, and IL-17 (Cassatella et al. ,2019). Additionally, IL-10 producing neutrophils in mouse models have been reported (Bazzoni et al. ,2010; Tsuda et al. ,2004; Kasten et al. ,2010; Ocuin et al. ,2011; Perobelli et al. ,2016); however, their existence in humans is still controversial (Tamassia et al. ,2018; Cassatella et al. ,2019; De Santo et al. ,2011; Acorci-Valério et al. ,2010). Therefore, we focused next on the IL-10 producing subsets of neutrophils during lung inflammation.

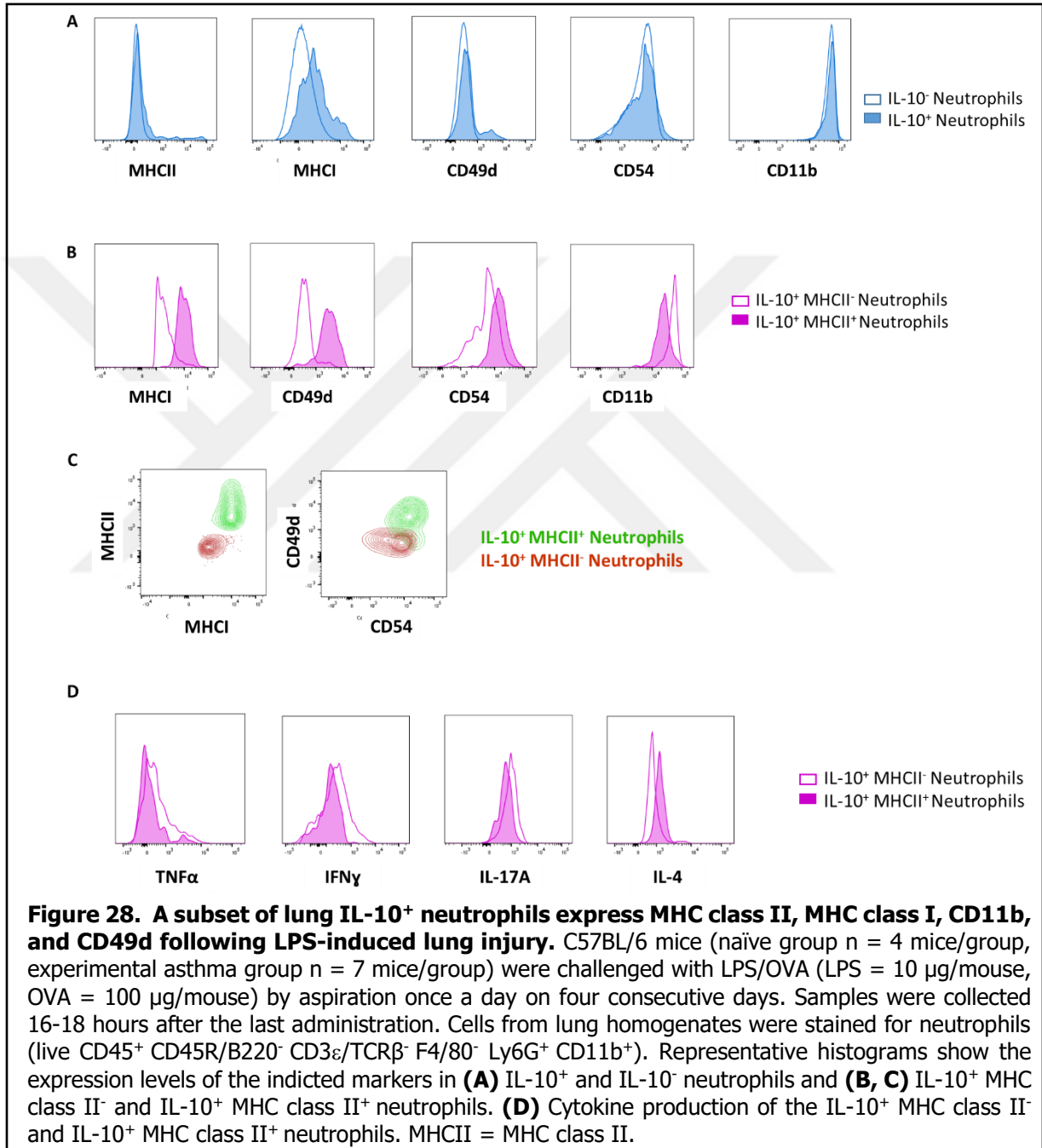
4.5.1. Neutrophil characterization during LPS-induced lung inflammations

To investigate neutrophil subsets during lung inflammations, we used the LPS-induced lung injury model, which is known to induce highly neutrophilic lung inflammation (Katsura et al. 2015; Donovan et al. 2015) (**Figure 27A**). We verified that the model is characterized by significant neutrophilia (**Figure 27B**). To identify neutrophil subsets, we co-stained the cells with the markers CD11b, CD49d, CD54, CD62L, CD117/cKit, CD86, CD182/CXCR2, MHC class I, and MHC class II which were previously implicated for characterization of subpopulation of neutrophils. We found that neutrophils in the inflamed lungs expressed high levels of CD54 (**Figure 27C**) and CD49d (**Figure 27D**) and most of the CD49d⁺ CD54⁺ neutrophils also expressed MHC class I and II (**Figure 27E**).

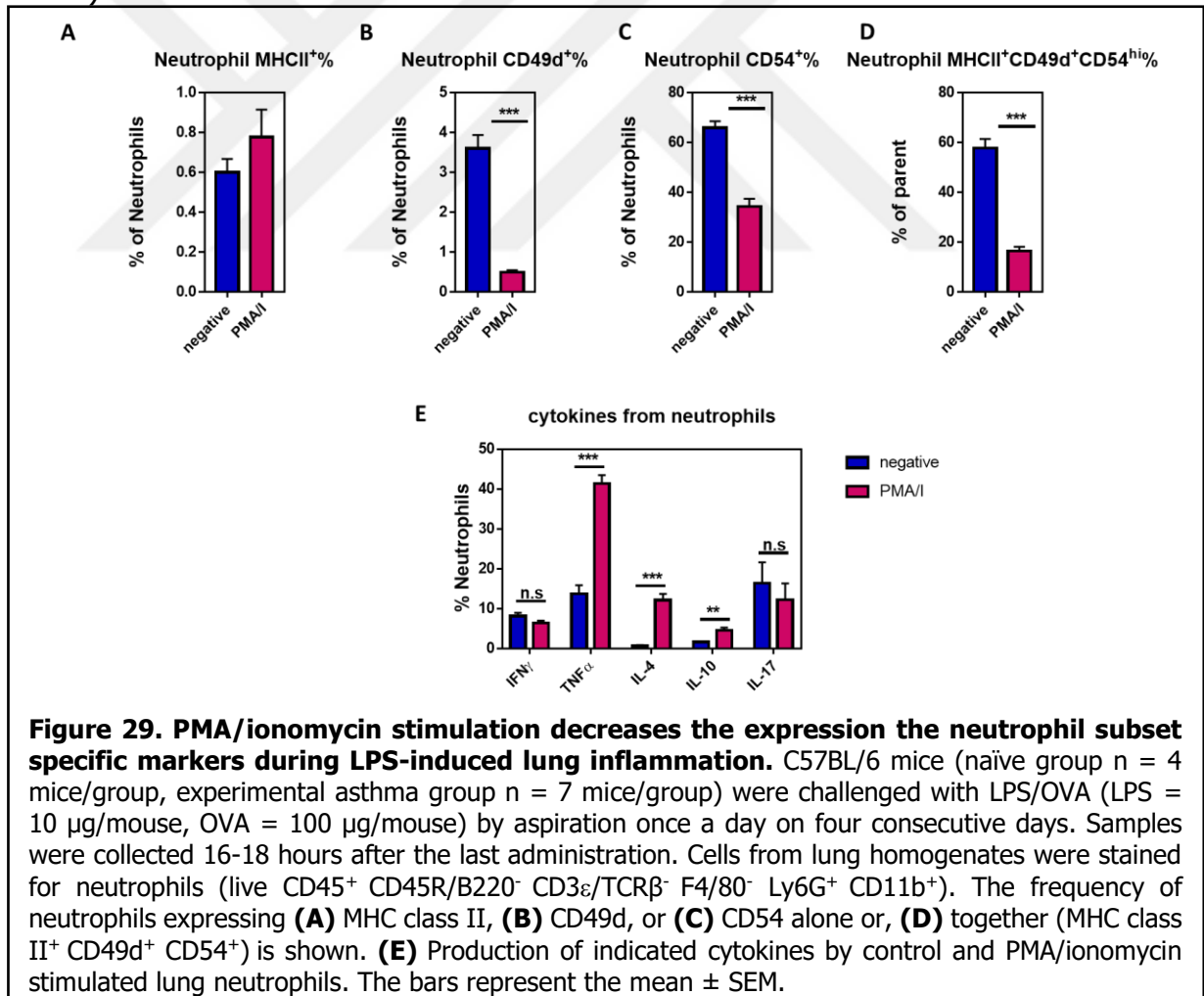


When focusing on the IL-10⁺ neutrophils, we found that they express slightly more MHC class II and MHC class I during lung inflammation (**Figure 28A**). The IL-10⁺ neutrophils that expressed MHC class II also had upregulated CD49d, CD54, and downregulated CD11b (**Figure 28B, C**). Recent literature indicates that functional distinct neutrophil subsets exist. For examples, it was reported that neutrophils could act as professional antigen-presenting cells (Tsuda et al. ,2004; Abdallah et al. ,2011), that they interact with T cells to either prime differentiation to Th1 or Th17 cells *in vitro* (Abdallah et al. ,2011), or that they suppress Th1 effector functions in *Brucella abortus* infection (Barquero-Calvo et al. ,2013).

Additionally, we found that these MHC class II⁺ neutrophils produced only IL-10 and IL-4, and not IFN γ , IL-17, or TNF (**Figure 28D**).

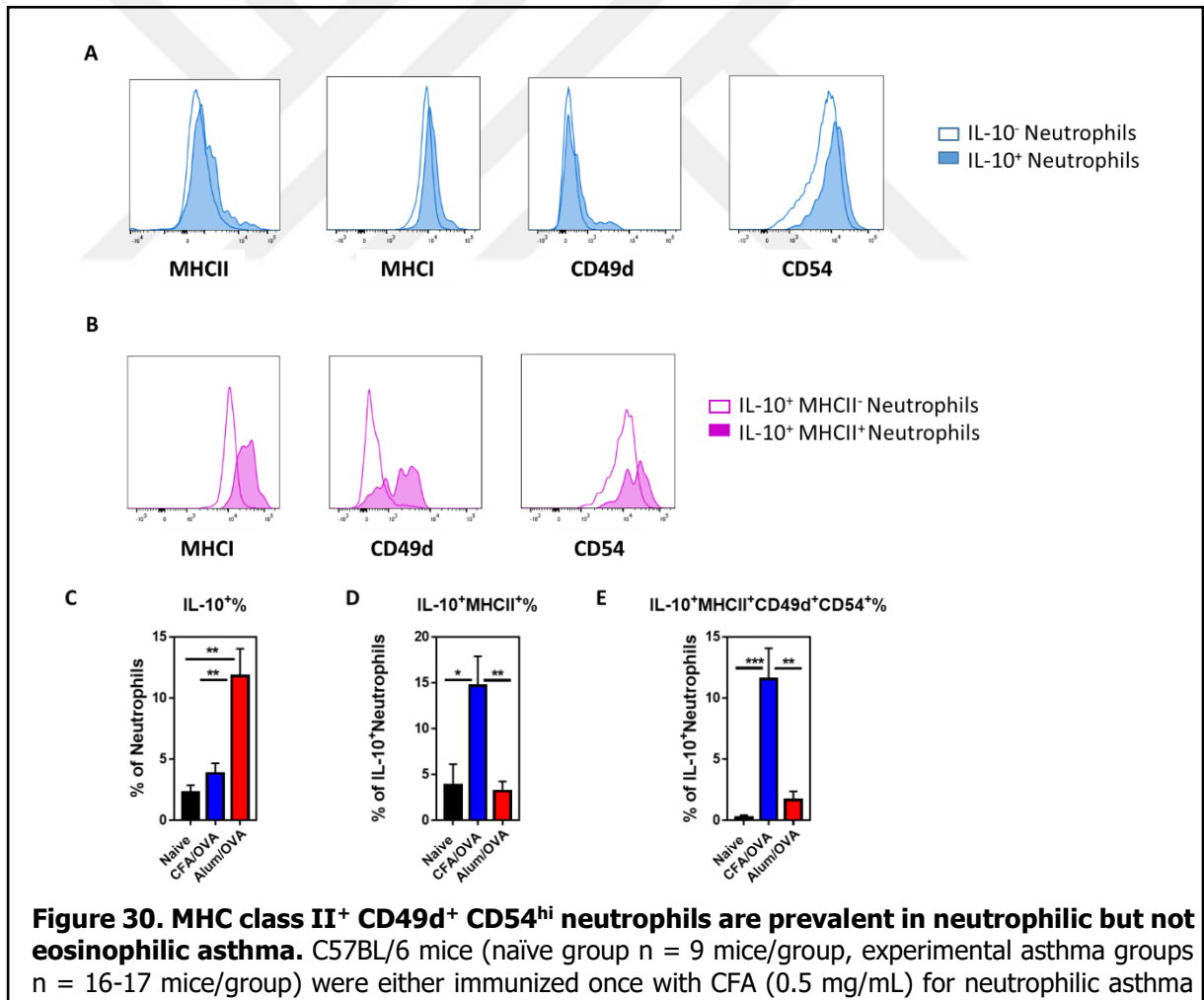


While measuring the cytokine production of neutrophils, we noticed that PMA/ionomycin stimulation caused a reduction of the CD54 (**Figure 29B**) and CD49d (**Figure 29C**) expression levels, which affected the analysis of neutrophil subsets. In contrast, the PMA/ionomycin stimulation did not significantly change MHC class II expression on neutrophils (**Figure 29A**). Due to the down-regulation of the CD54 and CD49d expression, the frequency of the MHC class II⁺ CD49d⁺ CD54⁺ specific 'effector' neutrophil population was reduced significantly (**Figure 29D**). Additionally, *ex vivo* PMA/ionomycin stimulation, induced TNF, IL-10, and IL-4 production by the neutrophils, without inducing IFN γ or IL-17A production (**Figure 29E**).



4.5.2. IL-10 releasing neutrophils in asthma endotypes

We analyzed the neutrophil subsets in the inflamed lungs of mice with adjuvant-induced neutrophilic and eosinophilic asthma (**Figure 30**). We verified that the IL-10⁺ lung neutrophils also express MHC class II, MHC class I, CD49d, and CD54 in this inflammation model (**Figure 30A, B**). The frequency of IL-10⁺ neutrophils was significantly higher in eosinophilic than in neutrophilic asthma (**Figure 30C**). In contrast, the frequency of the MHC class II⁺ CD49d⁺ CD54^{hi} neutrophil subset was significantly higher in neutrophilic than eosinophilic asthma (**Figure 30D, E**).



or three times with Alum (1 mg/mouse) for eosinophilic asthma along with the model antigen OVA (20 µg/mouse). Three weeks after the first administration, the experimental groups were challenged with OVA (50 µg/mouse) daily total of two times by aspiration. Samples were collected 16-18 hours post-challenge. Cells lung homogenates were stained for neutrophils (live CD45⁺ CD45R/CD19⁻ Ly6G⁺ CD11b⁺). Expression of MHC class II (MHCII), MHC class I (MHCI), CD49d, and CD54 on **(A)** IL-10⁺ and IL-10⁻ neutrophils and on **(B)** IL-10⁺ MHC class II⁺ and IL-10⁺ MHC class II⁻ neutrophils mice with neutrophilic lung inflammation. Summary data of the frequency of **(C)** IL-10⁺ **(D)** IL-10⁺ MHC class II⁺ (MHCII), and of **(E)** IL-10⁺ MHC class II⁺ CD49d⁺ CD54⁺ neutrophils from the lungs of indicated experimental groups. The bars represent the mean ±SEM. Combined data from three independent experiments are shown.

We next addressed whether this IL-10⁺ MHC class II⁺ neutrophil population is unique to the lung. Therefore, we analyzed neutrophils isolated from the lung, blood, mediastinal lymph nodes (mLN), spleen, and the bone marrow (BM) and measured the expression of MHC class I, MHC class II, CD49d, CD54, CD86, CD62L, and CD182 which were previously used to define subpopulations of neutrophils (Silvestre-roig et al. ,2017; Whittaker Hawkins et al. ,2017; Hosoki et al. ,2016; Tsuda et al. ,2004). We found that the frequency of neutrophils was significantly increased in the bone marrow and lungs of mice in the neutrophilic asthma group, whereas neutrophilia was rare in the mLNs and spleens (**Figure 31A**). Interestingly, the neutrophils recruited to the lungs during neutrophilic asthma were CD54⁺ (**Figure 31B**), whereas the neutrophils in the eosinophilic lungs were largely CD49d⁺ (**Figure 31C**). IL-10⁺ neutrophils appeared to be unique to the inflamed lungs (**Figure 31D**). Furthermore, the MHC class II⁺ CD49d⁺ CD54^{hi} neutrophils were particularly frequent in the lungs during neutrophilic inflammation (**Figure 31E, F**).

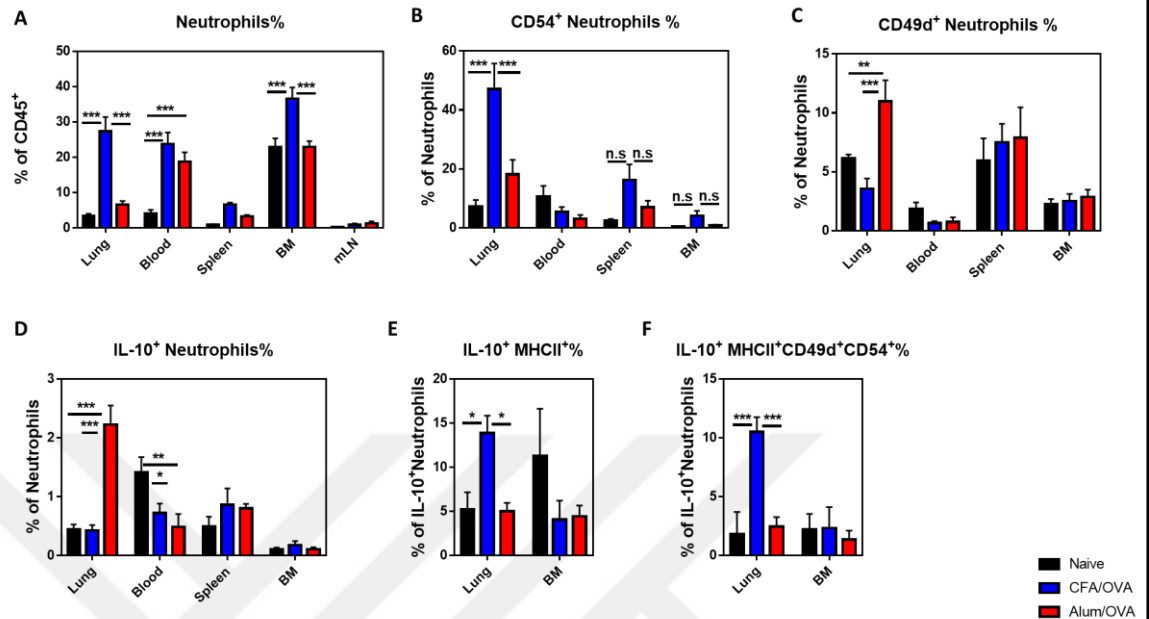


Figure 31. Neutrophil subset distributions among blood, bone marrow, lung, mLN and spleen during eosinophilic vs. neutrophilic asthma endotypes. C57BL/6 mice (naïve group n = 6 mice/group, experimental asthma groups n = 11-12 mice/group) were either immunized once with CFA (0.5 mg/mL) for neutrophilic asthma or three times with Alum (1 mg/mouse) for eosinophilic asthma along with the model antigen OVA (20 µg/mouse). Three weeks after the first administration, the experimental groups were challenged with OVA (50 µg/mouse) daily for two consecutive days by aspiration. Samples were collected 16-18 hours post-challenge. Cells purified from lung, blood, spleen, bone marrow (BM) and mediastinal lymph node (mLN) were analyzed for **(A)** the frequency of neutrophils (live CD45⁺ CD45R/CD19⁻ Ly6G⁺ CD11b⁺) and the expression of **(B)** CD54 and **(C)** CD49d on these neutrophils. The frequency of **(D)** IL-10⁺, **(E)** IL-10⁺ MHC class II⁺(MHCII), and **(F)** IL-10⁺ MHC class II⁺ CD49d⁺ CD54⁺ neutrophils from the indicated organs and mice. The bars represent the mean ± SEM. Combined data from two independent experiments are shown.

5. Discussion

We induced in the mouse neutrophilic and eosinophilic asthma with the aid of two adjuvants (Bogaert et al. ,2011; Dejager et al. ,2015) or two different concentration of LPS (Whitehead et al. ,2014; Mcalees et al. ,2015). We then directly compared in great detail the immune responses (i) between neutrophilic and eosinophilic asthma, (ii) between the two forms of neutrophilic airway inflammations, and (iii) between BALF and lung tissue. Furthermore, (iv) we assessed the role of NKT cells in neutrophilic asthma using $\text{J}\alpha 18^{-/-}$ mice and (iv) we characterized the IL-10 producing cells in the inflamed lungs.

Our data demonstrate;

- (i) that the BALF provides a good immunological representation of the inflamed lung,
- (ii) that asthma endotypes can be characterized by the distinct distribution of several myeloid cells, besides eosinophils and neutrophils; namely pDCs, IMs, and ExMs,
- (iii) that Th17 cells, but not NKT17 cells, as well as germinal center B cells were significantly expanded in neutrophilic asthma,
- (iv) that NKT cells do not contribute to the pathogenesis of neutrophilic asthma.

Airway inflammations have long been modeled *in vivo* in animals to gain insight into the pathogenesis, progression, and treatment options. Despite their intrinsic limitations, such animal models are instrumental for our understanding of complex human diseases. Prime/boost immunization of mice with ovalbumin (OVA) together with the adjuvant Alum (aluminum hydroxide) is a well-described approach to induce a type-2 immune response in the airways, including antigen-specific IgEs, hypersensitivity responses, and airway remodeling (Ray et al. ,2017). Clinically relevant allergens, such as extracts of house dust mites (HDM), have also been used in animal models (Radermecker et al. ,2019; Woo et al. ,2018). However, their use is hampered by technical difficulties and a lack of standardization, which reduces reproducibility (Cyphert-Daly et al. ,2019). For neutrophilic asthma, less work and fewer animal models have been reported (Fallon et al. ,2020). The protocols for the

adjuvant-induced eosinophilic (Alum/OVA) and neutrophilic (CFA/OVA) asthma models were based on published work, and our own data confirm that this approach is best suited to compare asthma-like eosinophilic and neutrophilic lung inflammation side-by-side.

Basophils (Korošec et al. ,2018; Sawaguchi et al. ,2012; Wakahara et al. ,2013) and mast cells (Nakae et al. ,2007; Chai et al. ,2011; Reber et al. ,2015; Sawaguchi et al. ,2012) are associated with allergen-induced airway inflammation due to IgE-mediated effector functions. In line with these reports, we noted a clear increase in basophils (Figure 8B) and mast cells (Figure 8C) in the lungs of mice with eosinophilic (Alum/OVA). Furthermore, our data indicate that basophils and mast cells do not migrate to the BALF during allergen-induced lung inflammation. To our knowledge, this is the first report showing this directly for eosinophilic and neutrophilic asthma. In Th2-low (neutrophilic) asthma, mast cells located in the proximal airway epithelium (Carroll et al. ,2002; ,2002) and the submucosal region (Balzar et al. ,2011), whereas in Th2-high (eosinophilic) asthma, they were found in the intraepithelial region (Carroll et al. ,2002; ,2002). Therefore, it seems possible that the location of the mast cells, and potentially the basophils, in the inflamed lung limits their propensity to migrate to the BALF.

In allergic, eosinophilic airway inflammation, the antigen presentation by pulmonary CD11b⁺ cDCs and CD103⁺ cDCs is essential for the induction of the Th2 response (Lambrecht et al. ,1998; ,2000; Hammad et al. ,2010). Although pDCs have the ability, similar to other pulmonary DCs, to take up and process antigens (H. Maazi et al. ,2013), they prevent the differentiation of effector T cells, and depletion of pDCs could exacerbate lung inflammation in an LPS-induced asthma model (Heer et al. ,2004; Kool et al. ,2009). Additionally, pDCs are associated with the induction of type 1 immune responses and, for example, protect from viral bronchiolitis (JP Lynch et al. ,2018) and suppress ILC2 activity via IFN α during

fungus-induced allergic asthma (Hadi Maazi et al. ,2018). Similar to the literature, we found that conventional pulmonary DCs, including CD11b⁺ and CD103⁺ cDCs, are significantly expanded in eosinophilic asthma (Figure 8D, E). In contrast, in neutrophilic asthma, pDCs were the only DCs that infiltrated the alveolar space of the inflamed lung (Figure 8F). Although pDCs have been reported to increase upon allergen challenge in the BALF (Bratke et al. ,2007) and the induced sputum (Dua et al. ,2010) of asthmatic patients, to our knowledge, no study compared their frequency in different endotypes. Interestingly, the increase of pDCs in the BALF was not accompanied by increased pDC population in the lung itself (Figure 8F).

Macrophages are important innate immune cells involved in tissue homeostasis and host defense. Lung macrophage subsets were initially defined by their location: alveolar macrophages (AMs) are mainly found in the alveolar lumen, whereas interstitial macrophages (IMs) reside in the lung interstitium (Byrne et al. ,2016; Zhou et al. ,2018). AMs are essential for the repair of lung injuries induced, for example, by physical damage (Machado-Aranda et al. ,2014), LPS (Maus et al. ,2006; Mould et al. ,2017), or infections (Taut et al. ,2008). Consequently, AMs are also protective during allergic airway inflammation (Zasłona et al. ,2014) and lung fibrosis (Misharin et al. ,2017). In contrast, IMs, which constitute approx. 9% of the macrophages in a healthy lung (Gibbins et al. ,2017), have primarily pro-inflammatory functions. For example, they aggravate allergic inflammation (Bedoret et al. ,2009; Sabatel et al. ,2017) and fibrosis (Meziani et al. ,2018; Zhou et al. ,2018). During lung inflammations, blood-monocytes can infiltrate the lung and give rise to a third lung macrophage population, Ly6C^{hi} exudate macrophages (ExMs). ExMs are mainly pro-inflammatory and have been described, for example, during lung inflammations caused by diphtheria toxin (Osterholzer et al. ,2013), LPS (Tighe et al. ,2011), and infections with bacteria (Taut et al. ,2008; Herbold et al. ,2010), fungi (Osterholzer et al. ,2011), or viruses (Lin et al. ,2008).

Due to their functional differences, it is crucial to analyze macrophages on the subset level during pulmonary diseases. When we analyzed AMs, IMs, and ExMs from the BALF and the inflamed lung of mice with eosinophilic and neutrophilic asthma, several changes were apparent. In line with previous findings (He et al. ,2016; Mathie et al. ,2015; Marriott et al. ,2006), AMs considerably declined during both types of lung inflammation (Figure 9A). Importantly, we found that the frequency of IMs and ExMs distinguishes the two asthma endotypes, with IMs being expanded more in eosinophilic asthma (Figure 9B) and ExMs being more prevalent in neutrophilic asthma (Figure 9C). Further analysis of the IMs based on surface markers (Gibbings et al. ,2017; Chakarov et al. ,2019) showed that the IMs in both endotypes mainly consisted of the CD11c^{lo} MHC class II^{neg} population (Figure 10 A, B), which have high phagocytic activity (Gibbings et al. ,2017). It will be necessary to clarify if the differences in the distribution of macrophage populations can be used as biomarkers to stratify asthma patients.

Analyzing T cells, we noted the increase of Th17 cells and IL-17 production in neutrophilic asthma (Figure 12A, B), which is in line with previous reports (Al-Ramli et al. ,2009; Ray et al. ,2017), but we could not demonstrate any difference of the T helper cells subsets Th1, Th2, or Treg populations between the two asthma endotypes (Figure 12A, B).

iBALTs (inducible bronchus-associated lymphoid tissues) are tertiary lymphoid tissues (Pitzalis et al. ,2014), structurally similar to germinal centers in the lymph nodes (Foo et al. ,2010), which are formed in the airways during lung inflammation, for example, following infections (Halle et al. ,2009; Geurtsvankessel et al. ,2009) or during chronic diseases, like COPD (chronic obstructive pulmonary disease) (Demoor et al. ,2008; John-Schuster et al. ,2014). In allergic asthma models, iBALTs support the Th2 cell responses induced by fungal infection (Ghosh et al. ,2015) or LPS (Shinoda et al. ,2017). Within iBALTs, germinal center (GC) B cells can act as

APCs by presenting inhaled antigens to T cells and can drive their differentiation into Th2 cells (Wypych et al. ,2018). Conversely, antigen-specific Th2 cells can support iBALT formation (Hirahara et al. ,2018). However, the role of iBALT in neutrophilic asthma and how it would compare to eosinophilic asthma is not known. We provide here, to our knowledge, the first direct comparison of GC B cells and iBALTs in these two asthma endotypes. Histological examination did not indicate that the size of the iBALTs differed between the two endotypes (data not shown). However, we found that in neutrophilic but not eosinophilic asthma, the frequency of GC B cells was significantly increased (Figure 14C) and that they produce more IFN γ following activation (Figure 14B). These data suggest that activated B cells, locally in the inflamed lung, might contribute to neutrophilic asthma pathology.

NKT cells are involved in the regulation of many disease pathologies ranging from autoimmune diseases like rheumatoid arthritis and diabetes (Nel et al. ,2016) to bacterial/viral infections (Wingender et al. ,2011; Gorski et al. ,2013; Ishikawa et al. ,2010), asthma and allergy (Omid Akbari et al. ,2003; Hachem et al. ,2005; Lisbonne et al. ,2003; Matsuda et al. ,2010; O Akbari et al. ,2010). Although NKT cells are not frequent in the pulmonary niche, studies showed that NKT cells play a protective role against the pulmonary pathogens *S. pneumonia* (Thanabalasuriar et al. ,2016) and influenza (Gorski et al. ,2013; Ho et al. ,2008; Ishikawa et al. ,2010; Kok et al. ,2012). However, allergen-induced, eosinophilic asthma studies showed that NKT cell deficiency abolished airway hyperresponsiveness and asthma disease phenotype (Lombardi et al. ,2010; Akbari et al. ,2003; JO Kim et al. ,2004; Shasby et al. ,2002; Lisbonne et al. ,2003; Wingender et al. ,2011). Furthermore, when NKT cells were activated with the antigen α GalCer (Okamoto et al. ,2010) they induced a protective response against eosinophilic asthma. These distinct functions of NKT cells in eosinophilic asthma have shown to be caused by the differential secretion of the cytokines IFN γ (Hachem et al. ,2005; Okamoto et al. ,2010) or TNF (Dejager et

al. ,2015). However, the exact mechanisms that promotes these different outcomes remain elusive. To determine whether *NKT* cells are involved in the pathogenesis of neutrophilic asthma, we immunized *NKT* cell-deficient *Jα18^{-/-}* mice along with their C57BL/6 controls with the previously adopted models for neutrophilic asthma (Figure 6A). We found that neither in CFA-induced (Figure 15) nor in LPS-induced (Figure 16) neutrophilic asthma *NKT* cell-deficiency affected the pulmonary neutrophil influx. In contrast to the Th17 cells, however, the frequency of *NKT*17 cells was decreased in neutrophilic asthma (Figure 16), suggesting that *NKT* cells might be less relevant for the pathology of this asthma endotype.

The regulatory cytokine IL-10 is involved in various lung diseases, including asthma, allergic airway disease (AAD), chronic obstructive pulmonary disease (COPD), and pulmonary infections (Branchett et al. ,2019). In the airways, FoxP3⁺ and FoxP3⁻ regulatory T cells (Branchett et al. ,2019), CD8⁺ T cells (Sun et al. ,2009), B cells, alveolar macrophages (AM) (Bracken et al. ,2015), interstitial macrophages (IM) (Kawano et al. ,2016), airway and alveolar epithelial cells (Branchett et al. ,2019), and airway-associated dendritic cells (DCs) (Branchett et al. ,2019) were shown to produce IL-10, which limited disease pathology. Specifically, IL-10⁺ B cells were shown to prevent casein- (HS Kim et al. ,2015) and HDM-induced (Faouzi Braza et al. ,2012) allergic airway inflammations. Furthermore, the frequency of regulatory T cells was reduced in both eosinophilic and neutrophilic asthma endotypes (Jang et al. ,2017).

However, due to the low percentage of IL-10⁺ cells within local cell populations and the low intensity of the flow cytometric staining of intracellular IL-10, the clear identification of IL-10 producing cells is challenging (Sag et al. ,2017). We optimized a procedure to better stain IL-10⁺ *NKT* cells by including an additional gradient centrifugation step in the cell homogenization procedure (Sag et al. ,2017). However, this approach reduced the already low number of live cells recoverable in

the lung even further (data not shown). Therefore, we decided to use a reporter mouse strain to detect IL-10-producing cells. The IL-10^{GFP} (VeRT-X) mouse line expresses an (IRES)-enhanced green fluorescent protein (eGFP) fusion protein downstream of the *Il10* gene located at exon 5 (Madan et al. ,2009). This strain was reported to successfully identify IL-10 producing lymphoid (Madan et al. ,2009; Bouabe et al. ,2012) and myeloid cells. Additional, we improved the measurable GFP-signal by adopting an optimized fixation method (Heinen et al. ,2014). Our analysis demonstrates that the frequency of IL-10⁺ T or B cells did not differ between the two asthma endotypes (Figure 18). In contrast, IL-10⁺ innate lymphoid cells (ILCs) were abundant in eosinophilic asthma (Figure 18). IL-10-producing ILCs (ILCreg) recently described an anti-inflammatory ILC subset (S Wang et al. ,2017). Although regulatory ILCs were identified in nasopharyngeal tissue of human subjects (Morita et al. ,2019), this is to our knowledge the first time that ILCregs are described outside of the intestine in murine models (Figure 18). The expansion of ILCregs specifically in eosinophilic asthma needs further investigation.

Neutrophils were suggested to be the primary source of IL-10 in the lung following *Klebsiella pneumoniae* ST258 infection (Peñaloza et al. ,2019). Furthermore, long-term exposure to house-dust mite (HDM) extracts suppressed allergic airway diseases via the IL-10 production by presumably FoxP3⁺ T cells and alveolar macrophages (Bracken et al. ,2015). Both studies analyzed IL-10 production with the IL10^{GFP} reporter mice by comparing the 'shift' in the GFP-signal in the experimental IL10^{GFP} mice to uninfected IL-10^{GFP} or C57BL/6 mice as a control. Following a similar strategy, we quantified IL-10 release from myeloid cells. We verified our results with intracellular cytokine staining in C57BL/6 mice due to discrepancies in our granulocyte-related data. Analyzing the IL-10 production during lung inflammation, we noticed that the background signal in the GFP-channel (505 nm - 550 nm) of leukocytes from lung, spleen, or mediastinal lymph node (mLN)

was comparable in non-inflamed controls of either C57BL/6 (WT) or IL-10^{GFP} mice (Figure 22A). In contrast, in the LPS-induced lung inflammation, this background tripled, particularly in the lungs (Figure 22B). Yet, the signal in the GFP-channel from WT and IL-10^{GFP} mouse-derived cells remained comparable for the percentage (Figure 21A, B) as well as the mean fluorescence intensity (MFI, Figure 22C), irrespective of the organ analysed and the inflammatory condition. Next, we confirmed that the IL10^{GFP} mice are suitable for CD4⁺ T cell (Figure 23A) and CD8⁺ T cell (Figure 23B). However, the IL10^{GFP} mice were not suitable to measure the IL-10 production from neutrophils (Figure 23C), macrophages (Figure 23D), Siglec-F⁺ cells (Figure 23E), pulmonary monocytes (Figure 23F), and pulmonary ILCs (Figure 23G), due to the substantial amount of background in the GFP-channel in cells from inflamed organs. This induced autofluorescence differed between cell types as Siglec-F > macrophage > neutrophil > monocyte > ILCs in both lung (Figure 24A) and spleen (Figure 25B). Additionally, we could not improve the specificity of the reporter-derived GFP-signal either by the arithmetic subtraction of GFP-signals of control (non-stimulated) C57BL/6 samples (data not shown) or by the staining with a secondary α GFP-AF488-antibody (Figure 24). We conclude that this strong increase in the false-positive GFP-signals masks the reporter-derived 'true' signal for myeloid cells during inflammations. This notion could have been missed previously due to a focus on lymphoid cells or due to a lack of the WT treated controls. Our data illustrates a general and important technical caveat using GFP-reporter lines for the analysis of myeloid cells and suggest that previous reports on effector functions of myeloid cells using such GFP-based reporters might require re-evaluation.

Neutrophils are the most abundant immune cells in the body. They have both effector and immunomodulatory function exerted by soluble factor release (Persson et al. ,2018; Baines et al. ,2010; Ley et al. ,2018), NET formation (Pham et al. ,2017;

Sanz-Moreno et al. ,2018; Khandpur et al. ,2013), antigen presentation (Li et al. ,2019; Potter et al. ,2001; Takashima et al. ,2015), and pathogen elimination (Acorci-Valério et al. ,2010; Tsuda et al. ,2004). Recent findings demonstrated that specific neutrophil subsets can survive *in vivo* for prolonged periods under pathological conditions (Deniset et al. ,2018; Silvestre-Roig et al. ,2016) by becoming resistant to apoptosis (Tian et al. ,2017; Grunwell et al. ,2019). Although the CS-resistance of non-Th2 asthma is usually attributed to the inability of the CS treatment to eliminate neutrophils, the U-BIORED cohort study found only one out of three group within severe CS-resistant asthma that had sputum neutrophilia (Lefaudeux et al. ,2017; Loza et al. ,2016). Consequently, neutrophil subsets with different functions have been implicated in severe airway inflammations. Thus, we analyzed how neutrophils in neutrophilic asthma affect the resolution and intensity of the lung inflammation. To this end, we first established the analysis of neutrophil subsets via surface markers in the inflamed lungs. Initially, neutrophil subsets were defined via the expression of CD49d and CD11b (Tsuda et al. ,2004), which are markers linked to reverse migration of neutrophils (Németh et al. ,2020; D Wu et al. ,2016; Oliveira et al. ,2016). Instead of elimination, activated neutrophils transverse through tissues and continue their effector functions, where in chronic inflammatory conditions carries out the immune response at a diverse site (Németh et al. ,2020; D Wu et al. ,2016; Oliveira et al. ,2016). Later, researchers also used the chemokine receptor CD182/CXCR2 responsible for neutrophil recruitment to the lung (X Wang et al. ,2020; Hosoki et al. ,2016; Gernez et al. ,2010), and inhibition of NETosis (Jose M. Adrover et al. ,2020). CD54/ICAM1, which is associated with neutrophil transmigration to the inflamed gut (Sumagin et al. ,2014), was also found to be involved in the neutrophil recruitment to the brain in experimental autoimmune encephalomyelitis (EAE) (Whittaker Hawkins et al. ,2017). Within various tissues, neutrophils found to express MHC class II, CD80, or CD86 markers (Li et al. ,2019;

Geng et al. ,2013; Abdallah et al. ,2011; Fites et al. ,2018; Barquero-Calvo et al. ,2013), which was linked to a specific neutrophil subset called DC-neutrophil hybrids or APC-like neutrophils (Geng et al. ,2013; Takashima et al. ,2015).

Using these markers, we identified neutrophil subsets in an acute LPS-induced lung injury model (Figure 27A). We found that in a highly neutrophilic lung inflammation, neutrophils expressed significantly more CD49d and CD54 (Figure 27B, C). These neutrophils also expressed MHC class II (Figure 27D), suggesting APC-like properties.

A suppressive neutrophil subset that can release IL-10 has been reported in mice (Bazzoni et al. ,2010; Tsuda et al. ,2004; Kasten et al. ,2010; Ocuin et al. ,2011; Perobelli et al. ,2016). However, the existence of such a neutrophil subsets in humans is still controversial (Tamassia et al. ,2018; Cassatella et al. ,2019; De Santo et al. ,2011; Acorci-Valério et al. ,2010). When we analysed the IL-10⁺ neutrophils, which we found in the LPS-induced lung injury model (Figure 27), further, we noticed that they expressed MHC class II, CD54, and CD49d (Figure 28A, B, C). Furthermore, they did not produce the pro-inflammatory cytokines IFN γ , TNF, or IL-17 (Figure 28D).

Next, we determined whether this neutrophil subset could also be found in mice with adjuvant-induced airway inflammation. We found that IL-10 producing neutrophils were more abundant in eosinophilic asthma than in neutrophilic asthma (Figure 30C). This result aligns with reports suggesting that IL-10 is essential to resolve allergic airway inflammation (Ogawa et al. ,2008; Branchett et al. ,2019). These IL-10⁺ neutrophils, similar to the LPS-induced acute lung injury model, expressed MHC class II along with CD49d and CD54 (Figure 28D, E). In allergic rhinitis patients, the composition of neutrophil subsets differed among nasopharyngeal biopsies, nasopharyngeal washes, and peripheral blood (Arebro et al. ,2017). To check whether the IL-10⁺ MHC class II⁺ CD49d⁺ CD54^{hi} neutrophils

we observed were unique to the inflamed lung, we analysed their presence among cells purified from the blood, bone marrow, lung, mLN, and the spleen of experimental mice (Figure 31). We found that the IL-10⁺ neutrophil subset was significantly expanded in the lung and bone marrow of mice with neutrophilic asthma compared to control mice or mice with eosinophilic asthma (Figure 31A). In contrast, they were relatively infrequent in the spleens and mLNs (Figure 31A). Furthermore, CD54⁺ neutrophils were specifically found in the lungs with neutrophilic inflammation (Figure 31B), whereas CD49d⁺ neutrophils were preferentially increased in the lungs with eosinophilic inflammation (Figure 31C). Finally, IL-10⁺ neutrophils were found only in the inflamed lung (Figure 31D) and the IL-10⁺ MHC class II⁺ CD49d⁺ CD54^{hi} subset was uniquely present during neutrophilic lung inflammation (Figure 31F).

With this study, we were able to identify an IL-10⁺ MHC class II⁺ CD49d⁺ CD54^{hi} neutrophil subsets that was uniquely found in the lungs with neutrophilic asthma. Further analysis regarding functional aspects and the effect of this neutrophil subsets on the severity of neutrophilic asthma needs to be carried out. Additionally, their presence in human subjects with respiratory disorders need to be verified. Nonetheless, our data suggest that these IL-10⁺ neutrophils could be a novel therapeutic target to treat corticosteroid-resistant asthma.

6. Conclusion and Future Directions

We induced in the mouse neutrophilic and eosinophilic asthma with the aid of two adjuvants (Bogaert et al. ,2011; Dejager et al. ,2015) or two different concentrations of LPS (Whitehead et al. ,2014; Mcalees et al. ,2015). We then directly compared in great detail the immune responses between neutrophilic and eosinophilic asthma. Furthermore, we assessed the role of *NKT* cells in neutrophilic asthma using *Jα18^{-/-}* mice. Our data demonstrate that the BALF provides a good immunological representation of the inflamed lung and neutrophilic asthma distinguished from eosinophilic asthma pDCs and ExMs populations. We also found that *NKT* cells do not affect the pathogenesis of neutrophilic asthma. However, *NKT1* in neutrophilic and *NKT17* in eosinophilic inflamed lungs were more abundant. We described the IL-10 secreting cells in two asthma endotypes and identified a subpopulation of neutrophils located lungs with extreme neutrophilia, which is IL-10⁺ and carries APC-specific markers (**Figure 32**).

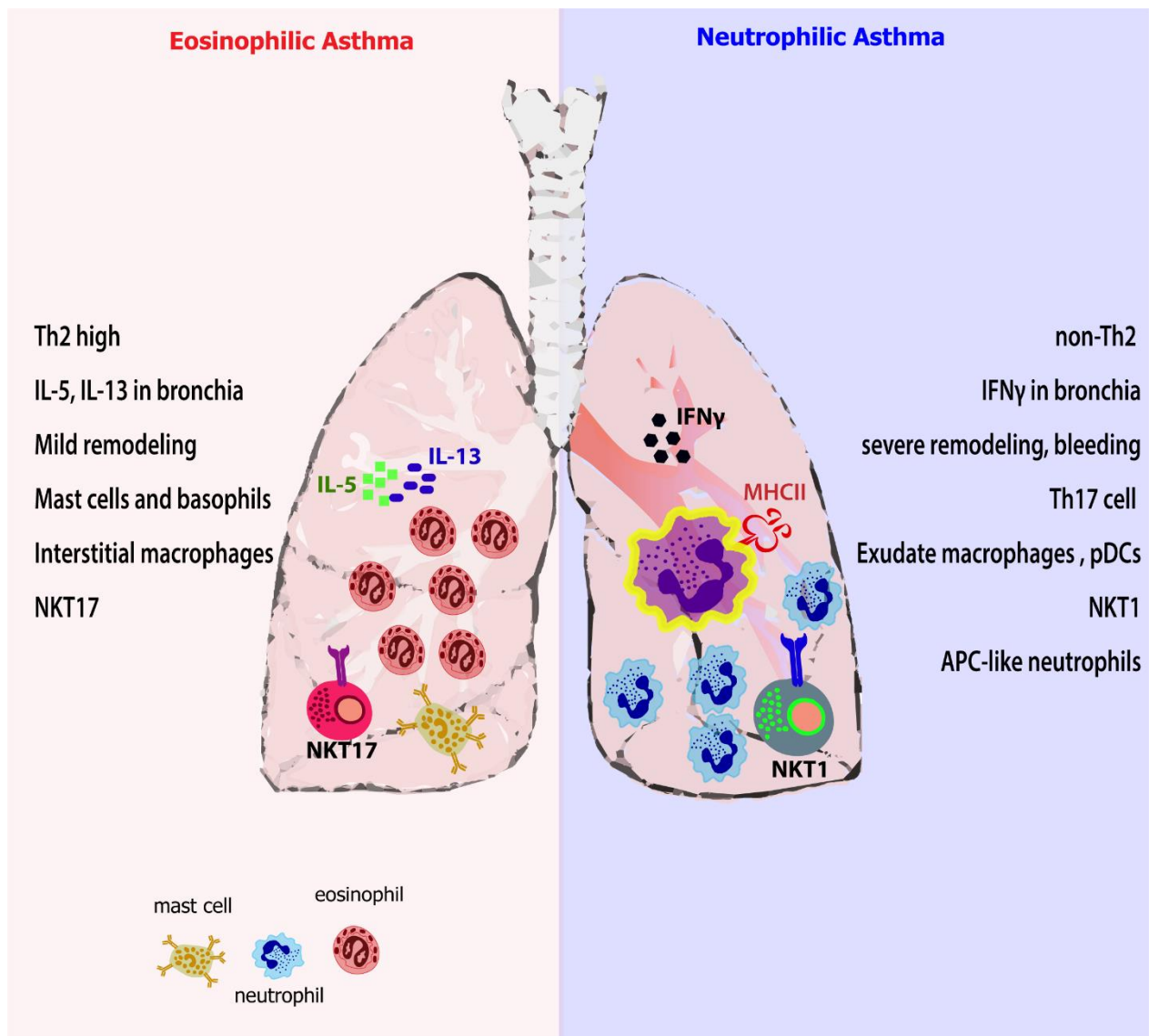


Figure 32. Conclusion summary

Questions remain;

1. Whether pDCs and ExM populations can be used as biomarkers for severe/ non-eosinophilic asthma patients?
2. How do ExMs and IMs affect the differential immune response induced during eosinophilic and neutrophilic airway inflammation?
3. What is the function of 'suppressive' APC-like neutrophils in neutrophilic asthma? Could this particular neutrophil subset also be identified in human asthma cases?

7. References

- Abdallah.DSA, Egan.CE, Butcher.BA, Denkers.EY. Mouse neutrophils are professional antigen-presenting cells programmed to instruct T h1 and T h17 T-cell differentiation. *International Immunology*, 2011. 23:(5): 317–26. <https://doi.org/10.1093/intimm/dxr007>.
- Acorci-Valério.MJ, Bordon-Graciani.AP, Dias-Melicio.LA, Assis Golim.M De, et al. Role of TLR2 and TLR4 in human neutrophil functions against paracoccidioides brasiliensis. *Scandinavian Journal of Immunology*, 2010. 71:(2): 99–108. <https://doi.org/10.1111/j.1365-3083.2009.02351.x>.
- Adrover.Jose M., Aroca-Crevillén.A, Crainiciuc.G, Ostos.F, et al. Programmed “disarming” of the neutrophil proteome reduces the magnitude of inflammation. *Nature Immunology*, 2020. 21:(2): 135–44. <https://doi.org/10.1038/s41590-019-0571-2>.
- Adrover.José M., Nicolás-Ávila.JA, Hidalgo.A. Aging: A Temporal Dimension for Neutrophils. *Trends in Immunology*, 2016. 37:(5): 334–45. <https://doi.org/10.1016/j.it.2016.03.005>.
- Agache.I, Akdis.C, Jutel.M, Virchow.JC. Untangling asthma phenotypes and endotypes. *Allergy: European Journal of Allergy and Clinical Immunology*, 2012. <https://doi.org/10.1111/j.1398-9995.2012.02832.x>.
- Agache.I, Cojanu.C, Rogozea.L. Endotype-driven approach for asthma. *Implementing Precision Medicine in Best Practices of Chronic Airway Diseases*, 2018., 45–49. <https://doi.org/10.1016/B978-0-12-813471-9.00014-1>.
- Agache.IO. From phenotypes to endotypes to asthma treatment. *Current Opinion in Allergy and Clinical Immunology*, 2013. 13:(3): 249–56. <https://doi.org/10.1097/ACI.0b013e32836093dd>.
- Akbari.O, Stock.P, Singh.AK, Lombardi.V, et al. PD-L1 and PD-L2 modulate airway inflammation and iNKT-cell-dependent airway hyperreactivity in opposing

- directions. *Mucosal Immunology*, 2010. 3:(1): 81–91.
<https://doi.org/10.1038/mi.2009.112>.
- Akbari.O, Umetsu.DT. Natural killer T cells and CD8(+) T cells are dispensable for T cell-dependent allergic airway inflammation - Reply. *Nature Medicine*, 2006. 12:(12): 1347.
- Akbari.Omid, Stock.P, Meyer.E, Kronenberg.M, et al. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nature Medicine*, 2003. 9:(5): 582–88.
<https://doi.org/10.1038/nm851>.
- Al-Ramli.W, Préfontaine.D, Chouiali.F, Martin.JG, et al. TH17-associated cytokines (IL-17A and IL-17F) in severe asthma. *Journal of Allergy and Clinical Immunology*, 2009. 123:(5): 1185–87.
<https://doi.org/10.1016/j.jaci.2009.02.024>.
- Albacker.LA, Chaudhary.V, Chang.Y-J, Kim.HY, et al. Invariant natural killer T cells recognize a fungal glycosphingolipid that can induce airway hyperreactivity. *Nature Medicine*, 2013. 19:(10): 1297–1304. <https://doi.org/10.1038/nm.3321>.
- Amu.S, Saunders.SP, Kronenberg.M, Mangan.NE, et al. Regulatory B cells prevent and reverse allergic airway inflammation via FoxP3-positive T regulatory cells in a murine model. *Journal of Allergy and Clinical Immunology*, 2010. 125:(5): 1114-1124.e8. <https://doi.org/10.1016/j.jaci.2010.01.018>.
- An.S, Jeon.YJ, Jo.A, Lim.HJ, et al. Initial Influenza Virus Replication Can Be Limited in Allergic Asthma Through Rapid Induction of Type III Interferons in Respiratory Epithelium, 2018. 9:(May): 1–14.
<https://doi.org/10.3389/fimmu.2018.00986>.
- Antolín-amérigo.D, Quirce.S. Non-eosinophilic asthma : current perspectives. *Journal of Asthma and Allergy*, 2018., 267–81.
- Arebro.J, Ekstedt.S, Hjalmarsson.E, Winqvist.O, et al. A possible role for neutrophils

- in allergic rhinitis revealed after cellular subclassification. *Scientific Reports*, 2017. 7: 1–9. <https://doi.org/10.1038/srep43568>.
- Baines.KJ, Simpson.JL, Bowden.NA, Scott.RJ, et al. Differential gene expression and cytokine production from neutrophils in asthma phenotypes. *European Respiratory Journal*, 2010. 35:(3): 522–31. <https://doi.org/10.1183/09031936.00027409>.
- Balzar.S, Fajt.ML, Comhair.SAA, Erzurum.SC, et al. Mast cell phenotype, location, and activation in severe asthma: Data from the Severe Asthma Research Program. *American Journal of Respiratory and Critical Care Medicine*, 2011. 183:(3): 299–309. <https://doi.org/10.1164/rccm.201002-0295OC>.
- Barquero-Calvo.E, Martirosyan.A, Ordoñez-Rueda.D, Arce-Gorvel.V, et al. Neutrophils Exert a Suppressive Effect on Th1 Responses to Intracellular Pathogen *Brucella abortus*. *PLoS Pathogens*, 2013. 9:(2). <https://doi.org/10.1371/journal.ppat.1003167>.
- Barrios.R. Animal Models of Lung Disease. In *Current Respiratory Medicine Reviews*, 2008., 11:144–49. https://doi.org/10.1007/978-0-387-72430-0_14.
- Bazzoni.F, Tamassia.N, Rossato.M, Cassatella.MA. Understanding the molecular mechanisms of the multifaceted IL-10-mediated anti-inflammatory response: Lessons from neutrophils. *European Journal of Immunology*, 2010. 40:(9): 2360–68. <https://doi.org/10.1002/eji.200940294>.
- Bedoret.D, Wallemacq.H, Marichal.T, Desmet.C, et al. Lung interstitial macrophages alter dendritic cell functions to prevent airway allergy in mice. *Journal of Clinical Investigation*, 2009. 119:(12): 3723–38. <https://doi.org/10.1172/JCI39717>.
- Bewley.MA, Preston.JA, Mohasin.M, Marriott.HM, et al. Impaired mitochondrial microbicidal responses in chronic obstructive pulmonary disease macrophages. *American Journal of Respiratory and Critical Care Medicine*, 2017. 196:(7): 845–55. <https://doi.org/10.1164/rccm.201608-1714OC>.

- Bogaert.P, Naessens.T, Koker.S de, Hennuy.B, et al. Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 2011. 300:(5): 679–90. <https://doi.org/10.1152/ajplung.00202.2010>.
- Böhm.L, Maxeiner.J, Meyer-Martin.H, Reuter.S, et al. IL-10 and Regulatory T Cells Cooperate in Allergen-Specific Immunotherapy To Ameliorate Allergic Asthma. *The Journal of Immunology*, 2015. 194:(3): 887–97. <https://doi.org/10.4049/jimmunol.1401612>.
- Bonnaud.P, Fabre.A, Frossard.N, Guignabert.C, et al. Optimising experimental research in respiratory diseases: An ERS statement. *European Respiratory Journal*, 2018. 51:(5): 1–52. <https://doi.org/10.1183/13993003.02133-2017>.
- Bouabe.H. Cytokine Reporter Mice: The Special Case of IL-10. *Scandinavian Journal of Immunology*, 2012. 75:(6): 553–67. <https://doi.org/10.1111/j.1365-3083.2012.02695.x>.
- Bracken.SJ, Adami.AJ, Szczepanek.SM, Ehsan.M, et al. Long-Term Exposure to House Dust Mite Leads to the Suppression of Allergic Airway Disease Despite Persistent Lung Inflammation. *International Archives of Allergy and Immunology*, 2015. 166:(4): 243–58. <https://doi.org/10.1159/000381058>.
- Branchett.WJ, Lloyd.CM. Regulatory cytokine function in the respiratory tract. *Mucosal Immunology*, 2019. 12:(3): 589–600. <https://doi.org/10.1038/s41385-019-0158-0>.
- Bratke.K, Lommatzsch.M, Julius.P, Kuepper.M, et al. Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge. *Thorax*, 2007. 62:(2): 168–75. <https://doi.org/10.1136/thx.2006.067793>.
- Braza.F, Chesne.J, Durand.M, Dirou.S, et al. A regulatory CD9 + B-cell subset inhibits HDM-induced allergic airway inflammation, 2015. 70:(2): 1421–31.

<https://doi.org/10.1111/all.12697>.

- Braza.Faouzi, Chesné.J, Mahay.G, Cheminant.M, et al. Deficiency of regulatory B cells in a house dust mite model of asthma. *Journal of Translational Medicine*, 2012. 10:(Suppl 3): P25. <https://doi.org/10.1186/1479-5876-10-S3-P25>.
- Busse.WW, Lemanske.RF, Gern.JE. Role of viral respiratory infections in asthma and asthma exacerbations. *The Lancet*, 2010. 376:(9743): 826–34. [https://doi.org/10.1016/S0140-6736\(10\)61380-3](https://doi.org/10.1016/S0140-6736(10)61380-3).
- Byrne.AJ, Maher.TM, Lloyd.CM. Pulmonary Macrophages: A New Therapeutic Pathway in Fibrosing Lung Disease? *Trends in Molecular Medicine*, 2016. 22:(4): 303–16. <https://doi.org/10.1016/j.molmed.2016.02.004>.
- Carroll.NG. Increased mast cells and neutrophils in submucosal mucous glands and mucus plugging in patients with asthma. *Thorax*, 2002. 57:(8): 677–82. <https://doi.org/10.1136/thorax.57.8.677>.
- Carroll.NG, Mutavdzic.S, James.AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. *European Respiratory Journal*, 2002. 19:(5): 879–85. <https://doi.org/10.1183/09031936.02.00275802>.
- Cassatella.MA, Östberg.NK, Tamassia.N, Soehnlein.O. Biological Roles of Neutrophil-Derived Granule Proteins and Cytokines. *Trends in Immunology*, 2019. 40:(7): 648–64. <https://doi.org/10.1016/j.it.2019.05.003>.
- Chai.OH, Han.EH, Lee.HK, Song.CH. Mast cells play a key role in Th2 cytokine-dependent asthma model through production of adhesion molecules by liberation of TNF- α . *Experimental and Molecular Medicine*, 2011. 43:(1): 35–43. <https://doi.org/10.3858/emm.2011.43.1.004>.
- Chakarov.S, Lim.HY, Tan.L, Lim.SY, et al. Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches. *Science*, 2019. 363:(6432). <https://doi.org/10.1126/science.aau0964>.
- Chambers.ES, Nanzer.AM, Pfeffer.PE, Richards.DF, et al. Distinct endotypes of

- steroid-resistant asthma characterized by IL-17A^{high} and IFN- γ ^{high} immunophenotypes: Potential benefits of calcitriol. *Journal of Allergy and Clinical Immunology*, 2015. 136:(3): 628-637.e4. <https://doi.org/10.1016/j.jaci.2015.01.026>.
- Chang.P-P, Barral.P, Fitch.J, Pratama.A, et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nature Immunology*, 2012. 13:(1): 35–43. <https://doi.org/10.1038/ni.2166>.
- Chesné.J, Braza.F, Mahay.G, Brouard.S, et al. IL-17 in severe asthma: Where do we stand? *American Journal of Respiratory and Critical Care Medicine*, 2014. 190:(10): 1094–1101. <https://doi.org/10.1164/rccm.201405-0859PP>.
- Chuang.Y, Wang.T-CT-CT, Jen.H-YH-YH, Alice.L, et al. α -Galactosylceramide-Induced Airway Eosinophilia Is Mediated through the Activation of NKT Cells. *The Journal of Immunology*, 2011. 186:(8): 4687–92. <https://doi.org/10.4049/jimmunol.1003659>.
- Coomes.SM, Kannan.Y, Pelly.VS, Entwistle.LJ, et al. CD4⁺ Th2 cells are directly regulated by IL-10 during allergic airway inflammation. *Mucosal Immunology*, 2017. 10:(1): 150–61. <https://doi.org/10.1038/mi.2016.47>.
- Coquet.JM, Chakravarti.S, Kyparissoudis.K, McNab.FW, et al. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4⁺ NK1.1⁺ NKT cell population. *Proceedings of the National Academy of Sciences of the United States of America*, 2008. 105:(32): 11287–92. <https://doi.org/10.1073/pnas.0801631105>.
- Cortjens.B, Ingelse.SA, Calis.JC, Vlaar.AP, et al. Neutrophil subset responses in infants with severe viral respiratory infection. *Clinical Immunology*, 2017. 176:(March): 100–106. <https://doi.org/10.1016/j.clim.2016.12.012>.
- Cyphert-Daly.JM, Yang.Z, Ingram.JL, Tighe.RM, et al. Physiologic response to chronic house dust mite exposure in mice is dependent on lot characteristics.

- Journal of Allergy and Clinical Immunology*, 2019. 144:(5): 1428-1432.e8.
<https://doi.org/10.1016/j.jaci.2019.07.019>.
- Dejager.L, Dendoncker.K, Eggermont.M, Souffriau.J, Hauwermeiren.F Van, Willart.M, Wonterghem.E Van, Naessens.T, Ballegeer.M, Vandevyver.S, Hammad.H, Lambrecht.B, Bosscher.K De, Grooten.J, Libert.C. Neutralizing TNF a restores glucocorticoid sensitivity in a mouse model of neutrophilic airway inflammation, 2015. 8:(6): 1212–25. <https://doi.org/10.1038/mi.2015.12>.
- Dejager.L, Dendoncker.K, Eggermont.M, Souffriau.J, Hauwermeiren.F Van, Willart.M, Wonterghem.E Van, Naessens.T, Ballegeer.M, Vandevyver.S, Hammad.H, Lambrecht.B, Bosscher.K De, Grooten.J, Libert.C, et al. Neutralizing TNF α restores glucocorticoid sensitivity in a mouse model of neutrophilic airway inflammation. *Mucosal Immunology*, 2015. 8:(6): 1212–25. <https://doi.org/10.1038/mi.2015.12>.
- Dejager.L, Dendoncker.K, Eggermont.M, Souffriau.J, Hauwermeiren.F Van, et al. Neutralizing TNF α restores glucocorticoid sensitivity in a mouse model of neutrophilic airway inflammation. *Mucosal Immunology*, 2015. 8:(6): 1212–25. <https://doi.org/10.1038/mi.2015.12>.
- Demoor.T, Bracke.KR, Maes.T, Vandooren.B, et al. Role of lymphotoxin- α in cigarette smoke-induced inflammation and lymphoid neogenesis. *European Respiratory Journal*, 2008. 34:(2): 405–16. <https://doi.org/10.1183/09031936.00101408>.
- Deniset.JF, Kubes.P. Neutrophil heterogeneity: Bona fide subsets or polarization states? *Journal of Leukocyte Biology*, 2018. 103:(5): 829–38. <https://doi.org/10.1002/JLB.3RI0917-361R>.
- Donovan.C, Royce.SG, Vlahos.R, Bourke.JE. Lipopolysaccharide does not alter small airway reactivity in mouse lung slices. *PLoS ONE*, 2015. 10:(3): 1–16. <https://doi.org/10.1371/journal.pone.0122069>.

- Dua.B, Watson.RM, Gauvreau.GM, O'Byrne.PM. Myeloid and plasmacytoid dendritic cells in induced sputum after allergen inhalation in subjects with asthma. *Journal of Allergy and Clinical Immunology*, 2010. 126:(1): 133–39. <https://doi.org/10.1016/j.jaci.2010.04.006>.
- Edwards.MR, Saglani.S, Schwarze.J, Skevaki.C, et al. Addressing unmet needs in understanding asthma mechanisms. *European Respiratory Journal*, 2017. 49:(5). <https://doi.org/10.1183/13993003.02448-2016>.
- Ekstedt.S, S  fholm.J, Geor  n.SK, Cardell.LO. Dividing neutrophils in subsets reveals a significant role for activated neutrophils in the development of airway hyperreactivity. *Clinical and Experimental Allergy*, 2019. 49:(3): 285–91. <https://doi.org/10.1111/cea.13311>.
- Evren.E, Ringqvist.E, Willinger.T. Origin and ontogeny of lung macrophages: from mice to humans. *Immunology*, 2020. 160:(2): 126–38. <https://doi.org/10.1111/imm.13154>.
- Fahy.J V. Type 2 inflammation in asthma-present in most, absent in many. *Nature Reviews Immunology*, 2015. Nature Publishing Group. <https://doi.org/10.1038/nri3786>.
- Fallon.PG, Schwartz.C. The high and lows of type 2 asthma and mouse models. *Journal of Allergy and Clinical Immunology*, 2020. 145:(2): 496–98. <https://doi.org/10.1016/j.jaci.2019.11.031>.
- Fites.JS, Gui.M, Kernien.JF, Negoro.P, et al. *An Unappreciated Role for Neutrophil-DC Hybrids in Immunity to Invasive Fungal Infections. PLoS Pathogens*, 2018. Vol. 14. <https://doi.org/10.1371/journal.ppat.1007073>.
- Foo.SY, Phipps.S. Regulation of inducible BALT formation and contribution to immunity and pathology. *Mucosal Immunology*, 2010. 3:(6): 537–44. <https://doi.org/10.1038/mi.2010.52>.
- Gause.WC, Wynn.TA, Allen.JE. Type 2 immunity and wound healing: evolutionary

- refinement of adaptive immunity by helminths. *Nature Publishing Group*, 2013. 13:(8): 607–14. <https://doi.org/10.1038/nri3476>.
- Geerdink.RJ, Pillay.J, Meyaard.L, Bont.L. Neutrophils in respiratory syncytial virus infection: A target for asthma prevention. *Journal of Allergy and Clinical Immunology*, 2015. 136:(4): 838–47. <https://doi.org/10.1016/j.jaci.2015.06.034>.
- Gelfand.EW, Joetham.A, Wang.M, Takeda.K, et al. Spectrum of T-lymphocyte activities regulating allergic lung inflammation. *Immunological Reviews*, 2017. 278:(1): 63–86. <https://doi.org/10.1111/imr.12561>.
- Geng.S, Matsushima.H, Okamoto.T, Yao.Y, et al. Emergence, origin, and function of neutrophil-dendritic cell hybrids in experimentally induced inflammatory lesions in mice. *Blood*, 2013. 121:(10): 1690–1700. <https://doi.org/10.1182/blood-2012-07-445197>.
- Gernez.Y, Tirouvanziam.R, Chanez.P. Neutrophils in chronic inflammatory airway diseases: Can we target them and how? *European Respiratory Journal*, 2010. 35:(3): 467–69. <https://doi.org/10.1183/09031936.00186109>.
- Geurtsvankessel.CH, Willart.MAM, Bergen.IM, Rijt.LS Van, et al. Dendritic cells are crucial for maintenance of tertiary lymphoid structures in the lung of influenza virus-infected mice. *Journal of Experimental Medicine*, 2009. 206:(11): 2339–49. <https://doi.org/10.1084/jem.20090410>.
- Ghosh.S, Hoselton.SA, Asbach.S V., Steffan.BN, et al. B lymphocytes regulate airway granulocytic inflammation and cytokine production in a murine model of fungal allergic asthma. *Cellular and Molecular Immunology*, 2015. 12:(2): 202–12. <https://doi.org/10.1038/cmi.2014.103>.
- Gibblings.SL, Thomas.SM, Atif.SM, McCubbrey.AL, et al. Three unique interstitial macrophages in the murine lung at steady state. *American Journal of Respiratory Cell and Molecular Biology*, 2017. 57:(1): 66–76.

- <https://doi.org/10.1165/rcmb.2016-0361oc>.
- Ginhoux.F, Guilliams.M. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity*, 2016. 44:(3): 439–49. <https://doi.org/10.1016/j.immuni.2016.02.024>.
- Global initiative for Asthma. Global Strategy for Asthma Management and Prevention, available from www.ginasthma.org, 2020. [https://doi.org/10.1016/s0335-7457\(96\)80056-6](https://doi.org/10.1016/s0335-7457(96)80056-6).
- Gorski.SA, Hahn.YS, Braciale.TJ. Group 2 Innate Lymphoid Cell Production of IL-5 Is Regulated by NKT Cells during Influenza Virus Infection. *PLoS Pathogens*, 2013. 9:(9). <https://doi.org/10.1371/journal.ppat.1003615>.
- Grunwell.JR, Stephenson.ST, Tirouvanziam.R, Brown.LAS, et al. Children with Neutrophil-Predominant Severe Asthma Have Proinflammatory Neutrophils With Enhanced Survival and Impaired Clearance. *Journal of Allergy and Clinical Immunology: In Practice*, 2019. 7:(2): 516-525.e6. <https://doi.org/10.1016/j.jaip.2018.08.024>.
- Gubernatorova.EO, Namakanova.OA, Tumanov.A V., Drutskaya.MS, et al. Mouse models of severe asthma for evaluation of therapeutic cytokine targeting. *Immunology Letters*, 2019. 207:(November 2018): 73–83. <https://doi.org/10.1016/j.imlet.2018.11.012>.
- Gupta.A, Dimeloe.S, Richards.DF, Chambers.ES, et al. Defective IL-10 expression and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant asthma. *Thorax*, 2014. 69:(6): 508–15. <https://doi.org/10.1136/thoraxjnl-2013-203421>.
- Hachem.P, Lisbonne.M, Michel.M-L, Diem.S, et al. α -Galactosylceramide-induced iNKT cells suppress experimental allergic asthma in sensitized mice: Role of IFN- γ . *European Journal of Immunology*, 2005. 35:(10): 2793–2802. <https://doi.org/10.1002/eji.200535268>.

- Halle.S, Dujardin.HC, Bakocevic.N, Fleige.H, et al. Induced bronchus-associated lymphoid tissue serves as a general priming site for T cells and is maintained by dendritic cells. *Journal of Experimental Medicine*, 2009. 206:(12): 2593–2601. <https://doi.org/10.1084/jem.20091472>.
- Hammad.H, Plantinga.M, Deswarte.K, Pouliot.P, et al. Inflammatory dendritic cells - not basophils - are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *Journal of Experimental Medicine*, 2010. 207:(10): 2097–2111. <https://doi.org/10.1084/jem.20101563>.
- Hampton.HR, Bailey.J, Tomura.M, Brink.R, et al. Microbe-dependent lymphatic migration of neutrophils modulates lymphocyte proliferation in lymph nodes. *Nature Communications*, 2015. 6:(1): 7139. <https://doi.org/10.1038/ncomms8139>.
- Han.X, Wang.S, Fan.Y, Yang.J, et al. Chlamydia Infection Induces ICOS Ligand-Expressing and IL-10-Producing Dendritic Cells That Can Inhibit Airway Inflammation and Mucus Overproduction Elicited by Allergen Challenge in BALB/c Mice . *The Journal of Immunology*, 2006. 176:(9): 5232–39. <https://doi.org/10.4049/jimmunol.176.9.5232>.
- Haspeslagh.E, Debeuf.N, Hammad.H, Lambrecht.BN. Murine models of allergic asthma. *Methods in Molecular Biology*, 2017. 1559: 121–36. https://doi.org/10.1007/978-1-4939-6786-5_10.
- Hayashi.N, Yoshimoto.T, Izuhara.K, Matsui.K, et al. T helper 1 cells stimulated with ovalbumin and IL-18 induce airway hyperresponsiveness and lung fibrosis by IFN- γ and IL-13 production. *PNAS*, 2007. 104:(37): 14765–70. <https://doi.org/10.1073/pnas.0706378104>.
- He.X, Qian.Y, Li.Z, Fan.EK, et al. TLR4-Upregulated IL-1 β and IL-1RI Promote Alveolar Macrophage Pyroptosis and Lung Inflammation through an Autocrine Mechanism. *Scientific Reports*, 2016. 6:(April): 1–11.

<https://doi.org/10.1038/srep31663>.

Heer.HJ De, Hammad.H, Soullié.T, Hijdra.D, et al. Essential Role of Lung Plasmacytoid Dendritic Cells in Preventing Asthmatic Reactions to Harmless Inhaled Antigen, 2004. 200:(1). <https://doi.org/10.1084/jem.20040035>.

Heinen.P, Wanke.F, Moos.S, Attig.S, et al. Improved Method to Retain Cytosolic Reporter Protein Fluorescence While Staining for Nuclear Proteins, 2014., no. 3. <https://doi.org/10.1002/cyto.a.22451>.

Herbold.W, Maus.R, Hahn.I, Ding.N, et al. Importance of CXC chemokine receptor 2 in alveolar neutrophil and exudate macrophage recruitment in response to pneumococcal lung infection. *Infection and Immunity*, 2010. 78:(6): 2620–30. <https://doi.org/10.1128/IAI.01169-09>.

Hinks.TSC, Brown.T, Lau.LCK, Rupani.H, et al. Multidimensional endotyping in patients with severe asthma reveals inflammatory heterogeneity in matrix metalloproteinases and chitinase 3–like protein 1. *Journal of Allergy and Clinical Immunology*, 2016. 138:(1): 61–75. <https://doi.org/10.1016/j.jaci.2015.11.020>.

Hirahara.K, Shinoda.K, Endo.Y, Ichikawa.T, et al. Maintenance of memory-type pathogenic Th2 cells in the pathophysiology of chronic airway inflammation. *Inflammation and Regeneration*, 2018. 38:(1): 8–11. <https://doi.org/10.1186/s41232-018-0067-8>.

Ho.L-PL, Denney.L, Luhn.K, Teoh.D, et al. Activation of invariant NKT cells enhances the innate immune response and improves the disease course in influenza A virus infection. *European Journal of Immunology*, 2008. 38:(7): 1913–22. <https://doi.org/10.1002/eji.200738017>.

Hodge.S, Hodge.G, Simpson.JL, Yang.IA, et al. Blood cytotoxic/inflammatory mediators in non-eosinophilic asthma. *Clinical and Experimental Allergy*, 2016. 46:(1): 60–70. <https://doi.org/10.1111/cea.12634>.

- Hopkin.JM. The diagnosis of asthma, a clinical syndrome. *Thorax*, 2012. 67:(7): 660–62. <https://doi.org/10.1136/thoraxjnl-2012-201825>.
- Hosoki.K, Aguilera-Aguirre.L, Brasier.AR, Kurosky.A, et al. Facilitation of allergic sensitization and allergic airway inflammation by pollen-induced innate neutrophil recruitment. *American Journal of Respiratory Cell and Molecular Biology*, 2016. 54:(1): 81–90. <https://doi.org/10.1165/rcmb.2015-0044OC>.
- Huang.YC, Weng.CM, Lee.MJ, Lin.SM, et al. Endotypes of severe allergic asthma patients who clinically benefit from anti-IgE therapy. *Clinical and Experimental Allergy*, 2019. 49:(1): 44–53. <https://doi.org/10.1111/cea.13248>.
- Hyde.EJ, Wakelin.KA, Daniels.NJ, Ghosh.S, et al. Similar immune mechanisms control experimental airway eosinophilia elicited by different allergens and treatment protocols. *BMC Immunology*, 2019. 20:(1): 1–14. <https://doi.org/10.1186/s12865-019-0295-y>.
- Im.JS, Arora.P, Bricard.G, Molano.A, et al. Article Kinetics and Cellular Site of Glycolipid Loading Control the Outcome of Natural Killer T Cell Activation. *Immunity*, 2009. 30:(6): 888–98. <https://doi.org/10.1016/j.immuni.2009.03.022>.
- Ishikawa.H, Tanaka.K, Kutsukake.E, Fukui.T, et al. IFN- γ production downstream of NKT cell activation in mice infected with influenza virus enhances the cytolytic activities of both NK cells and viral antigen-specific CD8⁺ T cells. *Virology*, 2010. 407:(2): 325–32. <https://doi.org/10.1016/j.virol.2010.08.030>.
- Iwasaki.A, Foxman.EF, Molony.RD. Early local immune defences in the respiratory tract. *Nature Reviews Immunology*, 2017. 17:(1): 7–20. <https://doi.org/10.1038/nri.2016.117>.
- Iwasaki.A, Medzhitov.R. Control of adaptive immunity by the innate immune system. *Nature Immunology*, 2015. 16:(4): 343–53. <https://doi.org/10.1038/ni.3123>.
- Jang.E, Nguyen.QT, Kim.S, Kim.D, et al. Lung-Infiltrating Foxp3⁺ Regulatory T Cells

- Are Quantitatively and Qualitatively Different during Eosinophilic and Neutrophilic Allergic Airway Inflammation but Essential To Control the Inflammation. *The Journal of Immunology*, 2017. 199:(12): 3943–51. <https://doi.org/10.4049/jimmunol.1700211>.
- John-Schuster.G, Hager.K, Conlon.TM, Irmiler.M, et al. Cigarette smoke-induced iBALT mediates macrophage activation in a B cell-dependent manner in COPD. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 2014. 307:(9): L692–706. <https://doi.org/10.1152/ajplung.00092.2014>.
- Kasten.KR, Muenzer.JT, Caldwell.CC. Neutrophils are significant producers of IL-10 during sepsis. *Biochemical and Biophysical Research Communications*, 2010. 393:(1): 28–31. <https://doi.org/10.1016/j.bbrc.2010.01.066>.
- Katsura.Y, Harada.N, Harada.S, Ishimori.A, et al. Characteristics of alveolar macrophages from murine models of OVA-induced allergic airway inflammation and LPS-induced acute airway inflammation. *Experimental Lung Research*, 2015. 41:(7): 370–82. <https://doi.org/10.3109/01902148.2015.1044137>.
- Kawakami.K, Yamamoto.N, Kinjo.Y, Miyagi.K, et al. Critical role of Valpha14+ natural killer T cells in the innate phase of host protection against Streptococcus pneumoniae infection. *European Journal of Immunology*, 2003. 33:(12): 3322–30. <https://doi.org/10.1002/eji.200324254>.
- Kawano.H, Kayama.H, Nakama.T, Hashimoto.T, et al. IL-10-producing lung interstitial macrophages prevent neutrophilic asthma. *International Immunology*, 2016. 28:(10): 489–501. <https://doi.org/10.1093/intimm/dxw012>.
- Kearley.J, Barker.JE, Robinson.DS, Lloyd.CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *Journal of Experimental Medicine*, 2005. 202:(11): 1539–47. <https://doi.org/10.1084/jem.20051166>.

- Khandpur.R, Carmona-Rivera.C, Vivekanandan-Giri.A, Gizinski.A, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Science Translational Medicine*, 2013. 5:(178): 178ra40. <https://doi.org/10.1126/scitranslmed.3005580>.
- Kim.A-R, Kim.HS, Kim.DK, Nam.ST, et al. Mesenteric IL-10-producing CD5+ regulatory B cells suppress cow's milk casein-induced allergic responses in mice. *Scientific Reports*, 2016. 6:(1): 19685. <https://doi.org/10.1038/srep19685>.
- Kim.HS, Kim.A, Kim.DK, Kim.HW, et al. Interleukin-10 – producing CD5 + B cells inhibit mast cells during immunoglobulin E – mediated allergic responses, 2015. 8:(368): 1–12.
- Kim.HY, Dekruyff.RH, Umetsu.DT. The many paths to asthma: Phenotype shaped by innate and adaptive immunity. *Nature Immunology*, 2010. 11:(7): 577–84. <https://doi.org/10.1038/ni.1892>.
- Kim.JH, Podstawka.J, Lou.Y, Li.L, et al. Aged polymorphonuclear leukocytes cause fibrotic interstitial lung disease in the absence of regulation by B cells article. *Nature Immunology*, 2018. 19:(2): 192–201. <https://doi.org/10.1038/s41590-017-0030-x>.
- Kim.JO, Kim.DH, Chang.WS, Hong.C, et al. Asthma is induced by intranasal coadministration of allergen and natural killer T-cell ligand in a mouse model. *Journal of Allergy and Clinical Immunology*, 2004. 114:(6): 1332–38. <https://doi.org/10.1016/j.jaci.2004.09.004>.
- King.IL, Fortier.A, Tighe.M, Dibble.J, et al. Invariant natural killer T cells direct B cell responses to cognate lipid antigen in an IL-21-dependent manner. *Nature Immunology*, 2012. 13:(1): 44–50. <https://doi.org/10.1038/ni.2172>.
- Kinjo.Y, Illarionov.P, Vela.JL, Pei.B, et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nature Immunology*, 2011. 12:(10): 966–74. <https://doi.org/10.1038/ni.2096>.

- Kok.WL, Denney.L, Benam.K, Cole.S, et al. Pivotal Advance: Invariant NKT cells reduce accumulation of inflammatory monocytes in the lungs and decrease immune-pathology during severe influenza A virus infection. *Journal of Leukocyte Biology*, 2012. 91:(3): 357–68. <https://doi.org/10.1189/jlb.0411184>.
- Kool.M, Nimwegen.M van, Willart.MAM, Muskens.F, et al. An Anti-Inflammatory Role for Plasmacytoid Dendritic Cells in Allergic Airway Inflammation. *The Journal of Immunology*, 2009. 183:(2): 1074–82. <https://doi.org/10.4049/jimmunol.0900471>.
- Korošec.P, Gibbs.BF, Rijavec.M, Custovic.A, et al. Important and specific role for basophils in acute allergic reactions. *Clinical and Experimental Allergy*, 2018. 48:(5): 502–12. <https://doi.org/10.1111/cea.13117>.
- Koya.T, Matsuda.H, Takeda.K, Matsubara.S, et al. IL-10-treated dendritic cells decrease airway hyperresponsiveness and airway inflammation in mice. *Journal of Allergy and Clinical Immunology*, 2007. 119:(5): 1241–50. <https://doi.org/10.1016/j.jaci.2007.01.039>.
- Kretschmer.S, Pieper.M, Hüttmann.G, Bölke.T, et al. Autofluorescence multiphoton microscopy for visualization of tissue morphology and cellular dynamics in murine and human airways. *Laboratory Investigation*, 2016. 96:(8): 918–31. <https://doi.org/10.1038/labinvest.2016.69>.
- Kuroda.E, Ozasa.K, Temizoz.B, Ohata.K, et al. Inhaled Fine Particles Induce Alveolar Macrophage Death and Interleukin-1 α Release to Promote Inducible Bronchus-Associated Lymphoid Tissue Formation. *Immunity*, 2016. 45:(6): 1299–1310. <https://doi.org/10.1016/j.immuni.2016.11.010>.
- Lambrecht.BN, Hammad.H. The immunology of asthma. *Nature Immunology*, 2015. 16:(1): 45–56. <https://doi.org/10.1038/ni.3049>.
- Lambrecht.BN, Salomon.B, Klatzmann.D, Pauwels.RA. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to

- inhaled antigen in sensitized mice. *Journal of Immunology*, 1998. 160:(8): 4090–97.
- Lambrecht.BN, Veerman.M De, Coyle.AJ, Gutierrez-Ramos.JC, et al. Myeloid dendritic cells induce TH2 responses to inhaled antigen, leading to eosinophilic airway inflammation. *Journal of Clinical Investigation*, 2000. 106:(4): 551–59. <https://doi.org/10.1172/JCI8107>.
- Lefaudeux.D, Meulder.B De, Loza.MJ, Pepper.N, et al. U-BIOPRED clinical adult asthma clusters linked to a subset of sputum omics. *Journal of Allergy and Clinical Immunology*, 2017. 139:(6): 1797–1807. <https://doi.org/10.1016/j.jaci.2016.08.048>.
- Ley.K, Hoffman.HM, Kubes.P, Cassatella.MA, et al. Neutrophils: New insights and open questions. *Science Immunology*, 2018. 3:(30). <https://doi.org/10.1126/sciimmunol.aat4579>.
- Li.Y, Wang.W, Yang.F, Xu.Y, et al. The regulatory roles of neutrophils in adaptive immunity. *Cell Communication and Signaling*, 2019. 17:(1): 1–11. <https://doi.org/10.1186/s12964-019-0471-y>.
- Lin.KL, Suzuki.Y, Nakano.H, Ramsburg.E, et al. CCR2 + Monocyte-Derived Dendritic Cells and Exudate Macrophages Produce Influenza-Induced Pulmonary Immune Pathology and Mortality . *The Journal of Immunology*, 2008. 180:(4): 2562–72. <https://doi.org/10.4049/jimmunol.180.4.2562>.
- Lisbonne.M, Diem.S, Castro.A De, Lefort.J, et al. Cutting edge: invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *Journal of Immunology (Baltimore, Md. : 1950)*, 2003. 171:(4): 1637–41. <https://doi.org/10.4049/jimmunol.171.4.1637>.
- Lombardi.V, Stock.P, Singh.AK, Kerzerho.J, et al. A CD1d-dependent antagonist inhibits the activation of invariant NKT cells and prevents development of

- allergen-induced airway hyperreactivity. *J Immunol*, 2010. 184:(4): 2107–15. <https://doi.org/10.4049/jimmunol.0901208>.
- Loza.MJ, Djukanovic.R, Chung.KF, Horowitz.D, et al. Validated and longitudinally stable asthma phenotypes based on cluster analysis of the ADEPT study. *Respiratory Research*, 2016. 17:(1): 1–21. <https://doi.org/10.1186/s12931-016-0482-9>.
- Lynch.JP, Werder.RB, Loh.Z, Sikder.MAA, et al. Plasmacytoid dendritic cells protect from viral bronchiolitis and asthma through semaphorin 4a-mediated T reg expansion. *Journal of Experimental Medicine*, 2018. 215:(2): 537–57. <https://doi.org/10.1084/jem.20170298>.
- Lynch.L, Michelet.X, Zhang.S, Brennan.PJ, et al. Regulatory iNKT cells lack expression of the transcription factor PLZF and control the homeostasis of T(reg) cells and macrophages in adipose tissue. *Nature Immunology*, 2015. 16:(1): 85–96. <https://doi.org/10.1038/ni.3047>.
- Maazi.H., Lam.J, Lombardi.V, Akbari.O. Role of plasmacytoid dendritic cell subsets in allergic asthma. *Allergy: European Journal of Allergy and Clinical Immunology*, 2013. 68:(6): 695–701. <https://doi.org/10.1111/all.12166>.
- Maazi.Hadi, Banie.H, Aleman Muench.GR, Patel.N, et al. Activated plasmacytoid dendritic cells regulate type 2 innate lymphoid cell-mediated airway hyperreactivity. *Journal of Allergy and Clinical Immunology*, 2018. 141:(3): 893-905.e6. <https://doi.org/10.1016/j.jaci.2017.04.043>.
- Machado-Aranda.D, Suresh.M V., Yu.B, Dolgachev.V, et al. Alveolar macrophage depletion increases the severity of acute inflammation following nonlethal unilateral lung contusion in mice. *Journal of Trauma and Acute Care Surgery*, 2014. 76:(4): 982–90. <https://doi.org/10.1097/TA.0000000000000163>.
- Madan.R, Demircik.F, Surianarayanan.S, Allen.JL, et al. Nonredundant Roles for B Cell-Derived IL-10 in Immune Counter-Regulation. *The Journal of Immunology*,

2009. 183:(4): 2312–20. <https://doi.org/10.4049/jimmunol.0900185>.
- Maltby.S, Tay.HL, Yang.M, Foster.PS. Mouse models of severe asthma: Understanding the mechanisms of steroid resistance, tissue remodelling and disease exacerbation. *Respirology*, 2017. 22:(5): 874–85. <https://doi.org/10.1111/resp.13052>.
- Marchi.LF, Sesti-Costa.R, Chedraoui-Silva.S, Mantovani.B. Comparison of four methods for the isolation of murine blood neutrophils with respect to the release of reactive oxygen and nitrogen species and the expression of immunological receptors. *Comparative Clinical Pathology*, 2014. 23:(5): 1469–76. <https://doi.org/10.1007/s00580-013-1808-3>.
- Margaroli.C, Garratt.LW, Horati.H, Dittrich.AS, et al. Elastase exocytosis by airway neutrophils is associated with early lung damage in children with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine*, 2019. 199:(7): 873–81. <https://doi.org/10.1164/rccm.201803-0442OC>.
- Marin.ND, Dunlap.MD, Kaushal.D, Khader.SA. Friend or Foe: The Protective and Pathological Roles of Inducible Bronchus-Associated Lymphoid Tissue in Pulmonary Diseases. *The Journal of Immunology*, 2019. 202:(9): 2519–26. <https://doi.org/10.4049/jimmunol.1801135>.
- Marriott.HM, Hellewell.PG, Cross.SS, Ince.PG, et al. Decreased Alveolar Macrophage Apoptosis Is Associated with Increased Pulmonary Inflammation in a Murine Model of Pneumococcal Pneumonia. *The Journal of Immunology*, 2006. 177:(9): 6480–88. <https://doi.org/10.4049/jimmunol.177.9.6480>.
- Mathie.SA, Dixon.KL, Walker.SA, Tyrrell.V, et al. Alveolar macrophages are sentinels of murine pulmonary homeostasis following inhaled antigen challenge. *Allergy: European Journal of Allergy and Clinical Immunology*, 2015. 70:(1): 80–89. <https://doi.org/10.1111/all.12536>.
- Matsuda.H, Takeda.K, Koya.T, Okamoto.M, et al. Plasticity of invariant NKT cell

- regulation of allergic airway disease is dependent on IFN-gamma production. *Journal of Immunology (Baltimore, Md.: 1950)*, 2010. 185:(1): 253–62. <https://doi.org/10.4049/jimmunol.0902301>.
- Maus.UA, Janzen.S, Wall.G, Srivastava.M, et al. Resident alveolar macrophages are replaced by recruited monocytes in response to endotoxin-induced lung inflammation. *American Journal of Respiratory Cell and Molecular Biology*, 2006. 35:(2): 227–35. <https://doi.org/10.1165/rcmb.2005-0241OC>.
- Mcalees.JW, Whitehead.GS, Harley.ITWW, Cappelletti.M, et al. Distinct Tlr4-expressing cell compartments control neutrophilic and eosinophilic airway inflammation. *Mucosal Immunol*, 2015. 8:(4): 863–73. <https://doi.org/10.1038/mi.2014.117>.
- McGrath.KW, Icitovic.N, Boushey.HA, Lazarus.SC, et al. A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. *American Journal of Respiratory and Critical Care Medicine*, 2012. 185:(6): 612–19. <https://doi.org/10.1164/rccm.201109-1640OC>.
- McQuattie-Pimentel.AC, Budinger.GRS, Ballinger.MN. Monocyte-derived Alveolar Macrophages: The Dark Side of Lung Repair? *American Journal of Respiratory Cell and Molecular Biology*, 2018. 58:(1): 5–6. <https://doi.org/10.1165/rcmb.2017-0328ED>.
- Meziani.L, Mondini.M, Petit.B, Boissonnas.A, et al. CSF1R inhibition prevents radiation pulmonary fibrosis by depletion of interstitial macrophages. *European Respiratory Journal*, 2018. 51:(3): 1–13. <https://doi.org/10.1183/13993003.02120-2017>.
- Michel.M-L, Keller.AC, Paget.C, Fujio.M, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *The Journal of Experimental Medicine*, 2007. 204:(5): 995–1001. <https://doi.org/10.1084/jem.20061551>.

- Misharin.A V., Morales-Nebreda.L, Reyfman.PA, Cuda.CM, et al. Monocyte-derived alveolar macrophages drive lung fibrosis and persist in the lung over the life span. *The Journal of Experimental Medicine*, 2017. 214:(8): 2387–2404. <https://doi.org/10.1084/jem.20162152>.
- Mitchell.AJ, Pradel.LC, Chasson.L, Rooijen.N Van, et al. Technical Advance: Autofluorescence as a tool for myeloid cell analysis. *Journal of Leukocyte Biology*, 2010. 88:(3): 597–603. <https://doi.org/10.1189/jlb.0310184>.
- Morita.H, Kubo.T, Rückert.B, Ravindran.A, et al. Induction of human regulatory innate lymphoid cells from group 2 innate lymphoid cells by retinoic acid. *Journal of Allergy and Clinical Immunology*, 2019. 143:(6): 2190-2201.e9. <https://doi.org/10.1016/j.jaci.2018.12.1018>.
- Moro.K, Ealey.KN, Kabata.H, Koyasu.S. Isolation and analysis of group 2 innate lymphoid cells in mice. *Nature Protocols*, 2015. 10:(5): 792–806. <https://doi.org/10.1038/nprot.2015.047>.
- Mould.KJ, Barthel.L, Mohning.MP, Thomas.SM, et al. Cell origin dictates programming of resident versus recruited macrophages during acute lung injury. *American Journal of Respiratory Cell and Molecular Biology*, 2017. 57:(3): 294–306. <https://doi.org/10.1165/rcmb.2017-0061OC>.
- Nakada.EM, Shan.J, Kinyanjui.MW, Fixman.ED. Adjuvant-dependent regulation of interleukin-17 expressing $\gamma\delta$ T cells and inhibition of Th2 responses in allergic airways disease. *Respiratory Research*, 2014. 15:(1): 1–14. <https://doi.org/10.1186/s12931-014-0090-5>.
- Nakae.S, Ho.LH, Yu.M, Monteforte.R, et al. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. *Journal of Allergy and Clinical Immunology*, 2007. 120:(1): 48–55. <https://doi.org/10.1016/j.jaci.2007.02.046>.
- Nel.I, Lehen.A. Defective Invariant Natural Killer T-Cell Suppression in Patients With

- Type 1 Diabetes. *Diabetes*, 2016. 65(8): 2121–23. <https://doi.org/10.2337/dbi16-0021>.
- Németh.T, Sperandio.M, Mócsai.A. Neutrophils as emerging therapeutic targets. *Nature Reviews Drug Discovery*, 2020. 19(4): 253–75. <https://doi.org/10.1038/s41573-019-0054-z>.
- Nicolás-Ávila.JÁ, Adrover.JM, Hidalgo.A. Neutrophils in Homeostasis, Immunity, and Cancer. *Immunity*, 2017. 46(1): 15–28. <https://doi.org/10.1016/j.immuni.2016.12.012>.
- Ntontsi.P, Samitas.K, Zervas.E, Gaga.M. Severe asthma: what is new in the new millennium. *Current Opinion in Allergy and Clinical Immunology*, 2020. 20(2): 202–7. <https://doi.org/10.1097/ACI.0000000000000623>.
- Ocuin.LM, Bamboat.ZM, Balachandran.VP, Cavnar.MJ, et al. Neutrophil IL-10 suppresses peritoneal inflammatory monocytes during polymicrobial sepsis. *Journal of Leukocyte Biology*, 2011. 89(3): 423–32. <https://doi.org/10.1189/jlb.0810479>.
- Ogawa.Y, Duru.E, Ameredes.B. Role of IL-10 in the Resolution of Airway Inflammation. *Current Molecular Medicine*, 2008. 8(5): 437–45. <https://doi.org/10.2174/156652408785160907>.
- Oh.JW, Seroogy.CM, Meyer.EH, Akbari.O, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *Journal of Allergy and Clinical Immunology*, 2002. 110(3): 460–68. <https://doi.org/10.1067/mai.2002.127512>.
- Okamoto.M, Shiraishi.Y, Miyahara.N, Dakhama.A, et al. Plasticity of Invariant NKT Cell Regulation of Allergic Airway Disease Is Dependent on IFN- γ Production. *J Immunol*, 2010., no. 185: 253–62. <https://doi.org/10.4049/jimmunol.0902301>.
- Oliveira.S De, Rosowski.EE, Huttenlocher.A. Neutrophil migration in infection and wound repair : going forward in reverse. *Nature Publishing Group*, 2016. 16(6):

- 378–91. <https://doi.org/10.1038/nri.2016.49>.
- Osterholzer.JJ, Chen.GH, Olszewski.MA, Zhang.YM, et al. Chemokine receptor 2-mediated accumulation of fungicidal exudate macrophages in mice that clear cryptococcal lung infection. *American Journal of Pathology*, 2011. 178:(1): 198–211. <https://doi.org/10.1016/j.ajpath.2010.11.006>.
- Osterholzer.JJ, Olszewski.MA, Murdock.BJ, Chen.G-H, et al. Implicating Exudate Macrophages and Ly-6C high Monocytes in CCR2-Dependent Lung Fibrosis following Gene-Targeted Alveolar Injury. *The Journal of Immunology*, 2013. 190:(7): 3447–57. <https://doi.org/10.4049/jimmunol.1200604>.
- Ouyang.W, O’Garra.A. IL-10 Family Cytokines IL-10 and IL-22: from Basic Science to Clinical Translation. *Immunity*, 2019. 50:(4): 871–91. <https://doi.org/10.1016/j.immuni.2019.03.020>.
- Panettieri.RA. The Role of Neutrophils in Asthma. *Immunology and Allergy Clinics of North America*, 2018. 38:(4): 629–38. <https://doi.org/10.1016/j.iac.2018.06.005>.
- Peñaloza.HF, Noguera.LP, Ahn.D, Vallejos.OP, et al. Interleukin-10 produced by Myeloid-Derived suppressor cells provides protection to Carbapenem-Resistant klebsiella pneumoniae sequence type 258 by enhancing its clearance in the airways. *Infection and Immunity*, 2019. 87:(5). <https://doi.org/10.1128/IAI.00665-18>.
- Perobelli.SM, Mercadante.ACT, Galvani.RG, Gonçalves-Silva.T, et al. G-CSF–Induced Suppressor IL-10 + Neutrophils Promote Regulatory T Cells That Inhibit Graft-Versus-Host Disease in a Long-Lasting and Specific Way . *The Journal of Immunology*, 2016. 197:(9): 3725–34. <https://doi.org/10.4049/jimmunol.1502023>.
- Persson.IM, Menzel.M, Ramu.S, Cerps.S, et al. IL-1 β mediates lung neutrophilia and IL-33 expression in a mouse model of viral-induced asthma exacerbation.

- Respiratory Research*, 2018. 19:(1): 1–10. <https://doi.org/10.1186/s12931-018-0725-z>.
- Peters.MC, Mekonnen.ZK, Yuan.S, Bhakta.NR, et al. Measures of gene expression in sputum cells can identify T H2-high and TH2-low subtypes of asthma. *Journal of Allergy and Clinical Immunology*, 2014. 133:(2). <https://doi.org/10.1016/j.jaci.2013.07.036>.
- Petsky.HL, Li.A, Chang.AB. Tailored interventions based on sputum eosinophils versus clinical symptoms for asthma in children and adults. *Cochrane Database of Systematic Reviews*, 2017. 2017:(8). <https://doi.org/10.1002/14651858.CD005603.pub3>.
- Pham.DL, Ban.GY, Kim.SH, Shin.YS, et al. Neutrophil autophagy and extracellular DNA traps contribute to airway inflammation in severe asthma. *Clinical and Experimental Allergy*, 2017. 47:(1): 57–70. <https://doi.org/10.1111/cea.12859>.
- Pitzalis.C, Jones.GW, Bombardieri.M, Jones.SA. Ectopic lymphoid-like structures in infection, cancer and autoimmunity. *Nature Reviews Immunology*, 2014. 14:(7): 447–62. <https://doi.org/10.1038/nri3700>.
- Postma.DS. Gender Differences in Asthma Development and Progression. *Gender Medicine*, 2007. 4:(SUPPL. 2): 133–46. [https://doi.org/10.1016/S1550-8579\(07\)80054-4](https://doi.org/10.1016/S1550-8579(07)80054-4).
- Potter.NS, Harding.C V. Neutrophils Process Exogenous Bacteria Via an Alternate Class I MHC Processing Pathway for Presentation of Peptides to T Lymphocytes. *The Journal of Immunology*, 2001. 167:(5): 2538–46. <https://doi.org/10.4049/jimmunol.167.5.2538>.
- Radermecker.C, Sabatel.C, Vanwinge.C, Ruscitti.C, et al. Locally instructed CXCR4hi neutrophils trigger environment-driven allergic asthma through the release of neutrophil extracellular traps. *Nature Immunology*, 2019. 20:(11): 1444–55. <https://doi.org/10.1038/s41590-019-0496-9>.

- Raeiszadeh Jahromi.S, Mahesh.PA, Jayaraj.BS, Madhunapantula.SR V., et al. Serum levels of IL-10, IL-17F and IL-33 in patients with asthma: A case-control study. *Journal of Asthma*, 2014. 51:(10): 1004–13. <https://doi.org/10.3109/02770903.2014.938353>.
- Ray.A, Kolls.JK. Neutrophilic Inflammation in Asthma and Association with Disease Severity. *Trends in Immunology*, 2017. 38:(12): 942–54. <https://doi.org/10.1016/j.it.2017.07.003>.
- Ray.A, Raundhal.M, Oriss.TB, Ray.P, et al. Current concepts of severe asthma. *Journal of Clinical Investigation*, 2016. 126:(7): 2394–2403. <https://doi.org/10.1172/JCI84144>.
- Reber.LL, Sibilano.R, Mukai.K, Galli.SJ. Potential effector and immunoregulatory functions of mast cells in mucosal immunity. *Mucosal Immunology*, 2015. 8:(3): 444–63. <https://doi.org/10.1038/mi.2014.131>.
- Robinson.DS. The role of the T cell in asthma. *Journal of Allergy and Clinical Immunology*, 2010. 126:(6): 1081–91. <https://doi.org/10.1016/j.jaci.2010.06.025>.
- Robledo-Avila.FH, Ruiz-Rosado.J de D, Brockman.KL, Kopp.BT, et al. Dysregulated Calcium Homeostasis in Cystic Fibrosis Neutrophils Leads to Deficient Antimicrobial Responses. *The Journal of Immunology*, 2018., ji1800076. <https://doi.org/10.4049/jimmunol.1800076>.
- Roederer.M, Darzynkiewicz.Z, Parks.DR. Guidelines for the presentation of flow cytometric data. *Methods in Cell Biology*, 2004. 2004:(75): 241–56. [https://doi.org/10.1016/s0091-679x\(04\)75010-4](https://doi.org/10.1016/s0091-679x(04)75010-4).
- Sabatel.C, Radermecker.C, Fievez.L, Paulissen.G, et al. Exposure to Bacterial CpG DNA Protects from Airway Allergic Inflammation by Expanding Regulatory Lung Interstitial Macrophages. *Immunity*, 2017. 46:(3): 457–73. <https://doi.org/10.1016/j.immuni.2017.02.016>.

- Sadik.CD, Kim.ND, Luster.AD. Neutrophils cascading their way to inflammation. *Trends in Immunology*, 2011. 32:(10): 452–60. <https://doi.org/10.1016/j.it.2011.06.008>.
- Sag.D, Krause.P, Hedrick.CC, Kronenberg.M, et al. IL-10 – producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *The Journal of Clinical Investigation*, 2014. 124:(9): 1–16. <https://doi.org/10.1172/JCI72308DS1>.
- Sag.D, Özkan.M, Kronenberg.M, Wingender.G. Improved Detection of Cytokines Produced by Invariant NKT Cells. *Scientific Reports*, 2017. 7:(1). <https://doi.org/10.1038/s41598-017-16832-1>.
- Samitas.K, Delimpoura.V, Zervas.E, Gaga.M. Anti-IgE treatment, airway inflammation and remodelling in severe allergic asthma: Current knowledge and future perspectives. *European Respiratory Review*, 2015. 24:(138): 594–601. <https://doi.org/10.1183/16000617.00001715>.
- Santo.C De, Salio.M, Dong.T, Reiter.Y, et al. Cerundolo et al. respond. *Nature Immunology*, 2011. 12:(11): 1018–20. <https://doi.org/10.1038/ni.2132>.
- Sanz-Moreno.V, Balkwill.FR. Mets and NETs: the Awakening Force. *Immunity*, 2018. 49:(5): 798–800. <https://doi.org/10.1016/j.immuni.2018.11.009>.
- Sawaguchi.M, Tanaka.S, Nakatani.Y, Harada.Y, et al. Role of Mast Cells and Basophils in IgE Responses and in Allergic Airway Hyperresponsiveness. *The Journal of Immunology*, 2012. 188:(4): 1809–18. <https://doi.org/10.4049/jimmunol.1101746>.
- Shasby.DM, Ries.DR, Shasby.SS, Winter.MC. Histamine stimulates phosphorylation of adherens junction proteins and alters their link to vimentin. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 2002. 282:(6 26-6): L1330–38. <https://doi.org/10.1152/ajplung.00329.2001>.
- Shimizu.K, Sato.Y, Shinga.J, Watanabe.T, et al. KLRG+ invariant natural killer T cells are long-lived effectors. *Proceedings of the National Academy of Sciences of the*

- United States of America*, 2014. 111:(34): 12474–79.
<https://doi.org/10.1073/pnas.1406240111>.
- Shinoda.K, Hirahara.K, Nakayama.T. Maintenance of pathogenic Th2 cells in allergic disorders. *Allergology International*, 2017. 66:(3): 369–76.
<https://doi.org/10.1016/j.alit.2017.03.005>.
- Siebert.JC, Inokuma.M, Waid.DM, Pennock.ND, et al. An analytical workflow for investigating cytokine profiles. *Cytometry Part A*, 2008. 73:(4): 289–98.
<https://doi.org/10.1002/cyto.a.20509>.
- Silvestre-Roig.C, Fridlender.ZG, Glogauer.M, Scapini.P. Neutrophil Diversity in Health and Disease. *Trends in Immunology*, 2019. 40:(7): 565–83.
<https://doi.org/10.1016/j.it.2019.04.012>.
- Silvestre-roig.C, Hidalgo.A, Soehnlein.O. Review Article Neutrophil heterogeneity: implications for homeostasis and pathogenesis, 2017. 127:(18): 2173–82.
<https://doi.org/10.1182/blood-2016-01-688887>.The.
- Silvestre-Roig.C, Hidalgo.A, Soehnlein.O. Neutrophil heterogeneity: Implications for homeostasis and pathogenesis. *Blood*, 2016. <https://doi.org/10.1182/blood-2016-01-688887>.
- Simpson.JL, Grissell.T V., Douwes.J, Scott.RJ, et al. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax*, 2007. 62:(3): 211–18.
<https://doi.org/10.1136/thx.2006.061358>.
- Simpson.JL, Scott.R, Boyle.MJ, Gibson.PG. Inflammatory subtypes in asthma: Assessment and identification using induced sputum. *Respirology*, 2006. 11:(1): 54–61. <https://doi.org/10.1111/j.1440-1843.2006.00784.x>.
- Stokes.JR, Casale.TB. Characterization of asthma endotypes: implications for therapy. *Annals of Allergy, Asthma and Immunology*, 2016. 117:(2): 121–25.
<https://doi.org/10.1016/j.anai.2016.05.016>.
- Sugita.K, Sokolowska.M, Akdis.CA. *Key Points for Moving the Endotypes Field*

Forward. Implementing Precision Medicine in Best Practices of Chronic Airway Diseases, 2019. Elsevier Inc. <https://doi.org/10.1016/b978-0-12-813471-9.00020-7>.

- Sumagin.R, Robin.AZ, Nusrat.A, Parkos.CA. Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment. *Mucosal Immunology*, 2014. 7:(4): 905–15. <https://doi.org/10.1038/mi.2013.106>.
- Sun.J, Madan.R, Karp.CL, Braciale.TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. *Nature Medicine*, 2009. 15:(3): 277–84. <https://doi.org/10.1038/nm.1929>.
- Sze.E, Bhalla.A, Nair.P. Mechanisms and therapeutic strategies for non-T2 asthma. *Allergy: European Journal of Allergy and Clinical Immunology*, 2020. 75:(2): 311–25. <https://doi.org/10.1111/all.13985>.
- Takashima.A, Yao.Y. Neutrophil plasticity: acquisition of phenotype and functionality of antigen-presenting cell. *Journal of Leukocyte Biology*, 2015. 98:(4): 489–96. <https://doi.org/10.1189/jlb.1MR1014-502R>.
- Tamassia.N, Bianchetto-Aguilera.F, Arruda-Silva.F, Gardiman.E, et al. Cytokine production by human neutrophils: Revisiting the “dark side of the moon.” *European Journal of Clinical Investigation*, 2018. 48:(May): 1–10. <https://doi.org/10.1111/eci.12952>.
- Taut.K, Winter.C, Briles.DE, Paton.JC, et al. Macrophage turnover kinetics in the lungs of mice infected with *Streptococcus pneumoniae*. *American Journal of Respiratory Cell and Molecular Biology*, 2008. 38:(1): 105–13. <https://doi.org/10.1165/rcmb.2007-0132OC>.
- Teo.SM, Mok.D, Pham.K, Kusel.M, et al. The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host and Microbe*, 2015. 17:(5): 704–15.

<https://doi.org/10.1016/j.chom.2015.03.008>.

- Thanabalasuriar.A, Neupane.AS, Wang.J, Krummel.MF, et al. iNKT Cell Emigration out of the Lung Vasculature Requires Neutrophils and Monocyte-Derived Dendritic Cells in Inflammation Article iNKT Cell Emigration out of the Lung Vasculature Requires Neutrophils and Monocyte-Derived Dendritic Cells in Inflammation. *CellReports*, 2016. 16:(12): 3260–72. <https://doi.org/10.1016/j.celrep.2016.07.052>.
- Tian.B ping, Xia.L xia, Bao.Z qiang, Zhang.H, et al. Bcl-2 inhibitors reduce steroid-insensitive airway inflammation. *Journal of Allergy and Clinical Immunology*, 2017. 140:(2): 418–30. <https://doi.org/10.1016/j.jaci.2016.11.027>.
- Tighe.RM, Liang.J, Liu.N, Jung.Y, et al. Recruited exudative macrophages selectively produce CXCL10 following noninfectious lung injury. *American Journal of Respiratory Cell and Molecular Biology*, 2011. 45:(4): 781–88. <https://doi.org/10.1165/rcmb.2010-0471OC>.
- Tighe.RM, Misharin.A V., Jakubzick.C V., Brinkman.R, et al. Improving the quality and reproducibility of flow cytometry in the lung. *American Journal of Respiratory Cell and Molecular Biology*, 2019. 61:(2): 150–61. <https://doi.org/10.1165/rcmb.2019-0191ST>.
- Tonti.E, Fedeli.M, Napolitano.A, Iannacone.M, et al. Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of CD4(+) T cell help. *Journal of Immunology (Baltimore, Md. : 1950)*, 2012. 188:(7): 3217–22. <https://doi.org/10.4049/jimmunol.1103501>.
- Tsuda.Y, Takahashi.H, Kobayashi.M, Hanafusa.T, et al. Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant *Staphylococcus aureus*. *Immunity*, 2004. 21:(2): 215–26. <https://doi.org/10.1016/j.immuni.2004.07.006>.
- Ubags.NDJ, Suratt.BT. Isolation and characterization of mouse neutrophils. *Methods*

- in Molecular Biology*, 2018. 1809: 45–57. https://doi.org/10.1007/978-1-4939-8570-8_4.
- Upham.JW, Denburg.JA, O’Byrne.PM. Rapid response of circulating myeloid dendritic cells to inhaled allergen in asthmatic subjects. *Clinical and Experimental Allergy*, 2002. 32:(6): 818–23. <https://doi.org/10.1046/j.1365-2222.2002.01375.x>.
- Verduyn.M, Botto.G, Jaubert.J, Lier.C, et al. Serum IgG Concentrations in Adult Patients Experiencing Virus-Induced Severe Asthma Exacerbations. *Journal of Allergy and Clinical Immunology: In Practice*, 2019. 7:(5): 1507-1513.e1. <https://doi.org/10.1016/j.jaip.2018.12.028>.
- Wadhwa.R, Dua.K, Adcock.IM, Horvat.JC, et al. Cellular mechanisms underlying steroid-resistant asthma. *European Respiratory Review*, 2019. 28:(153): 190096. <https://doi.org/10.1183/16000617.0096-2019>.
- Wakahara.K, Van.VQ, Baba.N, Bégin.P, et al. Basophils are recruited to inflamed lungs and exacerbate memory Th2 responses in mice and humans. *Allergy: European Journal of Allergy and Clinical Immunology*, 2013. 68:(2): 180–89. <https://doi.org/10.1111/all.12072>.
- Wang.S, Xia.P, Chen.Y, Yin.Z, et al. Regulatory Innate Lymphoid Cells Control Innate Intestinal Inflammation. *Cell*, 2017. 171:(1): 201-216.e18. <https://doi.org/10.1016/j.cell.2017.07.027>.
- Wang.X, Li.Y, Li.L, Jiao.Z, et al. Porcine CXCR1/2 antagonist CXCL8(3–72)G31P inhibits lung inflammation in LPS-challenged mice. *Scientific Reports*, 2020. 10:(1): 1–10. <https://doi.org/10.1038/s41598-020-57737-w>.
- Wenzel.SE. Asthma phenotypes: The evolution from clinical to molecular approaches. *Nature Medicine*, 2012. Nature Publishing Group. <https://doi.org/10.1038/nm.2678>.
- Whitehead.GS, Thomas.SY, Cook.DN. Modulation of Distinct Asthmatic Phenotypes in Mice by Dose-Dependent Inhalation of Microbial Products. *Environmental*

- Health Perspectives*, 2014. 122:(1): 34–42.
<https://doi.org/10.1289/ehp.1307280>.
- Whittaker Hawkins.RF, Patenaude.A, Dumas.A, Jain.R, et al. ICAM1+ neutrophils promote chronic inflammation via ASPRV1 in B cell–dependent autoimmune encephalomyelitis. *JCI Insight*, 2017. 2:(23).
<https://doi.org/10.1172/jci.insight.96882>.
- Williams.AE, Chambers.RC. The mercurial nature of neutrophils: Still an enigma in ARDS? *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 2014. 306:(3). <https://doi.org/10.1152/ajplung.00311.2013>.
- Wilson.RH, Whitehead.GS, Nakano.H, Free.ME, et al. Allergic Sensitization through the Airway Primes Th17-dependent Neutrophilia and Airway Hyperresponsiveness. *American Journal of Respiratory and Critical Care Medicine*, 2009. 180:(8): 720–30. <https://doi.org/10.1164/rccm.200904-0573OC>.
- Wingender.G., Birkholz. a. M, Sag.D, Farber.E, et al. Selective Conditions Are Required for the Induction of Invariant NKT Cell Hyporesponsiveness by Antigenic Stimulation. *The Journal of Immunology*, 2015. 195:(8): 3838–48.
<https://doi.org/10.4049/jimmunol.1500203>.
- Wingender.Gerhard, Rogers.P, Batzer.G, Lee.MS, et al. Invariant NKT cells are required for airway inflammation induced by environmental antigens. *The Journal of Experimental Medicine*, 2011. 208:(6): 1151–62.
<https://doi.org/10.1084/jem.20102229>.
- Wingender.Gerhard, Sag.D, Kronenberg.M. NKT10 cells: a novel i NKT cell subset. *Oncotarget*, 2015. 6:(29): 26552–53.
<https://doi.org/10.18632/oncotarget.5270>.
- Woo.LN, Guo.WY, Wang.X, Young.A, et al. A 4-Week Model of House Dust Mite (HDM) Induced Allergic Airways Inflammation with Airway Remodeling, 2018.,

- no. December 2017: 1–11. <https://doi.org/10.1038/s41598-018-24574-x>.
- Woodfin.A, Beyrau.M, Voisin.MB, Ma.B, et al. ICAM-1-expressing neutrophils exhibit enhanced effector functions in murine models of endotoxemia. *Blood*, 2016. 127:(7): 898–907. <https://doi.org/10.1182/blood-2015-08-664995>.
- Wu.D, Zeng.Y, Fan.Y, Wu.J, et al. Reverse-migrated neutrophils regulated by JAM-C are involved in acute pancreatitis-associated lung injury. *Scientific Reports*, 2016. 6:(June 2015): 1–15. <https://doi.org/10.1038/srep20545>.
- Wu.W, Bleecker.E, Moore.W, Busse.WW, et al. Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. *Journal of Allergy and Clinical Immunology*, 2014. 133:(5): 1280–88. <https://doi.org/10.1016/j.jaci.2013.11.042>.
- Wypych.TP, Marzi.R, Wu.GF, Lanzavecchia.A, et al. Role of B cells in TH cell responses in a mouse model of asthma. *Journal of Allergy and Clinical Immunology*, 2018. 141:(4): 1395–1410. <https://doi.org/10.1016/j.jaci.2017.09.001>.
- Xystrakis.E, Kusumakar.S, Boswell.S, Peek.E, et al. Reversing the defective induction of IL-10 – secreting regulatory T cells in glucocorticoid-resistant asthma patients. *Journal of Clinical Investigation*, 2006. 116:(1): 146–55. <https://doi.org/10.1172/JCI21759.146>.
- Zasłona.Z, Przybranowski.S, Wilke.C, Rooijen.N van, et al. Resident Alveolar Macrophages Suppress, whereas Recruited Monocytes Promote, Allergic Lung Inflammation in Murine Models of Asthma. *The Journal of Immunology*, 2014. 193:(8): 4245–53. <https://doi.org/10.4049/jimmunol.1400580>.
- Zhao.H, Aoshi.T, Kawai.S, Mori.Y, et al. Olfactory Plays a Key Role in Spatiotemporal Pathogenesis of Cerebral Malaria. *Cell Host & Microbe*, 2014. 15:(5): 551–63. <https://doi.org/10.1016/j.chom.2014.04.008>.
- Zhao.J, Lloyd.CM, Noble.A. See COMMENTARY page XX Th17 responses in chronic

allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. *Mucosal Immunology*, 2012. 6:(2): 335–46. <https://doi.org/10.1038/mi.2012.76>.

Zhou.X, Moore.BB. Location or origin? What is critical for macrophage propagation of lung fibrosis? *European Respiratory Journal*, 2018. 51:(3): 1–4. <https://doi.org/10.1183/13993003.00103-2018>.



8. Addenda

8.1 ARBIS CV

MÜGE ÖZKAN

Personal Information

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Education Information

29 September 2016 - Now (4 Year 4 Month)
Doktora, Doktora, DOKUZ EYLÜL ÜNİVERSİTESİ, TÜRKİYE
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Diploma Number: -
Cumulative Grade Point Average: 3.9 / 4.0

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Yüksek Lisans, Tezli Program (İkinci Öğretim), OSAKA UNIVERSITY, JAPONYA
OSAKA UNIVERSITY, OSAKA UNIVERSITY
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Hemozoin's Adjuvanticity
Thesis Subject: Natural Hemozoin Is A Monomeric Heme-derived Crystal And Is
Synthesized By Plasmodium Parasites During Erythrocytic Stage Of Their Life
Cycle. Synthetic Hemozoin (shz) Previously Described As A Biologically Active
Compound And Can Modulate Immune Responses. However, How Shz Exerts Its
Adjuvant Properties Remains Elusive. We Found Two Major Mechanisms. Firstly,
Upon Immunization With Shz, B Cell Responses Are Reduced In Myd88-deficient
Mice. Interestingly, Immunohistochemical Analysis Of Lymph Node Sections
Revealed That Shz, As Well As Antigen Accumulated In Macrophages, Around B
Cell Follicular Areas, Suggested Their Possible Interaction With Both T Follicular
Helper Cells (tfh) And B Cells Are Important For The Adjuvanticity. Secondly, We
Found That Shz Immunization Together With DnaseI Treatment Abolishes Immune
Responses And Hereby We Describe Shz Also Causes Host Dna Release, And
Tbk1- A Kinase Involved In Cytosolic Dna Sensing Mechanism- Is Involved In The
Adjuvanticity.
Date: 2015
null

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TEKNİK PERSONEL, DİĞER (Osaka Üniversitesi)

01 January 2015 - 01 January 2015 (1 Month) (Yarı Zamanlı)
ARAŞTIRMA GÖREVLİSİ, DİĞER (Osaka Üniversitesi)

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MOLEKÜLER BİYOLÖJİ VE GENETİK BÖLÜMÜ

Foreign Language Information

İNGİLİZCE (Reading: İyi, Writing: İyi, Speaking: İyi)

JAPONCA (Reading: Başlangıç, Writing: Başlangıç, Speaking: Orta)

FRANSIZCA (Reading: İyi, Writing: Orta, Speaking: Orta)

Scientific Technological Activity Fields

Keywords

İmmün Yanıt
Otoimmün Hastalıklar
Solunum Sistemi Hastalıkları
Bulaşıcı Hastalıklar

R&D Competency

Books

M. ÖZKAN, MODEL HASTALIKLAR PLASMODİUM ENFEKSİYONLARINDA
PATOGENEZ, P. D. S. B. P. D. H. A. P. D. B. [Editors], ENFEKSİYON PATOGENEZİ VE
BAĞIŞIKLIK, ISBN: 978-605-5013-62-2, TÜRKİYE: AKADEMİ , 19 May 2015,
Kitapta Bölüm.

Articles

D. SAG, M. OZKAN, M. KRONENBERG & G. WİNGENDER, Improved Detection of
Cytokines Produced by Invariant NKT Cells, SCIENTIFIC REPORTS, 2017, 2045-
2322, 7.

A. J. HOBRO, A. J. HOBRO, N. PAVİLLON, N. PAVİLLON, K. FUJİTA, K. FUJİTA, M.
OZKAN, M. OZKAN, C. COBAN, C. COBAN, N. I. SMİTH & N. I. SMİTH, Label-free
Raman imaging of the macrophage response to the malaria pigment hemozoin,
ANALYST, 2015, 0003-2654, 140, 7, 2350-2359.

H. ZHAO, H. ZHAO, T. AOSHİ, T. AOSHİ, S. KAWAİ, S. KAWAİ, Y. MORİ, Y. MORİ, A.
KONİSHİ, A. KONİSHİ, M. OZKAN, M. OZKAN, Y. FUJİTA, Y. FUJİTA, Y. HASEDA, Y.
HASEDA, M. SHİMİZU, M. SHİMİZU, M. KOHYAMA, M. KOHYAMA, K. KOBİYAMA, K.
KOBİYAMA, K. ETO, K. ETO, J. NABEKURA, J. NABEKURA, T. HORİİ, T. HORİİ, T.
ISHİNO, T. ISHİNO, M. YUDA, M. YUDA, H. HEMMİ, H. HEMMİ, T. KAİSHO, T.
KAİSHO, S. AKİRA, S. AKİRA, M. KİNOSHİTA, M. KİNOSHİTA, K. TOHYAMA, K.
TOHYAMA, Y. YOSHİOKA, Y. YOSHİOKA, K. J. ISHİİ, K. J. ISHİİ, C. COBAN & C. COBAN,
Olfactory Plays a Key Role in Spatiotemporal Pathogenesis of Cerebral Malaria,
CELL HOST & MICROBE, 2014, 1931-3128, 15, 5, 551-563.

M. OEZKAN, M. OEZKAN, P. B. C. MUTİSO, P. B. C. MUTİSO, L. NAHAR, L. NAHAR, P.
LİU, P. LİU, S. BROWN, S. BROWN, W. WANG, W. WANG, S. D. SARKER & S. D.
SARKER, Zanthoxylum usambarense (Engl.) Kokwaro (Rutaceae) Extracts Inhibit

the Growth of the Breast Cancer Cell Lines MDA-MB-231 and MCF-7, But Not the Brain Tumour Cell Line U251 In Vitro, PHYTOTHERAPY RESEARCH, 2013, 0951-418X, 27, 5, 787-790.

Conference Papers

M. ÖZKAN, Y. C. ESKİÖCAK & G. WINGENDER, Titled: "INVARIANT NATURAL KILLER T (iNKT) CELLS ARE NOT ESSENTIAL FOR MURINE NEUTROPHILIC ASTHMA PATHOGENESIS", Sözlü Sunum, International Molecular Immunology and Immunogenetics Congress IV, 27 April 2019, 29 April 2019.

M. ÖZKAN, MODE OF ACTION OF HEMOZOİN AS A VACCINE ADJUVANT, Poster Sunumu, THE 8TH ADJUVANT RESEARCH CONSORTIUM MEETING, 20 January 2015, 20 January 2015.

M. ÖZKAN, MECHANISM OF ACTION OF HEMOZOİN AS A VACCINE ADJUVANT, Sözlü Sunum, THE 43RD ANNUAL MEETING OF THE JAPANESE SOCIETY FOR IMMUNOLOGY, 10 December 2014, 10 December 2014.

M. ÖZKAN, SYNTHETIC HEMOZOİN AS AN ADJUVANT., Poster Sunumu, THE 7TH ADJUVANT RESEARCH CONSORTIUM MEETING, 20 January 2014, 20 January 2014.

Projects

1001 - ARAŞTIRMA, BURSİYER, NAİF CD4+T HÜCRELERİNDEN TH17 FENOTİPİNDE EFEKTÖR T HÜCRELERİNİN OLUŞTURULMASI VE TH17 FARKLIŞMASINDA ROL OYNAYAN MİKRORNA'LARIN BELİRLENMESİ, Enforcing Institution: KURULUŞ GÜNCELLENMESİ GEREKİYOR, Sponsor Institution: , 01 October 2010, 01 April 2014.

TÜBİTAK Scholarships and Fundings

Project Info

110T412, Naif CD4+T Hücrelerinden Th17 Fenotipinde Efektör T Hücrelerinin Oluşturulması ve Th17 Farklılaşmasında Rol Oynayan MikroRNA'ların Belirlenmesi, 1001 - Araştırma, Burslu, Sonuçlandı, ARDEB, KBAG - Kimya Biyoloji Araştırma Destek Grubu, Project Participation/Leave Dates: 12/1/11 - 2/1/13, Project Start/End Dates: 10/1/10 - 4/1/14.

BİDEB Supports

MÜGE ÖZKAN, Araştırma Burs ve Destekleri Müdürlüğü, 2247-C Stajyer Araştırmacı Burs Programı (STAR), Destekleniyor, 2020 - 1, 6/1/20 - 1/31/21.

Number of Panelist/Observer/Reporter Jobs

Refereeing/Panelist/Outer Consultant Count	ARDEB/BİDEB 0	TEYDEB 0	Total 0
Number of Observer/Consultant Jobs	ARDEB/BİDEB 0	TEYDEB 0	Total 0
Number of Reporter Jobs	ARDEB/BİDEB 0	TEYDEB 0	Total 0

8.2 Publication list

Sag, D., Özkan M., Kronenberg M., Wingender G. Improved Detection of Cytokines Produced by Invariant NKT Cells. *Scientific Reports*, 2017;7(1).<https://doi.org/10.1038/s41598-017-16832-1>

Hobro A. J., Pavillion N., Katsumasa F., Ozkan M., Coban C., Smith N. I. Label-free raman imaging of the macrophage response to the malaria pigment hemozoin. *Analyst*, 2015; 140(7):2350-9. doi: 10.1039/c4an01850h.

Zhao H., Aoshi T., Kawai S., Mori Y., Konishi A., Ozkan M., Fujita Y., Haseda Y., Shimizu M., Kohyama M., Kobiyama K., Eto K., Nabekura J., Horii T., Ishino T., Yuda M., Hemmi H., Kaisho T., Akira T., Kinoshita M., Tohyama K., Yoshioka Y., Ishii K.J., Coban C. Olfactory Plays a Key Role in Spatiotemporal Pathogenesis of Cerebral Malaria. *Cell Host Microbe*, 2014; 15(5):551-63. doi: 10.1016/j.chom.2014.04.008.

Özkan M., Mutiso P. B. C., Nahar L., Liu P., Brown S., Wang W., Sarker S. D. Zanthoxylum usambarens (Engl.) Kokwaro (Rutaceae) Extracts Inhibit the Growth of the Breast Cancer Cell Lines MDA-MB-231 and MCF-7, But Not the Brain Tumour Cell Line U251 In Vitro. *Phytotherapy Research*, 2013; 27(5):787-90. doi: 10.1002/ptr.4775.

8.3 Ethical committee reports



DOKUZ EYLÜL ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU

35340, Inciraltı, İzmir-232 4122234

Gündem No/ Toplantı No/Yıl : 03/05/2016
Toplantı Tarihi : 01 Mart 2016

İzmir Uluslararası Biyotıp ve Genom Enstitüsü Müdürlüğü'ne

10/2016 Protokol No'lu; Müdürlüğünüz Öğretim Üyelerinden Yard.Doç.Dr.Gerhard WINGENDER yürütücüsü olduğu "iNKT Hücrelerinin Solunum Yolu Aşırı Duyarlılığı Tepkileri Sırasında Hücre-Hücre Etkileşimleri (Cell-Cell Interactions of iNKT Cells During Airway Hypersensitivity)" isimli; DVM MSc Aslı ÇELİK'in araştırmacı olduğu, projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.



**İZMİR BİYOTIP VE GENOM MERKEZİ
HAYVAN DENEYLERİ YEREL ETİK KURULU
(İBG-HADYEK)
KARARI**

TOPLANTI TARİHİ	13/02/2019	TOPLANTI GÜNÜ	Çarşamba
TOPLANTI SAYISI	2	TOPLANTI SAATİ	15.30

Assoc. Prof. Gerhard Windenger,

05/2019 Protokol No'lu; yürütücüsü olduğunuz “The role of invariant natural killer T (iNK) cells in neutrophilic, Th1-driven, asthma ” başlıklı projenin uygulanmasında etik açıdan sakıncı olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.