Center for Pathophysiology, Infectiology and Immunology

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Institute of Specific Prophylaxis and Tropical Medicine

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γδ T cells in Allergy and the Cross-Talk with Obesity and Oral Tolerance Development

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Abstract

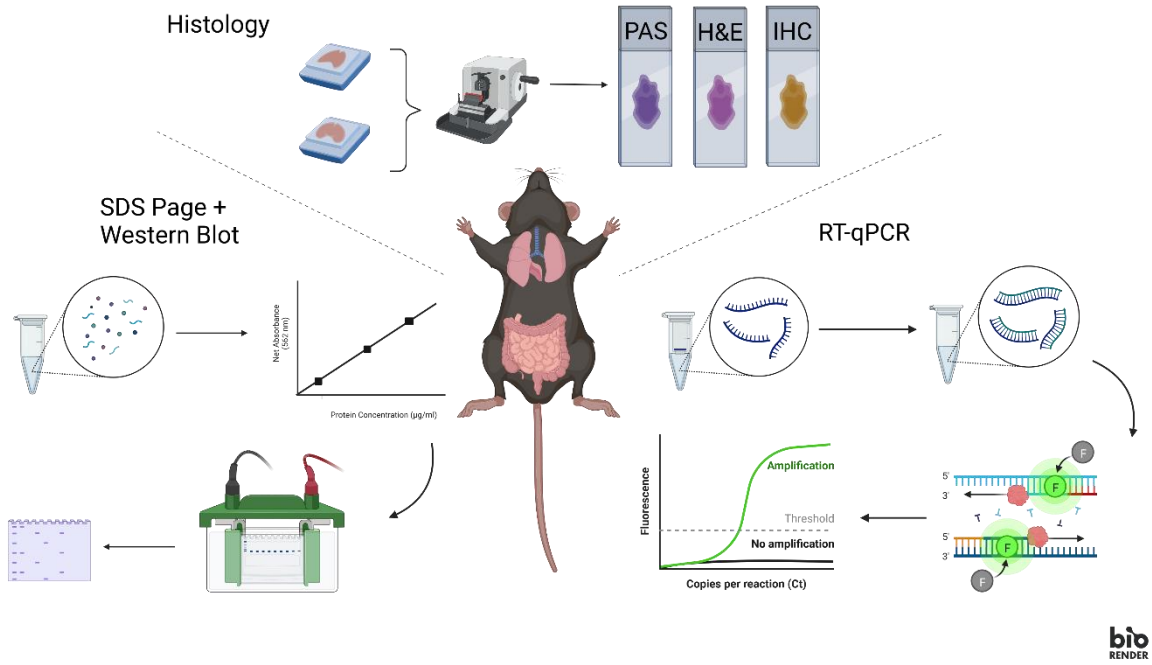
Background: γδ T cells belong to lymphocytes and participate in immune defense by repairing tissues, recruiting leukocytes, lysing cells, and maintaining tissue homeostasis. However, their function is disrupted in the case of allergies and obesity. Previously we induced immune tolerance to ovalbumin (OVA) in obese and lean mice. Here, we hypothesize that γδ T-cells could play a role in allergy development and immune tolerance, and these processes depend on the used diet.

Methods: Male C57BL/6 mice were fed a high-fat- (HFD) or standard chow diet (STD) for nine weeks, followed by immunization and challenge with OVA. Tolerance was induced orally or intranasally with OVA before sensitization. Lung and gut tissue were taken and used for analysis. The γδ T cell receptor gene expression was determined by RNA isolation, reverse transcription into cDNA, and quantitative polymerase chain reaction (PCR). Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were used for histological analysis. Immunohistochemistry was performed to localize γδ T cells in the lung and gut samples. Western Blotting was used to identify γδ TCR proteins extracted from the tissues.

Results: The gene expression of γδ T cell receptor in the lung tissue of allergic obese mice showed a significant reduction of γδ T cells compared to lean mice. In addition, intranasal treatment with OVA increased γδ T cell mRNA in HFD- and STD-fed mice. We observed no significant differences in the gut tissue γδ T cell gene expression levels between the different groups. Immunohistochemistry staining revealed γδ T cell populations in lung and gut tissue of HFD- and STD-fed mice. The Western blot analysis confirmed the expression of γδ at protein levels in all treatment groups of HFD-fed mice in the lung.

Conclusion: In allergic HFD mice, the gene expression level of γδ T cells in the lung was significantly reduced, which might lead to losing their protective potential against allergens. Intranasal treatment showed a significant increase of γδ T cells in the lung compared to the other treatment groups. This finding might indicate that γδ T cells are involved in tolerance development in the intranasal but not in the oral tolerance model. Understanding the mechanisms that mediate a cross-talk between obesity, allergy, and tolerance may lead to the identification of novel treatments for morbidities associated with a Westernized lifestyle.

Graphical Abstract



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1. Introduction

1.1. Allergy

Allergies dramatically increased in the last few decades, affecting more than 60 million people worldwide [1]. The exact reasons behind this phenomenon are far from complete understanding. However, urbanization and a westernized lifestyle are linked to a rising frequency of respiratory allergies. Epidemiologic studies showed that people who live in urban areas have an increased frequency of respiratory allergy compared to people living in rural areas [2].

The most common allergy phenotype is allergic asthma, and young people are more affected by it. Allergic asthma is defined as sensitization to environmental allergens, resulting in airway obstruction, chronic airway inflammation, and airway hyperresponsiveness (AHR) [3; 4].

Allergies are known to have an imbalance of Th1- and Th2-type of the immune response. Th1 cells secrete interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor β (TNF-β), which promote macrophage's activation and production of opsonizing antibodies and are rather IgE suppressive [5; 6]. Whereas, Th2 cells amplify the allergic inflammation by producing IL-4 (enhancing IgE synthesis), IL-5 (increasing eosinophil growth and differentiation), IL-9 (enhancing mast cell differentiation), and IL-13 (increasing mucus production and inducing airway hyperreactivity) [7].

Damage in the airway mucosa and an impaired mucociliary clearance induced by air pollution might facilitate the uptake of allergens into the immune system [8]. Allergens are antigens that can cause an allergic reaction [9]. B cells get activated by specific allergens and produce the immunoglobulin (Ig) E antibody. IgE-producing B cells are critical in allergic inflammation because increased IgE levels lead to further activation of Th2 cells. The IgE also binds to the high-affinity Fcε receptors on macrophages, eosinophils, and basophils, thereby sensitizing these cells to antigen exposure [9].

Many cells of the immune system are involved in response to allergens. These include eosinophils, mast cells, neutrophils, and T cells. Eosinophils and T cells are significant sources of cytokines in asthma [9]. Eosinophils produce a wide range of cytotoxic proteins, inflammatory cytokines, and chemokines, leading to epithelial tissue damage [9; 10]. It was

shown that T cells have an essential part in allergy and asthma. T cells are differentiated based on the receptors they express. There is αβ expressing T cells and γδ expressing T cells. However, their specific functions are still unclear. γδ T cells are elevated in bacterial, parasitic, viral infection, joint inflammation, autoimmune responses, and eosinophilic granuloma. Further details about γδ T cells are discussed below. Both γδ and αβ T cells secrete similar cytokines, express a similar activation, and can lyse antigen-bearing target cells [11].

1.2. Obesity

Not only allergic diseases but obesity is also significantly increasing in the Western world. Obesity is growing due to urbanization, better food supply and diet, and reduced physical activity in developing countries [12].

Diseases caused by obesity are type 2 diabetes, cardiac disease, insulin resistance, impaired glucose tolerance, and increased susceptibility to infection [13; 14].

Being overweight means having excess body weight compared to the same gender and height. The body mass index is defined as BMI; kg/m^2 [15]. The body weight is built from adipose tissue. The adipose tissue is an endocrine organ, producing hormones regulating body metabolism [14]. It performs various functions like thermal insulation, tissue repair, thermogenesis, and secretion of anti-microbial peptides and cytokines. The adipose tissue can be divided into brown adipose tissue (BAT), which is involved in thermoregulation, and white adipose tissue (WAT), which comprises the majority of adipose tissue in adults [16].

The adipose tissue is not only important for physiologic responses to fasting and feeding, but it also harbors 80-90 % of the innate immune system [17]. Disturbances can lead to inflammation and insulin resistance. The majority of cells which comprises the adipose tissue are lymphocytes such as type 2 innate lymphoid cells (ILC2s), invariant natural killer T cells (iNKT), natural killer (NK) cells, and γδ T cells. In lean individuals, those cells maintain an anti-inflammatory environment by secreting type 2 cytokines that support the function and survival of eosinophils, macrophages, and T regulatory (Treg) cells. Immune cells are sensitive to inflammation. During inflammation it comes to a shift in the balance of anti-inflammatory towards pro-inflammatory cytokines, impairing glucose handling, insulin

production, and insulin signaling in obesity [17; 18]. T cells also seem involved in obesity inflammation, although the mechanisms remain unclear [16].

Usually, inflammation is tightly regulated, but in an uncontrolled environment such as obesity, chronic inflammation negatively impacts lymphocytes, and inflammatory mediators like TNF-α and IL-6 get released [14; 19].

Epidemiological studies found that obesity is increasing the incidence of asthma. However, the connections are not known yet. It could be due to alterations in the respiratory airways and breathing caused by obesity. The excess weight could lead to a ventilation-perfusion mismatch [15]. Another possibility could be that the excessive fatty tissue produces cytokines such as TNF-α, IL-6, and IL-10, which have been implicated in the inflammatory response in the airway of asthma patients [20]. In animal models of asthma, a link between obesity and asthma was found. It was shown that obesity lowered the threshold for allergic sensitization, which led to an increase in IgE and eosinophilia. An ovalbumin challenge in obese mice also resulted in a higher eosinophil accumulation [21].

Despite all these connections, it is still unclear how weight gain influences the development of asthmatic diseases. So far, losing weight is the only way to improve the symptoms. Losing weight improves lung function, respiratory muscle function, gas exchange, sleep quality, and daytime sleepiness. Reducing excessive weight can potentially lower morbidity and mortality of obesity-induced respiratory complications [15].

1.3. Oral and Intranasal Tolerance

The dramatic increase in allergic and obese diseases led to the quest to search for novel treatment strategies. So far, the only causative treatment for allergies is specific immunotherapy, which is only efficacious in young patients [22]. One therapeutic approach to finding suitable treatments for allergy in conjunction to obesity is to induce mucosal tolerance via the oral or intranasal route. This approach has attracted increasing attention in recent years because it has been shown that immunoinflammatory disorders were down-regulated.

Mucosal surfaces can be found in the gastrointestinal, respiratory, and genito-urinary tracts. The mucosa is constantly threatened by harmful pathogens and antigens such as food, airborne antigens, or commensal bacteria [23].

Therefore, the mucosal immune system has developed two defence mechanisms. First, the induction of immunity and defence of mucosal pathogens. Secondly the induction and maintenance of tolerance to environmental antigens and bacterial flora. Failure of tolerance induction is believed to lead to allergies and food enteropathies [24].

An allergen needs to be presented via oral or intranasal route to induce immune tolerance [7]. Therefore, the antigen must access antigen-presenting cells (APCs) by penetrating the mucus layer. In animal models, it was reported that oral and nasal tolerance improved many conditions by the induction of Tregs. Activated Tregs secrete transforming growth factor β (TGF-β) or IL-10 at the target organ. It was shown that low doses of antigen induce regulatory Tregs, whereas higher doses favor the induction of anergy or deletion of Tregs [25].

T lymphocytes are the primary cell type involved in the induction of immune tolerance [26]. γδ T cells down-regulate the immune response in or during various inflammatory disorders [23]. γδ T cells can do this by recognizing stressed epithelial cells and by producing cytokines and thereby keeping tissue homeostasis by specifically suppressing IgE responses to OVA.

The exact mechanisms and cellular functions of induced tolerance by antigens are not well understood. Still, it was shown that it has long-lasting effects and affects many aspects of immunity [27].

1.4. γδ T cells

γδ T cells, together with αβ T cells and B cells, belong to the lymphocytes and are conserved in all but most primitive vertebrates suggesting that each cell population contributes to the host immune system [28].

All three cell types use somatic DNA rearrangement to assemble the genes for their cell-surface receptors. The use of a variable (V), diversity (D), and joining (J) elements give γδ T-, B-, and αβ T-cells great potential for diversity [29].

Most αβ T cells are CD4+ and CD8+, but γδ T cells have the CD4- CD8- double-negative phenotype predominantly [30].

Compared to αβ T cells, γδ T cells make up only a small population (1-5 %) in the blood. However, γδ T cells are more widespread within epithelial tissues, such as skin, intestine, and the respiratory and reproductive tract, where they make up 50 % of the T cells [31].

γδ T cells are responsible for tissue surveillance and maintenance. They serve as the first line of defense in the epithelial tissues and respond to signals from damaged epithelial cells [32].

They express pattern recognition receptors (PRRs) that stimulate their rapid activation and secretion of cytokines [33]. γδ T cells accumulate at sites of inflammation and infection and produce Th1- and Th2 cytokines [34]. Once activated, γδ T cells lyse transformed cells and increase the efficacy of wound healing [35].

γδ T cells are also known as a bridge between the innate and adaptive immune system because they can cross-talk with other immune cells such as NK cells, B cells, and αβ T cells to maintain tissue homeostasis [34].

Although γδ T cells are identified as critical immunoregulatory cells, their specific role in the immune response remains elusive, and it is still debated whether γδ T cells act in a pro- or anti-inflammatory manner [34; 36].

1.4.1. Development of γδ T cells

αβ- and γδ T cells develop from a common thymocyte precursor in the thymus. However, γδ T cells are the first T lymphocytes to emigrate from the thymus. Most of those cells reside in epithelial tissues such as skin, intestine, lung, and reproductive tracts [35].

Developing thymocytes expressing the γδ T cell receptor (TCR) already acquire functional competence in the thymus. In young mice, it was shown that γδ T cells are required for host resistance against pathogens. Adult mice needed αβ T cells for sufficient protection [37].

γδ TCRs are generated like other lymphocytes through somatic rearrangement of V (variable), D (diversity), and J (Joining) segments. γδ T cells are a homogenous population. However, they express distinct antigen recognition repertoires from one tissue to another (Table 1). These so-called invariant γδ T cell subsets arise from early waves of fetal γδ thymocytes [38]

Table 1: Invariant γδ T cells subsets of lung and gut from mice.

Species	Peripheral location	Predominant V gene segment usage
mouse	Lung epithelia	Vγ4 and Vγ6
	Gut epithelia	Vγ7Vδ4, Vγ7Vδ5, Vγ7Vδ6

1.4.2. γδ TCR antigen recognition

The γδ T cell's ability to perform so many different functions is due to how its receptor is formed. Within the area of the VDJ segments is the so-called complementarity-determining region 3 (CDR3). CDR3 sites are created by variations in the number of the V, D, and J elements. This variability is caused by random nucleotide insertion or deletion and results in extensive length and sequence heterogeneity. Therefore, γδ T cells have a greater potential diversity compared to αβ T cells because they can use multiple tandem copies of their D elements [30].

Additionally, the CDR3 length of the γδ TCR is similar to those of immunoglobulin. The CDR3 length of the δ chain is long and variable, while one of the γ chains is short and constrained. αβ T cells, on the other hand, have a nearly identical length of their α and β chains, and their length distributions are constrained [28].

Besides the unique structure of the γδ TCR, the T cell has additional receptors on its surface, such as Toll-like receptors (TLRs) and natural killer receptors (NKRs). The receptors act separately or additively to the particular γδ T cell effector functions.

γδ T cells also recognize other proteins than MHC molecules like soluble proteins. Soluble proteins are, for example, bacterial proteins like tetanus toxoid, staphylococcal enterotoxin A (SEA), and bacterial superantigens. In addition, several responses to smaller peptide antigens, phospholipids, and non-peptidic antigens, called phosphoantigens, have been reported. Responses to peptides as short as seven amino acids were found. Such short peptides are called heat shock proteins. Heat shock proteins (HSPs) do not need antigen processing [39].

All these points above allow the γδ T cell to respond much faster to pathogens or damaged tissues without requiring antigen processing and antigen-presenting cells, such as B cells,

macrophages, or dendritic cells (DCs). Also, the expression of different homing receptors further enhances the antigen specificity of the T cells in a given tissue and enables their rapid response to cell stress. This allows greater flexibility than the classical αβ T cell response, which requires TCR and co-stimulatory receptors to generate signals of appropriate strengths [38; 40].

1.4.3. The function of γδ T cells in the immune response

Epithelial γδ T cells have many different effector functions. To maintain tissue homeostasis, they produce immunomodulatory cytokines like TNF-α, IFN-γ, IL-4, IL-5, IL-13, and IL-17 against viruses, intracellular pathogens, bacteria, and fungi. They can kill activated or transformed cells via death-inducing receptors such as FAS or by releasing perforin and granzyme. They contribute to pathogen clearance through the production of granulysin and defensins or indirectly by antibacterial functions or activating other immune cells and epithelial cells. γδ T cells can produce immunosuppressive cytokines and downmodulate the innate and adaptive immune system. γδ T cells promote tissue healing through the production of growth factors (keratinocyte growth factor 1 (KGF1) and KGF2) and survival factors (epidermal growth factor 1 (EGF1). However, all the functions and responses of γδ T cells underlay different immune processes like inflammation, autoimmunity, allergy, and asthma [38]. However, γδ T cells can also negatively regulate these processes. For example, when pathogen clearance is accomplished, γδ T cells terminate macrophage, DCs, or T cell responses by producing cytokines such as TGF-β and IL-10 [38].

1.4.4. Early producers of inflammatory cytokines

γδ T cells take part in many processes in the immune system by producing various cytokines and chemokines. They can produce Th1-like cytokines such as IFN-γ and TNF-α to contribute to reduced survival of tumor cells but also Th2-like cytokines like IL-4 in allergic patients. γδ T cells produce cytokines such as KGF and connective tissue growth factor (CTGF) to maintain tissue integrity. Besides, they are also interacting with other immune cells. Some γδ T cells secrete IL-10 to expand CD8+ T cell expansion and regulate the TNF-α secretion by the activated CD8+ T cells. It was shown that γδ T cells have characteristics of antigen-presenting cells. They can process antigens and present those to αβ T cells or

induce naïve αβ T cells proliferation and differentiation [41]. They provide help for B cells and present antigens to produce IgM, IgG, and IgA antibodies. In mouse models of AHR, it was shown that DCs could induce γδ T cell effector functions by producing IL-23 [38]. This enhances the production of IL-17 by γδ T cells. IL-17 is required to initiate the inflammatory response by recruiting neutrophils and macrophages to the damaged tissues to induce wound healing [42].

1.4.5. γδ T cells in the airways

The lung epithelium is constantly exposed to airborne particles and pathogens. Pulmonary γδ T cells contribute to the maintenance and homeostasis of mucosal immunity in the lungs. Compared to other epithelial tissues such as the intestines and epidermis, the population of pulmonary γδ T cells is tiny. It makes up only 5-10 % of all T lymphocytes in a healthy lung. γδ T cells are broadly distributed over the lungs except for the airway mucosa. However, they show an intrinsic preference for macrophages and dendritic cells, to better mediate their protective and regulatory role [43].

Their major role is to defend the lung epithelium from antigens and pathogens by recruiting other cells of the innate immune system and suppressing lung tissue inflammation. γδ T cells contribute to the maintenance by promoting epithelial growth. By damage or stress in the lungs of mice, γδ T cells regulate the infiltration of other cells like αβ T cells and myeloid suppressor cells into the epithelium [13]. Hence, they show functional plasticity by inducing either inflammation or protection in the tissue [19].

1.4.6. γδ T cells in allergic inflammation

Allergic asthma is a chronic inflammatory disease resulting in increased infiltration of inflammatory cells. Allergy is associated with a predominantly Th2 response [44]. γδ T cells are known to have a role in the development of allergic inflammation as effector and immunoregulatory cells via the production of cytokines, which recruit other immune cells such as T cells, eosinophils, neutrophils, and mast cells [45; 46].

For diseases that cause damage to epithelial tissues, levels of γδ T cells are often elevated [44]. γδ T cells can produce Th17-type cytokines and Th2-type cytokines like IL-4, IL-5, and

IL-13, suggesting that they enhance airway allergic inflammation and AHR [46]. Subsets of γδ T cells were involved in allergic reactions. However, the exact mechanism of the involvement of γδ T cells is controversial [47]. Since different subsets of γδ T cells can show other functions, depending on the tissue where they reside and the TCR they express [46].

Exposure to an antigen increased allergen-specific Th2-type γδ T cells, which amplified the immune response by secreting IL-4 and IL-5 [48]. This leads to the infiltration of eosinophils, B cells, and mast cells in the mucosa to the site of inflammation [49]. Mainly, eosinophils play a significant role in driving Th2 responses and promoting IgE synthesis [50].

However, pulmonary γδ T cells have been found to have opposing roles in allergic inflammation in the airways [19]. In immunized mice with repeated intranasal OVA challenges, there was a significant increase in eosinophils and CD4+ and CD8+ T lymphocytes. γδ T cells released IL-4, which may contribute to the initiation of Th2 immune responses [50]. An increased number of γδ T cells was also found in the nasal mucosa of humans with allergic rhinitis. These cells participated in allergic inflammation by producing Th2 cytokines [51].

It was shown that γδ T cells could regulate IgE responses negatively and positively. γδ T cells are an alternative source of IL-4 which promotes IgE production. Those IgE-enhancing γδ T cells expand and undergo further development in unimmunized subjects during allergic induction. Also, γδ T cells help B cells secrete allergen-specific IgE, by promoting Ig class switch or providing activation signals [46]. However, γδ T cells were also found to inhibit an IgE response in an antigen-specific manner [6].

The primary effector function of γδ T cells is maintaining homeostasis in the surrounding environment. They do this by releasing IFN- γ. IFN-γ producing γδ T cells were found to inhibit responses in the airways, suggesting that γδ T cells have anti-inflammatory properties [52]. For example, γδ T cells regulate the IgE responsiveness to inhaled antigens by a high production of IFN-γ and thereby suppressing the pathogenic Th2 response in allergic asthma [46].

In murine models of asthma, a similar number of γδ T cells were found in asthmatic as in healthy patients, suggesting that an excess of γδ T cells might not cause inflammation. This subset of γδ T cells produced IL-17A and reduced airway AHR in patients giving the T cells an anti-inflammatory role [13].

Activation of Th-17-like γδ T cells in OVA-treated mice showed reduced AHR, decreased number of eosinophils, but an increase of neutrophils in the airways. IL-17 is a cytokine that takes part in the pathogenesis of asthma. However, the production of IL-17 seems to be dose-dependent, with low doses increasing and high doses decreasing AHR [53].

Although many studies reported that γδ T cells are enriched in asthmatic airways and probably promote allergic inflammation in asthma, it was impossible to define their exact role in the disease [54].

1.4.7. γδ T cells in obesity

Obesity can lead to non-healing injuries, susceptibility to infection, and diseases due to chronic low-grade inflammation. Inflammation can result in epithelial tissue dysfunction, negatively impacting the innate and adaptive immune system [19].

The intestinal epithelium provides a barrier between the luminal contents and the lamina propria. It enables a cross-talk between the immune cells and the microbiota. The epithelium is characterized by rapidly renewing tissue; therefore, an effective barrier function is required to maintain gut homeostasis. A dysfunction in the tissue leads to inflammation driven by bacterial invasion of the mucosal surface. Chronic inflammation is characterized by elevated levels of inflammatory mediators such as IL-1β, IL-6, and TNF-α. Those factors are produced by adipocytes and immune cells in the adipose tissue [16]. The HFD intake facilitates the uptake of intestinal antigens and can contribute to chronic inflammation in obese subjects [19].

In lean hosts, γδ IELs maintain intestinal epithelial homeostasis by producing growth factors such as KGF-1 [16]. KGF-1 induces epithelial cell proliferation and repair on the epithelium [13]. In addition, IELs produce IL-17A, IL-22, and IL-33. IL-22 leads to the secretion of anti-microbial peptides and contributes to intestinal epithelial repair [55]. IL-33 is essential for the downstream effects on Treg cell accumulation and thermoregulation. IL-17A is a critical regulator of thermogenesis [17]. γδ IELs can actively migrate through the intestinal epithelium and are in close contact with other immune cells, which allows extensive tissue surveillance. Overall, γδ T cells have a central role in epidermal barrier maintenance, which is negatively impacted by obesity [55].

γδ T cells are sensitive to inflammation, and their function might become impaired [19]. Once inflammation becomes chronic, obese subjects showed a reduced number of γδ T cells. In mice, γδ T cells show a reduced ability to produce cytokines and growth factors at the wounding [13]. Deficiencies in the number of γδ T cells and their production of IL-17A showed that mice lost their ability to survive after a cold challenge [17]. Adipose-resident γδ T cells produce elevated levels of IL-17 in obese mice, negatively affecting glucose metabolism and cholesterol homeostasis. Also, dysfunctional γδ T cells in obesity produce TGF-β, which contributes to the loss of Treg cells [19]. A mouse study fed a ketogenic diet (KD) long-term showed a depleted number of γδ T cells. KD is exceptionally high in fat and very low in carbohydrates. On the other hand, short-term KD feeding improved metabolic control and activated γδ T cells. However, it is still unclear how γδ T cells become activated in response to KD [33].

A reduced number of γδ T cells in mice resulted in impaired oral tolerance. Oral tolerance is necessary for nonresponsiveness to food and protein intake and is an essential feature of the mucosal immune system. Defects in this process result in allergic sensitization to food proteins [46].

Also, diet and microbiota could influence the function of the IELs and should be investigated further [55]. Although much is known about the role of γδ T cells in epithelial barrier maintenance, less is known about their activation in response to tissue damage in the gut [16].

Additionally, the increased inflammation in obesity impairs the function of the lung mucosal tissue resulting in susceptibility to AHR and infection. However, whether pulmonary γδ T cells are affected in obesity is unknown, but they are a critical subset to consider due to their regulatory and inflammatory functions [19].

2. Aims

Purpose:

The purpose of this master thesis was to comparatively investigate whether there are differences between the abundance of γδ T cells in allergy and oral tolerance in obese and lean mice cohorts by using OVA-treated mice's lung and gut tissue.

Hypothesis:

We hypothesized that γδ T cells could play a role in allergy development and immune tolerance, and these processes depended on the used diet.

Aims:

1. To investigate the abundance of γδ T cells in obese, obese allergic, obese allergic tolerized, and non-treated mice, as well as in the respective lean controls,
2. To examine the degree of inflammation via histology in obese, obese allergic, obese allergic tolerized, and non-treated mice, and in the respective lean controls,
3. To establish a PCR protocol for quantification of γδ T cells in the lung and gut and measure the gene expression,
4. To establish a Western blot protocol for visualization of γδ TCR in the lysates of mouse's lung and gut,
5. To establish an immunohistochemistry protocol for visualization of γδ T cells in the mouse's lung and gut.

3. Materials and Methods

3.1. OVA-induced allergic airway inflammation mouse model

3.1.1. Animals and Ethics

28-41 days old male C57BL/6J mice were obtained from Chares River Laboratories (Sulzfeld, Germany). Mice were acclimatized in an individually ventilated caging system with a standard diet (STD) and water *ad libitum* for 14 days upon delivery. The Animal Experimentation Committee of the Medical University of Vienna and the Federal Ministry of Science and Research approved all experiments (Approval No. BMBWF-66.009/0277-V/3b/2019).

3.1.2. Diet-induced obesity mouse model

C57BL/6J mice were fed ad libitum either with mouse high-fat diet (HFD) (Brogaarden; Lynge, Denmark) (fat 60%; carbohydrates 29%; protein 20%) or STD (LASvendi) (fat 4.3%; protein 16.9%; fibre 4.3%; ash 7%; nitrogen-free extract 55.5% and dry matter 88%) for 87 days. The weight of the mice was monitored weekly, and the food was changed twice a week.

3.1.3. Model of tolerance induction

After 53 days, mice were treated either with albumin from chicken egg white (OVA) (Sigma-Aldrich, Walldorf, Germany) in bicarbonate (HCO_3^-) buffer or HCO_3 buffer alone. OVA and phosphate-buffered saline (PBS) was administered intranasally (1 mg OVA in 30 µl HCO_3 buffer) or orally (5 mg OVA in 300 µl HCO_3 buffer) (Figure 1).

3.1.4. Model of OVA sensitization and challenge

On day 64, mice were sensitized by intraperitoneal (i.p.) injections of PBS or OVA (in PBS (25 µg in 50 µl) three times in an interval of three weeks, followed by intranasal challenge with PBS or OVA in PBS (20 µg in 20 µl) for three consecutive days. Intranasal administrations were performed under anesthesia with 5 % (v/v) isoflurane in compressed air in an anesthetic induction chamber. On days 88 and 89, mice were euthanized by exposure to an overdose of sevoflurane, and organs were harvested and processed (Figure 2).

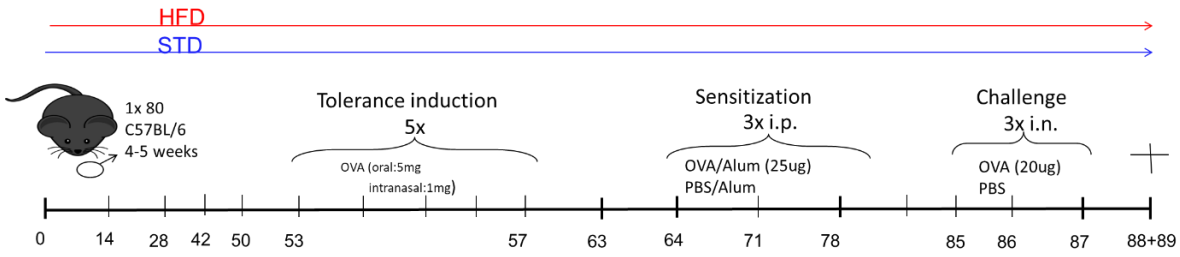


Figure 1: Experimental set-up of OVA-induced allergic airway mouse model.. Male C57BL/6 mice were fed a high-fat- (HFD) or standard chow diet (STD) for nine weeks, followed by tolerance induction, immunization and challenge with ovalbumin (OVA). Tolerance was induced orally or intranasally with OVA before sensitization.

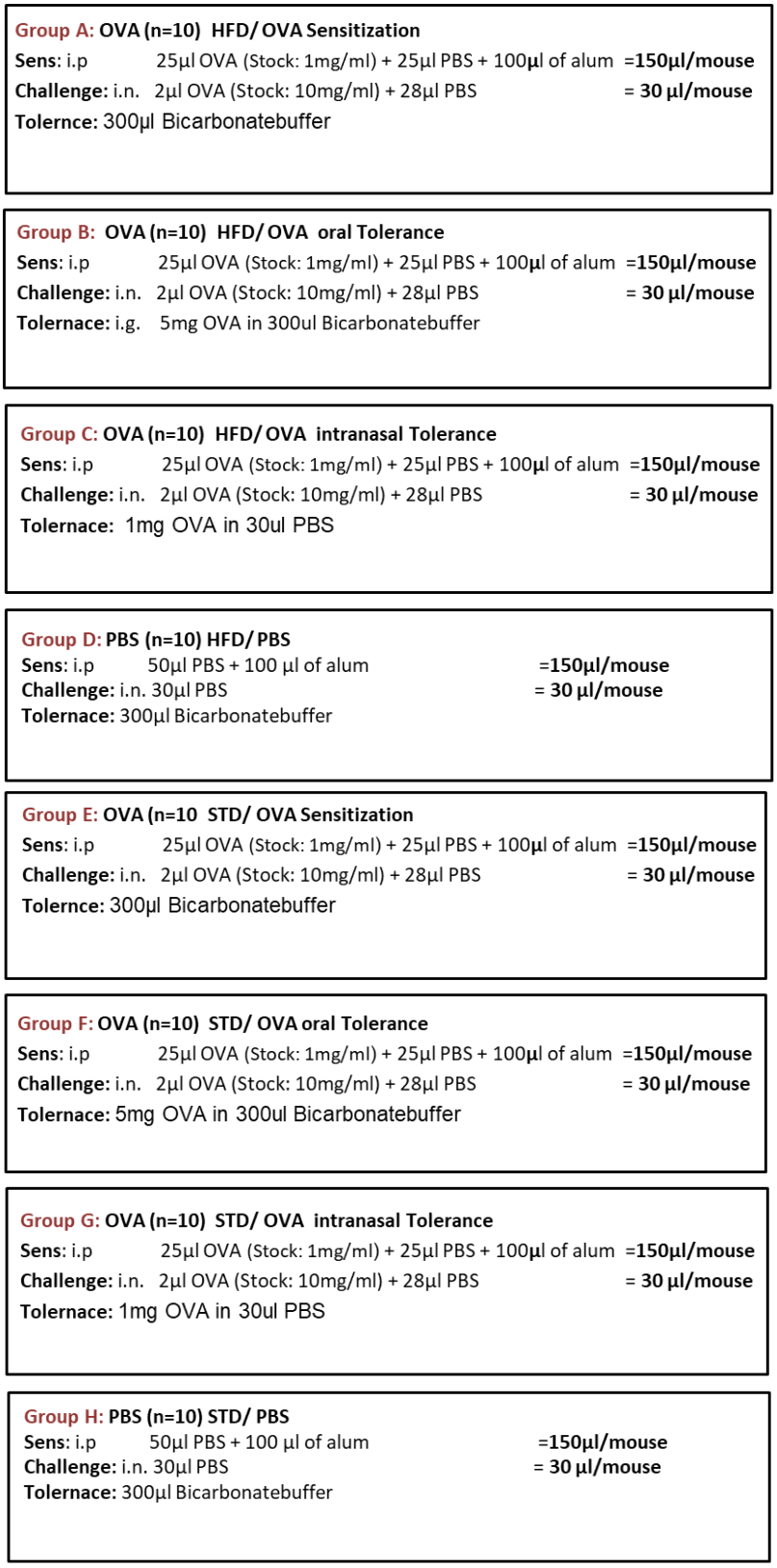


Figure 2: Overview of HFD-and STD-fed mice treated either with OVA, PBS, orally, or intranasally.

3.1.5. RNA isolation

Total RNA was extracted from lung and gut tissue with innuPREP RNA Mini Kit 2.0 (Analytik Jena) following the supplier's protocol. A maximum of 20 mg of the tissues were placed into a homogenizer tube (Peqlab) with 450 µl Lysis Solution RL (Analytik Jena) and homogenized for 20 seconds at 5500 rpm (Precellys® Evolution, Bertin Instruments). After lysis, the material was spined down by centrifugation at maximum speed for 1 minute. The supernatant of the lysed sample was transferred onto the Spin Filter D and centrifuged at 11,000 rpm for 2 minutes. An equal volume of 70 % ethanol was added to the sample and placed onto the Spin Filter R. It was centrifuged at 11,000 rpm for 2 minutes. 500 µl of Washing Solution HS (Analytik Jena) were added to the Spin Filter R and centrifuged at 11,000 rpm for 1 minute. 700 µl of Washing Solution LS (Analytik Jena) was added to the Spin Filter R and centrifuged again at 11,000 rpm for 1 minute. The tubes were centrifuged at 11,000 rpm for 2 minutes to remove all traces of ethanol. For elution, 30 µl of RNase-free water was added and centrifuged at 11,000 rpm for 1 minute. This step was repeated with another 30 µl of RNase-free water. NanoDrop 2000 (PreqLab) was used to measure the purity and concentration of the RNA in the sample (Figure 3).

3.1.6. Reverse transcription and qRT-PCR

PCR techniques give genetic information through the amplification of DNA sequences, starting with a low number of target copies. Although the analysis does not provide information on the biologically active product of genes, a correlation between the function of a protein and the expression pattern of its gene was found. Quantitative real-time (qRT) PCR has two significant advantages: the use of standard curves, which allows a comparison between experiments, and the use of internal standards, which shows variation in the template starting amounts [56].

qRT-PCR determines the initial number of copies of the template DNA with accuracy and high sensitivity. First, RNA needs to be isolated from the tissue of interest and reverse transcribed into cDNA. An aliquot of the cDNA is then used as a template for the qRT-PCR reactions [57].

In qRT-PCR, both target and reference genes are directly used. Compared to conventional PCR, qRT-PCR measures the amplification product using a fluorescent reporter molecule like SYBR Green. SYBR Green I dye is a fluorescent DNA binding dye that binds to any double-stranded DNA. The fluorescent signal increases with each PCR amplification cycle. The intensity is measured in real-time at each cycle. The threshold cycle (Ct) is the value at which the fluorescence crosses the threshold and is used for quantitative measurement [56; 58]. The more nucleic acids are in the sample, the sooner is a significant increase in fluorescence [59]. The Ct value is then used to calculate the initial DNA of the sample. The results get normalized using so-called reference genes. Reference genes should be consistently expressed since any variation in their expression would affect the final result. It is, therefore, necessary to use good reference genes to ensure the accuracy of qRT-PCR [56; 58].

Before reverse transcription, genomic DNA was removed from the samples using DNase I (Thermo Fisher Scientific). 1 µl of DNase I and 1 µl of 10x reaction buffer with $MgCl_2$ (Thermo Fisher Scientific) were added to 1 µg of RNA and incubated at 37 °C for 45min. Then 1 µl of 50 mM EDTA was added and incubated at 65 °C for 10 minutes. The prepared RNA was used for reverse transcription. The iScript cDNA Synthesis Kit (Bio-Rad) was used for the transcription into cDNA. To each sample 4 µl of 5x iScript Reaction Mix and 1 µl iScript Reverse Transcriptase were added. For a total volume of 20 µl the respective amount of nuclease-free water was added. The complete reaction mix was incubated in a Thermal cycler (Bio-Rad T100™), further described in the table below.

Table 2: Reaction Protocol for reverse transcription

Priming	5 minutes at 25 °C
Reverse Transcription	20 minutes at 46 °C
RT inactivation	1 minute at 95 °C
Optional step	Hold at 4 °C

Lastly, the cDNA was further used for quantitative real-time (qRT) PCR (Figure 3). The components used for the master mix are listed in the table 3. The qRT-PCR mix was prepared in a clear 96-well TW-MW plate (Biozym). A LightCycler® 480 System and

LightCycler SYBR Green I Master mix was used. The following pairs of primers were used for amplification: *Cd3g*, 5'-ACATCAAACCCCCTGCAAGT-3' (forward) and 5'-GCCGGATATGGTGCCTATGT-3' (reverse); *GADPH*, 5'-AACTTTGGCATTGTGGAAGG-3' (forward) and 5'-ACACATTGGGGGTAGGAACA-3' (reverse); *β-actin*, 5'-GCTCTTTTCCAGCCTTCCTT-3' (forward) and 5'-CTTCTGCATCCTGTCAGCAA-3' (reverse) (Eurofins Genomics). Change in Fold expression was calculated by the ΔΔ C_t method, and GADPH and β-actin were used as reference genes.

Table 3: Master Mix for qRT-PCR

Mastermix (LightCycler® 480 SYBR Green I Master, Roche)	7.5 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
Nuclease-free water (LightCycler® 480 SYBR Green I Master, Roche)	4.5 µl
cDNA	2 µl

Table 4: Reaction Protocol for qRT-PCR

	Temperature	Time
Denaturation	95 °C	4 minutes
Amplification	95 °C	30 seconds
	60 °C	40x 30 seconds
	72 °C	3 seconds
Melting	95 °C	5 seconds
	65 °C	1 minute
	97 °C	continuous

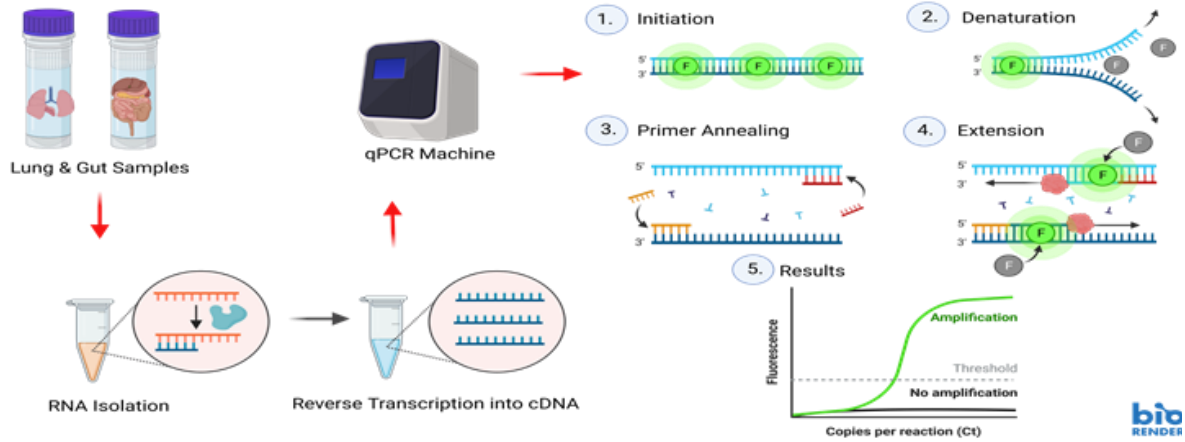


Figure 3: Determination of γδ T cell receptor gene expression. Lung and gut tissue were taken and used for analysis. The γδ T cell receptor gene expression was determined by RNA isolation, reverse transcription into cDNA, and quantitative polymerase chain reaction (PCR). Created with BioRender.com

3.1.7. Histology of lung and gut tissue

Histology studies cells and tissues in microscopic detail for pathological interpretation. Using light, fluorescence, or electron microscopes, samples or specimens are examined to investigate if the cells are healthy or diseased. Thin slices of tissues are prepared and analyzed using different staining methods [60].

Hematoxylin and eosin (H&E) is tissues' most used histology stain. It highlights the structures of cells and tissues. Hematoxylin is positively charged and stains the cell nuclei blue, whereas eosin is negatively charged and stains the cytoplasm of cells pink [60; 61].

Periodic acid-Schiff (PAS) staining is often used in diagnostics of histopathology. It is widely used in liver and muscle diseases and demonstrates polysaccharides, mucin, glycogen, specific glycoproteins, and glycolipids in a bright magenta pink to red [60].

Immunohistochemistry (IHC) is often used in pathology for morphologic diagnosis. It is a method for protein localization in paraffin-embedded or formalin-fixed cells and tissues. IHC requires care in many steps, including tissue fixation, antibodies used, and details in the IHC protocol. Various problems like nonspecific background or other staining problems can occur [62].

The concept behind IHC is the binding of an antibody to a specific antigen in tissue sections. When an antibody-antigen binding occurs, a colored chemical reaction is visible by light microscopy [63] (Figure 5).

In formalin fixation, amino acids can go into a conformational change of the epitope of interest. To avoid this, heat is induced for epitope retrieval. Also, the buffer with the correct pH plays a substantial role. Some antigens are retrievable in a wide pH range, while others only at extreme pHs. However, the higher the pH is, the harsher it acts on the tissue. Devices that can be used for heat applications include microwave ovens, pressure cookers, steamers, autoclaves, and water baths.

For visualization of the antigen-antibody reaction, a label is needed. Therefore, antibodies are labeled with enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP). Combined with an appropriate substrate and chromogen, it results in a colored stain. This process can occur directly as a one step with a primary antibody conjugated with a label or indirectly with multiple antibodies. The indirect method used an unlabeled primary antibody and a second layer conjugated with a label that acts against the primary antibody [63; 64].

Selecting the suitable antibody is one of the most critical steps for a successful IHC result. Antibodies are made by immunizing animals such as mice, rabbits, goats, horses, etc., with purified antigens. There are two forms of antibodies, polyclonal and monoclonal antibodies. Polyclonal antibodies are produced in multiple animal species and have higher affinity and broader reactivity but lower specificity than monoclonal antibodies. For this reason, monoclonal antibodies are preferred. It is also essential to take into account the species which is to be examined and the animal source of the antibody to avoid cross-reactions [63; 64].

Lung and gut tissues were taken from the mice, fixed in formalin, and embedded in paraffin. Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were used for histological analysis. Immunohistochemistry was performed to localize γδ T cells in the lung and gut samples (Figure 4). For all histological staining methods, the paraffin-embedded tissues were cut using a Microm HM 355 S histological microtome (Thermo Fisher Scientific Inc.) at 3 microns in thickness, then placed on positively charged Superfrost-Plus micro slides (Thermo Fisher Scientific Inc.). Before proceeding to the staining, the slides were incubated for 1 hour at 56 °C. The slides were deparaffinized three times for 10 minutes in

xylene and then rehydrated in the sequence of ethanol solutions (99%, 96%, 80%, 70%, and 50%) for 3 minutes each, followed by distilled water for 5 minutes. After mounting, the sections were scanned using a Tissue FAXS scanning system (Tissue Gnostics, Austria).

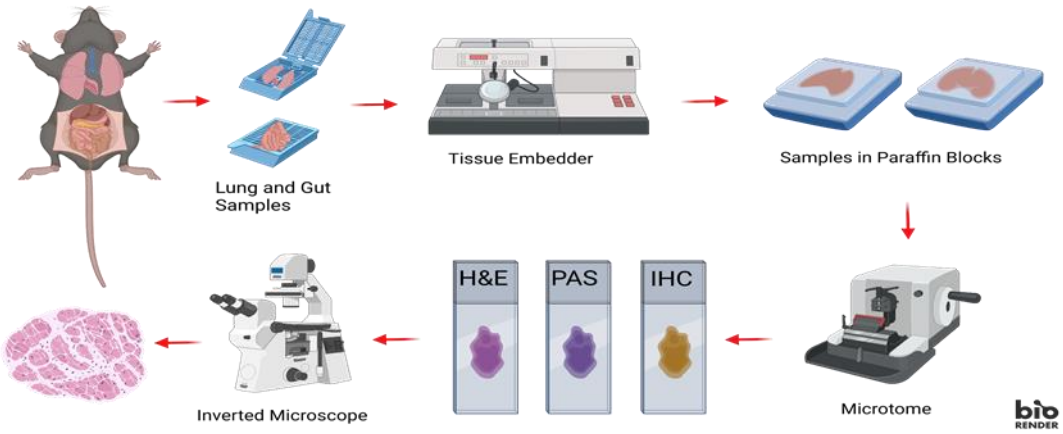


Figure 4: Histological Methods. Hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were used for histological analysis. Immunohistochemistry was performed to localize γδ T cells in the lung and gut samples. Created with BioRender.com

3.1.8. Hematoxylin and Eosin staining

After rehydration, tissue sections were stained with Hämalaun (Mayer) concentration (Prod. No. 109249) for 10 minutes and washed three times in distilled water. Slides were dipped shortly into 1 % HCl (Carl Roth GmbH) in 70 % EtOH and washed in running tap water for 7 minutes. Afterward, slides were incubated in eosin solution (Sigma-Aldrich) for 25 seconds and rinsed in distilled water. The tissue sections were dehydrated in ascending alcohol concentration series (80 %, 96 %, 99 %) by dipping into each concentration three times and incubated in xylene for 5 minutes. The slides were mounted (Histomount Mounting Solution, Life Technologies), sealed with a cover slip, and dried overnight. Lung scoring was performed to determine tissue changes in the respiratory tract between allergic and non-allergic mice. Therefore, scores were defined according to the severity of inflammation of the damaged tissue in the lung. Scoring grades are: "0"-normal, "1"-mild, "2"-moderate, "3"-severe. The classification was performed blindly.

3.1.9. Periodic Acid-Schiff staining

After rehydration, the slides were immersed in periodic acid solution (Periodic Acid-Schiff (PAS) Kit; Sigma-Aldrich) for 5 minutes at room temperature and rinsed in several changes in distilled water. Slides were incubated in Schiff's reagent for 15 minutes and washed under running tap water for 5 minutes. The tissue sections were counterstained in Hematoxylin Solution for 90 seconds, dehydrated in ascending alcohol series, and mounted (Histomount Mounting Solution, Life Technologies). Goblet cell counting was performed blinded by randomly choosing 15 regions of the tissue and counting the goblet cells within those regions.

3.1.10. Immunohistochemistry Staining

The samples were incubated in a citrate buffer (10 mM Sodium citrate, pH. 6.0) for 9 minutes in the microwave and cooled down with cold running tap water for 10 minutes to unmask the antigen. The slides were washed two times for 5 minutes in 1x PBS plus 0.025 % Triton X-100 with gentle agitation to allow the antibodies to access the intracellular antigens. The samples were first blocked in 0.3 % H_2O_2/absolute EtOH for 10 minutes and then for 2 hours with 10 % BSA to reduce the nonspecific staining. To detect γδ T cells, the slides were incubated overnight at 4 °C in a humid chamber with a primary antibody (Anti-TCR gamma+ TCR delta antibody; Abcam ab231545) diluted 1:100 in 1 % BSA. Slides were rinsed 2x5 minutes in PBS 0.025 % Triton with gentle agitation. Secondary antibody (Rabbit Anti-Hamster IgG (H+L) Antibody, HRP Conjugate, cross-adsorbed; Life Technologies) with conjugated enzymes diluted 1:1000 in 1 % BSA was applied (Figure 5). As negative controls, samples were stained with secondary antibodies alone. The tissue sections were rinsed three times for 5 min in 1x PBS and counterstained with DAB (Dako) until either a brown color was seen or up to 10 minutes. Slides are washed briefly in distilled water and then counterstained with hematoxylin for 10 minutes. Slides were dipped shortly into 1% HCl (Carl Roth GmbH) in 70 % EtOH and washed in running tap water for 7 minutes. The tissue sections were dehydrated in ascending alcohol concentration series (80 %, 96 %, 99 %) by dipping into each concentration three times and incubated in xylene for 5 minutes. The slides were mounted (Histomount Mounting Solution, Life Technologies), sealed with a cover slip, and dried overnight.

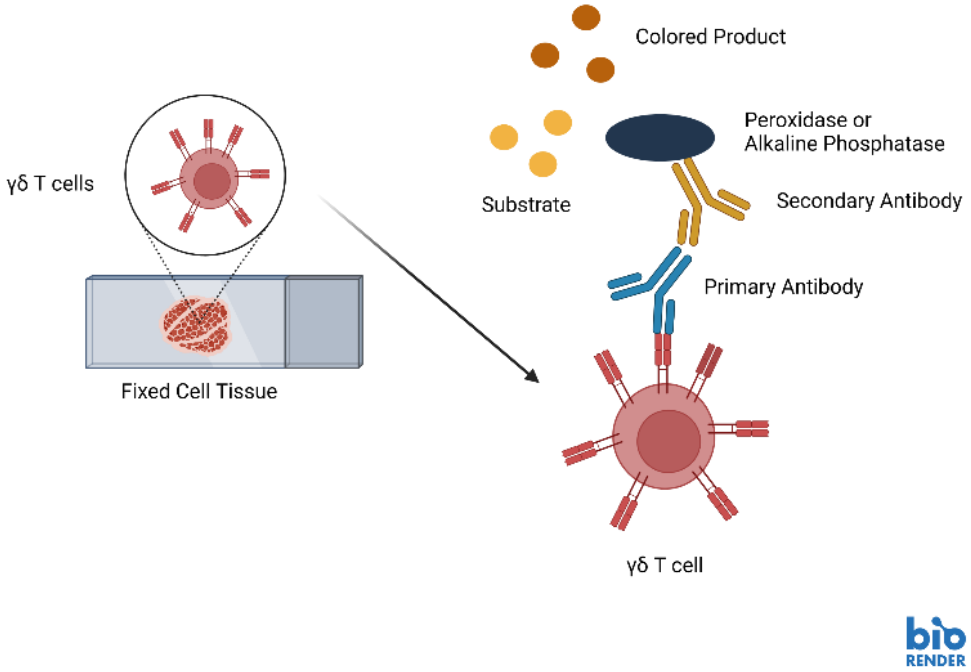


Figure 5: Immunohistochemistry used to localize γδ T cells in the tissues. To specifically detect γδ T cells in the lung and gut tissue, a primary antibody and a secondary antibody with a conjugated enzyme were used.

3.1.11. Protein Extraction

For protein extraction, lung and gut tissues were weighed, cut, and washed 2x with 450 µl of ice-cold 1x PBS. The tissue was transferred into homogenizer tubes (Peqlab), and 450 µl of RIPA buffer (Thermo Fisher Scientific) was added. Samples were homogenized for 30 seconds at 6500 rpm (Precellys® Evolution, Bertin Instruments). After lysis, the material was spined down by centrifugation at maximum speed for 1 minute, and the lysate was transferred into new Eppendorf tubes. The samples were incubated on ice for 30 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4 °C. The supernatant was transferred into a new tube, and the pellet was discarded. Determining of the protein concentration in the samples was performed according to the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific) protocol. Afterward, the samples were stored at -80 °C.

3.1.12. SDS-PAGE

For Coomassie stain, 39 µl of the samples together with 15 µl of NuPAGE LDS sample buffer (Thermo Fisher Scientific) and 6 µl of NuPAGE reducing agent (Thermo Fisher Scientific) were heated up to 100 °C for 10 minutes. 20 µg of the cell lysates were used per well to get ideal bands, 20 µl of the prepared samples were loaded into the wells of the gels (NuPAGE, 4-12%, Bis-Tris; Thermo Fisher Scientific) and 7 µl of the PageRuler (10-180 kDa, Prestained Protein Ladder; Thermo Fisher Scientific). First, the gel was running at 120 volts until the end of the stacking gel and afterward at 200 volts and 125 milliamperes for 60 minutes. After the run was completed, the gel was stained with Simply Blue Safe Stain (Thermo Fisher Scientific) based on the manufacturer's instructions. The gels were analyzed by ChemiDoc™ Touch Imaging System (Bio-Rad) (Figure 6).

3.1.13. Western Blot

Western blot (WB) is a semi-quantitative technique to identify specific proteins. It is widely used in diagnostic methods for various infections and autoimmune and oncological diseases. Using gel electrophoresis, WB is based on separating proteins by their molecular weight (MW). Afterward, the proteins are transferred onto a membrane exposed to antibodies specific to the protein of interest [65]. This technique allows for studying proteins' presence, relative abundance, molecular mass, and posttranslational modifications [66].

Protein extraction is required to detect specific proteins. Proteins can be extracted from samples, such as animal and herbal tissues or cells [67]. This can be performed mechanical or chemical. Mechanical methods are sonication and homogenization with glass or metallic beads. Chemical methods include buffers that solubilize the proteins, such as sodium dodecyl sulfate (SDS). One of the most widely used buffers is radioimmunoprecipitation assay (RIPA). The purpose of lysing buffers is to maintain the protein structures using protease inhibitors [65].

Before performing the electrophoresis, the protein content needs to be quantified by determining the protein concentration. Lowry, Bradford, and bicinchoninic acid (BCA) are the most used methods for quantifying proteins. Those are colorimetric assays causing a color change proportional to the amount of protein in the sample [65; 68].

After the protein concentration is determined, the samples are diluted in a loading buffer. The loading buffer contains glycerol so that the samples sink into the gel wells, bromophenol blue dye to indicate how far the separation has progressed, and SDS, to denature the proteins and give them a negative charge [68].

The gel used for WB consists of stacking and separating gel. The stacking gel has a lower acrylamide concentration which causes the proteins to form defined bands. The separating gel has a higher polyacrylamide content causing the proteins to separate by size. The smaller the proteins, the faster they travel through the gel than the larger proteins [67].

The proteins need to be transferred from the gel onto solid support like a membrane for the immunodetection of specific proteins. There are two membranes: nitrocellulose and polyvinylidene difluoride (PVDF) [65]. The PVDF membrane is more robust, provides better binding, and can be used for reprobing. However, the background is higher than in nitrocellulose. Nitrocellulose is used for its high protein affinity but cannot be used for reprobing [67].

The transfer can be performed under wet or semi-dry conditions. Both systems are characterized by close contact between the gel and the membrane [65]. In the wet transfer, the gel-membrane sandwich is placed into a cassette which is then put in a buffer tank and subjected to an electric field. In the semi-dry transfer, the sandwich is put on electrode plates which generate the electric field [68]. After the transfer, the membrane is blocked to avoid nonspecific and empty sides. For blocking non-fat milk and bovine serum albumin (BSA) are the most widely used. Afterward, the membrane is incubated with the primary antibody specific for the protein of interest. As in IHC, monoclonal and polyclonal antibodies can be used [65].

Immunodetection can be performed by chemiluminescence and fluorescence. Chemiluminescence uses peroxidase or phosphatase enzymes conjugated to secondary antibodies. After activation, light is generated by a reaction of luminescent compounds (luminol). In fluorescent immunodetection, the secondary antibody has a conjugated fluorophore and emits light when excited [65].

For the wet transfer, the gel and a nitrocellulose membrane were soaked in a transfer buffer (NuPAGE; Thermo Fisher Scientific) containing 10 % methanol and then blotted for 1 hour at 30 volts and 400 milliamperes using the X-Cell II Blot Module. For staining the bands for γδ T cells, the following steps were performed after the protocol of SuperSignal™ West Atto

Ultimate Sensitivity Substrate (Thermo Fisher Scientific). After blotting, the membrane was washed in deionized water for 5 minutes under gentle agitation to remove all transfer buffer. The membrane was blocked with 5 % PBS-skim milk for 1 hour at room temperature with side-to-side shaking. After blocking, the membrane was incubated with the primary antibody (Purified anti-mouse TCR γ/δ Antibody; BioLegend; clone GL3) diluted 1:1,000 in 10 % BSA at 4 °C overnight. Afterward, the membrane was washed 3x with PBS containing 0.05 % Tween 20 detergent and then incubated with the secondary antibody (Rabbit Anti-Hamster IgG (H+L) Antibody, HRP Conjugate, cross-adsorbed; Life Technologies) HRP-conjugate diluted 1:2,000 in 1 % BSA for 1 hour at room temperature. The membrane was washed six times for 5 minutes each in 0.05% PBS-Tween20 to remove any unbound secondary antibody conjugate. Then, the membrane was incubated with the substrate working solution (Thermo Fisher Scientific) for 5 minutes. As a control, a western blot was performed to stain for β-actin protein. After the transfer, the membrane was washed with 1x PBS for 15 minutes, and then blocked with PBS-skim milk of 2 % for one hour. The membrane was washed two times for 5 minutes with PBS-tween 0.05 % and one time for 5 minutes with 1x PBS. The membrane was then incubated with the examined primary antibody (Anti-β-Actin; ZooMab clone 6L12) diluted 1:1,000 in 1 % BSA at 4 °C overnight. The membrane was washed two times for 5 minutes with PBS-tween 0.05 % and one time for 5 minutes with 1x PBS and incubated for 1 hour at room temperature with a respective secondary antibody (Anti-Rabbit IgG with Alkaline Phosphatase; Sigma Aldrich SLBC3108). The washing steps as described before were repeated, and then the membrane was incubated with the substrate solution 1Step-NBT/BCIP (Thermo Fisher Scientific) to develop the reaction. The data was acquired by ChemiDoc™ Touch Imaging System (Bio-Rad) (Figure 6).

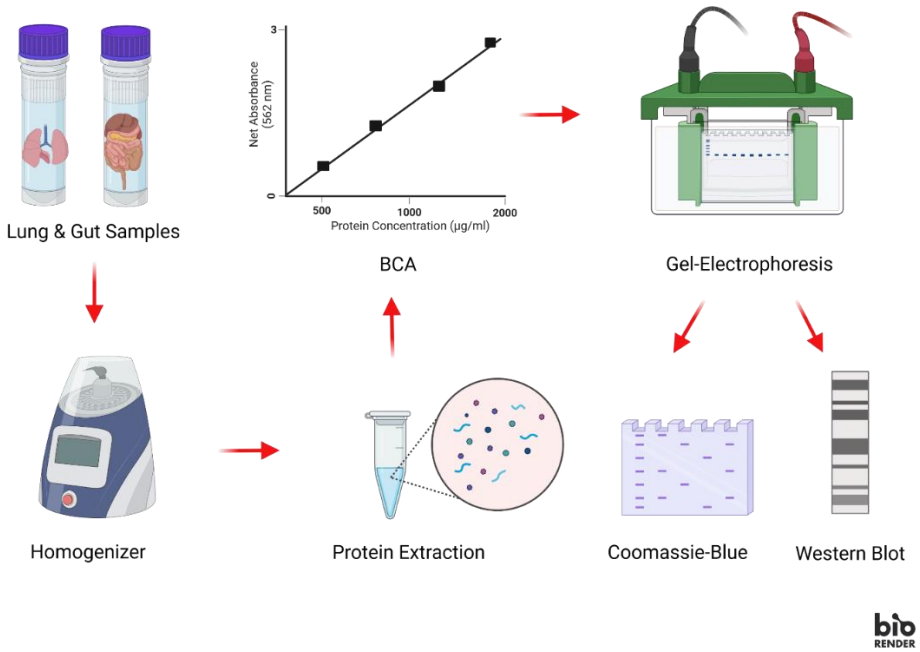


Figure 6: Methodology for protein concentration determination. SDS-Page and Western blot were performed to determine how much protein was produced. Created with BioRender.com

3.2. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 9.3.1. All results were represented as the mean ± SD. The γδ T cell gene expression between the different groups was compared using one-way ANOVA followed by Tukey's correction for multiple comparisons and an unpaired t-test. The significance of the histologic data was tested by two-way ANOVA followed by Tukey's correction for multiple comparisons. P-values < 0.05 were considered significant: $p \leq 0.05$= *, $p \leq 0.01$ = **, $p \leq 0.001$= ***.

3.3. Media and Solutions

Table 5: Listed are all media and solutions which were used for the experiments

Media and Solution	**Company**
Tri-Sodium Citrate Buffer (pH 6.0)	
Tri-Sodium citrate 2.94g	Sigma-Aldrich
dH_2O 1,000 ml	
Wash Medium IHC	
1x PBS 50 ml	Sigma-Aldrich
0.025 % Triton-X 12.5 µl	Sigma-Aldrich
10 % BSA Blocking Buffer	
BSA 5g	Sigma-Aldrich
dH_2O 50 ml	
1 % BSA Blocking Buffer	
10 % BSA 1 ml	
1x PBS 9 ml	
0.3 % H_2O_2/absolute EtOH buffer	
H_2O_2 30 µl	
absolute EtOH 10 ml	Carl Roth GmbH
0.5 % Eosin Working Solution	
Eosin 100 ml	Sigma-Aldrich
Glacial acetic acid 0.5 ml	Applichem Gmbh
1 % HCl in 70 % EtOH	
37 % HCl 4.1 ml	Carl Roth GmbH
70% EtOH 145.9 ml	Carl Roth GmbH
Lysis Buffer	
0.15 M NaCl 4.38 g	Sigma-Aldrich
5 mM EDTA 0.93 g	Carl Roth GmbH
10 mM Tris-HCl 0.79 g	Carl Roth GmbH
1% Triton-X 100 5 ml	Sigma-Aldrich
dH_2O 500 ml	
Wash Medium WB	
1x PBS 500 ml	
0.05 % Tween 20 250 µl	Sigma-Aldrich

4. Results

4.1. Intranasal tolerization upregulated γδ T cells gene expression in the lung of obese and lean mice

We measured the gene expression of the γ-chain of γδ TCR genes in the lung and gut samples of obese allergic and allergic tolerized mice cohort to determine whether there was different expression of γδ T cells, which would give us a hint about their involvement in allergy, and immune tolerance development. We aimed to investigate if there are differences between HFD- and STD-fed mice and if γδ T cell populations in different organs respond differently to the treatments. We isolated RNA from the lungs and gut and reverse-transcribed it into cDNA. The cDNA was then used for qRT-PCR to detect a change in gene expression. We found an increase trend in gene expression in the lungs of intranasally treated HFD- and STD fed mice compared to all other groups. However, only in STD-fed mice, the gene expression increased significantly (Figure 7 A, B) (Figure 8 C).

In obese allergic mice, the mRNA of γδ T cells was down-regulated compared to lean mice. In lean allergic mice, we found a slight increase (Figure 8 A). Orally tolerized mice showed no change in gene expression and exhibited similar results to the control groups (Figure 8 B, D). The γδ T cells of those mice seem to stay in homeostasis and are not influenced by the diet or the treatment.

On the other hand, we found no differences in γδ T cell populations gene expression in the gut for all tested groups, indicating that the γδ T cell were able to maintain the tissue homeostasis in this organ and were not influenced by the treatments or the diet (Figure 7 C, D), (Figure 9 A-D).

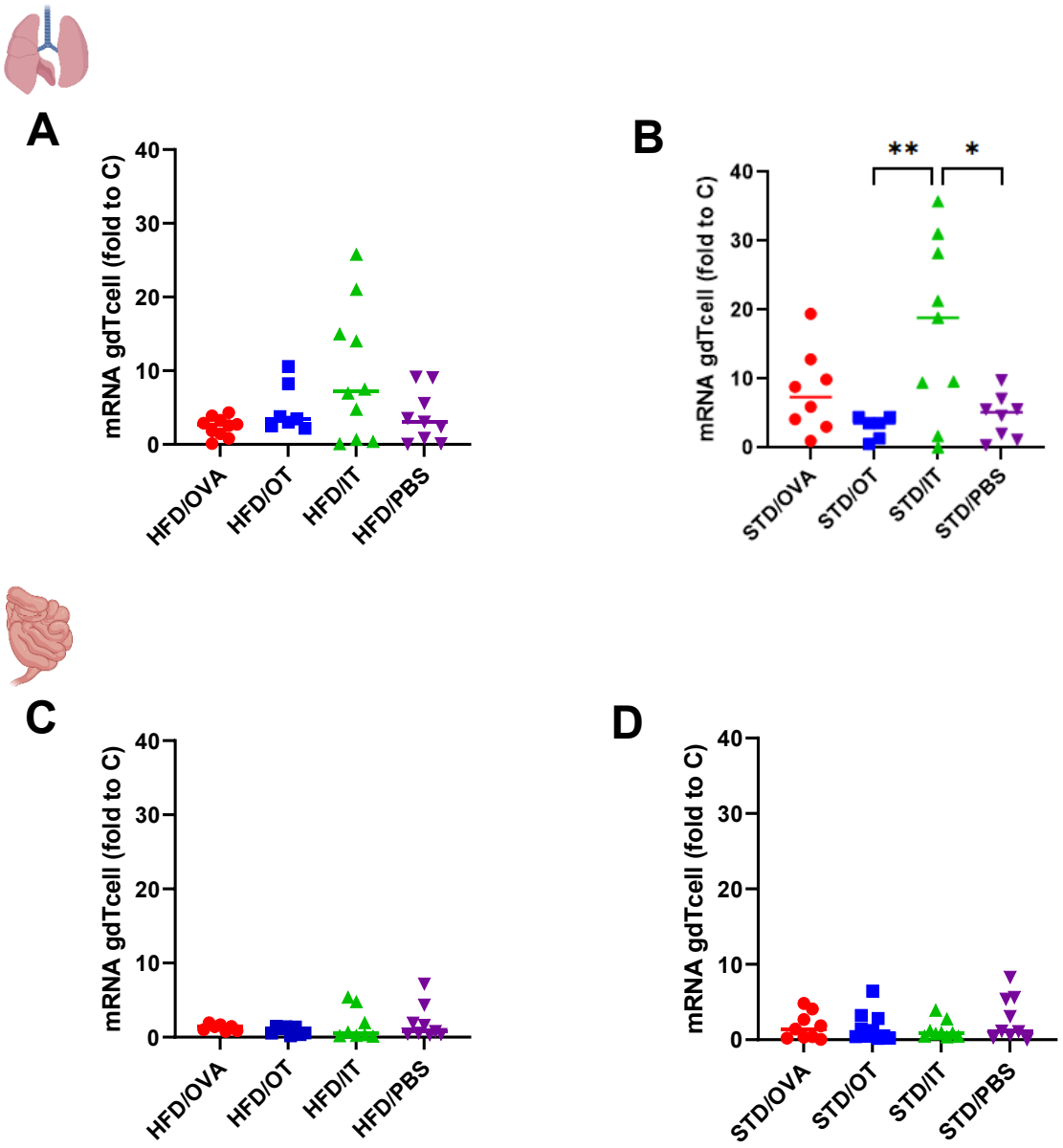


Figure 7: Gene expression analysis of γδ T cells in the lung and gut. To investigate if the gene expression of γδ T cells might be affected by the treatments and the diet, the RNA got isolated from lung and gut tissues and was reverse transcribed into cDNA. The cDNA was then used to perform qRT-PCR to measure the change in gene expression. A, B) Intranasal treatment with OVA increased γδ T cell mRNA in the lung of HFD- and STD-fed mice. Although only in STD-fed mice was this result statistically significant compared to orally tolerized mice and sham control. C, D) No significant differences in the gut tissue γδ T cell gene expression levels between the different groups were observed. The statistical analysis was done using a one-way ANOVA followed by Tukey's correction for multiple comparisons. *p< 0.05, **p< 0.01, ***p< 0.001.

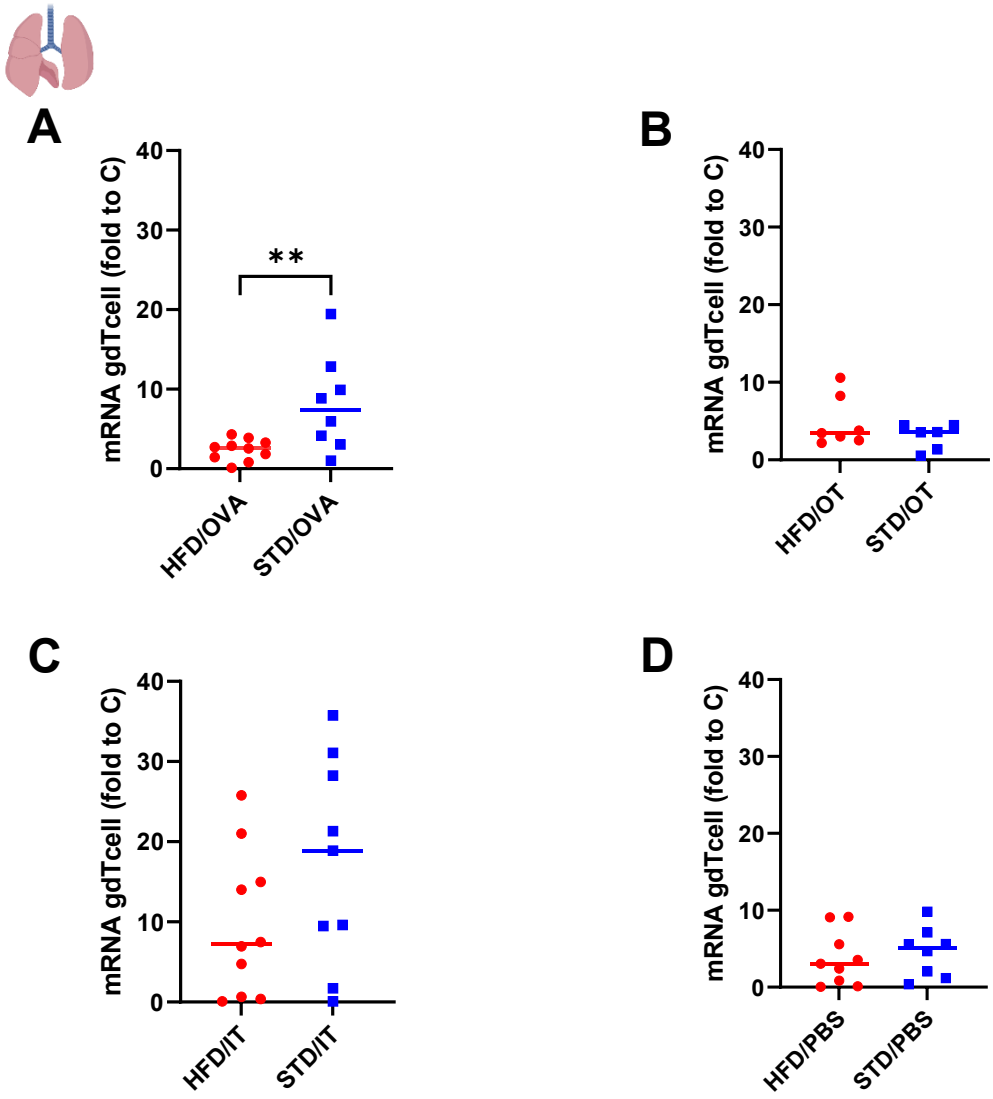


Figure 8: Comparison of the gene expression of γδ T cells in the lung between the diets. A) The gene expression of γδ T cells in the lung tissue of allergic obese mice showed a significant reduction of γδ T cells compared to lean mice. B) Oral tolerized mice had no change in γδ T cell gene expression as well as D) the control group. C) Intranasal treatment with OVA increased γδ T cell mRNA in HFD- and STD-fed mice. The statistical analysis was done by using an unpaired t-test. *p< 0.05, **p< 0.01, ***p< 0.001.

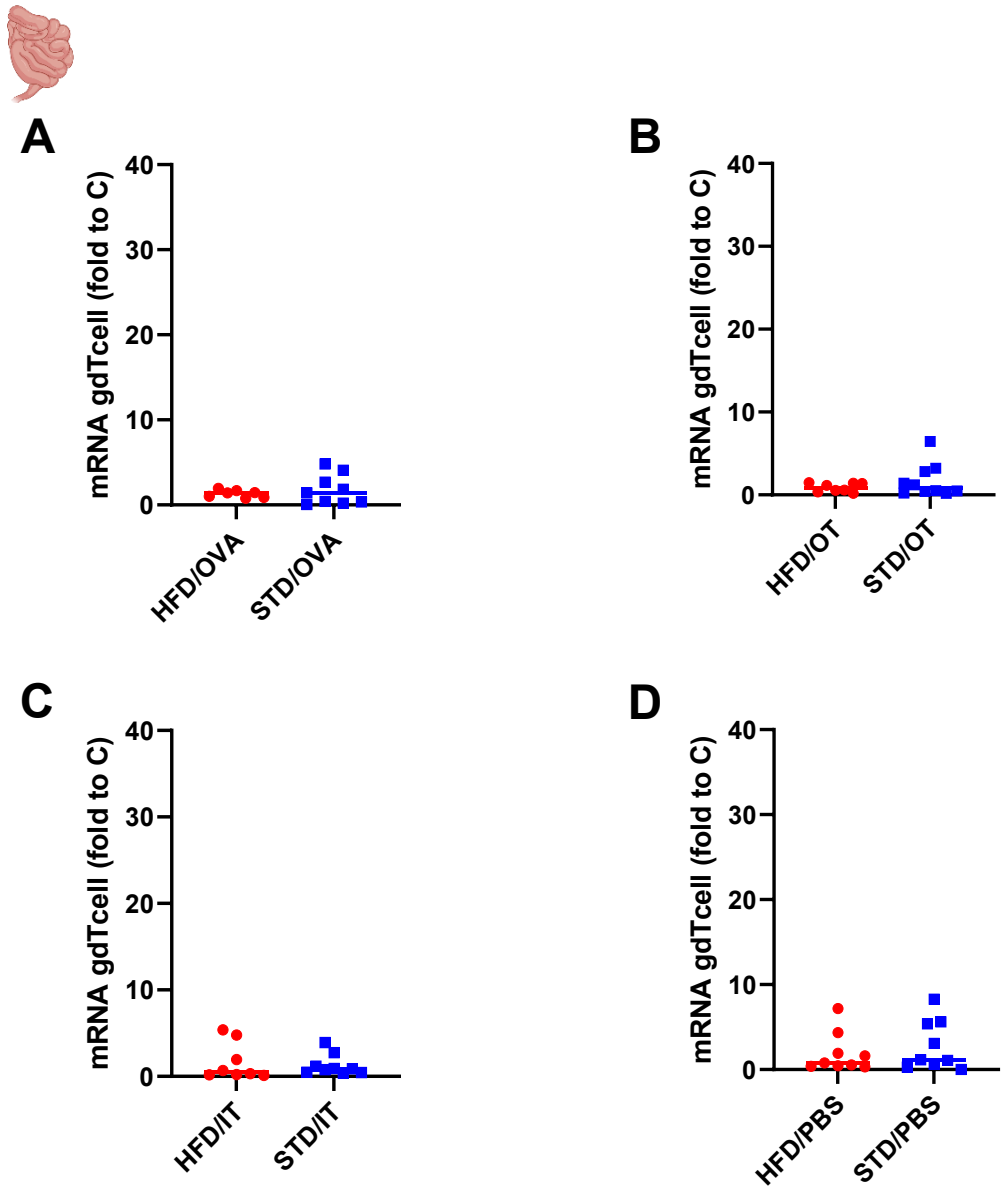


Figure 9: Comparison of the gene expression of γδ T cells in the gut between the diets. A-D) No differences in the gut tissue of γδ T cell gene expression levels between the different groups and diets were observed. The statistical analysis was done by using an unpaired t-test. *p< 0.05, **p< 0.01, ***p< 0.001.

4.2. Histological analysis of lung and gut tissue in obese and tolerized-obese cohort and respective controls

Infiltrating eosinophils are a central player in allergic asthma and the pathogenesis of this disease. The recruitment to the sites of allergic reaction in the lung is made via adhesion receptors, activating factors like cytokines and chemokines, and leukocytes which regulate the expression and release of the activating factors. Antigen-induced T cells activated through macrophages or other antigen-presenting cells could contribute to the pathological process. A correlation was found between the level of cytokines released by T cells and the degree of tissue damage mediated by eosinophils [69].

To assess the severity of the tissue damage in our mouse model, we performed blinded lung scoring. Therefore, the lung tissue was stained using H&E (Figure 10, 11). Based on the literature, it was measured how heavily the inflammation in the lung is i.e. number of infiltrating eosinophils. Therefore, scoring grades were allocated: "0"-normal, "1"-mild, "2"-moderate, "3"-severe.

As expected, all OVA-treated groups showed a significant increase in inflammation score compared to the control group. However, we observed no significant differences between the diets. The sham groups showed normal to mild inflammation. The HFD-fed mice of the control group showed a slightly higher increase in inflammation than the STD group (Figure 14 A).

In asthma patients, it was found that goblet cells contribute mucins to airway secretions. PAS staining of tissues allows clear visualization of goblet cells. So far, PAS staining's facilitated pathological studies of goblet cells in humans and experimental animals [70].

Here, we investigated if diet, treatments, or allergies influence the goblet cells. Therefore, performed PAS staining in the lung and gut tissues to visualize the goblet cells (Figure 10, 11, 12, 13). We selected 15 random regions of a lung or gut tissue and counted the goblet cells within those regions. The goblet cells in the gut seem not to be affected since no changes in their numbers were found (Figure 14 C). However, in the lung tissue, the sham control showed fewer goblet cells than the OVA-treated mice, where an increase can be seen (Figure 14 B).

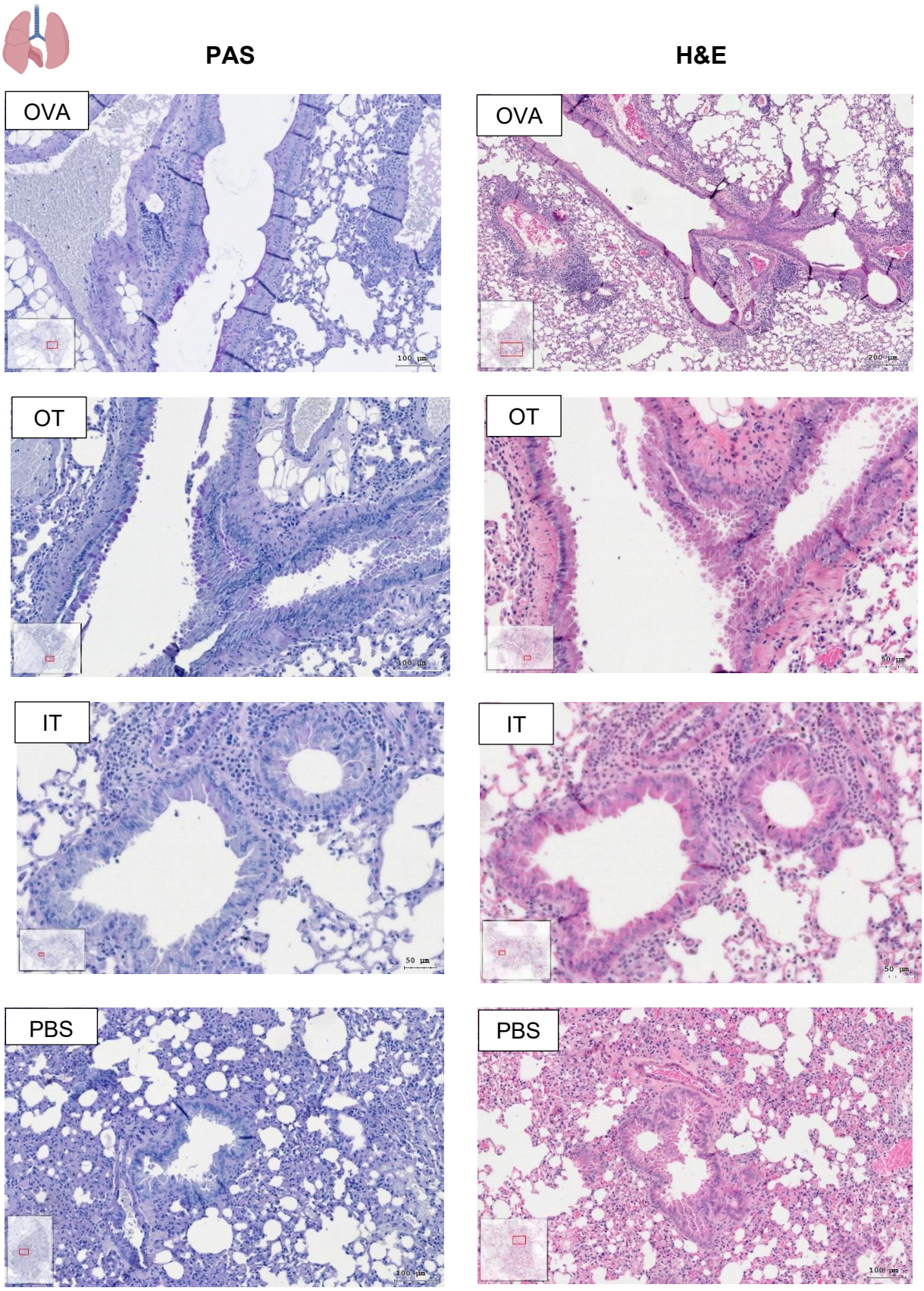


Figure 10: PAS and H&E Staining of HFD-fed mice of the lung

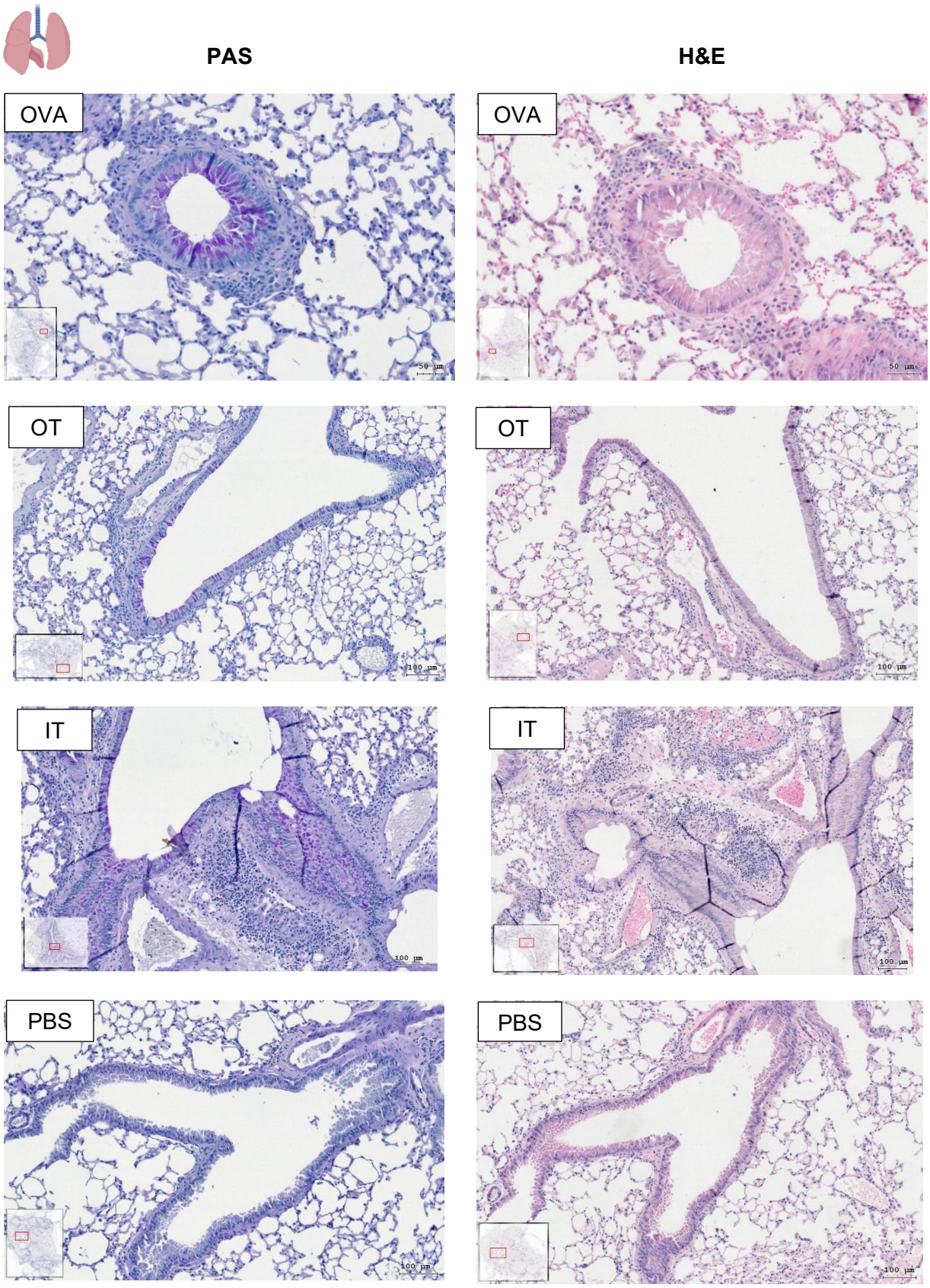


Figure 11: PAS and H&E Staining of STD-fed mice of the lung

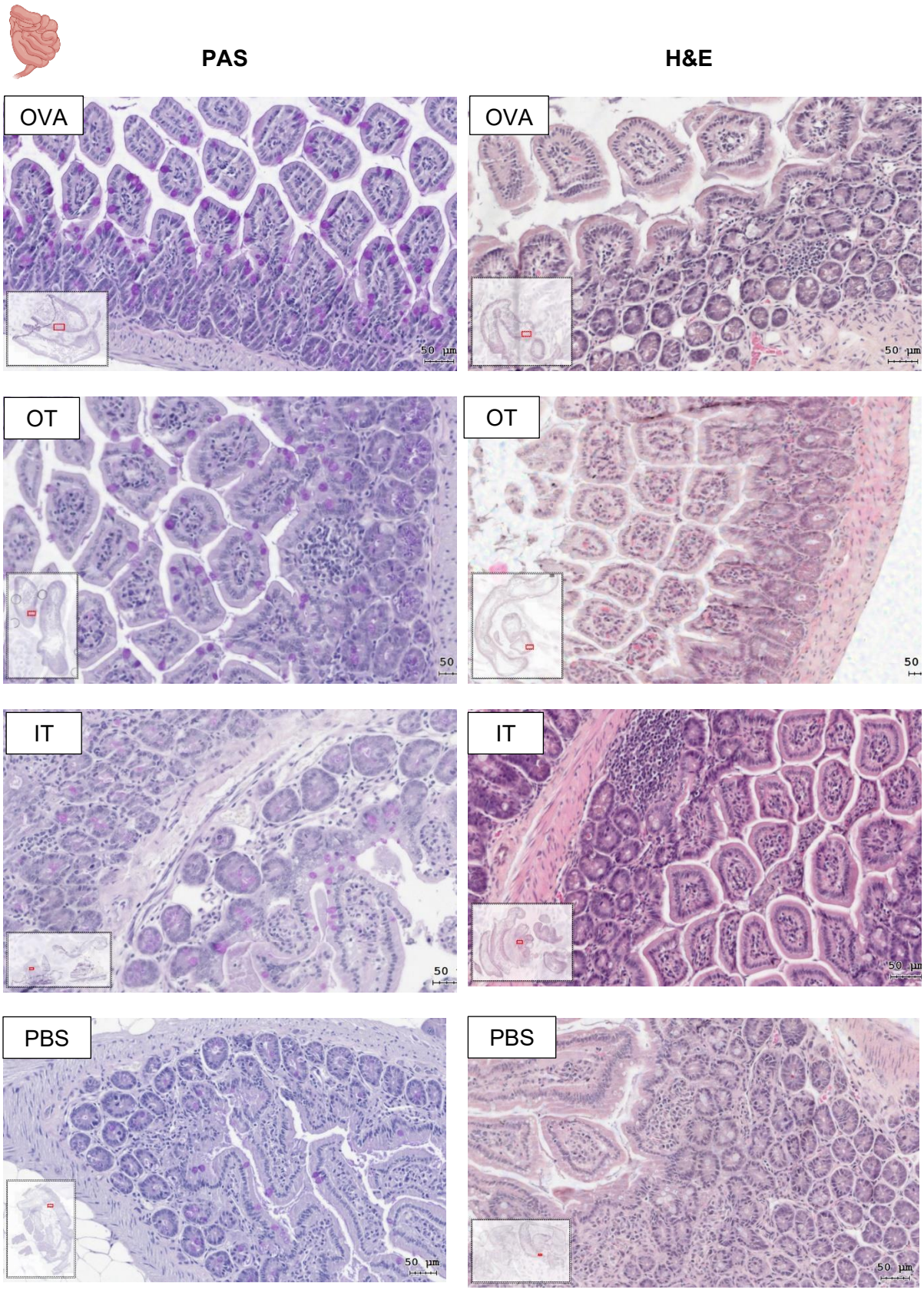


Figure 12: PAS and H&E of HFD-fed mice of the gut.

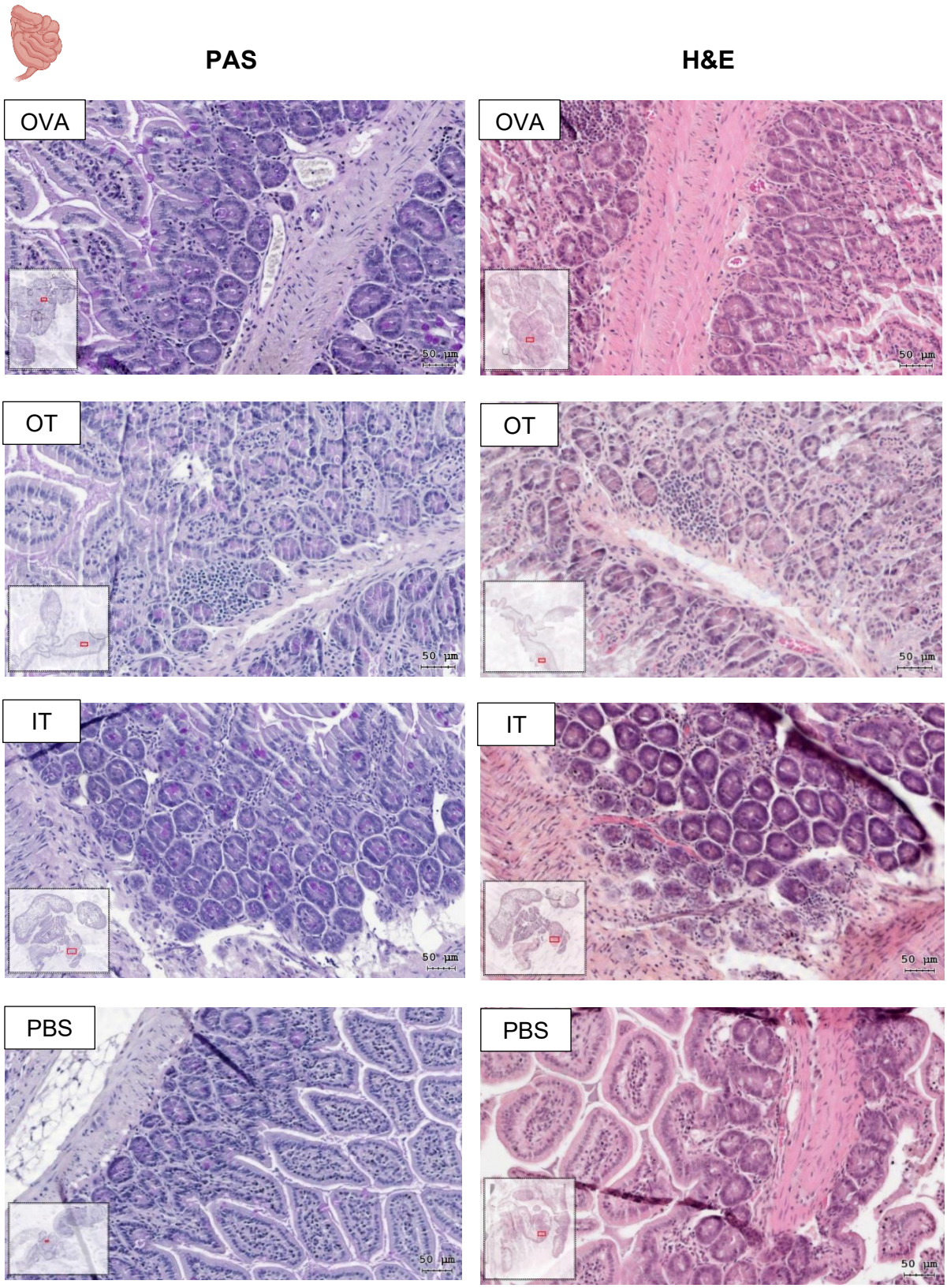
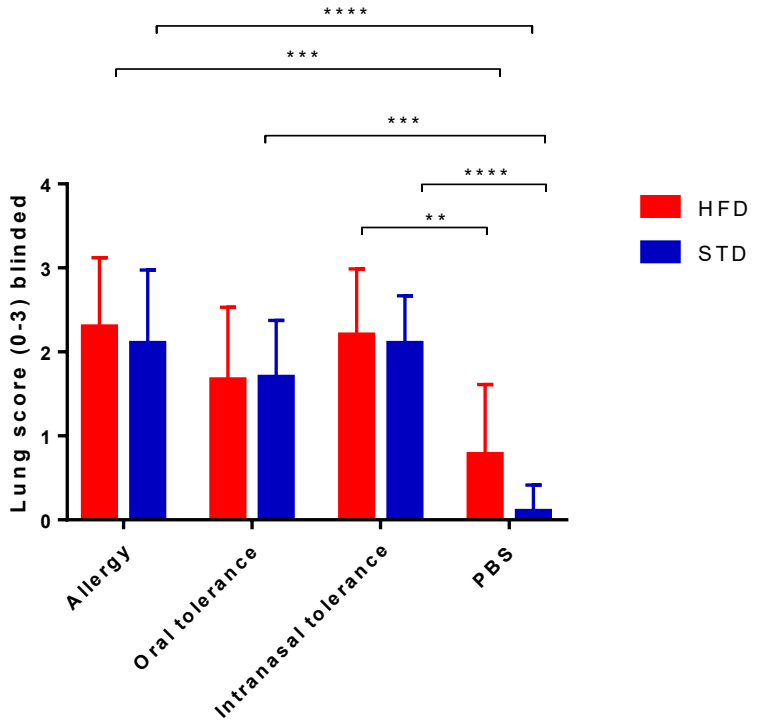
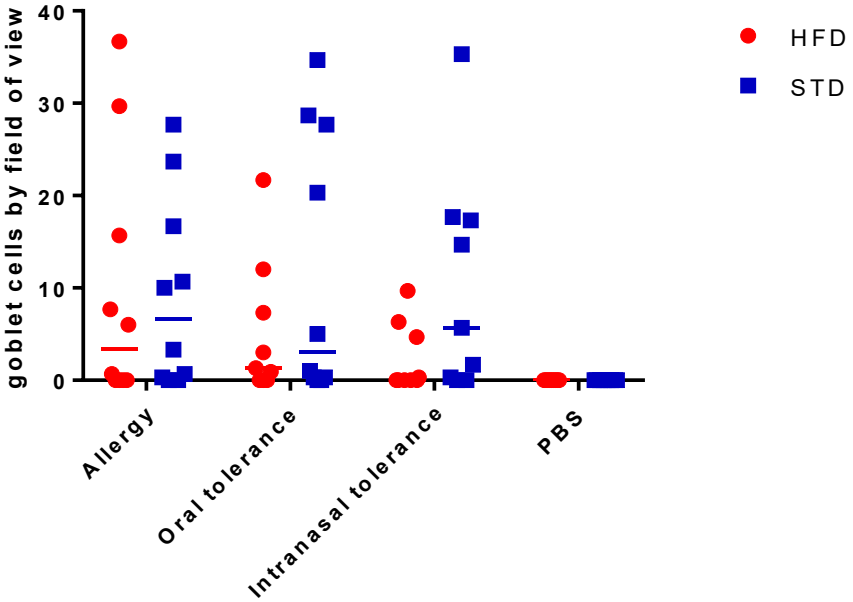


Figure 13: PAS and H&E Staining of STD-fed mice of the gut.

A


****
***
***
****
**
Lung score (0-3) blinded
0
1
2
3
4
Allergy
Oral tolerance
Intranasal tolerance
PBS
HFD
STD

B


goblet cells by field of view
0
10
20
30
40
Allergy
Oral tolerance
Intranasal tolerance
PBS
HFD
STD

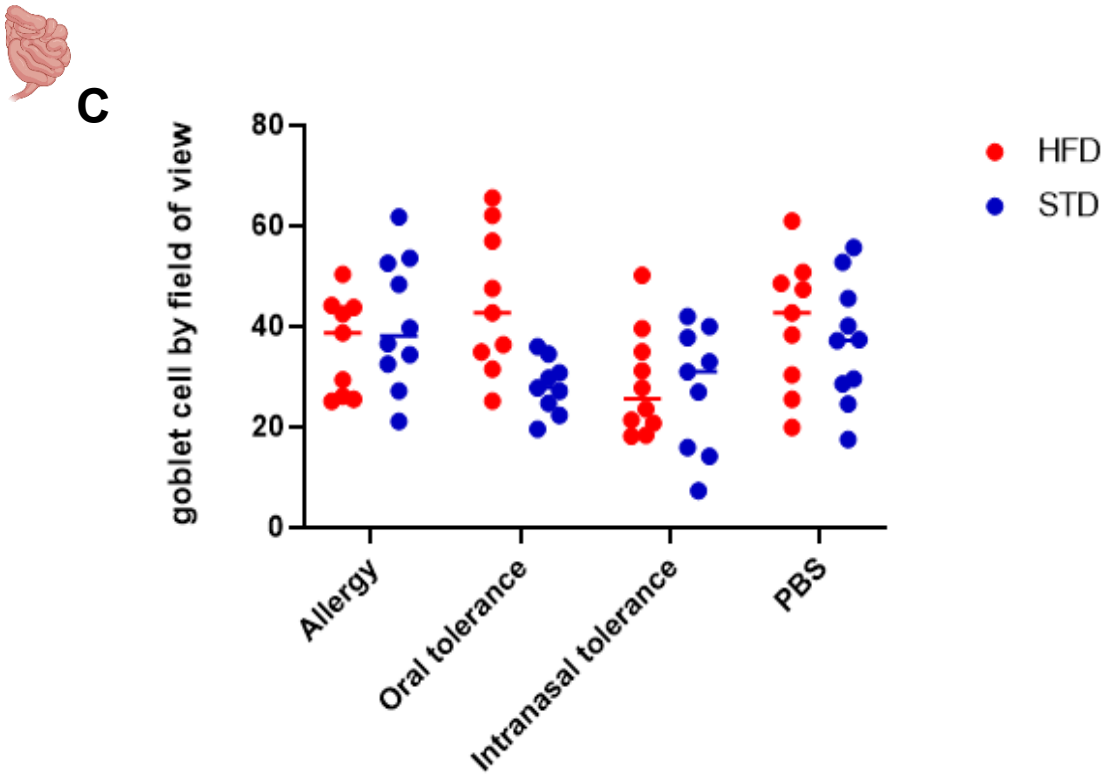


Figure 14: Histological analysis of the lung and gut tissue by counting goblet cells and scoring lung inflammation. A) lung scoring was performed blinded to define the severity of inflammation in the lung. Scoring grades are: "0"-normal, "1"-mild, "2"-moderate, "3"-severe. All OVA-treated groups showed a significant increase in inflammation compared to the control group. However, there are no significant differences between the diets. Even between HFD and STD in the control group, an increase in inflammation in can be seen. PAS staining was performed to see if the immunoregulation of the goblet cells might be influenced by allergy, obesity, and tolerance induction. Fifteen random regions were selected to count the goblet cells by field of view. B) No differences in the goblet cell numbers between the groups were found in the lung. However, the control group seems to have fewer goblet cells in the tissue. C) The number of goblet cells in the gut was not influenced by either the diet or the treatments. The statistical analysis of the histologic data was done by two-way ANOVA followed by Tukey's correction for multiple comparisons. *p< 0.05, **p< 0.01, ***p< 0.001.

4.3. Histological analysis of the abundance of γδ T cells in lung and gut tissue

To detect γδ T cells in the lung and gut tissues of all group's enzyme-labeled antibodies were used. When positively associated with the substrate and the enzyme, a brown color forms around the cell. Due to the extremely low density of distribution of γδ T cells a variety of problems can occur like nonspecific labeling of cells and tissues. Therefore, proper controls

are required for the specificity of the antibody staining. For the control, the secondary antibody alone was added to the sample. No positively stained cells were found in the controls (Figure 15 B). Positively stained γδ T cells were found in both organs, and the majority of γδ T cells were allocated to nonalveolar regions in the lung (Figure 15 A).

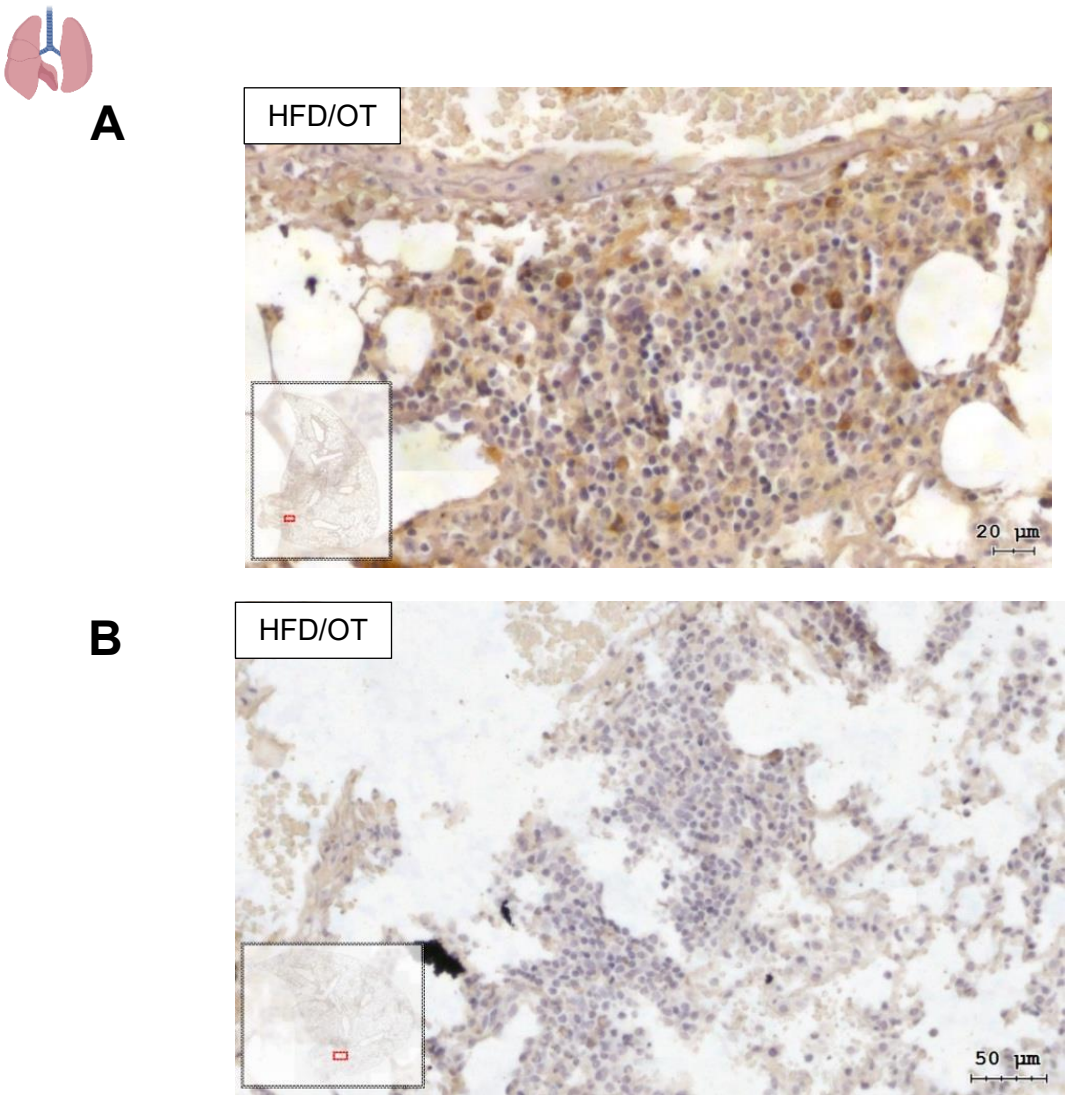


Figure 15: Immunohistochemistry of γδ T cells in the lung of obese orally tolerized mice. A) Shows positively stained γδ T cells in the lung of HFD-fed oral tolerized mice. A primary antibody (Anti-TCR gamma+ TCR delta antibody; Abcam ab231545) diluted 1:100 in 1 % BSA and a secondary antibody (Rabbit Anti-Hamster IgG (H+L) Antibody, HRP Conjugate, cross-adsorbed; Life Technologies) HRP-conjugate diluted 1:100,000 in 1 % BSA was used for detection. B) Shows the control of the same region as figure A. Here, no primary antibody was added.

4.4. Identification of γδ T cells in lung and gut mouse tissue by Western Blot

Western blotting was used to determine the number of γδ TCRs in the different treatment groups. First, proteins were extracted and separated using gel electrophoresis. These were then stained using Coomassie Blue staining. In the lung, the proteins in all samples were evenly separated and stained with the same intensity (Figure 16A). In the intestine, however, an evident degradation of the proteins can be seen since weaker signals are present here (Figure 17A). This can also be seen from the western blot analysis of the β-actin protein in the gut (Figure 17B). Here, the protein in the samples was degraded.

Western blot analysis of the β-actin protein in the lungs and gut was performed to determine expression, as this protein was used as a reference gene in the qRT-PCR. Uniform expression of β-actin can be seen in the lungs (Figure 16B). However, it was broken down in the gut, and no precise analysis could be carried out.

In addition, a western blot analysis was also performed to determine how much γδ TCR is present and whether these results agree with the gene expression analysis. Since the proteins in the samples were broken down in the gut, only the lungs were examined here for comparison. The Western blot analysis confirmed the expression of γδ at protein levels in all treatment groups of HFD-fed mice in the lung (Figure 18A).

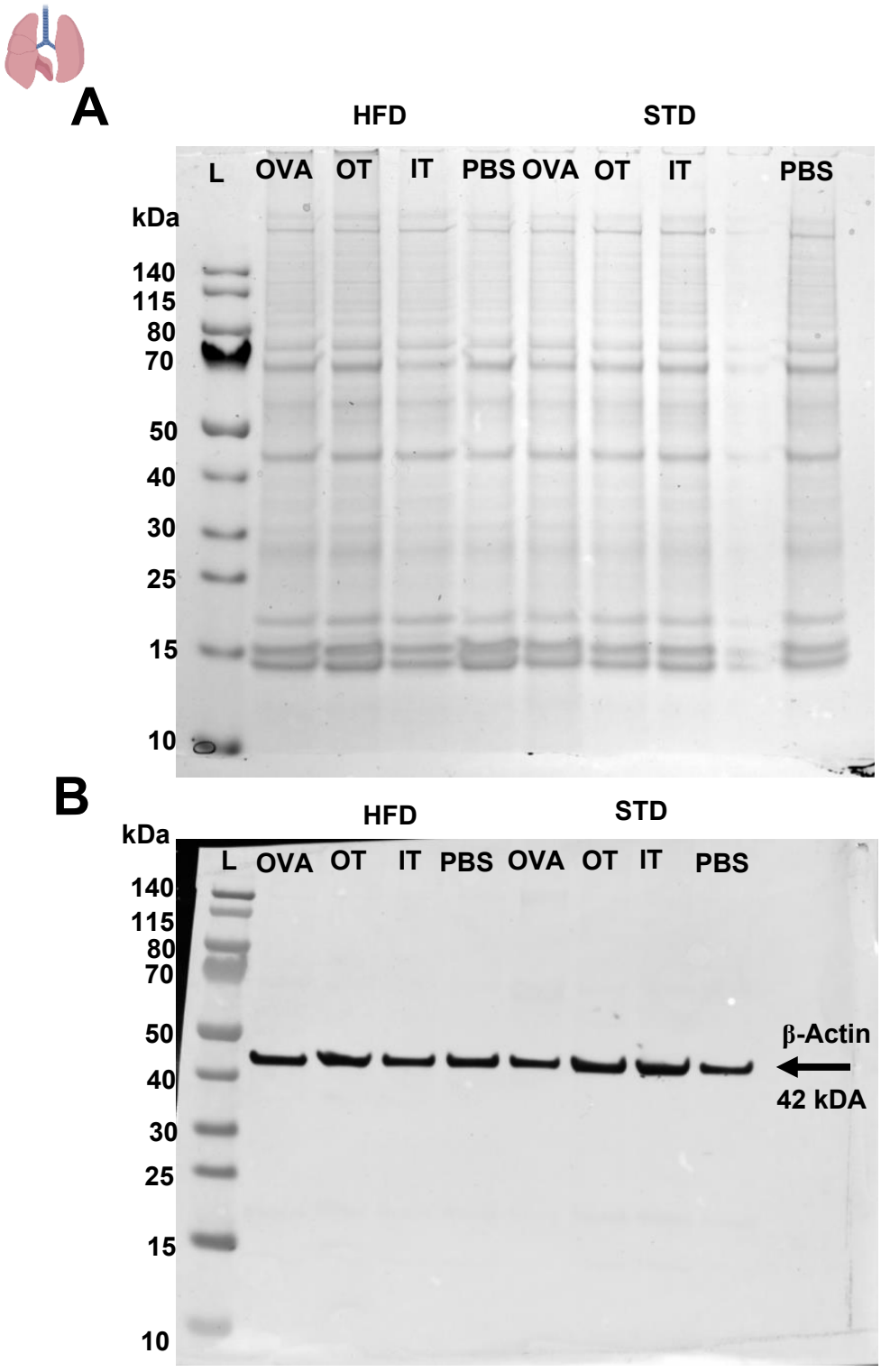


Figure 16: Coomassie Blue Staining and Western blot of lung samples. Proteins were extracted from lung samples and used for Coomassie Blue staining and Western blot analysis. A) Coomassie Blue staining shows an equal intensity of proteins between all treatment groups in the lung. B) Since β-actin was used as a reference gene in qRT-PCR, a western blot analysis was performed to confirm uniform expression of the protein. Roughly the same amount of protein was produced in the lungs in all groups.

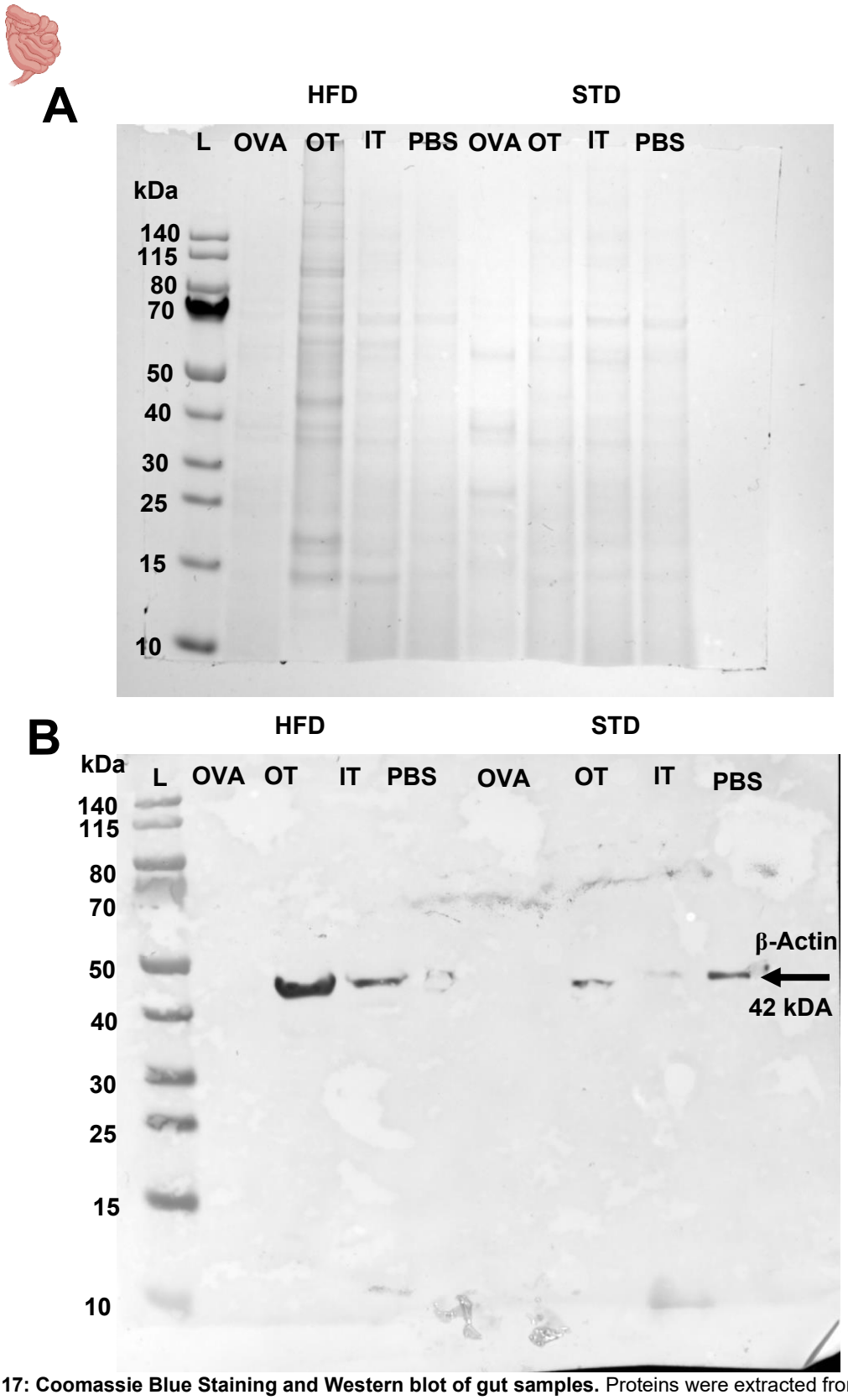


Figure 17: Coomassie Blue Staining and Western blot of gut samples. Proteins were extracted from gut samples and used for Coomassie Blue staining and Western blot analysis. A) Coomassie Blue staining shows degradation of proteins in the treatment groups. B) Western blotting of β-actin confirmed the breakdown of the protein. Therefore, no precise analysis could be carried out in the gut.

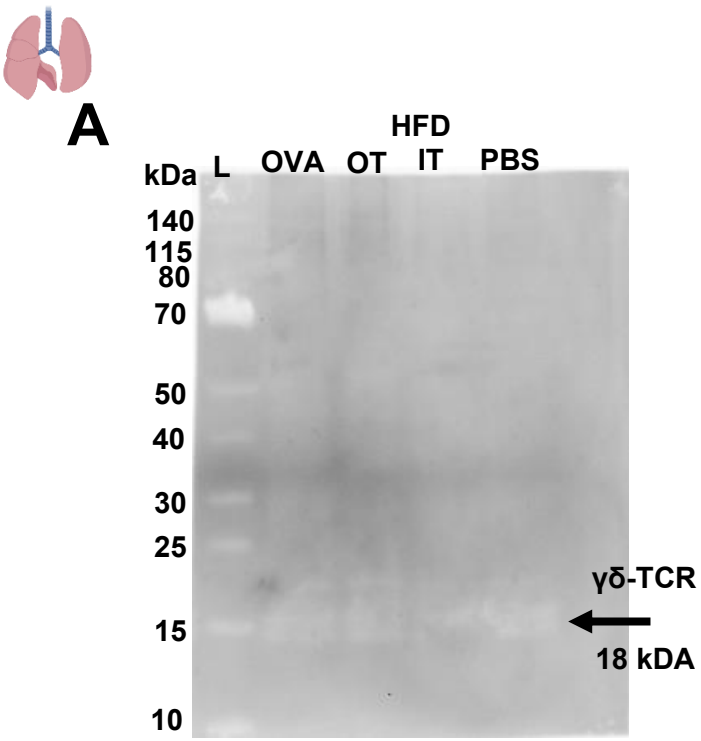


Figure 18: Western Blotting of γδ TCR. A western blot analysis was performed to determine how much γδ TCR is present in the samples and if the amount agrees with the gene expression analysis. For the specific detection, the membrane was incubated with a primary antibody (Purified anti-mouse TCR γ/δ; BioLegend; clone GL3) diluted 1:1,000 in 10 % BSA and a secondary antibody (Rabbit Anti-Hamster IgG (H+L) Antibody, HRP Conjugate, cross-adsorbed; Life Technologies) diluted 1:2,000 in 1 % BSA. The analysis confirmed the expression of γδ at protein levels in all treatment groups of HFD-fed mice in the lung.

5. Discussion

Previous studies focusing on γδ T cells showed that these cells are not a homogenous population and are composed of different subsets with opposite roles in the immune response [10]. It has been shown that in sensitized mice challenged with OVA, Vγ1+ γδ T cells enhance AHR, whereas Vγ4+ γδ T cells suppress AHR [6]. This suggests that γδ T cells of different phenotypes could be involved and could even have opposite functions in airway allergic inflammation [46]. The work of this master thesis is based on previous findings of our group exploring the prophylactic and therapeutic inhibition of allergic airway inflammation by probiotic *E. coli* 083. We showed that the intranasal delivery of *E. coli* 083 suppressed AHR in BALB/c mice, which was associated with a significant increase in the number of γδ T cells [71].

Although the functions of γδ T cells are known, their exact role in allergy associated with obesity is still unclear. To study obesity and allergy in parallel, we had to switch to another mouse strain: C57BL/6 because BALB/c mice, although a gold standard for allergy experiments, did not develop an obese phenotype to the HFD-feeding. In contrast, HFD-feeding induced a strong obese phenotype in the C57BL/6 mouse strain, and our OVA sensitization and challenge protocol triggered a robust Th2 response. Differences between these two mouse strains might be explained by distinctions in their immunometabolic parameters [72].

Our gene expression analysis data showed that the mRNA of γδ T cells was significantly upregulated in intranasally-treated lean allergic C57BL/6 mice. It is still unknown, and it was not foreseen to be elucidated in this thesis, whether they act pro-or anti-inflammatory. However, a previous study focusing on γδ T cells showed they work pro-inflammatory in ovalbumin-induced allergic airway inflammation by producing several Th1, Th2, and Th17 cytokines and chemokines [41].

Upon activation, γδ T cells initiate Th2 responses via IL-4 production and drive the immunoglobulin isotype switch. An increase in IgE leads to eosinophil tissue accumulation and airway hyperresponsiveness. Eosinophil infiltration is a hallmark of allergic and helminthic diseases and is coordinated among others from γδ T cells [10]. Our hematoxylin and eosin staining revealed that the tissue in the lungs was severely inflamed in all OVA-treated groups, as well as in the overweight groups. Only the lean sham control showed no

increase in tissue inflammation. According to the literature, little is known about the influence of obesity on allergic eosinophil infiltration. It was shown that diet-induced obesity leads to enhanced eosinophil traffic into the lung tissue [73]. Further analysis is required to determine whether γδ T cells have any role in these processes.

An additional characteristic feature of asthma is goblet hyperplasia. It consists of rapid secretion of mucin onto the airway surface, leading to a compromised mucociliary transport and airflow obstruction and thus contributes to respiratory disease [74]. To investigate if this is also the case in our mouse model, tissues of the lung and gut were stained with PAS. Then the goblet cells were counted. Our data showed no change in the number of goblet cells in the gut. However, an increase of goblet cells was found in the lungs in all OVA-treated groups. Since this finding does not provide any information about how much mucus was produced by the cells, further experiments which would measure the mucus production would shed light about it.

We also found a slight decrease in the mRNA of γδ T cells gene expression in obese allergic mice compared to lean allergic mice. At this point we could not state that this was due to an impairment in their function or if γδ T cells get outcompeted during the disease, although this would be plausible to hypothesize. Other authors found that a decrease of γδ and αβ T cells leads to a reduction in HFD-induced weight gain, and this reduction resulted in a decrease in inflammation and adipose tissue distribution [18]. Similar results were found in mice fed a short-term KD: the metabolic control was improved, and tissue-resident γδ T cells supported tissue repair and homeostasis [33].

Finally, we examined whether γδ T cells could contribute to oral or intranasal immune tolerance in allergic obese and lean mice. In intranasally treated mice, the gene expression of γδ T cells was significantly upregulated, while in orally treated mice, the gene expression was comparable to the sham control. The exact mechanism in tolerance development induced by antigens is not well understood. Studies showed that systemic tolerance induced by exposure of the respiratory mucosa to OVA activates γδ and CD8+ T cells [27]. It still remains to be determined if the γδ T cells recognize antigens such as OVA or if they get triggered by other immune cells, but they still play an active role in this process. Mucosal induction is a very attractive avenue to treat diseases, and further research could help develop new treatments.

Despite these results' positive perspectives, they should be viewed with caution. There are significant differences between animal and human physiology and immunology, even in the structure of the γδ T cell. The building of the γδ TCR uses different VDJ segments. In humans, mainly Vδ segments are used, while in mice, the Vγ segments [53].

The genetic background of the mouse used as a model for this experiment also needs to be considered. C57BL/6 mice are prone to Th1 immune responses, and it is easier to induce immune tolerance [75]. Also, many experimental aspects such as the duration of allergen exposure, route of antigen administration, age, sex, etc. need to be considered when interpreting those findings.

Another important part of my thesis was optimizing the protocols before I could start to analyse the samples. Accurate work and precision are necessary for successful results in the laboratory. The correct handling of protocols must also be considered because protocols that have already been optimized may not be suitable for your project and must therefore be adapted. To examine the γδ T cells in the lung and gut of of C57BL/6 mice model, the protocols for qRT- PCR, IHC, and Western blot were developed or optimized during this project. However, many obstacles were encountered before achieving good results.

Discussion supplement: methods troubleshooting

Evaluation of qRT-PCR analysis

RNA isolation, reverse transcription, and qRT-PCR were used to determine the gene expression of γδ T cells. The methods mentioned were carried out using the manufacturer's protocols; only minor optimizations were made. However, several primers for γδ TCR were designed and tested for specificity and sensitivity. The best primer was selected, and a dilution series was tested. Only the appropriate settings for the primer, such as the temperature, had to be optimized.

The same optimization was also done for the primers of the reference genes. Reference genes are used as internal reaction controls and have different sequences as the target. Therefore, the reference genes need to fulfill some important criteria. Its expression level should remain constant, and the variability in its expression between tissues should be

minimal. Genes that meet those conditions are fundamental metabolism genes called Housekeeping Genes [76].

In this project, the two housekeeping genes, *GADPH* and *β-actin* were chosen as the reference genes for the PCR analysis. It is recommended to use at least two reference genes since the use of one may lead to relatively large errors. However, discrepancies were found with *GADPH* as a reference gene. It was used in this experiment nonetheless since it was shown that *GADPH* showed no changes in expression in obesity [76].

Troubleshooting IHC

IHC requires care in many steps, and various problems can occur without carefully performing the experiments. Here, we established a protocol for IHC to precisely locate γδ proteins in the lung and gut tissues. Critical parameters one should pay attention to are differences in using fixed versus unfixed tissue, antibody dilution, primary antibodies, antigen retrieval, secondary markers, fluorescent markers, nonfluorescent markers, detection systems, and controls [64].

Selecting a suitable antibody is essential for successful results in IHC and WB. Suppliers provide a data sheet about the respective antibodies. These show how specific and sensitive the antibody is and in which processes it has already been used [77]. Different primary unlabelled antibodies were ordered to detect γδ T cells for IHC and WB. Therefore, a suitable secondary antibody with a conjugated chromogen was ordered, specifically binding to the primary antibody. Primary antibodies to γδ T cells are mainly produced in hamsters and only a few in mice. The antibodies produced in mice cannot be used because cross-reaction can occur. This complicates the search for a suitable secondary antibody. In addition, the available primary antibodies were not yet tested for Western blot. In the case of IHC, these primary antibodies were used with frozen tissue but not paraffin-embedded tissue. For this reason, it was unknown whether the antigen of interest would be lost during fixation and processing.

A dilution series was performed for both primary and secondary antibodies to ensure optimal results. Therefore, the manufacturer’s recommendations were used as a starting point.

For both methods, IHC and WB, the staining of the γδ protein failed for a long time, and troubleshooting was performed.

In IHC, no positive staining was found for some time. To ensure that this was not due to the antibodies freezing and thawing, aliquots were made or diluted 1:1 with glycerol. The dilution series has also been expanded to the recommendations of the manufacturer. Many factors can contribute to an experiment failing. Especially at IHC, an optimal temperature, antigen retrieval buffer reagent pH, incubation time, and the heating device are of great importance and need proper adjustment [62]. Another possibility would be that the antibody was not fully optimized. Following a standard protocol, a trisodium citrate buffer with a pH of 6.0 was used for antigen retrieval.

Some antibodies or antigens can be temperature sensitive and may be decreased at higher temperatures. Nonspecific staining can also be caused by the wrong temperature [62].

Also, there were some problems with the background. To avoid increasing the background, the samples were always kept moist and stored in a box with high humidity. In this project, a microwave was used. Microwave heat application is not recommended because the temperature and potential evaporation cannot be easily controlled [64]. To avoid potential evaporation, the heating was stopped after three minutes, and samples were controlled. This was repeated two additional times.

Another way to improve background staining would be to incubate the primary antibody at 37° C with a shorter incubation time than at 4° C overnight or to reduce the substrate-chromogen incubation time. Here, DAB was used for staining since it should not be affected by organic solvents in alcohol-based counterstains or mounting media [62]. As soon as a brown color is visible, the reaction should be stopped. Variation in time could cause deviation in the results.

However, one of the most critical points is to differentiate nonspecific background and specific antigen staining. This is especially important for untested antibodies and can be done using different controls [64]. In this project, the secondary antibody alone was used to test the specificity of the primary antibody.

Although extensive troubleshooting was performed, only a few samples showed positive staining. A reason could be that the antigen levels are too low, and a more sensitive method might be necessary. Especially for γδ T cells, it was found that the cells have an extremely low density of distribution, often less than one cell per field of view [43].

Troubleshooting Western Blot

No γδ proteins have been examined directly from a tissue yet using western blots. Instead, cell sorting was carried out beforehand [78]. Therefore, this work explicitly established a protocol for γδ T cells. As with immunohistochemistry, there were difficulties in achieving a perfect result. Therefore, troubleshooting was carried out.

First, the protocol for protein extraction was adjusted. Since whole lungs and gut tissues were used, mechanical dissection was chosen. The samples were broken up using a homogenizer and beads. The RIPA buffer was used as the lysis buffer. According to the standard protocol, 200 µl should be added to 10 mg of tissue. However, since the protein concentration was still very high, 450 µl of buffer was added instead. The concentration of the proteins was determined using BCA.

Before specifically looking for γδ T cells using Western Blot, control experiments were performed, and a standard protocol was tested. β-actin was chosen as a negative control to confirm that the staining is not nonspecific. An attempt was also made to carry out a positive control using a purified protein of a known target protein. However, this did not work and should be further tested. Furthermore, β-actin was also taken since it was used as a reference gene in the PCR analysis and should therefore be evenly expressed, so there is no variation. This was confirmed using a western blot in the lungs since all treated groups had a uniform β-actin protein expression.

However, this was not the case with the gut samples. No uniform formation of β-actin proteins could be found here. However, this is not because the protein is expressed differently in the gut but because the proteins in the samples were degraded, resulting in an irregular coloration. A Coomassie blue staining confirmed this. In the Coomassie blue staining, it was easy to see that the proteins were degraded shortly after extraction. To prevent this, a protease/phosphatase inhibitor mix was added to the RIPA lysis buffer. Compared to before, only half of the protein concentration was determined using BCA. Therefore, it was suspected that the inhibitors destroyed degrading enzymes. However, Coomassie blue staining showed no improvement from the samples with the inhibitors. So far, no solution has been found, and further troubleshooting must be carried out. Uneven areas of the staining were also visible on the western blot of the gut samples. This could be due to not carefully removing all air bubbles when forming the gel/membrane sandwich. It could also be due to insufficient solution added during incubation or uneven agitation.

Just as with immunohistochemistry, a wide variety of primary antibodies were tested specifically for γδ proteins in Western Blot, as well as various dilutions. There were difficulties here because no suitable antibody could be found, since there are no antibodies for γδ that have been tested for Western blotting. Therefore, the same primary antibodies were tested by immunohistochemistry for western blot.

Initially, the same standard protocol for γδ was used as for β-actin analysis. However, no bands were found here for γδ proteins. Since there are still no recommended antibodies for western blot analysis, it was unclear whether they were even suitable for a long time. It could also be due to the incorrect dilution of the antibody. Since it is known that γδ T cells make up only a tiny population of the immune system, it was assumed that very little protein would also be present in the sample. Therefore, the standard protocol was switched to a chemiluminescence protocol. This enables proteins to be stained from a few femtograms to high attograms. First, the dilutions suggested by the manufacturer of the chemiluminescence kit were tested. No bands were found here either. Therefore, the dilutions recommended by the antibody manufacturer were tested again. This time white bands were detected on one of the primary antibodies. White bands indicate that there is too much horseradish peroxidase in the system. The best antibody dilutions for primary and secondary should still be tested to obtain a better result.

Furthermore, only one western blot was performed for γδ T cells in lung samples, as degradation of proteins was found in gut samples. Also, only samples from lungs obtained from HFD-fed mice were tested. After the optimization of the antibodies, a western blot would have to be tested with all samples from the lungs, and analysis would have to be carried out if there is a change in the expression of the γδ protein.

It must be considered that the results are only semi-quantitative using the western blot analysis. The method allows a comparison of protein levels but not an absolute measure of quantity. There could be variations in loading and transfer between the samples in the separate lanes in different blots. Also, the signal generated by detection is not linear across the concentration range of the samples. Therefore, the differences should be standardized before a comparison can be made [67].

6. Conclusion

The significant discovery of this work was that γδ T cells are involved in developing intranasal tolerance. Since the γδ T cells in mice treated orally did not change, more focus should be placed on intranasal treatment in the future. This is particularly true in overweight individuals since the increase in gene expression in γδ T cells was lower than in lean mice. To better adapt the treatment method for obesity, one should find out why γδ T cells were down-regulated and whether this is more beneficial for obese individuals than to increase the number of γδ T cells. However, we hypothesize that targeting γδ T cells to develop intranasal tolerance is a promising treatment modality for asthma patients, particularly the elderly who are unresponsive to current methods.

The execution of this experiments and its results showed the importance of correctly handling protocols. Even if there are already detailed protocols for a method, it is crucial to reconsider them and adapt them optimally to the respective actual experiment, because only minor deviations can lead to significant variations and errors. It is essential to go through each step carefully. However, should problems occur, specific troubleshooting is an advantage. Although we encountered many obstacles when performing the protocols, most problems were solved, and good results were obtained.

7. Summary

Most γδ T cells are found in epithelial tissues throughout the body. They are responsible for maintaining surveillance and homeostasis. In allergic subjects, the number of γδ T cells is enhanced, indicating an additional role in mediating inflammation. However, their specific role in the immune response remains elusive. We investigate the role of γδ T cells in allergy in cross-talk to obesity and immune tolerance development using an allergic airway inflammation model in C57BL/6 mice. Mice fed an HFD or STD were sensitized and challenged with OVA. The analysis showed that γδ T cells were distributed all over the epithelial tissue of the organs. However, in the lung, they were allocated preferably to nonalveolar regions. Western blotting confirmed the expression of γδ at protein levels in all treatment groups of HFD-fed mice in the lung. Obese allergic mice showed a reduction of γδ TCR mRNA expression in the lung, whereas, in lean mice, the gene expression was significantly increased. Intranasal-treated mice had the highest increase of γδ T cell expression in HFD- and STD-fed mice. These results lead to hypothesis that obesity could negatively affect γδ T cells in their function. On the other hand, allergy enhances the number of γδ T cells which might contribute to allergic airway inflammation. Also, γδ T cells were increased in intranasal tolerance but not in the oral tolerance model, indicating that they might have a role in intranasal tolerance development. Understanding the mechanisms that mediate a cross-talk between obesity, allergy, and tolerance may lead to the identification of novel treatments for morbidities associated with a Westernized lifestyle.

8. Zusammenfassung

Die meisten γδ-T-Zellen werden in Epithelgeweben im ganzen Körper gefunden. Sie sind für die Aufrechterhaltung der Homöostase verantwortlich. Bei Allergikern ist die Zahl der γδ-T-Zellen erhöht, was auf eine zusätzliche Rolle bei der Entwicklung von Entzündungen hinweist. Ihre spezifische Rolle bei der Immunantwort bleibt jedoch unklar. Wir untersuchen die Rolle von γδ-T-Zellen bei Allergien in Verbindung mit Fettleibigkeit und der Entwicklung einer Immuntoleranz unter Verwendung eines allergischen Atemwegsentzündungsmodells in C57BL/6-Mäusen. Mit HFD oder STD gefütterte Mäuse wurden sensibilisiert und mit OVA provoziert. Die Analyse zeigte, dass γδ T-Zellen über das gesamte Epithelgewebe der Organe verteilt waren. In der Lunge wurden sie jedoch bevorzugt den nicht-alveolären Regionen zugeordnet. Western Blotting bestätigte die Expression von γδ auf Proteinniveau in allen Behandlungsgruppen von HFD-gefütterten Mäusen in der Lunge. Übergewichtige allergische Mäuse zeigten eine Verringerung der γδ-TCR-mRNA-Expression in der Lunge, während bei schlanken Mäusen die Genexpression signifikant erhöht war. Intranasal behandelte Mäuse hatten den höchsten Anstieg der γδ-T-Zell-Expression bei HFD- und STD-gefütterten Mäusen. Diese Ergebnisse weisen darauf hin, dass Fettleibigkeit γδ-T-Zellen in ihrer Funktion negativ beeinflusst. Andererseits erhöht eine Allergie die Anzahl von γδ-T-Zellen, die zu einer allergischen Atemwegsentzündung beitragen könnten. Außerdem waren γδ-T-Zellen in der intranasalen Toleranz erhöht, aber nicht im oralen Toleranzmodell, was darauf hindeutet, dass sie eine Rolle bei der Entwicklung der intranasalen Toleranz spielen könnten. Das Verständnis der Mechanismen, die eine Wechselwirkung zwischen Adipositas, Allergie und Toleranz vermitteln, kann zur Identifizierung neuartiger Behandlungen für Morbiditäten führen, die mit einem westlichten Lebensstil verbunden sind.

9. References

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10. List of abbreviations

AHR - Airway hyperresponsiveness
AMP - Anti-microbial peptide
AP - Alkaline phosphatase
APC - Antigen-presenting cell
BAT - Brown adipose tissue
BCA - Bicinchoninic acid assay
BSA - Bovine serum albumin
CDR - Complementarity-determining region
CTGF - Connective tissue growth factor
DAB - 3,3'-diaminobenzidine
DC - Dendritic cell
EGF - Epidermal growth factor
H&E - Haematoxylin and Eosin
HSP - Heat shock protein
HFD - High-fat diet
HRP - Horseradish peroxidase
Ig - Immunoglobulin
IHC - Immunohistochemistry
ILC - Innate lymphoid cell
IFNγ - Interferon-gamma
IL - Interleukin
IEL - Intraepithelial lymphocyte
iNKT - Invariant natural killer T cells
KGF - Keratinocyte growth factor
KD - Ketogenic diet
MW - Molecular weight
NK - Natural killer cell
NKG2D - Natural killer group 2 member D
NKR - Natural killer receptor
OVA - Ovalbumin
PAS - Periodic-acid Schiff
PBS - Phosphate-buffered saline
PVDF - Polyvinylidene difluoride
qRT-PCR - quantitative real-time PCR
RIPA - Radioimmunoprecipitation *assay*
RT- Room temperature
SEA - Staphylococcal entertoxin A
STD - Standard-fat diet
SDS - Sodium dodecyl sulfate
TCR - T cell receptor
TLR - Toll-like receptor
TGF - Transforming growth factor
Treg - T regulatory cell
TNF - Tumor necrosis factor

WAT - White adipose tissue
WB - Western Blot

11. List of figures

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