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38th CYCLE

Use of the cellular cultures and flow cytometry for the study of chronic lung diseases: a translational research project

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Summary

Abstract

Eosinophils are pleiotropic granulocytes implicated in host defense against parasites and modulation of Th2 inflammatory responses. Their involvement is relevant in chronic lung diseases, such as asthma and COPD, where they contribute to the release of pro-inflammatory mediators and tissue damage. Recent studies described two distinct eosinophilic subtypes: resident eosinophils (rEos), involved in tissue homeostasis, and inflammatory eosinophils (iEos), recruited in response to pathogenic stimuli in inflammatory sites. These two subtypes can be distinguished by the expression of CD62L, being elevated ($CD62L^{high}$) in residents and reduced ($CD62L^{low}$) in inflammatory eosinophils.

This thesis aimed to optimize an experimental model of eosinophil maturation by differentiating EoL-1 cells with butyric acid (BA), and to investigate the effects of IL-5, LPS, and macrophage-derived supernatants (M0, M1, and M2) on eosinophil differentiation toward rEos or iEos phenotypes. In parallel, it aimed to develop and validate a human whole-blood assay, for the flow cytometry characterization of eosinophil subsets in asthma and COPD.

Methods

Commercial EoL-1 cell line was stimulated with different concentration of BA at different time-points and subsequently exposed to IL-5, LPS, or supernatants from polarized macrophages (M0, M1, M2). Morphological, phenotypic and molecular characteristics were assessed by microscopy, flow cytometry, and RT-PCR. In parallel, a whole-blood flow cytometry assay was developed and validated for the identification of rEos and iEos in human peripheral blood.

Results

The stimulation with BA, 500 µM for 120 hours, was the most effective in inducing differentiation of EoL-1 cells, as confirmed by the microscopic analysis that showed nuclear remodeling, increased cell dimension and granularity.

Flow cytometry analysis confirmed that BA-treated EoL-1 cells expressed a significant increase of eosinophils population markers, such as Siglec-8 and CCR3, and of functional molecules, such as CD62L and IL5Rα. At the same time, gene expression analysis confirmed the presence of transcripts characteristic of mature eosinophils, such as GATA1, ECP and CCR3.

IL-5 and LPS did not further enhance maturation but selectively modulated inflammatory mediators (IL-5, IL-8, IL-13). Exposure to macrophage-polarized supernatants influenced eosinophil differentiation: M0, M1 and M2 supernatants reduced CD62L and IL5Rα, increased CCR3, and enhanced pro-inflammatory gene expression, thus driving cells toward an iEos-like phenotype.

In human peripheral blood, the validated flow cytometry assay showed low intra- and inter-sample variability and stability of samples up to 4 hours at room temperature. Evaluation of eosinophils in peripheral blood demonstrated significantly higher iEos and lower rEos proportions in both asthmatic and COPD patients compared to controls, while CCR3 and IL5Rα levels remained unchanged.

Conclusion

In conclusion, the results of this thesis indicate that treatment with butyric acid represents an effective experimental strategy for inducing the maturation of the EoL-1 cells.

The integration of butyric acid treatment with specific inflammatory stimuli derived from differentiated macrophages (M0, M1, and M2) drove the cells toward a pro-inflammatory phenotype.

Finally, a whole-blood assay for discriminating eosinophils subsets in human blood samples was validated. The findings obtained indicate that inflammatory eosinophils were increased in asthma and COPD supporting their contribution in airway inflammation.

Therefore, the findings of this thesis provide valuable insights into the cellular and molecular mechanisms regulating eosinophil function in chronic inflammatory lung

diseases such as asthma and COPD, while also offering new opportunities for identifying potential therapeutic targets and developing innovative intervention strategies.

1. Introduction

1.1 Eosinophils

1.1.1 Eosinophils: origin and maturation

Eosinophils are terminally differentiated granulocytes characterized by a bilobed nucleus and by the presence in their cytoplasm of specific secondary granules containing toxic cationic proteins (Figure 1). Eosinophils granules contain a crystalloid core made of major basic proteins 1 and 2 (MBP1 and MBP2) and a matrix composed of eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin [1]. The granules also store numerous cytokines, enzymes and growth factors. Other prominent features of eosinophils include primary granules that contain Charcot–Leyden crystal protein (also known as galectin 10 and eosinophil lysophospholipase) and lipid bodies, which are the sites of synthesis of cysteinyl leukotrienes, thromboxane and prostaglandins.

Blood eosinophils in various mammals (rabbits, dogs, and humans) were identified by Paul Ehrlich in 1879 using eosin staining. Following this initial observation, several studies have explored the multiple biological functions of eosinophils.

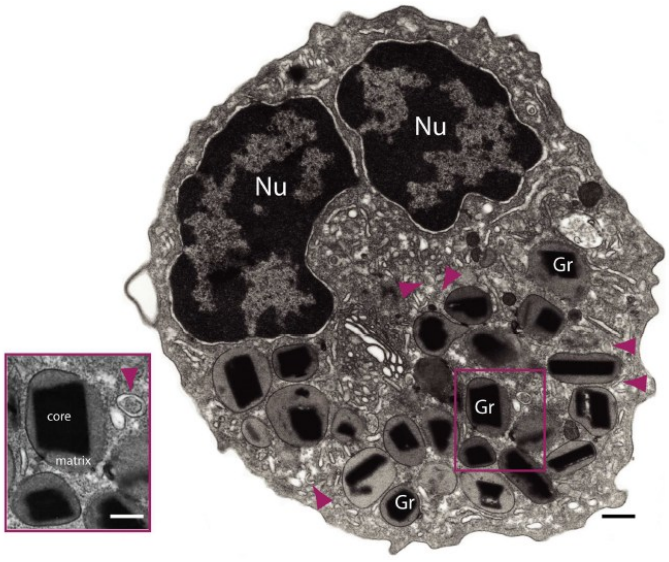


Figure 1: Transmission electron microscopy of a human eosinophil. Nu = bilobed nucleus; Gr = granules. Arrowheads indicate large tubular carriers. Bars: 500nm; 300nm (inset) [2].

Eosinophils account for about 1–5% of peripheral blood leukocytes in healthy individuals. They are produced in the bone marrow and, under homeostatic conditions, migrate into peripheral circulation as mature cells identified as Siglec-8$^+$ CCR3$^+$ F4/80$^+$ CD62L$^+$ [3]. When mature, they have a relatively short lifespan of about 18 hours [4].

As indicate in Figure 2, mature eosinophils derive from bone marrow pluripotential stem cells, which differentiate into eosinophil progenitors marked by CD34$^+$IL5Rα$^+$ expression [1], under control of key transcription factors, mainly GATA1 (a zinc finger family member), C/EBOα (a CCAAT/enhancer-binding protein family member), PU.1 (an ETS family member), and XBP1 [1, 5-9]. In eosinophilic differentiation, the transcription factors GATA1, GATA2, C/EBPα, C/EBPβ, C/EBPε, PU.1, and Stat5 cooperatively and synergistically play important roles in the expression of eosinophilic-specific genes [8, 10-14]. Among them, GATA-1 [15], GATA-2 [16], and C/EBPβ [17] are acetylated by CBP/p300, PCAF or GCN5, resulting in an increase in DNA-binding activity.

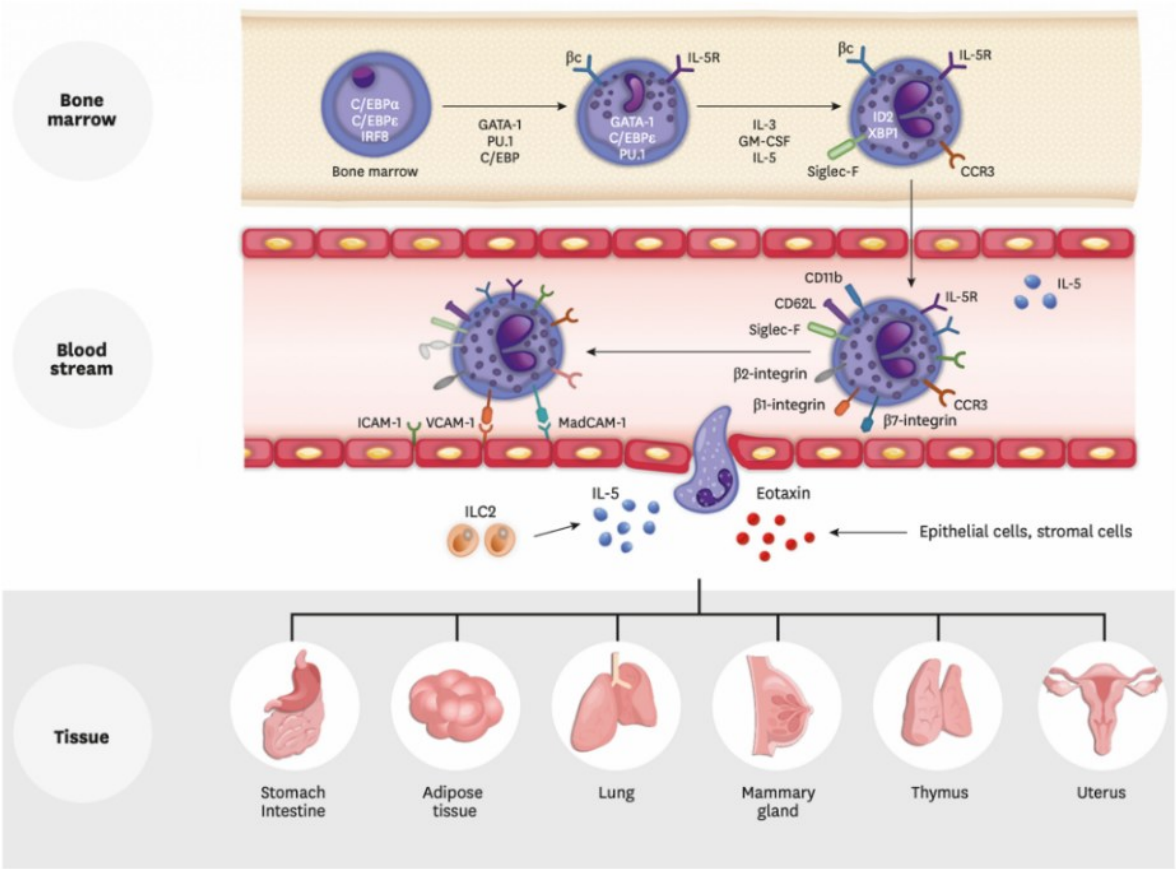


Figure 2. Eosinophil Development Process – From Bone Marrow to Tissues [18]. The image refers to eosinophils maturation in a mouse model that express Siglec-F as population marker.

1.1.2 Eosinophils: proliferation and recruitment

The differentiation, proliferation and accumulation of eosinophils in the tissue are modulated by a complex interaction between survival-promoting factors such as IL-5, IL-3, and GM-CSF and inhibitor signals. IL-5 is the most specific cytokine released to induce the selective differentiation and migration of eosinophils from the bone marrow during inflammation. During allergic inflammation, in addition to type 2 CD4+ T lymphocytes (Th2) cells, innate lymphoid cells (ILC2) are one of the main sources of IL-5 [19].

Recruitment into tissues is further regulated by chemokines such as eotaxin-1 (CCL11), together with IL-4, IL-13 and other adhesion molecules. Once recruited, eosinophils tend to localize in specific sites, such as the gastrointestinal tract, adipose tissue, lungs, thymus and female reproductive system. Under physiological conditions, only a small proportion of eosinophils leave the bone marrow; however, in Th2 responses, such as during helminth infections or in allergic diseases such as asthma, eosinophilopoiesis can be significantly amplified [1, 5].

In addition to the circulating population, a significant proportion of eosinophils reside permanently in tissues, where they play key roles in local homeostasis. The non-esophageal gastrointestinal tract is home to most of these resident eosinophils (20–30%), which contribute to the maintenance of IgA-positive plasma cells, the development of Peyer's patches and the production of intestinal mucus [20]. In other areas, such as adipose tissue and the reproductive system, eosinophils participate in regulatory processes that demonstrate their multifunctionality. Despite being present in small percentages (< 1%) in the lung, eosinophils play a central role in both tissue physiology and the pathogenesis of chronic airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD).

1.1.3 Eosinophils: degranulation

Degranulation is a key step in the function of eosinophils, during which the contents of their granules are released into the extracellular space in response to specific stimuli. The release of eosinophil granules content can occur in different ways (Figure 3) [2].

The degranulation from granulocytes (such as eosinophils) classically occurs as exocytosis process, whereby intracellular granules fuse with the plasma membrane and engage in a wholesale release of granule contents, or in more extreme instances as

compound exocytosis, whereby intracellular granules fuse together prior to fusion with the plasma membrane and release of their combined contents. Although degranulation via classic and compound exocytosis is observed upon interaction with very large metazoan parasites, in most other physiologically relevant scenarios eosinophils either differentially and progressively secrete their granule-stored contents through a vesicle-dependent process termed piecemeal degranulation (PMD) or deposit intact granules directly into the tissue through a distinctive mode of cell death, termed eosinophil cytolysis.

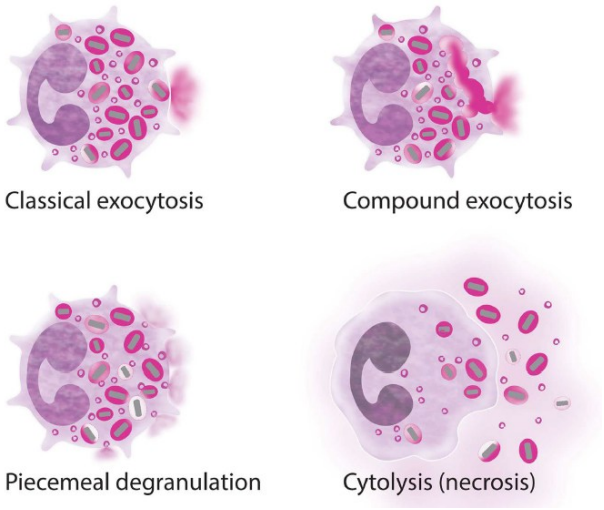


Figure 3. Processes of eosinophils secretion: classical exocytosis, compound exocytosis, piecemeal degranulation and cytolysis (necrosis) [2].

1.1.4 Eosinophils: physiopathological functions

Eosinophils exert a wide range of functions that can be grouped in: cytotoxic effector functions, immunoregulation and tissue homeostasis (Figure 4). The cytotoxic functions of eosinophils comprise host protection against diverse pathogens and involvement in pathophysiologic conditions like allergy and autoimmune diseases, as well as cancer. Eosinophils take part in immunoregulation by modulating Th1/Th2 balance and functions of lymphocytes, dendritic cells, neutrophils, mast cells, and natural killer cells, as well as participating in antigen presentation. Additionally, eosinophils perform homeostatic functions in a variety of tissues and play a role in wound healing and tissue remodeling [21, 22].

They play a leading role in both the acute and chronic phases of inflammation and can be considered the "ultimate effectors" of the inflammatory response, since in addition to

releasing cytokines and growth factors, they also release preformed mediators as well as cytotoxic and oxidizing substances. This cytotoxic tissue damage contributes to the inflammatory condition documented in Th2-high asthma and other eosinophil-associated disorders (Hypereosinophilic syndrome - HES, Eosinophilic granulomatosis with polyangiitis - EGPA, chronic rhinosinusitis with nasal polyps - CRSwNP).

The role of eosinophils is not limited to the effector phase of Th2 responses but also seems to be important for the induction of adaptive immunity. Indeed, there is evidence of antigen presentation by eosinophils in some experimental models of Th2 responses against helminths or allergens [23-25], and recent studies have further shown that eosinophils can also promote Th2 immunity through EPO-mediated induction of dendritic cells activation and migration [26, 27].

Although eosinophils are typically considered circulating cells, a fraction of eosinophils reside stably in tissues, particularly in the thymus, adipose tissue, gastrointestinal tract, lungs, and in the female reproductive system. The nonesophageal portions of the gastrointestinal tract contains the highest number of resident eosinophils (20–30%), where they promote IgA class switching and the maintenance of IgA-expressions plasma cells [20, 28]. They also support the development of Peyers's patches and mucus production in the small intestine [20]. Adipose tissue and the lungs contain less than 4% and less than 1%, respectively. An eosinophil counts greater than 2% in sputum or greater than 3% in BAL (bronchoalveolar lavage) are indeed considered pathological. Resident eosinophils present in the adipose tissue produce IL-4, thereby favoring the polarization of adipose macrophages toward the alternatively activated phenotype [29]. In the thymus, rEos have the capacity to promote thymocyte apoptosis and are therefore thought to contribute to the process of negative T cell selection [30]. Finally, a role for resident eosinophils in preparing the uterus for pregnancy and in regulating mammary gland development has been proposed [31, 32].

Eosinophils also play a role in tissue repair and regeneration. For example, muscle injury promotes rapid recruitment of eosinophils to the site of inflammation and the release of IL-4, which activates FAPs (fibrocyte-adipocyte progenitors) that support the regeneration of damaged muscle fibers. Eosinophils can also induce angiogenesis by producing pro-

angiogenic mediators such as VEGF (vascular endothelial growth factor). IL-4 is further involved in promoting the proliferation of quiescent hepatocytes and in liver regeneration.

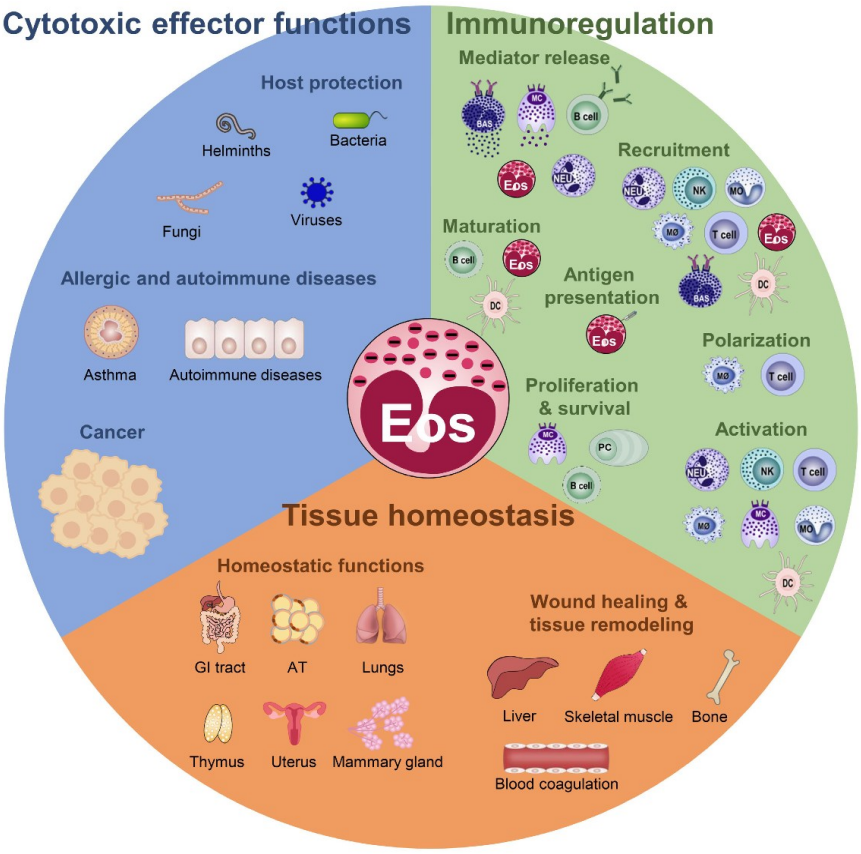


Figure 4. Schematic representation of eosinophils functions: cytotoxic effector functions, immunoregulation and tissue homeostasis [22]. AT = adipose tissue; BS = basophils; DC = dendritic cells; GI tract = gastrointestinal tract; M Ø = resting macrophages; MC = mast cells; MO = monocytes; NEU = neutrophils; NK = natural killer cells; PC = plasma cells.

1.2 Role of eosinophils in asthma and chronic obstructive pulmonary disease

Eosinophils, as effector and regulatory cells of the immune system, have a central position in the pathophysiology of various pulmonary disorders, particularly in obstructive diseases such as bronchial asthma and Chronic Obstructive Pulmonary Disease (COPD). In asthma, eosinophils are pivotal drivers of the airway inflammation that underlies the disease phenotype, whereas in COPD their role appears more heterogeneous and remains the subject of ongoing debate. Elucidating these differences is essential not only for advancing the understanding of the pathogenetic mechanisms underlying the two conditions, but also for the identification of novel therapeutic targets and the development of tailored treatment strategies.

1.2.1 Eosinophils in asthma

Asthma can be distinguished based on the presence of eosinophils, and is therefore classified into eosinophilic forms, which can be allergic or non-allergic, and non-eosinophilic forms. In eosinophilic asthma, regardless of whether it is allergic or not, eosinophils play a central role in pathogenesis: they release toxic proteins and mediators, such as IL-9 and IL-13 that cause contraction of the bronchial smooth muscle, increased vascular permeability, recruitment of immune cells and hypersecretion of mucus [33].

Among the various forms, allergic asthma is the most common variant and is generally induced by sensitization to environmental allergens, such as dust mites, pollen, fungal spores and animal hair. Once sensitization has occurred, clinical manifestations usually appear following subsequent exposure to the allergen. The onset of the immune response is mediated by the activation of allergen-specific Th2 cells, which stimulate IgE production. These induce the activation of mast cells and the consequent release of inflammatory mediators, while also promoting the recruitment of eosinophils in the lungs [34].

Th2 cells exert their action mainly through the secretion of various cytokines: IL-4 stimulates the differentiation of naive T cells into Th2 cells, while IL-5 promotes the maturation and release of eosinophils from the bone marrow [34]. Once activated, eosinophils can present allergenic antigens to Th2 cells, further amplifying the inflammatory response and promoting their own survival.

After activation, eosinophils release cytotoxic proteins including ECP which have been known to possess deleterious effects on tissues including the bronchial epithelium [35]. Recent studies have demonstrated that activation of eosinophils and increase of the release of ECP occur in patients naturally exposed to allergen. The elevated serum ECP concentrations decline consequent to effective therapy. ECP may therefore be useful in evaluating the treatment of asthmatic patients and as a marker for the efficacy of therapy [36].

In non-allergic eosinophilic asthma, however, the main mechanism does not depend on IgE production, but on the activation of ILC2 cells. These cells secrete cytokines such as IL-5 and IL-13, which contribute to aggravating and maintaining the eosinophilic inflammatory environment [33].

Although IgE-mediated and ILC2-mediated mechanisms are predominant respectively in severe allergic and non-allergic forms, eosinophils represent a point of convergence, being involved in both pathophysiology and airway damage. However, their specific role in the development of allergic asthma is not yet fully understood, despite extensive research in this area [33]. Some studies suggest that eosinophils may act as regulatory cells, modulating T cell responses and local tissue inflammation. In mouse models congenitally lacking eosinophils, for example, a significant reduction in Th2 responses following acute or chronic exposure to allergens has been observed.

A significant contribution in this regard comes from Jacobsen's study [37], which hypothesized a primary role for eosinophils in promoting the recruitment and accumulation of effector T cells in the lungs. To test this hypothesis, transgenic mice deficient in eosinophils (PHIL strain) were sensitized with ovalbumin (OVA) and compared with wild-type mice. In PHIL mice, unlike the controls, no pulmonary eosinophilia, increase in Th2 cytokines or adequate lymphocyte recruitment was observed after exposure to the allergen. In wild-type mice, eosinophil infiltration peaked on the fourth day and returned to baseline levels within two weeks, while lymphocytes followed a similar pattern but remained elevated for longer. The absence of eosinophils in PHILs compromised this kinetics, highlighting that their recruitment is necessary for lymphocyte recruitment and Th2 cytokine production [37].

Therefore, eosinophils seem to play a key role in asthma. Indeed, elevated blood eosinophil count is associated with poor outcomes and eosinophil count helps differentiate asthma endotypes. Moreover, several monoclonal antibodies to different cytokines (IL-5 and its receptor, IL-4 and IL-13), have been beneficial in the management of these patients, alleviating symptoms, decreasing exacerbation rates, and in some cases improving lung function [38].

1.2.1.1 Eosinophils subtypes in asthma: mouse model

In recent years, it has emerged that eosinophils do not constitute a homogeneous population but they can be distinguish in different subtypes with distinct functions and localizations: resident eosinophils (rEos) and inflammatory eosinophils (iEos) [39-42].

These eosinophils subsets were described for the first time by Mesnil et al. in 2016 in mouse model [39]. Although it cannot be excluded that rEos may transform into iEos [43,

44], this first study provides evidence that lung rEos and iEos represent distinct terminally differentiated eosinophil subsets with different features, morphological characteristics and functions. They reported that steady-state pulmonary rEos are IL-5-independent Siglec-F^{int}CD125^{int}CD62L^{+}CD101^{lo} cells with a ring-shaped nucleus. During house dust mite–induced airway allergy, rEos features remained unchanged, and were accompanied by recruited iEos, which were defined as IL-5–dependent Siglec-F^{hi}CD125^{int}CD62L^{-} CD101^{hi} cells with a segmented nucleus.

rEos and iEos were confined to different compartments of the lung: iEos were localized in the peribronchial areas, whereas rEos resided in the parenchyma, even during allergic inflammation. As the inflammation is thought to be mainly restricted to the peribronchial connective tissue in asthma [45], this observation could account for the fact that rEos were not at all affected by the inflammatory process. Due to their parenchymal localization, rEos are not affected by IL-5 during allergen-induced inflammation, because they are not reached by IL-5. It is indeed thought that IL-5– secreting cells such as effector Th2 cells are mainly infiltrating the peribronchial area [46, 47], where rEos are absent.

All these observations are consistent with a model in which blood rEos-like eosinophils are produced by default in the absence of IL-5 and rapidly home to the tissues where they exert homeostatic functions. In this model, IL-5 acts as an emergency cytokine that amplifies the production of iEos, which are then recruited to the sites of Th2 cell responses [46, 47], where they participate in the elimination of extracellular parasites and the exacerbation of inflammation. Such a model, if applicable to humans, could further explain why residual eosinophils are found in the lung [48], duodenal mucosa, or blood [49, 50] of eosinophilic patients treated with humanized α–IL-5 Abs.

Mesnil et al. then determined that the parenchymal eosinophils were not more numerous in the lungs of the asthmatic patients, in which peribronchial eosinophils were also found. These eosinophils found in asthmatic human lungs (Siglec-8^{+}CD62L^{+}IL-3R^{lo} cells) were phenotypically distinct from the iEos isolated from the sputa of eosinophilic asthmatic patients (Siglec-8^{+}CD62L^{lo}IL-3R^{hi} cells), confirming the importance of these observations also in human samples.

Nevertheless, the dependency of rEos on IL-5 is still a matter of debate. A recent study [44] demonstrated that the inhibition of IL-5 signaling in an asthma mouse model, exposed to house dust mite (HDM) and diesel exhaust particles (DEP), reduced both

pulmonary eosinophil subsets. Both rEos and iEos were present in lung tissue of HDM + DEP exposed mice and increased significantly upon combined HDM + DEP exposure. The percentage of rEos in lung tissue declined over time while the percentage of iEos increased. These data suggest that rEos may transform into iEos under inflammatory conditions. Anti-IL-5 treatment affected both eosinophil subsets in lung tissue of HDM + DEP exposed mice which is not in accordance with Mesnil et al. study, maybe due to the differences in experimental design.

1.2.1.2 Eosinophils subtypes in asthma: patients' analysis

After these initial observations in a mouse model, only a few studies were made about human eosinophils subtypes.

The first data proposing the existence of eosinophil endotyping in humans dates back to the 80s, describing the presence of both hypodense and normodense eosinophils [51, 52]. Hypodense eosinophils are detectable at increased numbers in severe eosinophilic asthma patients (blood, bronchoalveolar lavage, and lung tissue) and display increased survival, adhesion activity, oxygen metabolism, superoxide production, and antibody-dependent cytotoxicity following activation compared with normodense eosinophils. Although scarce and not conclusive, some data about the correspondence between iEos/rEos and hypodense/normodense cells are available in humans [53, 54].

Recent studies have focused on the role of CD62L in distinguishing eosinophil subsets. CD62L is a selectin adhesion molecule involved in leukocyte rolling, the first step of eosinophil recruitment into tissues, followed by adhesion and transmigration. Resident eosinophils (rEos) display high CD62L expression, consistent with their homeostatic role, whereas inflammatory eosinophils (iEos) show low but not absent expression, suggesting that rolling is still possible and that reverse migration into the circulation cannot be excluded [39, 55].

Matucci et al. [41] confirmed in humans the existence of two circulating eosinophil subsets, identified as $CD45^{+}Siglec\text{-}8^{+}CD16^{-}$ cells and differentiated by CD62L expression into iEos ($CD62L^{low}$) and rEos ($CD62L^{high}$). Severe eosinophilic asthma patients showed an increased proportion of iEos compared with healthy donors, allergic rhinitis, and non-severe asthma. Compared with rEos, iEos expressed lower levels of CRTH2, costimulatory molecules (CD28, CD86), and IL5R (CD125), but higher levels of CCR3. The

same subsets were also detected in nasal polyp tissue, where the elevated proportion of iEos in blood reflected their increased tissue infiltration. Eotaxin levels correlated with iEos accumulation, highlighting the role of the eotaxin/CCR3 axis in eosinophil recruitment. These findings indicate that iEos may serve as a biomarker of type 2 inflammation, as IL-5 is known to drive CD62L downregulation [56].

Furthermore, in vitro studies showed that IL-5 stimulation of eosinophils from healthy donors reduced CD62L expression, supporting the link between IL-5 signaling and iEos generation. These data reinforce the hypothesis of a common origin for eosinophil sub-phenotypes, in contrast with Mesnil et al. [39], who proposed distinct lineages for rEos and iEos in animal models.

1.2.2 Eosinophils in COPD

The role of eosinophils in COPD is controversial. On one hand, blood eosinophil count is the first biomarker that has been shown to relate directly to the beneficial effect of inhaled corticosteroids (ICSs) in reducing exacerbations [57]. Having more than 300 eosinophils/µl reliably predicts ICS effect, and this threshold is currently recommended in different guidelines and documents as valuable for implementing this therapy. On the other hand, blood eosinophil counts lower than 100 cells/µl suggest a relatively poor response to ICSs, thereby helping clinicians make informed therapeutic decisions. In contrast, clinical trials of monoclonal antibodies (mepolizumab and benralizumab) that significantly decreased blood eosinophil counts in patients with COPD with histories of exacerbation had minimal effects on exacerbation rates, health status, and lung function, suggesting that eosinophils per se may not represent a pivotal target for disease modifying therapy in COPD [58].

Therefore, a specific investigation of the phenotype of eosinophils could explain the differential response to the same biological agents between patients with asthma and patients with COPD.

1.2.2.1 Eosinophils subtypes in COPD

The characterization of eosinophilic subtypes has only recently been explored in COPD. The study of Cabrera López is one of the most important works in this field [42]. The researcher applied the criteria identified by Mesnil to distinguish resident eosinophils

(Siglec-8^{+}CD62L^{high}IL-3R^{low}) from inflammatory eosinophils (Siglec-8^{+}CD62L^{low}IL-3R^{high}) in the bloodstream. A comparison between patients with COPD and asthma revealed a marked difference in the distribution of eosinophil subpopulations. In subjects with COPD, the proportion of circulating inflammatory eosinophils averages around 0.5%, compared to approximately 25% in asthmatic patients, regardless of the use of inhaled corticosteroids or the severity of the disease [28]. Furthermore, in COPD, blood eosinophils are represented almost exclusively by the resident subpopulation, while inflammatory eosinophils are virtually absent.

1.3 EoL-1 *in vitro* model

Although studies on the differentiation of eosinophils and their roles in physiological and pathological conditions have progressed much recently, there are still many unanswered questions about eosinophils. The major problem in the study of human eosinophils is the difficulty of obtaining enough purified cells, since they constitute less than 5% of peripheral blood leukocytes. Moreover, eosinophils have a short in vitro lifespan of approximately 18 hours, which makes even more difficult to obtain informations about their differentiation and activation processes. For these reasons, an *in vitro* model based on human eosinophilic cell line represents a crucial way for exploring eosinophilic physiological and pathological roles.

The human leukemia cell line, EoL-1, has been established for the first time in 1985 by Saito et al. from the peripheral blood of a 33-year-old patient with Philadelphia chromosome-negative eosinophilic leukemia suffering from a six-year-long hypereosinophilia case terminating in blastic transformation [59]. Most of the EoL-1 cells had a blastic appearance without cytoplasmic granules and were morphologically similar to myeloblasts or monoblasts and only a small percentage (2%) of the cells contained granules.

EoL-1 cells under normal culture conditions show cytological features of myeloblasts, but their differentiation into eosinophilic granule-containing cells can be induced by different stimuli [60]. Therefore, the untreated EoL-1 cells should be useful for analyzing leukemic cell differentiation and the properties of malignant eosinophils, while the phenotypically differentiated EoL-1 cells function like normal eosinophils. In 2003, it was reported that peripheral cells from some patients with hypereosinophilic syndrome and EoL-1 cells

have a fusion gene generated by the interstitial deletion on chromosome 4 at q 12 of the fi p1-like 1 (FIP1L1) gene and platelet-derived growth factor receptor (PDGFR) A gene, termed FIP1L1-PDGFRA. The gene's product, FIP1L1-PDGFRα, is a constitutively activated tyrosine kinase and induces cell proliferation via the activation of Stat5 [61-63]. Therefore, EoL-1 cells have also contributed to research as a model for chronic eosinophilic leukemia expressing FIP1L1-PDGFRα [62].

1.3.1 EoL-1 differentiation to mature eosinophils

The acetylation and deacetylation of numerous proteins including histones and transcription factors occur in cells in response to various stimuli. The modifications are mediated by two different sets of enzymes, histone acetyltransferases (HATs) such as CBP/p300 and PCAF, and histone deacetylases (HDACs). HDACs are the enzymes that remove the acetate from lysine residues acetylated by HATs, and are grouped into the following four classes: I (HDAC 1, 2, 3 and 8), II (HDAC 4, 5, 6, 7, 9 and 10), III (Sirt 1, 2, 3, 4, 5, 6 and 7) and IV (HDAC 11). Acetylation by HATs is sustained while HDACs are inhibited by HDAC inhibitors, resulting in an accumulation of acetylated-proteins such as histones and transcription factors that induce various biological activities including differentiation, proliferation and apoptosis via gene expression. In 2007, Ishihara et al. demonstrated that sodium butyrate induces the differentiation of HL-60 clone 15 cells and EoL-1 cells into eosinophils by continuously inhibiting deacetylation using various histone deacetylase inhibitors, such as sodium butyrate, apicidin, and trichostatin A [64]. In particular, the stimulation with 500 μM *n*-butyrate induced a change in the cell -surface expression of the eosinophilic markers integrin β7, CCR1, and CCR3. The proliferation of EoL-1 cells was inhibited approximately of 75% after 8 days incubation with apicidin (100 nM) or *n*-butyrate (500 μM) compared with the cells incubated without HDAC inhibitor. However, *n*-butyrate (500 μM) decreased the viability of cells approximately 15% on day 8. Therefore, it is suggested that the inhibition of cell proliferation by apicidin and n-butyrate is due to the differentiation not but the cytotoxicity. The continuous acetylation of histones H4 and H3K14 by apicidin and n-butyrate contributes to the continuous recruitment and activation of a transcription factor complex leading to the expression of various gene products characterizing eosinophils including CCR1, CCR3 and integrin β7.

Therefore, continuous acetylation of histones is necessary for the differentiation of EoL-1 cells into eosinophils.

Butyric acid (BA) is a short-chain fatty acid that acts as a histone deacetylase (HDAC) inhibitor, in particular blocking HDAC of class I (Figure 5). Butyric acid has been shown to induce cellular differentiation of different mammalian cells [65-67] and also the differentiation of EoL-1 cells into mature eosinophil-like cells [68]. Jung investigated the differences between butyric acid and dbcAMP in EoL-1 stimulation to mature eosinophils. After treatment with butyric acid and dbcAMP, morphological signs of EoL-1 differentiation were observed, including nuclear lobulation and increased proportion of cytoplasm to nucleus [69]. However, compared to the group stimulated with dbcAMP, EoL-1 cells stimulated with butyric acid showed remarkable increase in apoptotic populations exhibiting cellular shrinkage and nucleus condensation [69]. Consistent with the morphological findings, stimulation with butyric acid significantly inhibited the proliferation of EoL-1 cells. The expression of PRG2, EPX, CCR3, IL5RA and GATA1 could be used as markers of mature eosinophils. Jung Y. demonstrated that 0.5 μM butyric acid effectively induced the expression of PRG2, EPX, CCR3, IL5RA, and GATA1 in EoL-1 cells than 100 μM dbcAMP in a time-dependent manner. Additionally, the effect of 100 μM dbcAMP stimulation was limited to the expression of PRG2 and EPX, which encode cytoplasmic granules of eosinophils. Collectively, these data indicate that compared to dbcAMP treatment, treatment with 0.5 μM butyric acid was more effective in inducing phenotypic maturation of EoL-1 cells, and that the treatment duration should be less than 5 days to preserve the viability of the stimulated cells.

As a further indication of maturation, Wang et al. demonstrated that extracellular ECP concentration and total ECP synthesis increased as the EoL-1 cells underwent differentiation induced by dbcAMP [70].

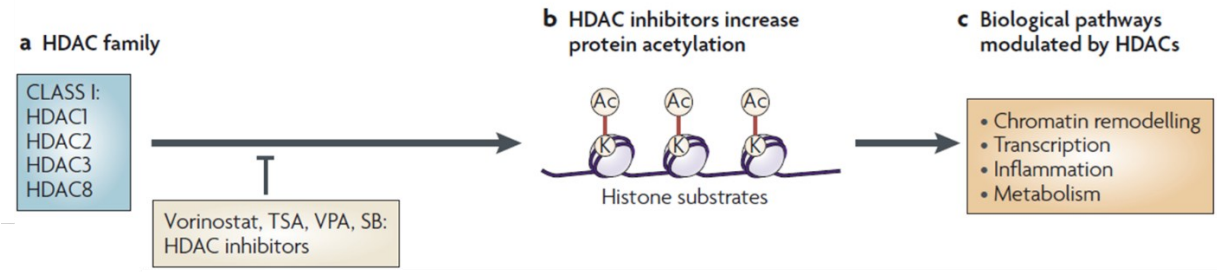


Figure 5. Schematic function and effect of histone deacetylase inhibitors. TSA = trichostatin A, VPA = valproic acid; SB = sodium butyrate. Image adapted from the paper by Kazantsev [71].

Although butyric acid is the most widely used stimulus for the EoL-1 differentiation to mature eosinophils, it was reported a wide variety of stimuli in literature including dimethylsulfoxide (DMSO) [59], TNF-α [72], G-CSF [73], alkaline medium [59] and a human adult T cell leukemia cell line (HIL-3)-derived factor [73]. High concentrations of EoL-1 cells in culture also induce slight eosinophil differentiation in association with a decrease of cell viability.

For these reasons, the EoL-1 cell line is a fundamental tool for studying the biology of eosinophils. It allows analysis of the molecular mechanisms underlying the maturation and function of these cells and constitutes a useful experimental model for investigating their role in allergic and eosinophilic diseases. The ability to manipulate EoL-1 cells under controlled laboratory conditions also offers a significant advantage over the use of primary eosinophils, confirming the importance of this cell line in the study of physiological and pathological processes.

1.4 Macrophages

Macrophages are innate immune cells that perform phagocytosis and eliminate pathogen as part of physiological processes [74]. Macrophages can reside in different organs. In the lungs, they are crucial for the organ development, maintenance of homeostasis, tissue repair and the balance between immune cell defense against invaders and tolerance to non-inflammatory stimuli [75]. They are the primary immune sentinels and protect the lung by phagocyting inhaled particulate, pathogens, surfactant, apoptotic cells, and cell debris [76].

In the lungs, macrophages reside in different anatomical compartments and can be divided into two different populations: alveolar and interstitial macrophages. The alveolar

macrophages (AM) are located in the lumen of the alveoli, while the interstitial macrophages (IM) are located between the lung epithelium and the capillaries. In the lungs, AM are the most abundant innate cells located in the alveolar space. AM are the first cells that phagocyte inhaled particular matter and can capture and transport antigens to the draining lymph nodes [77]. Moreover, AM are essential to lung homeostasis since they maintain lung biomechanics by capturing and metabolizing surfactants. Compared to AM, IM are not so abundant in the lungs and have a lower phagocytic potential. Moreover, IM were shown to be morphologically smaller and presents higher HLA-DR expression than AM [78].

Macrophages are plastic cells that can adapt on demand changing their physiology in response to different stimuli. Although too simplistic and mostly based on in vitro experiments, macrophages can be classified into distinct phenotypes in analogy of Th1 and Th2 responses called classically activated (M1) macrophages and alternative activated (M2) macrophages. M1 macrophages in response to lipopolysaccharide (LPS) and interferon-γ (IFNγ), display a pro-inflammatory phenotype, which promotes pathogen destruction via production of pro-inflammatory cytokines and reactive oxygen species. Whereas M2 macrophages arise in response to IL-4 and IL-10 and are capable of anti-inflammatory responses and repair damaged tissues. Studies have described different M2 subsets such as M2a involved in wound healing, M2b involved in immunoregulation, M2c involved in tissue remodeling and recently was described M2d a tumor associated macrophages (TAM) which is involved in tumor progression [79-81]. Therefore, these different phenotypes are associated with different immunoregulatory functions.

Indeed, our recent publication demonstrates that the M1 and M2 macrophages have different roles in intercellular communications [82]. In particular, we investigated the cross-talk with fibroblasts cells mediated by extracellular vesicles (EVs). The activation of the fibroblasts was assessed evaluating fibroblasts TGF-β production.

To address this issue, we developed an *in vitro* cell culture model starting from the monocyte cell line THP-1 to induce macrophage polarization into M0, M1 and M2 states using a well-established protocol [83], and we molecularly characterized polarized macrophages by Real-Time PCR through the use of specific polarization markers to assess polarization efficiency. The results clearly showed that the molecular signatures of M1 polarization - IRF1 and STAT1 mRNA expression - were significantly increased in M1

macrophages, and consistently also M2 marker Alox15 mRNA was highly expressed in M2 macrophages, indicating an efficient macrophages polarization. Furthermore, we isolated polarized macrophages-derived EVs from cell culture supernatant and stimulated human lung fibroblasts in vitro with EVs, to assess whether EVs could have a role in the modulation of TGF-β production. The results revealed that, compared with baseline conditions, the production of TGF-β in lung fibroblasts was higher after stimulation with EVs derived from polarized-macrophages. However, this increase was statistically significant only with EVs derived from M0 and M1, but not with M2. To find out why TGF-β production observed was different, we tried to consider the different content in miRNAs biological cargo, specifically investigating miRNA-425-5p that is involved in TGF-β regulation. Our results showed higher levels of miRNA-425-5p in M2 compared to both M1 and M0 derived EVs. This is consistent with the inhibitory function of the miRNA-425-5p on TGF-β production and with our observation that both M0 and M1, but not M2 macrophages-derived EVs induced a significant increase of TGF-β production in fibroblasts. Despite further studies are necessary to understand the role of macrophages in fibroblasts activation in physio pathological processes, we can conclude that this in vitro experimental model reproduces the different phenotypes and functions of M1 and M2 macrophages.

In this perspective, macrophages represent a key component in modulating the inflammatory response, as their polarization significantly shapes the type of immune reaction; due to this pivotal role as immunoregulators in chronic diseases such as asthma and COPD, a similar model was employed in this study to evaluate the effect of polarized macrophages on the activation and differentiation of eosinophils.

2. Aim

Eosinophils play a key role as immunological modulator in both physiological and pathological conditions. These granulocytes, derived from pluripotent stem cells in the bone marrow, undergo maturation, differentiation and migration guided by specific transcription factors and cytokines, especially IL-5. Despite progress in understanding their basic biology, the role of eosinophils in pathological mechanisms is still debated. Eosinophils are traditionally recognized for their role in host defense against helminth infections through migration to infection sites triggered by type 2 (T2) immune response signaling but are experiencing renewed scientific interest as target of biological therapies for both asthma and COPD.

Recent studies discovered that eosinophils could differentiate in two different phenotypes: resident IL-5 independent eosinophils (rEos) and inflammatory IL-5 dependent eosinophils (iEos). The dissimilar behavior of eosinophils is not completely clear and might be due in part to the different microenvironmental cues. Lung macrophages have the optimal properties to orchestrate and calibrate such microenvironmental cues, depending on their polarization in classically activated (pro-inflammatory – M1) macrophages and alternatively activated (anti-inflammatory – M2) macrophages.

Since eosinophils constitute less than 5% of peripheral blood leukocytes with a short lifespan of approximately 18 hours, the challenge of purifying an adequate number of cells for *in vitro* studies prevents a comprehensive understanding of their functional and biological properties. For these reasons, the use of the human eosinophilic leukemia cells (EoL-1) as an *in vitro* model represents a crucial way for exploring eosinophilic physiological and pathological roles. This investigation not only helps to understand the mechanisms of the diseases, but also has implications for treatment strategies for patients with asthma and COPD.

For these purposes, aims of my PhD project are:

1) to develop an *in vitro* model of mature eosinophils starting from EoL-1 cell line that allows a better understanding of eosinophils maturation and activation;
2) to investigate the role of immunological stimuli (IL-5 and LPS) on EoL-1 maturation and differentiation;

3) to investigate whether polarized macrophages (pro-inflammatory M1 or anti-inflammatory M2) are able to influence the eosinophils differentiation in inflammatory or resident subtypes;
4) to develop and validate a flow-cytometry assay on human whole blood to evaluate the presence and proportion of the eosinophil subtypes (iEos and rEos) in asthma and COPD patients.

3. Methods

3.1 Cell Culture

The human eosinophilic leukemia cell line EoL-1 and human monocyte cell line THP-1 were obtained from ECACC (Salisbury, UK) and cultured in standard medium RPMI media (Sigma-Aldrich), 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin (Lonza), and 1% glutamine (Euroclone) and maintained at 37°C, 5% CO2 in humidified tissue culture incubator. They both grow in suspension, singly or in small clusters.

3.1.1 EoL-1 maturation protocol

EoL-1 cells were differentiated into mature eosinophil-like cells following stimulation with butyric acid (BA) as previously described [84]. To optimize and achieve the highest proportion of mature cells while maintaining maximal viability, we evaluated different BA concentrations and stimulation durations. In particular, as indicated in Figure 6, EoL-1 cells were counted and seeded in 6 wells plate at a concentration of 500,000 cells/ml and starvested in medium without FBS for 24 hours. The cells were then incubated with butyric acid at 3 different concentrations (300 µM, 400 µM and 500 µM) (Sigma-Aldrich) diluted in complete medium with 10% FBS at 2 time points (72 and 120 hours).

At the end of the stimulation the cells were then washed in PBS, counted by the use of trypan blue solution for the viability evaluation, and stained with hematoxylin and eosin (H&E staining) for morphological analysis. All the experiments were performed at least 3 times.

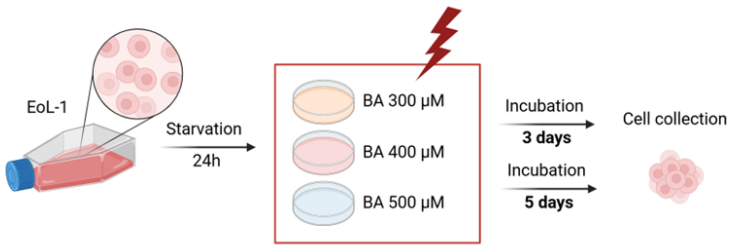


Figure 6. Schematic representation of the experimental protocol: stimulation of EoL-1 cells with 3 different concentrations of BA (300, 400 and 500 µM) at 2 time points (3 days and 5 days). BA = butyric acid.

3.1.2 EoL-1 stimulation with IL-5 or LPS

To assess further maturation and differentiation level, the cells were then stimulated with LPS or with IL-5, as Th1 and Th2 stimuli. In particular, after a stimulation with BA 500 µM for 4 days, the EoL-1 were treated with IL-5 (5 ng/ml and 10 ng/ml, Miltenyi Biotec) or with LPS (1 ng/ml and 5 ng/ml) in addition to BA and incubated for other 24 hours (Figure 7). Therefore, the stimulation with BA lasts in total for 5 days. At the end of the stimulation the cells were washed in PBS, counted by the use of trypan blue solution for the viability evaluation and prepared for the MTT assay, morphological analysis, flow cytometry evaluation and Real-Time PCR. The supernatants were also collected to evaluate the expression and release of a panel of cytokines by the use of a cytokine array, as described below. All the experiments were performed at least 3 times.

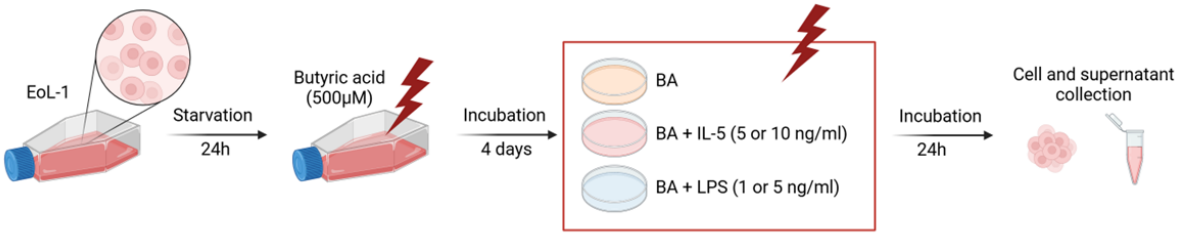


Figure 7. Schematic representation of the experimental protocol: stimulation of EoL-1 cells with IL-5 and LPS at 2 different concentrations. BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide.

3.1.3 THP-1 differentiation protocol

THP-1 monocytes were differentiated into resting macrophages (M0), classically activated macrophages (pro-inflammatory, M1), and alternatively activated macrophages (anti-inflammatory, M2) using a modified version of the protocol described by Park et al. [83]. Cells were counted and seeded in 12 wells plate at a concentration of 400,000 cells/ml. THP-1 monocytes are differentiated into resting macrophages (M0) with 48 hours incubation with 100nM phorbol 12-myristate 13-acetate (PMA, Sigma). After stimulation with PMA, cells were washed twice in PBS, and stimulated for 48 hours with IFNγ 40 ng/mL (SinoBiological) + LPS 40 ng/mL (Enzo Life Sciences) for M1 polarization or with IL-4 40 ng/mL (SinoBiological) for M2 polarization. All the experiments were performed at least 3 times to obtain an adequate volume of supernatant to use for subsequent experiments.

3.1.4 EoL-1 stimulation with macrophages supernatants

To assess the modulation of the inflammatory phenotype on EoL-1 cells, the cells were stimulated with macrophages supernatants. EoL-1 cells were primed to obtain mature eosinophils as previously reported and then stimulated with macrophages supernatant (M0, M1 and M2) at 3 different concentrations: 10%, 20% and 30% v/v (Figure 8). The cells were then incubated for 24 hours and collected to check the viability by trypan blue exclusion assay and MTT, to perform flow cytometry analysis and Real-Time PCR. All the experiments were performed at least 3 times.

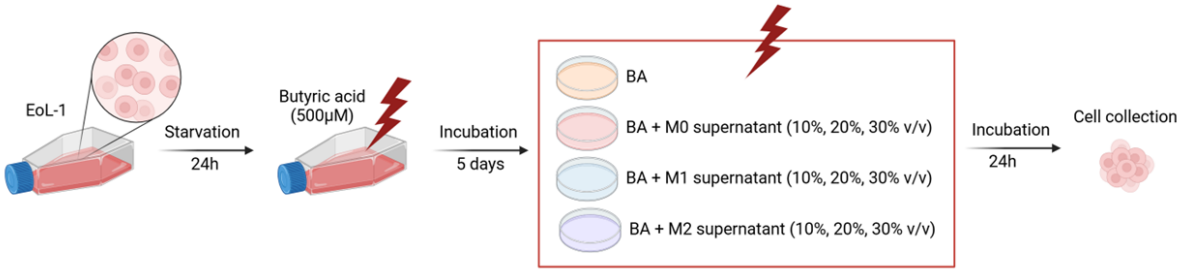


Figure 8. Schematic representation of the experimental protocol: stimulation of the mature cells with macrophages supernatants (M0, M1 and M2) at 3 different concentrations (10%, 20% and 30% v/v). BA = butyric acid.

3.2 MTT assay

Viability of EoL-1 cells was evaluated at the end of the experiments by performing the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, cultures were incubated with MTT solution (12 mM) for 4 h at 37 °C. Then formazan crystals were solubilized with dimethyl sulfoxide (DMSO). Absorbance of the dye was measured at a wavelength of 570 nm and recorded using a microplate photometer reader (Multiscan FC, Thermoscientific). The viability of the cells was expressed as fold change compared to reference condition.

3.3 Morphological analysis

To assess cellular maturation, we evaluated morphological changes, including nuclear modifications as well as alterations in cell size and cytoplasmic granularity. These parameters are widely recognized as hallmarks of eosinophil differentiation and functional maturation. For this morphological analysis, a suspension of 400,000 cells/ml

was centrifuged onto microscopic slides and subsequently stained using the Diff-Quick Kit (PanReac AppliChem), following the manufacturer's instructions. Briefly, the slides were first immersed in the Fixative solution, drained the surplus solution onto filter paper, then immersed in Solution I (eosin) and Solution II (hematoxylin) for the staining of nucleus and cytoplasm. Morphological analysis was performed using a Leica optical microscope at 40× magnification on Diff-Quick–stained cytological preparations. Non-consecutive fields were examined to ensure sample representativeness and minimize bias. Images were acquired and analyzed with the Leica Application Suite (LAS) software. For each slide, at least 100 cells were evaluated. Nuclear morphology, nuclear area, cell area, and the nucleus-to-cytoplasm ratio were quantified and used to determine the maturation stage.

3.4 Flow Cytometry

Flow cytometry was applied to extend morphological observations, providing a quantitative and reproducible assessment of EoL-1 maturation and inflammatory marker expression. This approach allows the analysis of large cell populations and the reliable identification of differentiation and activation states. Briefly, 100,000 cells were resuspended in PBS/FBS 1% and labeled for 20 minutes at room temperature (RT) in the dark with anti-CD45, CD16, Siglec-8, CD62L, IL5Rα and CCR3, monoclonal antibodies (Table 1). In particular, the eosinophils were identified as $CD45^{+}Siglec\text{-}8^{+}CD16^{-}$ cells. Based on CD62L expression, eosinophils were distinguished in inflammatory ($CD62L^{low}$) and resident ($CD62L^{high}$). In addition, the expression of the IL-5 receptor and CCR3 on the eosinophil surface were quantified.

A total of 10,000 events per sample were acquired on CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed with CytExpert (Software Version 1.2, Beckman Coulter) and reported as mean percentage ± standard deviation (SD) of positive cells or median of fluorescence intensity (MFI) ± SD.

Table 1. Monoclonal anti-human antibodies for eosinophils characterization by flow cytometry.

Antibody	Antigen	Manufacturer	Fluorochrome	REF	Clone
PE/Cy7 anti-human CD45	CD45	SONY	PE/Cy7	2120080	HI30
FITC anti-human CD16	CD16	SONY	FITC	2110030	3G8
PE anti-human Siglec-8	Siglec-8	SONY	PE	2335520	7C9
APC anti-human CD62L	CD62L	SONY	APC	2124050	DREG-56
IL5RA Monoclonal Antibody (26815) APC	IL5RA	Invitrogen	APC	MA5-23581	26815
Brilliant Violet 510 anti-human CD193 (CCR3)	CD193	SONY	BV510	2153610	5E8

FITC = fluorescein isothiocyanate; BV = Brillian Violet; PE = Phycoerythrin; APC = Allophycocyanin; Cy7 = Cyanine7.

3.5 Real-Time PCR

Flow cytometry was complemented with Real-Time PCR to study EoL-1 cultures. While flow cytometry profiles surface markers, Real-Time PCR detects gene expression changes linked to activation and polarization, providing a more complete picture of eosinophil responses to different stimuli. Briefly, total RNA was isolated from EoL-1 cells following the manufacturer's instructions using total RNA isolation kit (Qiagen, RNeasy Plus Mini Kit). Once extracted, RNA was reverse transcripted to obtain cDNA templates. Syber green Real-Time PCR was then performed to evaluate markers expression using Comparative Ct (ΔΔCt) method and normalized to GAPDH. In Table 2 are reported the primer sequences.

Table 2. Primer sequences used for all the Real-Time PCR performed.

Primer	Forward	Reverse
GATA-1	5'-TTGTCAGTAAACGGGCAGGTA-3'	5'-CTTGCGGTTTCGAGTCTGAAT-3'
ECP	5'-TTACGAGGGCTCAGTGGTTT-3'	5'-GTTCTGTTATGAGGGCAGCG-3'
CCR3	5'-GTCATCATGGCGGTGTTTTTC-3'	5'-CAGTGGGAGTAGGCGATCAC-3'
CD62L	5'-TTACCTCTGCATGTACCTTCATCTG-3'	5'-CACCCTCCTTAATCATTGAGAAACTT-3'
IL5Rα	5'-TTTGCCCTTCACGCCATTGAT-3'	5'-TGGATAGAGAGACGAGTTCCTTC-3'
CCL17	5'-GGGCTTCTCTGCAGCACATC-3'	5'-CCCTGGAGCAGTCCTCAGAT-3'
Eotaxin-3	5'-CCTCCTGAGTCTCCACCTTG-3'	5'-AAGGGGCTTGTGGCTGTATT-3'
IL-8	5'-CTGATTTCTGCAGCTCTGTG-3'	5'-GGGTGGAAAGGTTTGGAGTATG-3'
IL-13	5'-GCAGCCCTGGAATCCCTGAT-3'	5'-TGGTGTCTCGGACATGCAAGC-3'

IL-5	5'-TCTACTCATCGACTCTGCTGA-3'	5'-CCCTTGCACAGTTTGACTCTC-3'
GAPDH	5'-CTCCTGCACCACCAACTGCT-3'	5'-GGGCCATCCACAGTCTTCTG-3'

3.6 Cytokine array

Cell culture supernatants were collected to assess cytokine expression and release. The expression profile of 80 different cytokines (Figure 9) in each sample was assessed using a Human Cytokine Array kit following the instructions provided by the manufacturer (RayBiotech, AAH-CYT-5-8). Briefly, following an initial blocking step, the membranes were incubated with the samples overnight at 4°C. The membranes were then incubated with Biotinylated Antibody Cocktail for 1.5 hours and HRP-Streptavidin for other 2 hours before chemiluminescence detection. Chemiluminescence signals produced in proportion to the amount of cytokines were detected using a biomolecular imager (Alliance Atom, Uvitec), analyzed by densitometry software (ImageJ) and normalized on the positive control spots.

	A	B	C	D	E	F	G	H	I	J	K
1	POS	POS	POS	POS	NEG	NEG	ENA-78 (CXCL5)	G-CSF	GM-CSF	GRO a/b/g	GRO alpha (CXCL1)
2	I-309 (CCL1)	IL-1 alpha (IL-1 F1)	IL-1 beta (IL-1 F2)	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	IL-8 (CXCL8)	IL-10
3	IL-12 p40/p70	IL-13	IL-15	IFN-gamma	MCP-1 (CCL2)	MCP-2 (CCL8)	MCP-3 (CCL7)	M-CSF	MDC (CCL22)	MIG (CXCL9)	MIP-1 beta (CCL4)
4	MIP-1 delta	RANTES (CCL5)	SCF	SDF-1 alpha	TARC (CCL17)	TGF beta 1	TNF alpha	TNF beta (TNFSF1B)	EGF	IGF-1	Angiogenin
5	OSM	TPO	VEGF-A	PDGF-BB	Leptin	BDNF	BLC (CXCL13)	Ck beta 8-1 (CCL23)	Eotaxin-1 (CCL11)	Eotaxin-2 (CCL24)	Eotaxin-3 (CCL26)
6	FGF-4	FGF-6	FGF-7 (KGF)	FGF-9	FLT-3 Ligand	Fractalkine (CX3CL1)	GCP-2 (CXCL6)	GDNF	HGF	IGFBP-1	IGFBP-2
7	IGFBP-3	IGFBP-4	IL-16	IP-10 (CXCL10)	LIF	LIGHT (TNFSF14)	MCP-4 (CCL13)	MIF	MIP-3 alpha	NAP-2 (CXCL7)	NT-3
8	NT-4	OPN (SPP1)	OPG (TNFRSF11	PARC	PLGF	TGF beta 2	TGF beta 3	TIMP-1	TIMP-2	POS	POS

Figure 9. Cytokine profile analyzed by Human Cytokine Array kit (RayBiotech).

3.7 Asthmatic and COPD patients enrollment

Asthmatic and COPD patients, aged at least 18 years, have been enrolled from March 2024 among patients attending the outpatient clinics of the Pulmonology Unit (U.O.C. di Pneumologia) at the Padua University Hospital. Asthma population includes patients with a diagnosis of asthma made according to the latest version of the Global Initiative for asthma (GINA) guidelines (more than 1 year before study entry). COPD population includes patients with a diagnosis of COPD based on spirometry (FEV1/FVC < 0.70 post-bronchodilator) according to the latest version of the GOLD guidelines and a smoking history of > 10 pack-year**.** Healthy controls, with no history of asthma, COPD, or other respiratory diseases, were recruited from staff members of the Department of Cardiac, Thoracic, Vascular Sciences and Public Health at the University of Padua.

At recruitment, patients underwent a complete clinical evaluation with record of the clinical history (smoking, environmental and occupational exposures, allergies, drugs), past medical history (extrapulmonary comorbidities), treatment during the previous 12 months, pulmonary function test and blood cell counts.

The study was performed according to the International Conference on Harmonisation of Technical Requirements for Pharmaceuticals for Human Use E6 Guidelines for Good Clinical Practice and the principles of the Declaration of Helsinki. Each patient gave written informed consent.

At recruitment peripheral blood samples were collected:

- 1 vacutainer with EDTA anticoagulant (BD, K2EDTA) for eosinophils flow cytometry analysis, identification of eosinophils subtypes (iEos and rEos) and eosinophils characterization.
- 1 vacutainer with EDTA anticoagulant (BD, K2EDTA), for possible future analysis of the plasma samples. The vacutainer was centrifuged at 2500g for 10 minutes at 4°C. The plasma obtained was collected using a Pasteur pipette, taking care not to aspirate the underlying layer, and was frozen in aliquots at -80°C for long-term storage.

3.8 Flow cytometry analysis of eosinophils in peripheral blood

Whole blood was gently mixed by inverting the vacutainer 7-8 times and then aliquoted in 1.5 ml tubes (100 µl per tube). The fluorescent anti-human antibodies were then added to each aliquot as described in table 1 and incubated at room temperature (RT) in the dark for 20 minutes. At the end of the incubation, 1 ml of lysis buffer per tube was added for red blood cell lysis and incubated for 5 minutes. The samples were then centrifuged at 300g for 5 minutes with brake, the supernatants were discarded, and the cell pellets were resuspended in 500 µl of PBS for a washing step. The cell suspensions were centrifuged at 300g for 5 minutes with brake, the supernatants were discarded, and the cell pellets were resuspended in 500 µl of PBS. The samples were then immediately analyzed. The first step of the flow cytometry gating strategy (Figure 10) includes granulocytes identification based on the physical characteristics of the cells that are bigger and more complex compared to the lymphocytes and monocytes. The dimension and the complexity of the cells were defined by physical parameters: forward scatter (FSC) for cell dimension and side scatter (SSC) for cell complexity and granularity.

In the gate of granulocytes were then identified the neutrophils as $CD45^{+}Siglec\text{-}8^{-}CD16^{+}$ cells and the eosinophils population as $CD45^{+}Siglec\text{-}8^{+}CD16^{-}$ cells. The eosinophils were then distinguished in inflammatory and resident eosinophils based on the expression of CD62L: the inflammatory present low levels of CD62L and the resident high levels of this protein. In addition, the expression of the IL-5 receptor and CCR3 on the eosinophil surface were quantified.

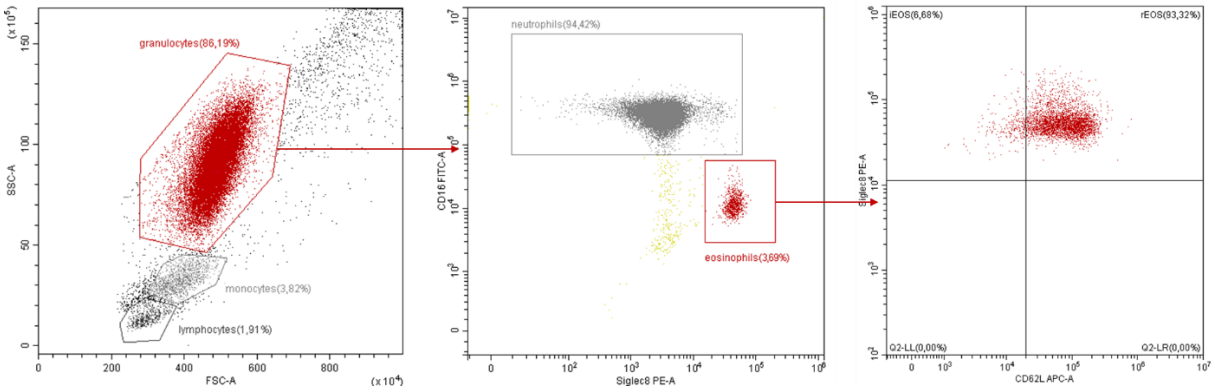


Figure 10. Flow cytometry gating strategy for eosinophils characterization. FSC = Forward Scatter; SSC = Side Scatter.

A total of 30,000 events per sample were acquired on CytoFLEX flow cytometer (Beckman Coulter). Data were analyzed with CytExpert (Software Version 1.2, Beckman Coulter) and reported as mean percentage ± standard deviation (SD) of positive cells or median of fluorescence intensity (MFI) ± SD.

3.9 Statistical Analysis

All data are presented as mean ± standard deviation (SD), unless otherwise specified. Statistical analyses were performed using SPSS (version 3.5.2), and graphs were generated with GraphPad Prism (version 9.4.0). A p-value < 0.05 was considered statistically significant. For in vitro experiments, comparisons between two groups were conducted using Student's t-test, while comparisons among more than two groups were analyzed by one-way ANOVA followed by Tukey's post hoc test. For patient data, comparisons between two independent groups were performed using the Mann–Whitney U test.

4. Results

4.1 Development of the EoL-1 cells maturation protocol

EoL-1 cells can be differentiated into mature eosinophil-like cells by butyric acid (BA) treatment. We tested the effects of different concentrations of butyric acid (300 µm, 400 µm and 500 µm) at two time points (72 hours and 120 hours) on cell viability and maturation.

Firstly, we checked the cell viability by trypan blue exclusion assay: after 72 hours the untreated cells present a viability of 92.58% ± 1.14 that progressively decreases with butyric acid treatment (BA 300 µm: 91% ± 0.86 butyric; BA 400 µm: 87.97% ± 1.97; BA 500 µm: 81.52% ± 6.77) (Figure 11A). After 120 hours the untreated cells viability is even lower (73.28% ± 6.75) and decreases further with butyric acid treatment (BA 300 µm: 68.39% ± 7.49; BA 400 µm: 60.08% ± 10.32; BA 500 µm: 53.02% ± 8.24) (Figure 11B). No significant differences were found between the treated and untreated conditions.

The viability of the cells treated with higher concentration of butyric acid and/or over 5 days was inadequate to deep in the morphological and functional characteristics of the cells.

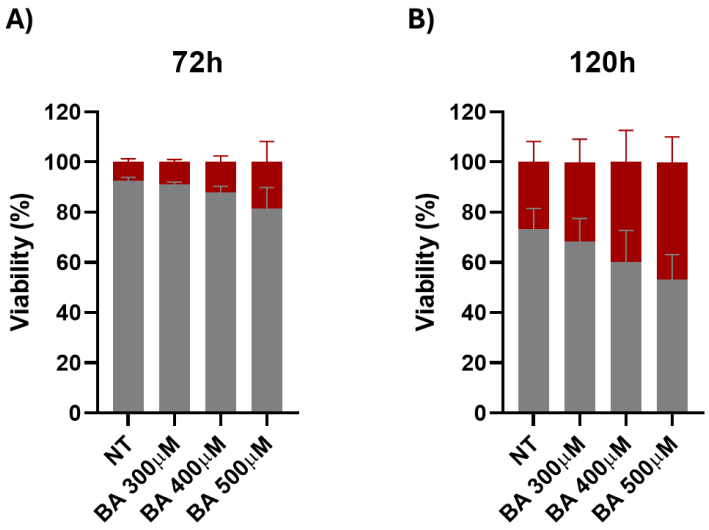


Figure 11. Viability of the EoL-1 cells by trypan blue exclusion assay evaluated at two time points: 72 hours (A) and 120 hours (B). NT = not treated cells; BA = butyric acid. Grey bar = percentage of live cells; red bar = percentage of dead cells. The data are presented as mean ± standard deviation of 3 independent experiments. One-way ANOVA followed by Tukey's post hoc test was applied.

The maturation of the cells was determined by the morphological changes of the nucleus. In particular, the nuclei became typically indented like kidney beans, which can be interpreted as eosinophilic differentiation. As shown in figure 12, after 72 hours of culture, a small proportion of the untreated cells (7.56% ± 0.42) presents a spontaneous maturation as described in the literature. With the stimulation of BA we observed a significant increase of the percentage of mature cells: 46.33% ± 1.22 with BA 300 µM treatment ($p = 0.01$), 53.62% ± 10.95 with BA 400 µM ($p = 0.0036$) and 67.50% ± 13.86 with BA 500 µM ($p = 0.0006$). After 120 hours of culture, we observed an increase of the percentage of maturation: 10.74% ± 2.65 of the untreated cells, 60.25% ± 4.39 with a stimulation of BA 300 µM ($p = 0.0002$), 67.23% ± 3.68 with BA 400 µM ($p < 0.0001$) and 72.73% ± 10.73 with BA 500 µM ($p < 0.0001$). For these reasons, the BA 500 µM for 5 days stimulation condition was selected for further experiments regarding the morphological and functional analysis.

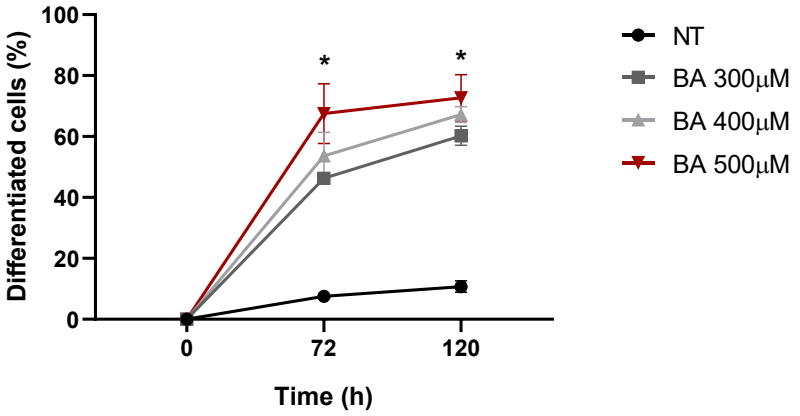


Figure 12. Percentage of differentiated cells based on morphological changes of the nuclei. NT = not treated cells; BA = butyric acid. The data are presented as mean ± standard deviation of 3 independent experiments. One-way ANOVA followed by Tukey's post hoc test was applied*; * = p significant vs NT, where * p < 0.05.*

4.2 Effect of IL-5 and LPS on EoL-1 maturation and differentiation

To further investigate the mechanisms that regulate the maturation and differentiation of EoL-1 cells to mature eosinophils, we tested different concentrations of inflammatory stimuli [interleukin 5 (IL-5) and lipopolysaccharide (LPS)], in addition to BA treatment: BA 500 µM + IL-5 5 ng/ml, BA 500 µM + IL-5 10 ng/ml, BA 500 µM + LPS 1 ng/ml, BA 500 µM +

LPS 5 ng/ml. In figure 13 are reported the cell culture images of the different tested conditions as illustrative purpose.

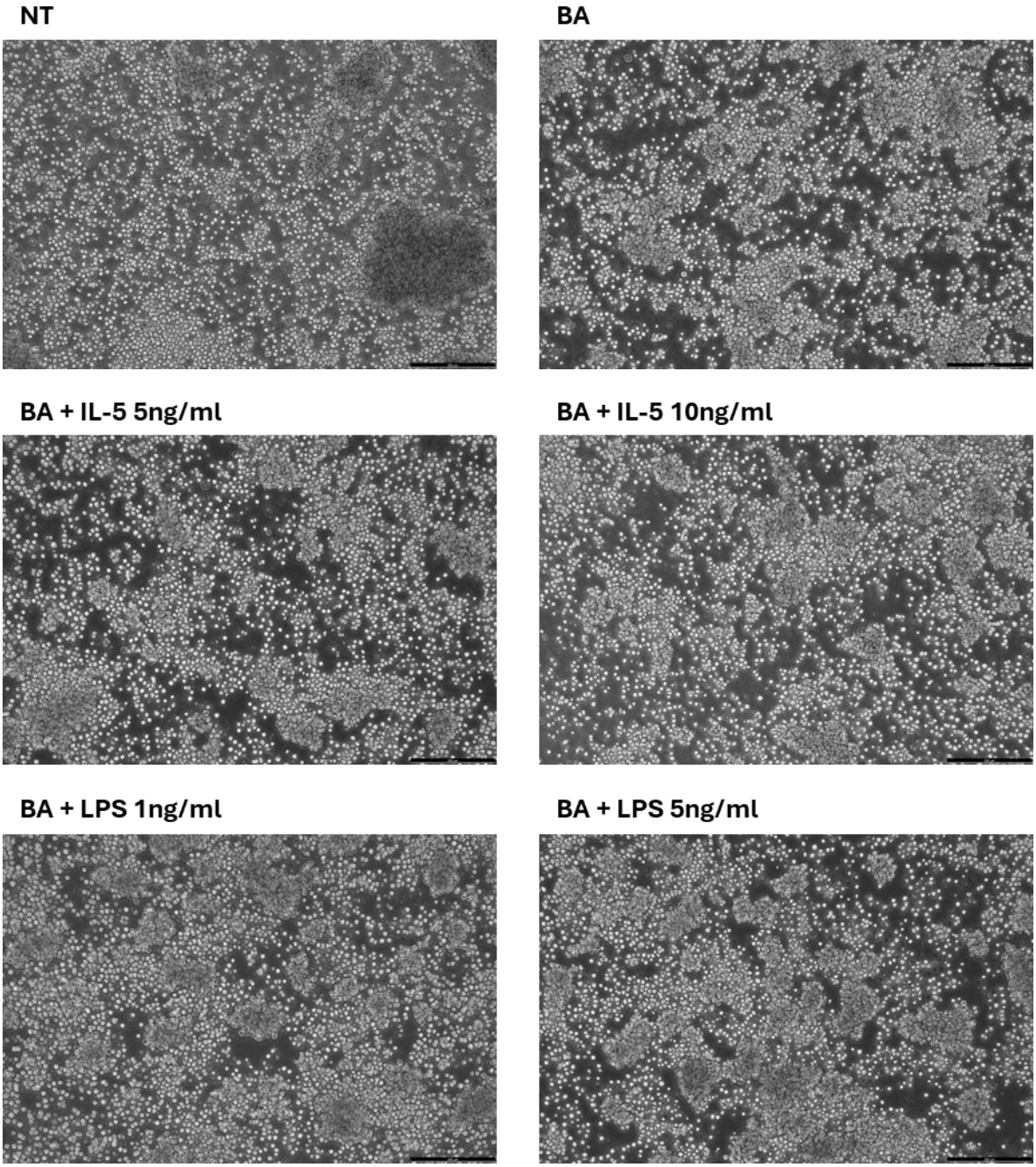


Figure 13. Optical microscope images of the tested conditions on EoL-1 cells culture in 6 well plates. The images were acquired at 10X magnification, a scale bar of 250µm is reported. NT = untreated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide.

4.2.1 Effect of IL-5 and LPS on viability and growth rate of differentiated EoL-1 cells

The viability of the cells obtained with the final protocol was evaluated by the use of the trypan blue exclusion assay and by MTT assay. In particular, we observed a viability of

80.92% ± 3.31 of the untreated cells and 75.88% ± 3.75 with a stimulation of butyric acid 500 µM. The stimulation with IL-5 does no further significantly decrease the viability: 75.81% ± 4.15 for IL-5 5 ng/ml and 74.98% ± 3.02 for IL-5 10 ng/ml conditions. The viability is 75.52% ± 4.32 for the LPS 1 ng/ml and 70.31% ± 2.92 for the LPS 5 ng/ml conditions. Only the highest concentration of LPS decreases significantly the cell viability ($p = 0.049$; Figure 14A).

Despite a good viability of the treated cells, the growth rate is significantly lower in the all the butyric acid-treated cells conditions compared to the untreated cells (Figure 14B). In particular, the untreated cells present a growth rate of 255.63% and the butyric acid-treated cells a growth rate of 76.88%, that is less than ⅓ of the physiological growth rate of the cells ($p = 0.0001$). The cells stimulated with BA + IL-5 5 ng/ml present a growth rate of 84.85% ($p = 0.0002$), 61.88% for the cells treated with BA + IL-5 10 ng/ml ($p = 0.0001$), 82.98% for BA + LPS 1 ng/ml-treated cells ($p = 0.0002$) and 30.62% for the cells treated with BA + LPS 5 ng/ml ($p < 0.0001$).

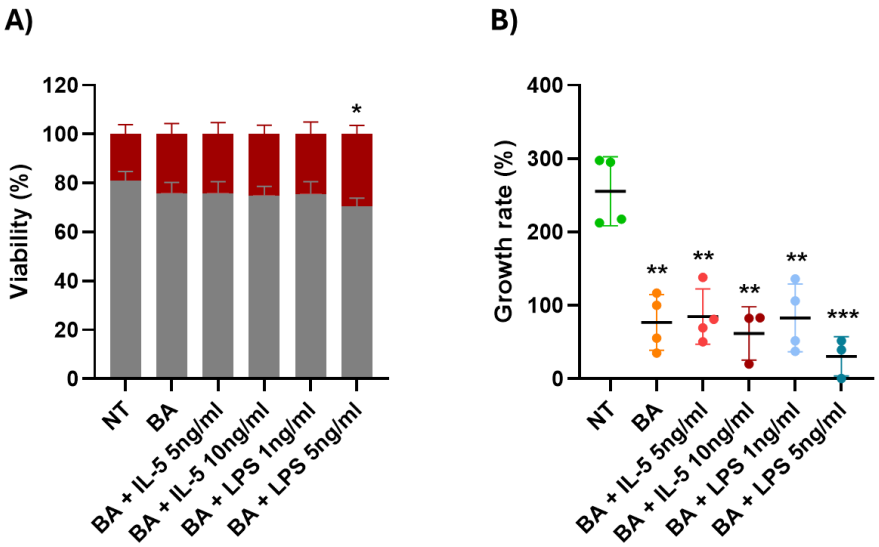


*Figure 14. A) Viability evaluated by trypan blue exclusion assay. Grey bar = percentage of live cells; red bar = percentage of dead cells. B) Cell growth rate (percentage). NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. The data are presented as mean ± standard deviation of 3 independent experiments. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs NT where *p < 0.05, **p < 0.01, ***p < 0.001.*

The MTT assay reflects the cell viability and the total amount of the cells. Indeed, as shown in figure 15, the viability of the BA-treated cells is significantly lower compared to

the untreated cells (59.90% ± 8.27; p < 0.0001). The cells stimulated with BA + IL-5 5 ng/ml present a viability of 59.07% ± 8.89 (p < 0.0001), 58.33% ± 6.75 for the cells treated with BA + IL-5 10 ng/ml, 56.94% ± 6.61 (p < 0.0001) for BA + LPS 1 ng/ml condition and 56.95% ± 7.86 (p < 0.0001) for the cells treated with BA + LPS 5 ng/ml.

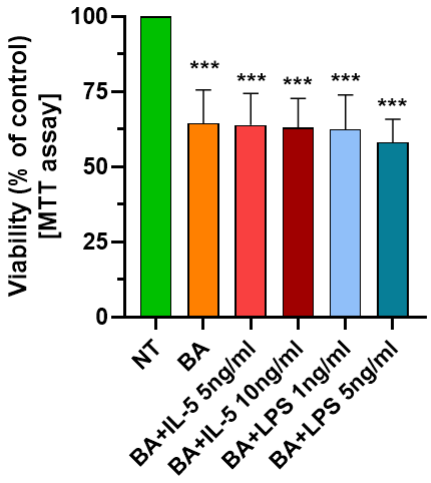
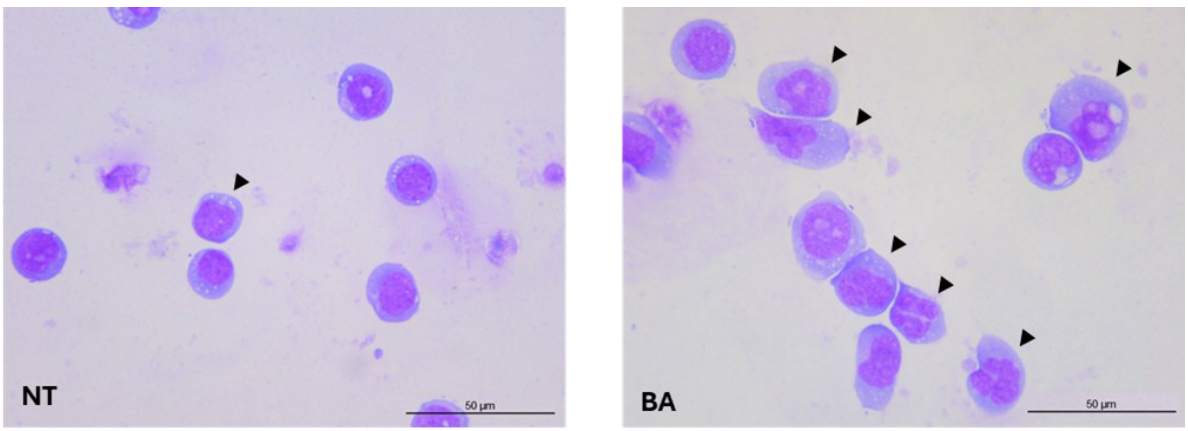


*Figure 15. Cell viability by MTT assay expressed as fold change compared to not treated cells (%). NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. The data are presented as mean ± standard deviation of 3 independent experiments. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs NT where ***p < 0.001.*

4.2.2 Effect of IL-5 and LPS on EoL-1 morphology

As previously mentioned, nuclear morphology, nuclear area, cell area, and the nucleus-to-cytoplasm ratio were quantified (Figure 16). The maturation of the cells was determined by the morphological changes of the nucleus.



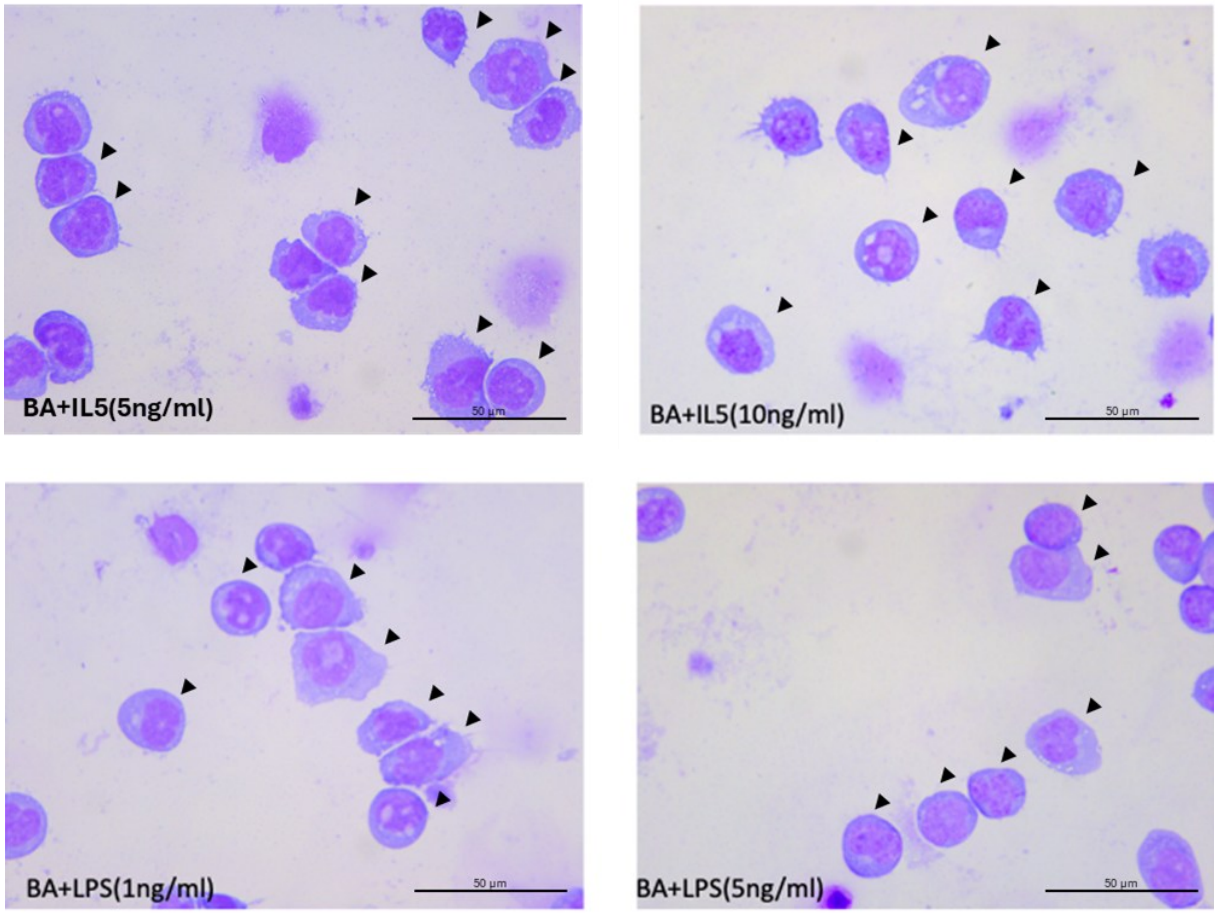


Figure 16. Optical microscope images of the tested condition on EoL-1 cells stained with hematoxylin and eosin (H&E). The images were acquired at 40X magnification, a scale bar of 50µm is reported. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. Black arrows indicate mature cells with morphological changes of the nuclei.

As shown in figure 17, the untreated cells present a percentage of spontaneously maturated cells of 17.75% ± 8.03. This percentage increases significantly with the butyric acid treatment (66.29% ± 9.94; $p < 0.0001$). The stimulation with IL-5 and LPS does not further increase the percentage of differentiated cells but it remains significantly higher compared to the untreated cells: 71.04 ± 5.22 for the BA + IL-5 5 ng/ml-treated cells ($p < 0.0001$), 62.82 ± 2.00 for the BA + IL-5 10 ng/ml-treated cells ($p < 0.0001$), 61.83 ± 4.16 for the BA + LPS 1 ng/ml-treated cells ($p < 0.0001$) and 64.01 ± 7.14 for the BA + LPS 5 ng/ml-treated cells ($p < 0.0001$).

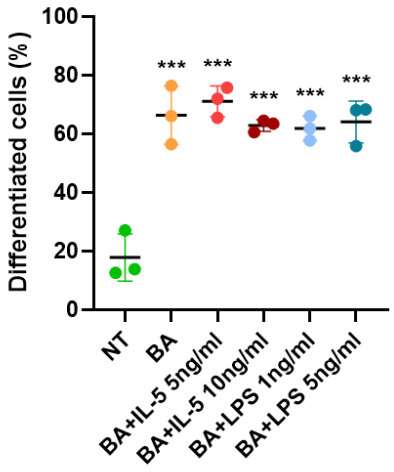


*Figure 17. Percentage of differentiated cells. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. The data are presented as mean ± standard deviation. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs NT where ***p < 0.001.*

To deep-in the morphological modifications of the cells due to the maturation treatment, we evaluated the area of the cells (Figure 18A), the area of the nuclei (Figure 18B) and the percentage of area covered by the nucleus (Figure 18C). The median dimension of the untreated cells is 278.1 μm^2 (122.4 - 650.9) and the treatment with butyric acid significantly increases the dimension of the cells to 377.7 μm^2 (214.5 - 720.5; $p < 0.0001$). The BA + IL-5-stimulated cells, both at a concentration of 5 ng/ml and 10 ng/ml, present a higher cell dimension compared to the untreated cells ($p < 0.0001$), although a significant decrease compared to the BA-treated cells [345.8 μm^2 (135.4 - 694.8); $p = 0.0002$ and 352.4 μm^2 (161.0 - 795.2); $p = 0.0006$, respectively]. The LPS stimulated cells present a significant higher dimension compared to the untreated cells [351.5 μm^2 (182.9 - 606.5); $p < 0.0001$ for the BA + LPS 1 ng/ml and 365.8 μm^2 (210.8 - 900.8); $p < 0.0001$ for the BA + LPS 5 ng/ml-treated cells]. Only the stimulation with LPS 1 ng/ml shows a significant lower dimension of the cells compared to the BA-treated cells ($p = 0.0398$) (Figure 36A).

The dimension of the nuclei was evaluated for each cell: the dimension of the nuclei of the BA-treated cells is significantly higher compared to the untreated cells [187.4 μm^2 (124.5 - 369.5) and 146.1 μm^2 (73.13 - 338.5) ($p < 0.0001$), respectively]. As for the cells size, IL-5 treatment causes a decrease of the dimension of the nuclei compared to the BA-treated cells both for the BA + IL-5 5 ng/ml condition [175.4 μm^2 (90.99 - 366.0); $p = 0.0004$] and for BA + IL-5 10 ng/ml condition [175.1 μm^2 (102.8 – 392.3)], that is significant

only for the BA + IL-5 5 ng/ml condition (p = 0.009). However, in both cases, the nuclear area remains significantly higher than the untreated control (p < 0.0001). The LPS-stimulated cells present a significant higher dimension of the nuclei compared to the untreated cells [181 μm^2 (103.9 - 369.4); p < 0.0001 for BA + LPS 1 ng/ml and 182.4 μm^2 (107 - 473.4); p < 0.0001 for BA + LPS 5 ng/ml] (Figure 36B).

As expected from the data shown, the nucleus area / cell area ratio follows the same trend of the dimension of the cells and the nuclei. In particular, the untreated cells present a median percentage of 52.23% (35.48 - 71.02) and butyric acid treatment significantly decreases the ratio to 49.88% (28.7 - 70.43; p = 0.003), although the difference is not so relevant. No significant differences were found for the BA + IL-5 stimulation and untreated cells [BA + IL-5 5 ng/ml: 50.90% (33.30 - 84.09); BA + IL-5 10 ng/ml: 51.25% (34.59 - 88.27)] but the percentage of BA + IL-5 5 ng/ml treated cells is significantly higher compared to the BA condition (p = 0.015). The stimulation with BA + LPS causes a lower percentage of the nucleus area / cell area ratio compared to the untreated cells: 50.39% (30.31 – 74.54; p = 0.038) for BA + LPS 1 ng/ml and 50.12% (34.04 - 74.21; p = 0.011) for BA + LPS 5 ng/ml (Figure 36C).

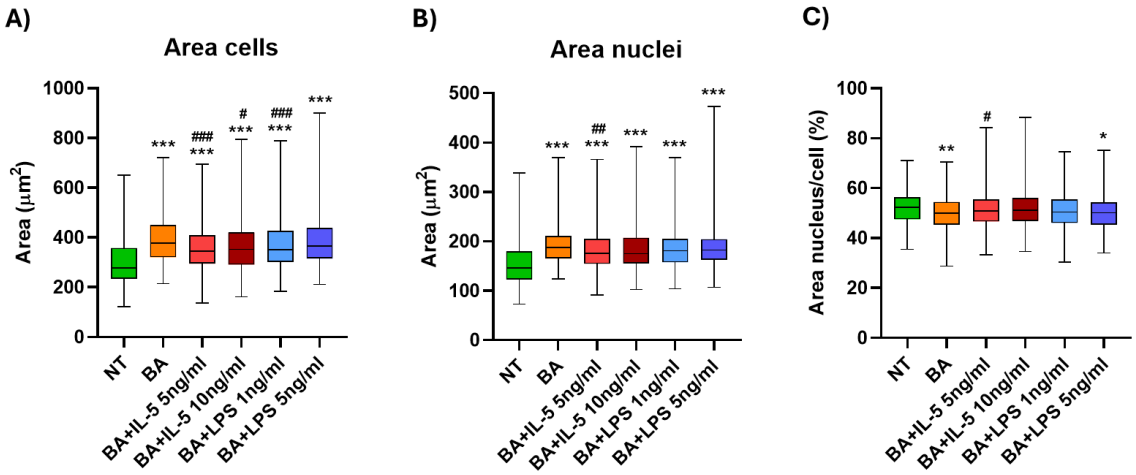


Figure 18. Morphological analysis of the EoL-1 cells: A) dimension of the cells, B) dimension of the nuclei, C) area of the nucleus / area of the cell ratio. Data are presented as box (25°-75° percentile and median) and whiskers (minimum and maximum values). NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied*; * = p significant vs NT where *p < 0.05, **p < 0.01, ***p < 0.001; # = p significant vs BA where #p < 0.05, ##p < 0.01, ###p < 0.001.*

We then evaluated the correlation between the dimensions of the cells and the dimension of the nuclei. Considering all the different conditions, the area of the nuclei correlates positively with the area of the cells ($r = 0.86$; $p < 0.0001$) (Figure 19A).

We obtained a positive correlation even when we consider each condition separately (Figure 19B): untreated cells ($r = 0.93$; $p < 0.0001$), BA-treated cells ($r = 0.85$; $p < 0.0001$), BA + IL-5 5 ng/ml-treated cells ($r = 0.82$; $p < 0.0001$), BA + IL-5 10 ng/ml-treated cells (; $r = 0.87$; $p < 0.0001$), BA +LPS 1 ng/ml-treated cells ($r = 0.8$; $p < 0.0001$) and BA +LPS 5 ng/ml-treated cells ($r = 0.81$; $p < 0.0001$).

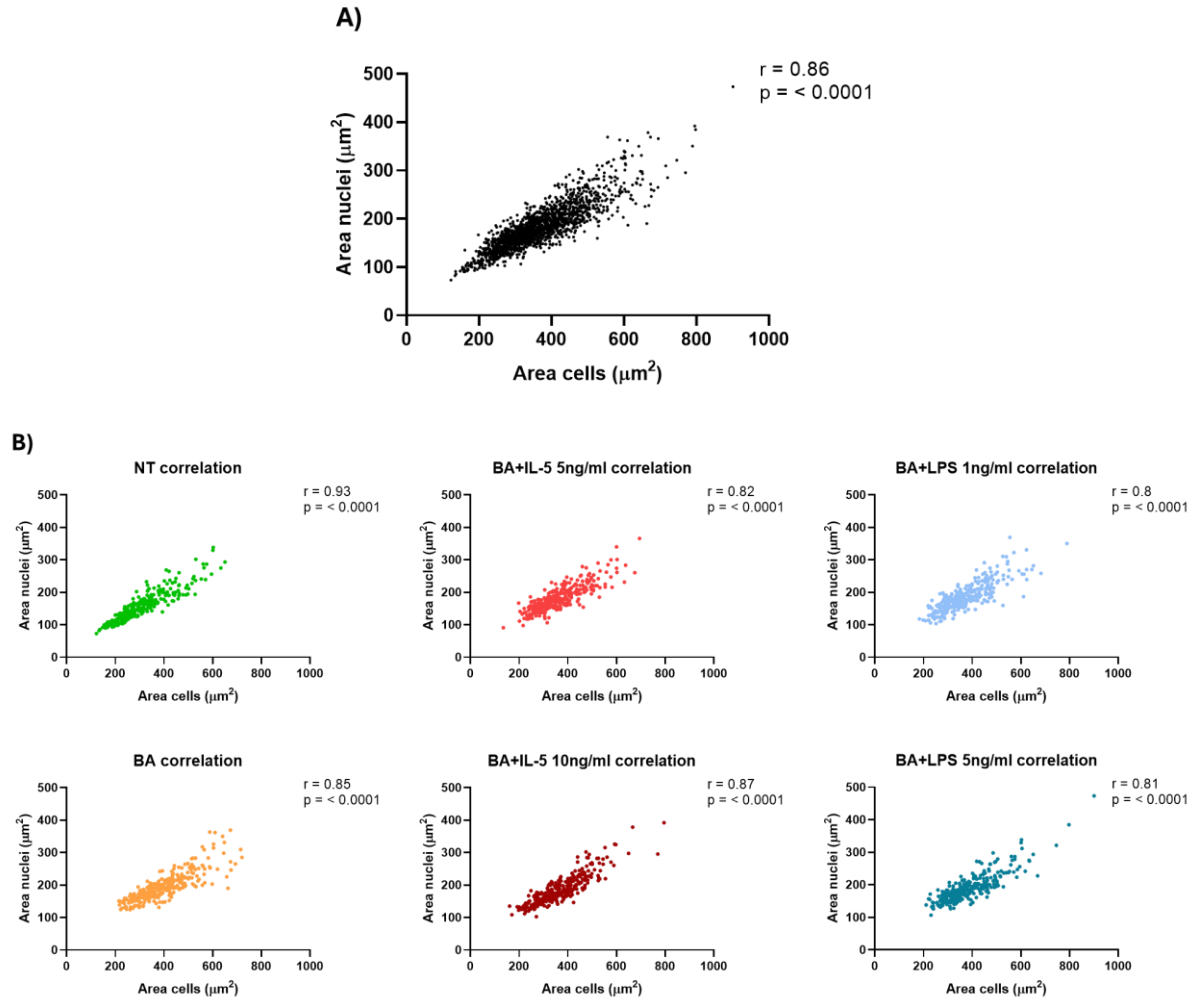


Figure 19. Correlation between the area of the cells and the dimension of the nuclei: A) correlation involving all the conditions; B) correlations evaluated for each condition. The data represent the measurements of the areas of each individual cell analyzed. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. Spearman's correlation was evaluated for each groups.

We also evaluated the number of "granules" per cell, identified in the cytoplasm as empty vacuoles (Figure 20). The stimulation with BA causes a trend of increase of the number of vacuoles compared to the untreated cells. In particular, the median number of granules present in the untreated cells is 3 (0 - 22) and 3 (0 - 21) for the BA-treated cells. The IL-5 and LPS treated cells do not show significant differences compared to the untreated cells: the BA + IL-5 stimulated cells present a median of 3 vacuoles (0 - 19); 2 (0 - 16) for IL-5 10 ng/ml; 2 (0 - 23) for BA + LPS 1ng/ml and 3 (0 - 22) for BA + LPS 5 ng/ml.

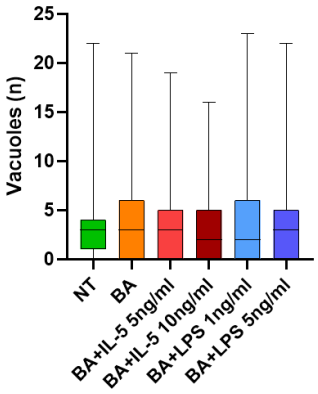


Figure 20. Number of vacuoles per cell. Data are presented as box (25°-75° percentile and median) and whiskers (minimum and maximum values). NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied.

4.2.3 Surface markers expression by flow cytometry analysis

A complete flow cytometry characterization was conducted to define the phenotype of the Eol-1 cells. As indicated by the morphological analysis, the treatment with butyric acid causes an increase of the physical properties of the cells, as an indicator of eosinophils maturation: we observed an increase of the dimension of the cells (FSC, Forward Scatter; Figure 21A) and a significant increase of the internal complexity and granularity of the cells (SSC, Side Scatter; p = 0.0003; Figure 21B). The stimulation with IL-5 or LPS does no further increase the dimension (FSC) and complexity of the cells (SSC) compared to the BA-treated cells. Both the IL-5 and LPS conditions still present significantly higher SSC values compared to the untreated cells: BA + IL5 5 ng/ml-treated cells (p = 0.0006), BA + IL5 10 ng/ml-treated cells (p = 0.0012), BA +LPS 1 ng/ml-treated cells (p = 0.0005) and BA +LPS 5 ng/ml-treated cells (p = 0.0013).

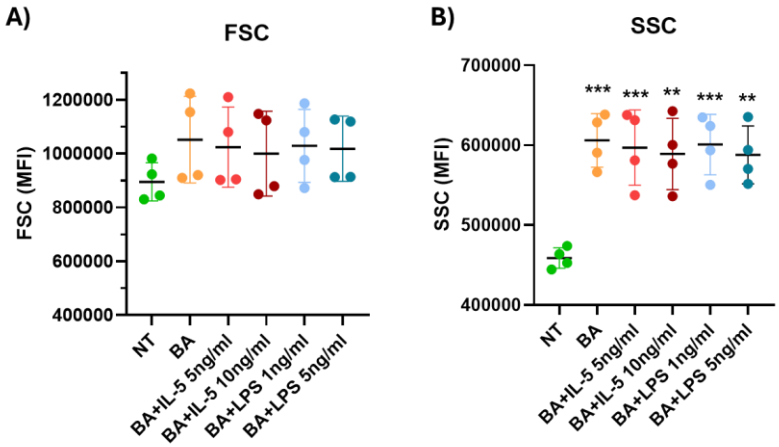
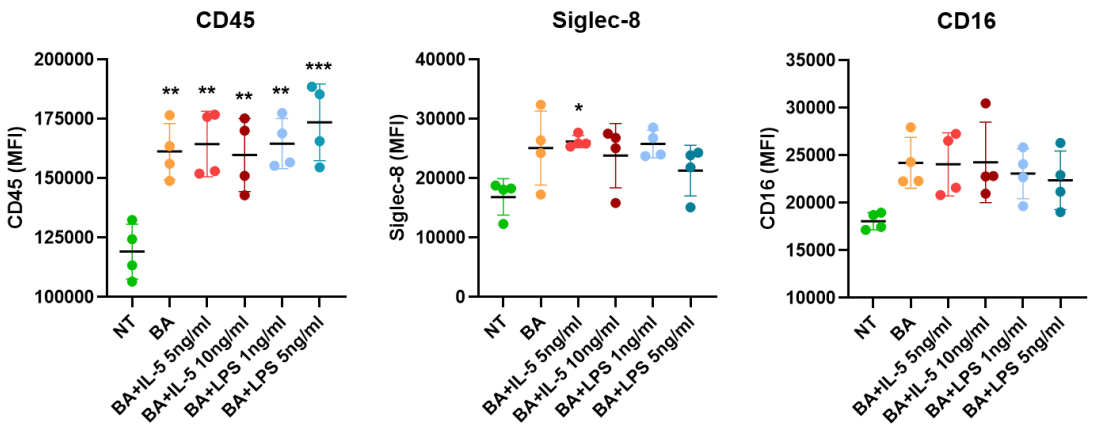


*Figure 21. Morphological parameters evaluated by flow cytometry analysis: A) Forward Scatter (FSC); B) Side Scatter (SSC). The data are presented as mean ± standard deviation. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs NT where **p < 0.01 and ***p<0.001.*

The eosinophils population in whole blood is identified as $CD45^{+}Siglec\text{-}8^{+}CD16^{-}$ cells. For this reason, we tested the same markers expression on the EoL-1 cells in terms of median of fluorescence (Figure 22). The butyric acid-treated cells present a significant higher expression of CD45, the marker of the leukocytes, compared to the untreated cells. The stimulation with IL-5 or LPS does no further increase the CD45 expression but confirms the significant differences compared to the untreated cells.

As shown in figure 22, we observed a trend of increased expression of Siglec-8, the marker of human eosinophils, for the cells stimulated with butyric acid compared to the untreated cells. The addition of IL-5 (5 or 10 ng/ml) or LPS (1 or 5 ng/ml) does not result in significant increase of Siglec-8 expression compared to BA condition, sustaining the significant higher expression compared to the untreated condition for BA + IL-5 5 ng/ml.

The expression of CD16, a neutrophil marker, presents a trend of increased expression after the treatment with BA compared to the untreated cells. The addition of IL-5 (5 or 10 ng/ml) or LPS (1 or 5 ng/ml) does not result in significant increase in CD16 expression compared to BA condition.



	NT	BA	BA + IL-5 5ng/ml	BA + IL-5 10ng/ml	BA + LPS 1ng/ml	BA + LPS 5ng/ml
CD45	119178 ± 11519	161276 ± 11804 (p = 0.0035)	164407 ± 13763 (p = 0.0017)	159787 ± 15349 (p = 0.0049)	164566 ± 10541 (p = 0.0017)	173537 ± 16176 (p = 0.0002)
Siglec-8	16865 ± 3047	25077 ± 6231 (ns)	26189 ± 1026 (p = 0.0048)	23820 ± 5413 (ns)	25772 ± 2311 (ns)	21289 ± 4254 (ns)
CD16	18076 ± 903.9	24201 ± 2679 (ns)	24055 ± 3315 (ns)	24263 ± 4230 (ns)	23068 ± 2614 (ns)	22366 ± 3073 (ns)

Figure 22. Eosinophils population markers expression evaluated by flow cytometry analysis and expressed as median of fluorescence (MFI): CD45, Siglec-8 and CD16. The data are presented as mean ± standard deviation. Raw data and p values are reported in the table below the graphs. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide; ns = not significant. One-way ANOVA followed by Tukey's post hoc test was applied*; * = p significant vs NT where *p < 0.05, **p < 0.01.*

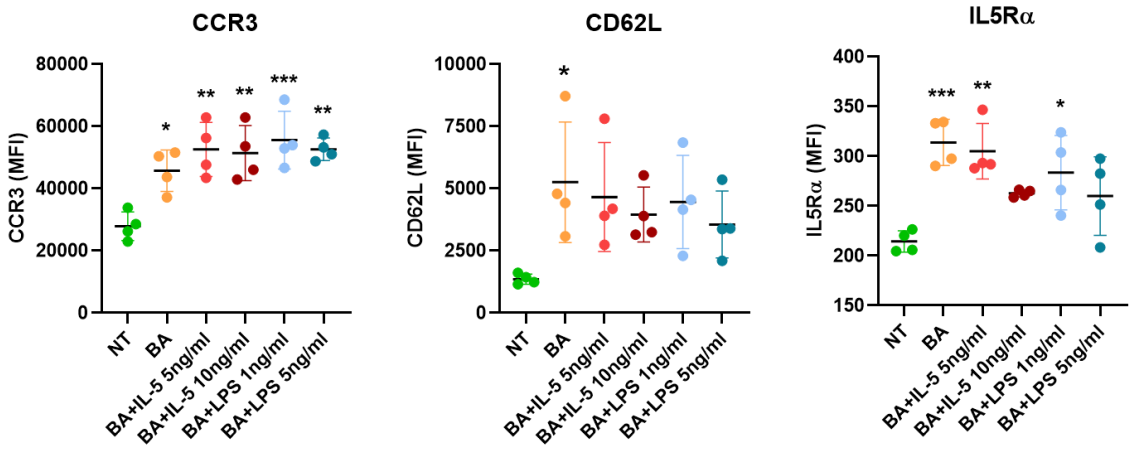
We then evaluated the expression of eosinophils functional markers: CCR3, CD62L and IL5Rα (IL-5 receptor α) (Figure 23).

The expression of the CCR3 receptor, a functional marker of eosinophils involved in chemokine-mediated cell migration, is significantly increased in cells treated with BA compared to untreated cells. The addition of IL-5 (5 or 10 ng/ml) or LPS (1 or 5 ng/ml) does not result in significant increase in CCR3 compared to BA condition, but confirms a significant higher expression compared to the control condition.

The expression of CD62L, a marker that distinguishes between inflammatory eosinophils (iEos) and resident eosinophils (rEos), is significantly increased in cells treated with BA compared to NT. The addition of IL-5 (5 or 10 ng/ml) or LPS (1 or 5 ng/ml) does not significantly alter CD62L levels compared to BA condition, but confirms a trend of increased expression compared to the NT condition.

Finally, was observed a significant increase of IL5Rα expression in cells treated with BA compared to NT. The addition of IL-5 (5 or 10 ng/ml) or LPS (1 or 5 ng/ml) does not

significantly alter IL5Rα levels compared to BA condition, maintaining a significant higher expression compared to the NT condition only for the conditions BA + IL-5 5ng/ml and BA + LPS 1ng/ml.



	NT	BA	BA + IL-5 5ng/ml	BA + IL-5 10ng/ml	BA + LPS 1ng/ml	BA + LPS 5ng/ml
CCR3	27905 ± 4578	45696 ± 6661 (p = 0.029)	52534 ± 8688 (p = 0.0018)	51351 ± 8880 (p = 0.0029)	55535 ± 9279 (p = 0.0005)	52573 ± 3638 (p = 0.0018)
CD62L	1358 ± 205.4	5251 ± 2420 (p = 0.04)	4658 ± 2188 (ns)	3955 ± 1099 (ns)	4461 ± 1871 (ns)	3554 ± 1344 (ns)
IL5Rα	214.4 ± 10.81	313.9 ± 23.26 (p = 0.0007)	305.0 ± 27.94 (p = 0.002)	262.8 ± 3.6 (ns)	283.6 ± 37.43 (p = 0.02)	260 ± 39.41 (ns)

Figure 23. Eosinophils functional markers expression evaluated by flow cytometry analysis and expressed as median of fluorescence (MFI): CCR3, CD62L and IL5Rα. The data are presented as mean ± standard deviation. Raw data and p values are reported in the table below the graphs. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied*; * = p significant vs NT where *p < 0.05, **p < 0.01 and ***p < 0.001*.

Considering the data obtained from the viability, morphological investigation and flow cytometry analysis, the gene expression of maturation markers and inflammatory mediators was performed by Real-Time PCR only for the highest concentration of IL-5 (10 ng/ml) and LPS (5 ng/ml) conditions.

4.2.4 Gene expression of maturation markers and inflammatory mediators

In accordance with the literature indications, we then checked the expression of the transcripts (GATA1, CCR3 and ECP) that are markers of eosinophils maturation (Figure 24). The expression of the GATA1 gene is absent in untreated cells. BA treatment induces

the expression of GATA1. The addition of IL-5 (10 ng/mL) or LPS (5 ng/mL) does not result in a significant increase in GATA1 expression compared to BA condition (fold change: 1.39 ± 0.16 and 1.17 ± 0.3, respectively).

The expression of the ECP gene, encoding the eosinophil cationic protein, is significantly increased after treatment with BA compared to NT (fold change: 3.2 ± 0.62; p = 0.0007). The addition of IL-5 (10 ng/ml) or LPS (5 ng/ml) does not significantly alter ECP levels compared to BA condition, but confirms the higher expression compared to the NT condition [fold change: BA + IL-5 10 ng/ml (3.42 ± 0.13); p = 0.0004 and BA + LPS 5 ng/ml (3.27 ± 0.51); p = 0.0006].

CCR3 gene expression is significantly increased in BA-treated cells compared to NT (fold change: 4.66 ± 1.33; p = 0.019). The addition of IL-5 (10 ng/ml) or LPS (5 ng/ml) does not significantly alter CCR3 levels compared to BA condition, sustaining the higher expression compared to the NT condition [fold change: BA + IL-5 (3.89 ± 1.14) and BA + LPS (5.51 ± 1.49)], that is significant only for the BA + LPS condition (p = 0.006).

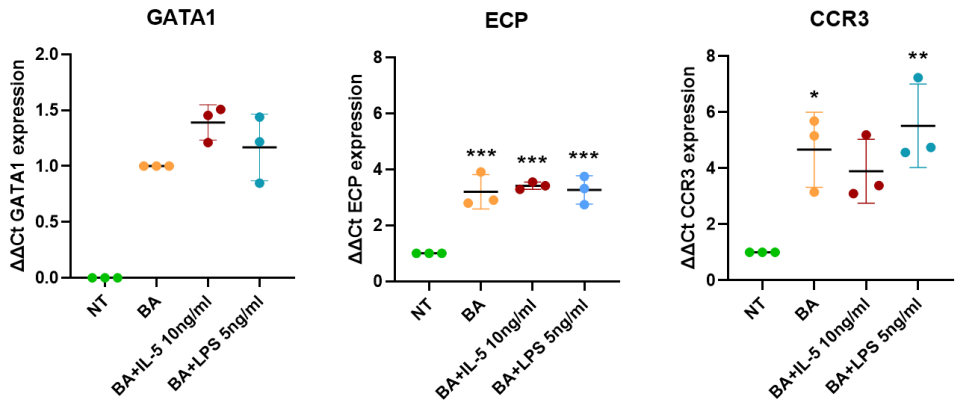


Figure 24. Eosinophils maturation markers expression evaluated by Real-Time PCR: GATA1, ECP and CCR3. The data are presented as mean ± standard deviation. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied*; * = p significant vs NT where *p < 0.05*.

Concerning the expression of functional and inflammatory mediators (Figure 25), a significant increase in CD62L expression was observed after treatment with BA compared to NT (fold change: 4.59 ± 1.23; p = 0.0017). The addition of IL-5 (10 ng/ml) or LPS (5 ng/ml) does not significantly alter the CD62L levels compared to BA condition, but confirms the increase compared to the NT condition [fold change: BA + IL-5 (4.6 ± 0.8); p = 0.0016 and BA + LPS (3.63 ± 0.31); p = 0.01).

The expression of the IL5Rα gene is significantly increased after treatment with BA compared to the untreated control (fold change: 12.19 ± 0.98; p = 0.0004); however, the combination of BA with IL-5 (10 ng/ml) results in a significant reduction expression compared to BA stimulation (p = 0.015), still maintaining a higher expression level than NT (fold change: 5.96 ± 1.66; p = 0.046). Stimulation with LPS (5 ng/ml) induce a significant increase compared to NT (fold change: 7.34 ± 3.18; p = 0.01).

The expression of the CCL17 gene, involved in the recruitment of immune cells, does not differ after treatment with BA compared to NT (fold change: 1.97 ± 0.26). The addition of IL-5 (10 ng/ml) or LPS (5 ng/ml) does not increase CCL17 levels compared to the untreated control.

The expression of the Eotaxin-3 gene, involved in the tissue recruitment of eosinophils, is not significantly increased compared to NT in any of the conditions tested (fold change: BA: 3.73 ± 4.5; BA + IL-5: 2.73 ± 1.75; BA + LPS: 2.08 ± 1.42).

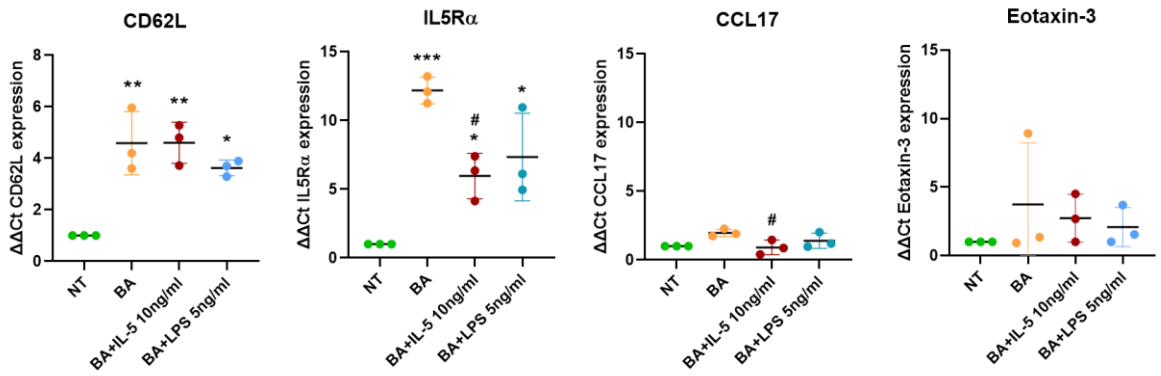


Figure 25. Functional mediators expression evaluated by Real-Time PCR: CD62L, IL5Rα, CCL17 and Eotaxin3. The data are presented as mean ± standard deviation. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied*; * = p significant vs NT where *p < 0.05, **p < 0.01 and ***p < 0.001; # = p significant vs BA where #p < 0.05.*

Among the pro-inflammatory cytokines (Figure 26), IL-8 gene expression is significantly increased only following treatment with BA + LPS (5 ng/ml) (fold change: 3.78 ± 0.67; p = 0.039) compared to the untreated cells, and similarly IL-13 gene expression is significantly increased only in the BA + LPS (5 ng/ml) condition (fold change: 3.45 ± 0.88; p = 0.04).

Finally, IL-5 gene expression shows significantly increased expression after treatment with BA + LPS (fold change: 6.38 ± 2.7) compared to NT and BA condition (p = 0.005 and p = 0.01, respectively).

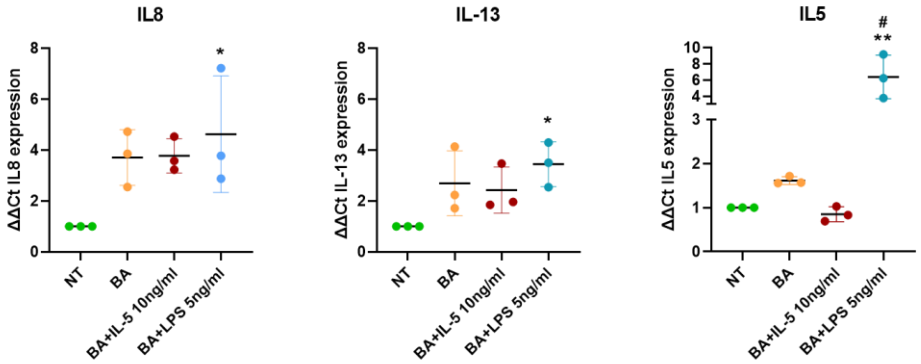


*Figure 26. Inflammatory mediators expression evaluated by Real-Time PCR: IL-8, IL-13 and IL-5. The data are presented as mean ± standard deviation. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs NT where *p < 0.05, **p < 0.01; # = p significant vs BA where #p < 0.05.*

4.2.5 Cytokine production and release

We tested the cytokine production and release in the supernatants for the untreated condition and after BA, BA + IL-5 10 ng/ml or BA + LPS 5 ng/ml stimulation. Among the 80 analyzed molecules, only a few showed consistent and detectable modulation of expression in at least one of the experimental conditions tested (Figure 27). In particular, IL-8 production is higher after the stimulation with BA + LPS (5 ng/mL), with a fold change of 5.71 ± 4.04, while the other conditions show no significant changes compared to the untreated control. In contrast, Eotaxin-3 expression is increased after BA + IL-5 (10 ng/ml) stimulation, with an average fold change of 5.31 ± 5.88. Finally, the angiogenin shows a trend of higher expression after BA + IL-5 (10 ng/mL) treatment, with a fold change of 3.73 ± 0.25 compared to untreated cells.

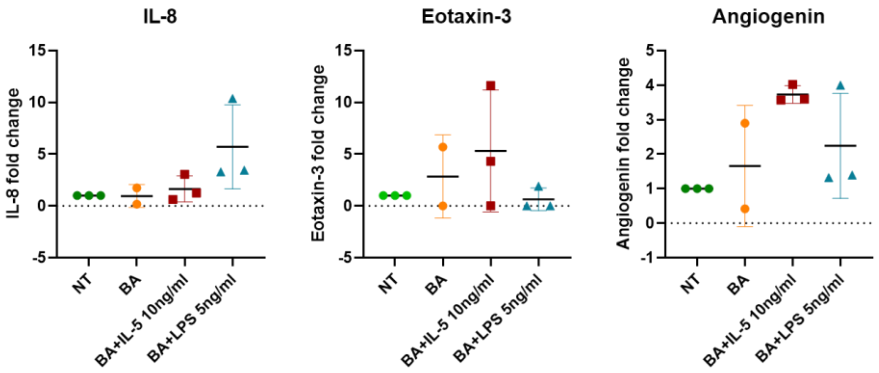


*Figure 27. Cytokine production expressed as fold change compared to not treated cells: IL-8, Eotaxin-3 and angiogenin. The data are presented as mean ± standard deviation. NT = not treated cells; BA = butyric acid; IL-5 = interleukin 5; LPS = lipopolysaccharide. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs NT where **p < 0.01.*

4.3 Effect of polarized macrophages supernatant on EoL-1 differentiation

The mature cells treated with BA 500 µm for 5 days were then stimulated with conditioned medium of M0, M1 or M2 macrophages, obtained from THP-1 differentiation. We tested different concentrations of macrophages supernatants: 10% v/v, 20% v/v and 30% v/v. In figure 28 the cell culture images of the different tested conditions are reported as illustrative purpose.

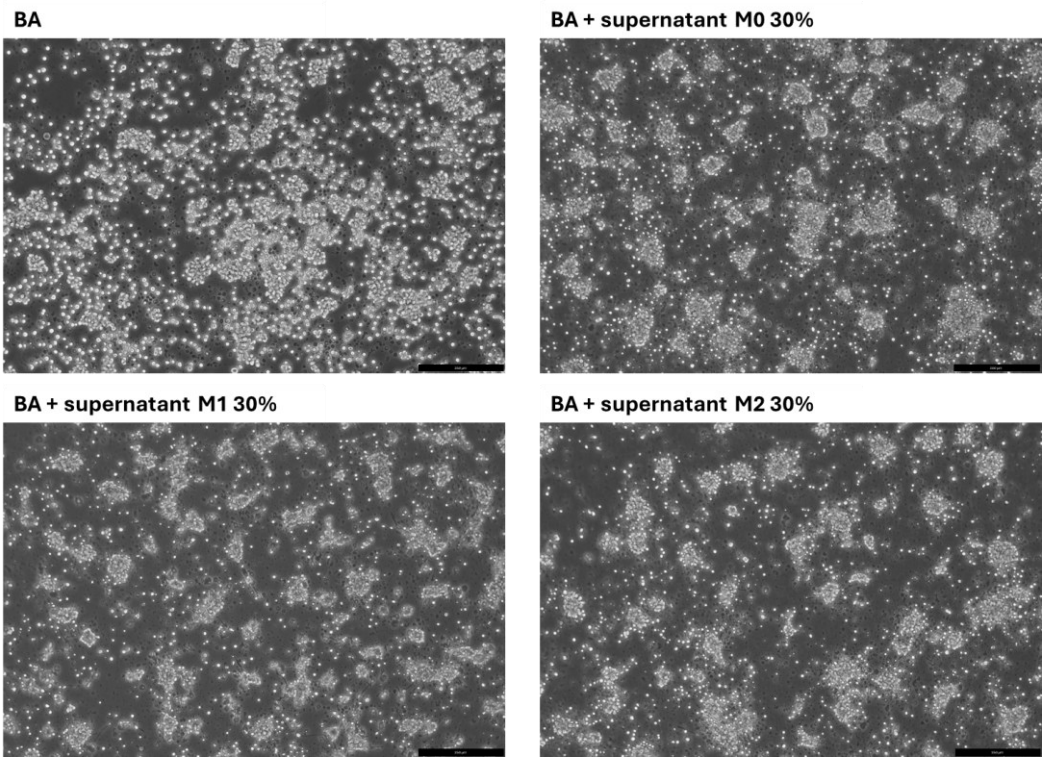


Figure 28. Optical microscope images of the tested condition on EoL-1 cells culture in 6 well plates. The images were acquired at 10X magnification, a scale bar of 250µm is reported. BA =

butyric acid treated cells; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages supernatant.

4.3.1 Effect of polarized macrophages supernatants on EoL-1 viability and growth rate

Figure 29A shows that the viability evaluated by Trypan blue exclusion assay for the BA condition is 73.73% ± 5.21. The stimulation with macrophages supernatants progressively reduces the viability in dose-dependent manner. In particular, with M0 supernatants stimulation it was observed a viability of 65.69% ± 6.50 with 10% v/v, 62.47% ± 10.84 with 20% v/v and 57.77% ± 6.93 with 30% v/v.

A similar trend was observed for the M1 and M2 supernatants stimulation. In particular, the M1 supernatant stimulation determines a viability of 62.63% ± 11.12 with 10% v/v, 61.67% ± 9.75 with 20% v/v, and 59.37% ± 2.12 with 30% v/v. As regards the M2 supernatant, a viability of 64.43% ± 7.97 was observed with 10% v/v, 63.00% ± 8.03 with 20% v/v and 61.00 ± 5.77 with 30% v/v. No significant differences were found between the BA-treated cells and the macrophages supernatants stimulated cells.

We then evaluated the proliferative capacity expressed as percentage of growth rate, as shown in Figure 29B. Cells treated with BA alone showed an increase in growth rate of 217.00%. In the presence of M0 supernatant, growth values of 209.70% with 10% v/v, 160.00% with 20% v/v, and 148.30% with 30% v/v were detected. The addition of M1 supernatant results in values of 178.00% with 10% v/v, 150.30% with 20% v/v, and 118.30% with 30% v/v, with no statistically significant differences compared to control condition.

In the presence of supernatant M2, growth values of 172.30% were observed with 10% v/v, 162.30% with 20% v/v, and 150.30% with 30% v/v, with no statistically significant differences compared to control condition.

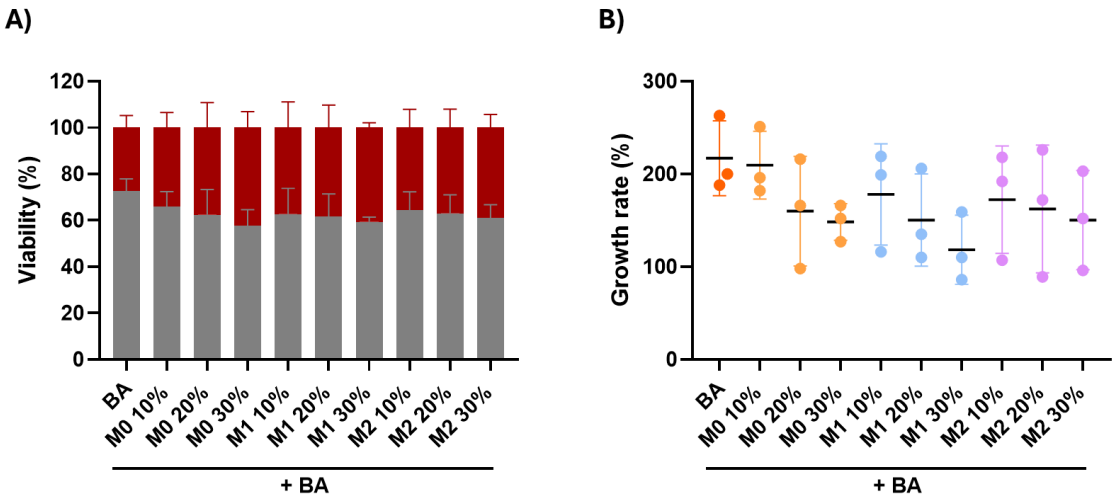


Figure 29. A) Cell viability evaluated by Trypan blue exclusion assay. Grey bar = percentage of live cells; red bar = percentage of dead cells. B) Cell growth rate (percentage). The data are presented as mean ± standard deviation. BA = butyric acid treated cells; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages supernatant. One-way ANOVA followed by Tukey's post hoc test was applied.

Compared to the control condition, cells treated with M0 supernatants show metabolic activity equal to 84.93% ± 9.38 (10% v/v; p = 0.005), 81.18% ± 8.85 (20% v/v; p = 0.0002) and 86.14% ± 8.05 (30% v/v; p = 0.018). In the presence of M1 supernatant, the values are 83.48% ± 12.88 (10% v/v; p = 0.0017), 81.89% ± 11.67 (20% v/v; p = 0.0003) and 87.47% ± 14.35 (30% v/v; p = 0.04). The cells treated with the M2 supernatant have a metabolic activity of 88.33% ± 13.12 (10% v/v; ns), 84.60% ± 7.36 (20% v/v; p = 0.0038) and 91.21% ± 11.66 (30% v/v; ns) (Figure 30).

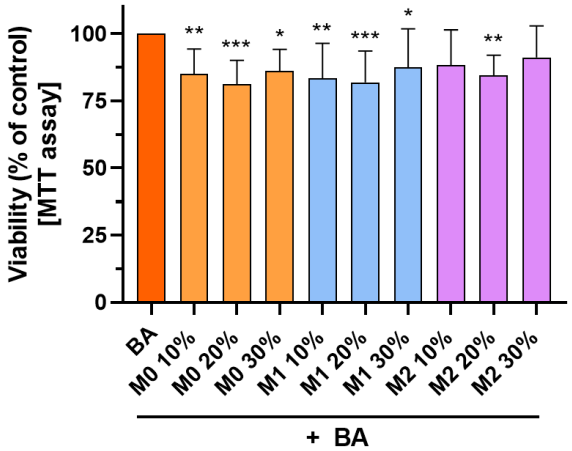


Figure 30. Cell viability evaluated by MTT assay expressed as fold change compared to BA-treated cells. The data are presented as mean ± standard deviation. BA = butyric acid treated cells; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages

*supernatant. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs BA where *p < 0.05, **p < 0.01, ***p < 0.001.*

4.3.2 Effect of polarized macrophages supernatants on the EoL-1 phenotype

In order to investigate the effect of macrophages supernatants on the differentiation of EoL-1 cells towards a resident or inflammatory phenotype, surface markers and gene expression of EoL-1 cells were analyzed after stimulation with BA and supernatants derived from macrophages differentiated into M0, M1 and M2. Due to the data obtained from the cell viability and growth rate, we tested the concentration of 30% v/v of macrophages supernatant to obtain the greatest inflammatory effect on EoL-1 cells.

4.3.2.1 Surface marker expression by flow cytometry analysis

Flow cytometry analysis revealed changes in morphological parameters and surface marker expression, both population and functional markers. In all conditions in which EoL-1 cells were treated with BA in combination with supernatants derived from M0, M1 or M2 macrophages (30% v/v), a significant reduction in both cell size (FSC; Figure 31A) and internal complexity (SSC; Figure 31B) was observed compared to treatment with BA. In particular, in the BA + M0 supernatant condition, there is a reduction in FSC ($p < 0.0001$) and SSC ($p = 0.008$) and a similar effect was observed in the BA + M1 supernatant condition, with a significant decrease in FSC ($p < 0.0001$) and SSC ($p = 0.012$). Treatment with BA + M2 supernatant also results in a reduction in both parameters, with lower FSC ($p < 0.0001$) and SSC ($p = 0.017$) values compared to the control condition.

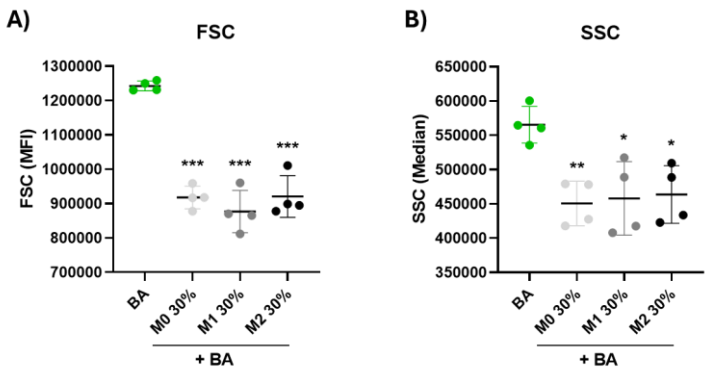


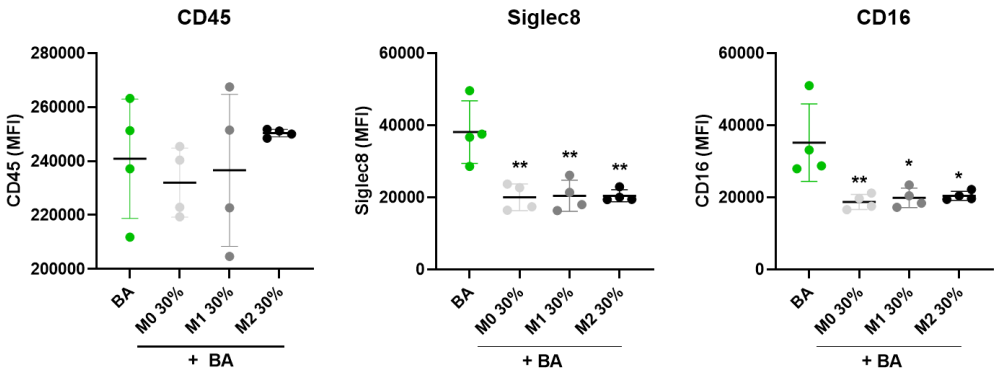
Figure 31. Morphological parameters evaluated by flow cytometry analysis: A) Forward Scatter (FSC); B) Side Scatter (SSC). The data are presented as mean ± standard deviation. BA = butyric

*acid; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages supernatant. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs BA where *p < 0.05, **p < 0.01, ***p < 0.001.*

The CD45 marker shows an average expression of 240878 ± 22103 (MFI) in BA condition. The addition of M0 supernatant or M1 supernatant does not result in significant changes. In the presence of M2 supernatant, an increased expression was observed, but this was not statistically significant.

The Siglec-8 marker has an average expression of 38152 ± 8659 (MFI) in cells treated with BA alone. The addition of supernatants results in a significant reduced expression of Siglec-8.

The CD16 marker has an average expression of 35240 ± 10751 (MFI) in cells treated with BA alone. The addition of supernatants results in a significant reduced expression of CD16.



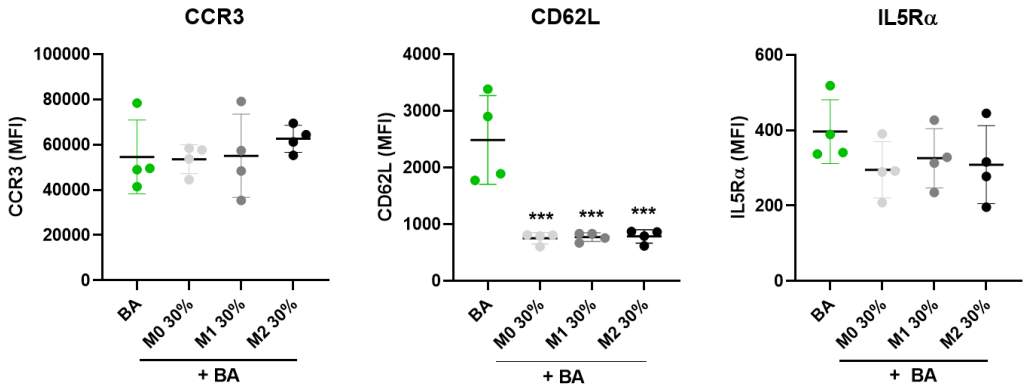
	BA	BA + M0 (30% v/v)	BA + M1 (30% v/v)	BA + M2 (30% v/v)
CD45	240878 ± 22103	232024 ± 12804 (ns)	236606 ± 28188 (ns)	250372 ± 1424 (ns)
Siglec-8	38152 ± 8659	20080 ± 3684 (p = 0.0019)	20499 ± 4328 (p = 0.002)	20496 ± 1680 (p = 0.002)
CD16	35240 ± 10751	18786 ± 2087 (p = 0.007)	19916 ± 2702 (p = 0.011)	20471 ± 1236 (p = 0.014)

*Figure 32. Population markers expression evaluated by flow cytometry analysis and expressed as median of fluorescence (MFI): CD45, Siglec-8, CD16. The data are presented as mean ± standard deviation. Raw data and p values are reported in the table below the graphs. BA = butyric acid; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages supernatant. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs BA where *p < 0.05, ** p < 0.01.*

The expression of the CCR3 in cells treated with supernatants does not differ significantly from that of cells treated with BA alone.

The CD62L had an average expression of 2488 ± 782.9 (MFI) in cells treated with BA alone. The addition of supernatants resulted in a significant reduction of CD62L expression.

The IL5Rα has an average expression of 396.5 ± 84.67 (MFI) in cells treated with BA alone. The addition of supernatants results in a non-significant reduced IL5Rα expression (Figure 33).



	BA	BA + M0 (30% v/v)	BA + M1 (30% v/v)	BA + M2 (30% v/v)
CCR3	54576 ± 16304	53568 ± 6383 (ns)	55071 ± 18402 (ns)	62585 ± 5931 (ns)
CD62L	2488 ± 782.9	753.9 ± 100.0 (p = 0.0003)	775.5 ± 77.07 (p = 0.0003)	787.1 ± 118.6 (p = 0.0003)
IL5Rα	396.5 ± 84.67	295.3 ± 74.59 (ns)	326.0 ± 78.85 (ns)	308.8 ± 103.7 (ns)

*Figure 33. Eosinophils functional markers expression evaluated by flow cytometry analysis and expressed as median of fluorescence (MFI): CCR3, CD62L and IL5Rα. The data are presented as mean ± standard deviation. Raw data and p values are reported in the table below the graphs. BA = butyric acid; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages supernatant. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs BA where ***p < 0.001.*

4.3.2.2 Gene expression of inflammatory mediators

To verify the effect of supernatants obtained from polarized macrophages on the gene expression of specific maturation, functional and inflammatory markers, an analysis of CCR3, CD62L, IL5Rα, IL-8, IL-13 and IL-5 transcripts was conducted.

CCR3 gene expression is increased in cells treated with BA and supernatants compared to BA stimulation alone [fold change: BA + M0 (16.50 ± 10.50), BA + M1 (23.49 ± 13.15) and BA + M2 (12.10 ± 7.08)]. CD62L gene expression is significantly reduced after treatment with BA and M0, M1 and M2 supernatants compared to BA stimulation alone [fold change: BA + M0 (0.071 ± 0.015; p < 0.0001), BA + M1 (0.081 ± 0.032; p < 0.0001) and BA + M2

(0.079 ± 0.027; p < 0.0001)]. Similarly, the expression of the IL5Rα gene is significantly reduced after combined treatment with BA and supernatants M0, M1 and M2 [fold change: BA + M0 (0.40 ± 0.02; p = 0.0013), BA + M1 (0.51 ± 0.14; p = 0.005) and BA + M2 (0.34 ± 0.07; p = 0.0007)] (Figure 34A).

Among the pro-inflammatory cytokines, IL-8 gene expression shows an increase, particularly evident in the BA + M1 condition (13.82 ± 9.58) compared to BA. IL-13 gene expression is increased after treatment with BA and M0, M1 and M2 supernatants compared to BA stimulation alone [fold change: BA + M0 (8.61 ± 7.95), BA + M1 (11.78 ± 8.58) and BA + M2 (8.91 ± 4.76)] (Figure 34B). Finally, IL-5 gene expression was increased in the BA + M0 (2.82 ± 2.04) and BA + M2 (2.80 ± 2.64) conditions, with high inter-sample variability, while it does not show changes in the BA + M1 condition (1.36 ± 1.03).

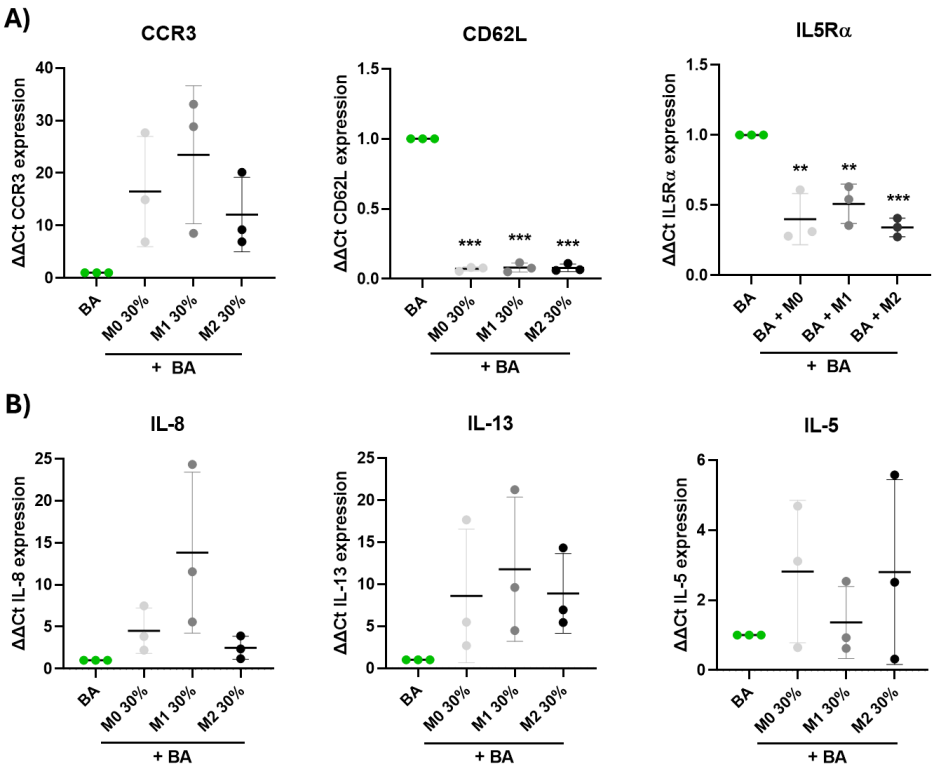


*Figure 34. Inflammatory mediators expression evaluated by Real-Time PCR: A) CCR3, CD62L, IL5Rα; B) IL-8, IL-13 and IL-5. The data are presented as mean ± standard deviation. BA = butyric acid; M0 = M0 macrophages supernatant; M1 = M1 macrophages supernatant; M2 = M2 macrophages supernatant. One-way ANOVA followed by Tukey's post hoc test was applied; * = p significant vs BA where **p < 0.01 and ***p < 0.001.*

To summarize, the results of this *in vitro* model indicate that treatment with butyric acid represents an effective experimental strategy for inducing the differentiation of the EoL-1

cell line and the stimulation with inflammatory stimuli (IL-5 and LPS) did not further increase the maturation of eosinophils in this experimental setting.

The integration of this treatment with inflammatory stimuli derived from differentiated macrophages (M0, M1, and M2) drove the cells toward a pro-inflammatory phenotype, as evidenced by increased CCR3 expression and decreased CD62L and IL5Rα.

4.4 Flow cytometry analysis of eosinophils in human peripheral blood

For this part of the thesis, 51 asthmatic patients and 16 healthy subjects (controls) were enrolled. There were no demographic differences between these two groups. Asthmatic patients had lower FEV1 and FEV1/FVC compared to the healthy controls ($p < 0.0001$). Eosinophils in whole blood, identified using flow cytometry analysis as $CD45^{+}$Siglec-$8^{+}CD16^{-}$ cells were then distinguished in inflammatory (low levels of CD62L) and resident (high levels of CD62L) eosinophils.

First of all, we settled the method of analysis investigating the coefficient of variation and the blood samples stability. The coefficients of variation (CV%) for both intra- and inter-sample measurement were calculated using the key parameter of our analysis (MFI of CD62L on eosinophils population) at two different time points: immediately after blood collection and 4 hours later. The intra sample CV is 3.03% ± 2.54 at time 0 and 2.25% ± 0.6 for the samples evaluated 4 hours later. The inter sample CV is 4.51% ± 3.19 at time 0 and 5.95% ± 1.91 for the samples evaluated 4 hours after.
These values align with the specifications provided in the instrument's user manual. Therefore, only larger differences between groups should be considered statistically significant in our analysis.

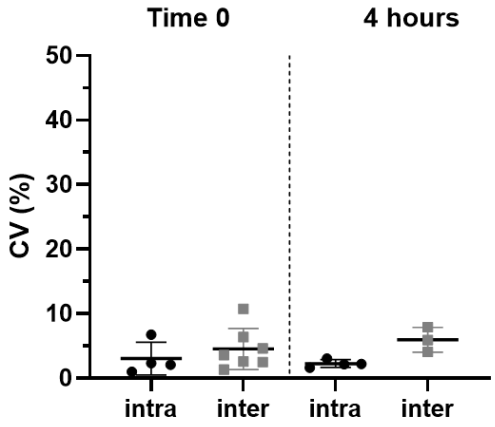


Figure 35. Coefficient of variation (CV%) for intra- and inter-sample measurements, evaluated at two different time points (time 0 and 4 hours).

The blood samples remain stable for up to 4 hours at room temperature (RT) following peripheral blood collection, as the flow cytometry analysis consistently yields the same

MFI for CD62L on eosinophils across different time points within this period (Figure 36A). The slight variations are compatible with the coefficient of variation. In figure 36B are reported the stability analysis performed for healthy subjects and asthma patients at time 0 and 4 hours after blood collection.

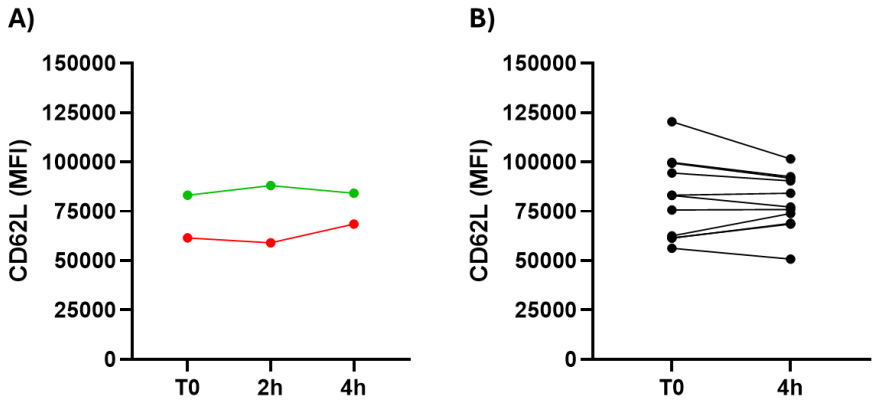


Figure 36. A) CD62L median of fluorescence intensity (MFI) of eosinophils evaluated at time 0 and at two time points: 2 hours and 4 hours after blood collection. Green points = healthy subject; red points = asthma patient. B) CD62L median of fluorescence intensity (MFI) of eosinophils evaluated at time 0 and 4 hours after blood collection.

4.4.1 Flow cytometry eosinophil analysis in asthmatic subjects

The percentage of iEos is significantly higher in asthmatic patients compared to the healthy controls [Median (range) 5.72 (0.81 – 22.22) vs 3.07 (1.12 – 25.33); p = 0.012] (Figure 37A). Correspondingly, the percentage of rEos is significantly reduced in the asthmatic patients compared to the healthy controls [94.28; (77.78 – 99.19) vs 96.94 (74.67 – 98.88); p = 0.012)] (Figure 37B). As the identification of inflammatory and resident eosinophils is based on the CD62L expression, the eosinophils median of fluorescence of CD62L is significantly higher in healthy subjects compared to asthmatic patients [97984 (61497 – 142956) vs 85349 (56349 – 124178); p = 0.019] (Figure 37C).

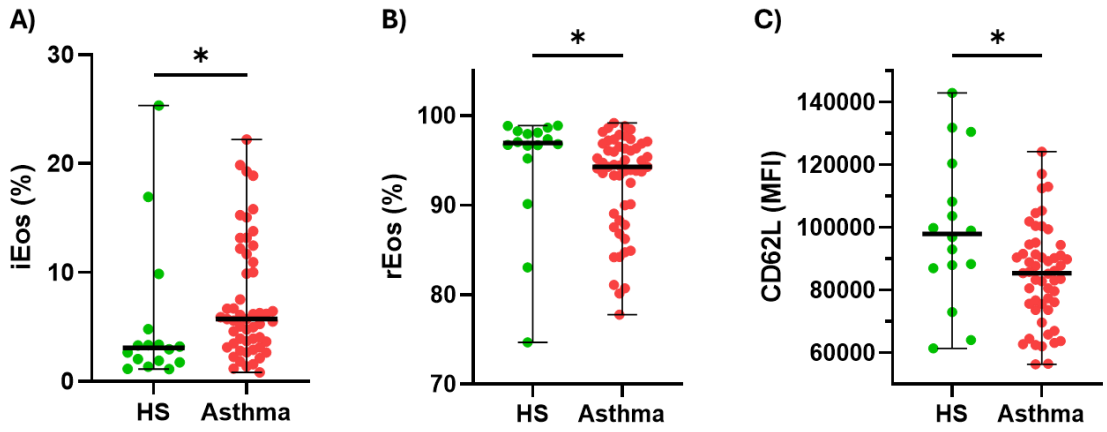


*Figure 37. Flow cytometry characterization of eosinophils in whole blood: relative distribution of eosinophils subsets. A) relative percentage of inflammatory eosinophils, B) relative percentage of resident eosinophils, C) CD62L median of fluorescence (MFI) of eosinophils. Green dots = healthy subjects (HS); red dots = asthmatic patients. Data are presented as scatter dot plot with median and range lines (minimum and maximum values). Mann-Whitney test was applied. * $p < 0.05$.*

The eosinophils expression of CCR3 and IL5Rα was then evaluated. The eosinophils median of fluorescence intensity of CCR3 is 291899 (167279 – 345709) for the healthy controls and 271319 (150575 – 426941) for asthma patients (Figure 38A). The eosinophils median of fluorescence intensity of IL5Rα is 1337 (624.6 – 2420) for healthy controls and 1405 (962.9 – 2624) for asthma patients (Figure 38B). No significant differences were found between the groups.

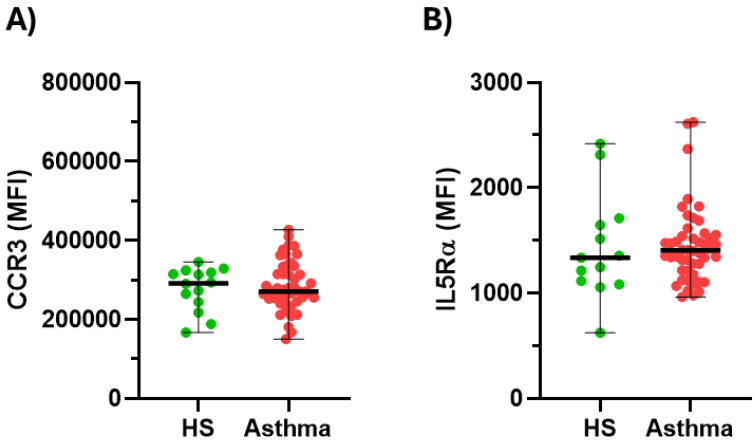


Figure 38. Median of fluorescence (MFI) of CCR3 (A) and IL5Rα (B) of eosinophils. Green dots = healthy subjects (HS); red dots = asthmatic patients. Data are presented as scatter dot plot with median and range lines (minimum and maximum values). Mann-Whitney test was applied.

4.4.2 Flow cytometry eosinophil analysis in COPD subjects

A small group of COPD patients (n = 6) was enrolled in the study to compare the eosinophils phenotype with healthy subjects.

Percentage of iEos is significantly higher in COPD patients compared to controls [11.19 (7.35 – 24.56) vs 3.07 (1.12 – 25.33); p = 0.003]. In the same way, the percentage of rEos of COPD patients is significantly lower compared to healthy controls [87.36 (75.44 – 92.65) vs 96.94 (74.67 – 98.88); p = 0.003]. The expression of CD62L follows the same trend: COPD patients present a significant lower CD62L expression compared to healthy controls [63086 (47217 – 106409) vs 97984 (61497 – 142956); p = 0.018].

The eosinophils CCR3 expression of COPD patients is not significantly different from healthy controls [256553 (135271 – 371503) and 291899 (167279 – 345709) respectively]. Similarly, also the eosinophils IL5Rα expression of COPD patients does not differ from the healthy controls one [1337 (624.6 – 2420) and 1480 (1102 – 2004) respectively].

Discussion

Eosinophils are pleiotropic granulocytes that play a key role both in the acute and chronic phase of inflammation. Eosinophils are traditionally recognized for their role in host defense against helminth infections through migration to infection sites triggered by type 2 (T2) immune response signaling but their functions range from cytotoxic effector to immunoregulation and tissue homeostasis [21, 22].

Recent studies discovered that eosinophils can be classified in two different subtypes: resident eosinophils (rEos) and inflammatory eosinophils (iEos), with different localization and functions. In particular, rEos, characterized by a high expression of CD62L, have a role in the tissue homeostasis while iEos, characterized by low expression of CD62L, mediate inflammatory processes [39].

Although the presence of the two subtypes of eosinophils has been described, less is known about the molecular mechanisms that lead to the establishment of inflammatory and resident eosinophils in humans. Since eosinophils constitute less than 5% of peripheral blood leukocytes and have a short lifespan, it is particularly difficult to study these cells *in vitro*. For these reasons, the use of the commercial cell line EoL-1 represents a valid option as eosinophil *in vitro* model, since it allows to obtain a stable and reproducible mature eosinophils population to study the molecular mechanisms that regulate their maturation and function. EoL-1 cells are immature myeloblasts cells isolated from a patient with eosinophilic leukemia that can be differentiated in mature cells by the use of butyric acid, an histone deacetylases inhibitor [59]. Despite a large use of these cells in the literature, a unique maturation protocol and a complete characterization of the cells is not clearly established.

In this study, we used human cell line EoL-1, treated with butyric acid (BA), to develop and characterize an in vitro model of eosinophilic differentiation.

Our results demonstrated that butyric acid treatment induces a maturation process indicated by a modification of the nuclear morphology, obtaining a bilobed nucleus. In particular, among the experimental conditions tested, the stimulation with BA 500 µM for 120 hours is the most effective in promoting differentiation, while maintaining an adequate level of cell viability. Consistent with the acquisition of a more mature phenotype, the analysis of viability and cell proliferation highlighted that the butyric acid treatment, while ensuring relatively high survival, significantly reduces the proliferative

capacity of EoL-1 cells. This phenomenon is consistent with observations made in other cell maturation models, in which the loss of replicative capacity is a crucial step in the transition from an immature, proliferative state to a functionally specialized state. Therefore, the decrease in proliferation not only confirms the effectiveness of the treatment in the modulation of the differentiation process, but also represents an indirect indicator of the acquisition of a more advanced cell stage.

Morphological characterization of cells treated with butyric acid revealed changes consistent with the progressive acquisition of a mature eosinophilic phenotype. In particular, was observed a marked nuclear segmentation, a morphological parameter indicative of an advanced stage of differentiation, associated with an increase of intracytoplasmic complexity, likely related to a higher density of specific granules typical of the eosinophil line.

BA-treated EoL-1 cells showed a significant increase in the expression of surface markers typical of the eosinophil population, such as Siglec-8 and CCR3, and of functional molecules, including CD62L and IL5Rα. At the same time, gene expression analysis confirmed the presence of transcripts characteristic of mature eosinophils, such as GATA1, ECP and CCR3, together with pro-inflammatory mediators and immune response regulators, such as IL-8 and IL-13.

Taken together, these findings suggest that this experimental protocol allows to obtain a cellular model that reproduces key aspects of the eosinophil maturation process, making it a highly useful experimental tool for functional and potentially translational studies.

Using this maturation protocol, we then evaluated the impact of inflammatory stimuli on the maturation process of the EoL-1 cells. In particular, the addition of lipopolysaccharide (LPS) and interleukin-5 (IL-5) aimed to reproduce in vitro conditions compatible with Th1/Th2-type inflammation, verifying the potential ability of these mediators to promote a functional and morphological differentiation compared to treatment with butyric acid alone. The morphological analysis revealed that the stimulation with IL-5 and LPS does not further increase the maturation of the cells. Furthermore, the growth rate evaluation, the eosinophils markers expression and maturation gene expression do not show any significant modifications compared to BA-treated cells. Finally, molecular analysis showed that cells treated with LPS tend to express higher levels of cytokines typically associated with inflammatory responses, such as IL-13 and IL-5. This data suggests that

LPS does not act directly as a promoter of eosinophil maturation, but rather as an inducer of inflammatory response cytokines. Therefore, our results indicate that these mediators, while contributing to the overall immune context, are not sufficient on their own, in this experimental model, to promote the maturation of EoL-1 cells.

The nature of the immune microenvironmental cues might influence the development of the different eosinophil's phenotypes (iEos and rEos). In this contest, macrophages have the optimal properties to orchestrate such microenvironmental cues, depending on their polarization. Indeed, we have recently shown that the classically activated (M1) macrophages and the alternatively activated (M2) macrophages have different roles in intercellular communications [82]. M1 macrophages, obtained from IFN-γ and LPS stimulation, are typically associated with inflammatory processes and the M2 macrophages, differentiated after IL-4 stimulation, are involved in anti-inflammatory processes and tissue remodeling. For this reason, the hypothesis of this study is that the M1 and M2 macrophages could differently regulate eosinophils differentiation in inflammatory and resident eosinophils.

The results obtained stimulating Eol-1 with supernatants derived from M0, M1 or M2 macrophage, showed a dose-dependent reduction in cell viability and proliferation. Furthermore, phenotypical and molecular analysis revealed a modulation in the expression of eosinophilic markers and genes associated with the inflammatory response. In particular, a reduction in CD62L expression was detected in all experimental conditions, with an increase of the CCR3 expression and a reduction of IL5Rα compared to the control condition.

This cellular inflammatory phenotype is associated with an increased expression of pro-inflammatory cytokines, such as IL-8 and IL-13, especially in samples stimulated with M1 supernatants. These results confirm the central role of the inflammatory microenvironment in modulating the differentiation processes and functions of eosinophils. However, while suggesting a tendency towards a more inflammatory profile in the presence of mediators derived from M1 macrophages these data do not allow to definitively outline a clear phenotypic differentiation (rEos-like and iEos-like) of EoL-1 cells in response to the different macrophages supernatants (M0, M1 and M2).

Eosinophils, as effector and regulatory cells of the immune system, have a central role in the pathophysiology of various pulmonary disorders, particularly in obstructive diseases such as bronchial asthma and chronic obstructive pulmonary disease (COPD).

Blood eosinophil level is one of the most important biomarkers of bronchial asthma and recently it has been described as a risk factor for COPD exacerbations [85]. Eosinophils are indeed experiencing renewed scientific interest as target of biological therapies for both asthma and COPD.

In this thesis, the characterization of circulating eosinophil subtypes in asthmatic and COPD patients and healthy control subjects were addressed using flow cytometry.

A preliminary step was the method validation. The intra- and inter-sample coefficients of variation for CD62L expression on eosinophils were within acceptable ranges and in line with those reported in the literature, confirming the reproducibility and robustness of the analysis. Furthermore, peripheral blood samples proved stable up to four hours at room temperature, ensuring that the observed differences across groups were not attributable to pre-analytical variability.

In asthmatic patients, iEos were significantly increased than in healthy subjects, with a corresponding reduction in rEos. Importantly, the reduced median fluorescence intensity (MFI) of CD62L in asthmatic patients further supports the predominance of inflammatory eosinophils, consistent with the chronic inflammatory milieu typical of asthma.

Interestingly, the analysis of eosinophil surface markers IL5Rα and CCR3 did not reveal significant differences between asthmatic patients and controls. This finding may indicate that these receptors, while essential for eosinophil recruitment and activation, are not substantially modulated at the circulating eosinophil level, at least under the conditions studied. It is possible that their regulation occurs primarily within the tissue microenvironment rather than in peripheral blood. Indeed, the low levels of IL5Rα, observed in blood eosinophils in this study, are in agreement with those reported in previous studies.

The small cohort of COPD patients analyzed revealed a similar trend to that observed in asthma, with a significantly higher percentage of iEos, reduced rEos, and lower CD62L expression compared to controls. Despite this, as observed in asthmatic patients, the eosinophils CCR3 and IL5Rα expression of COPD patients is not significantly different from healthy controls.

Although COPD is not classically considered an eosinophil-driven disease, our data suggest that eosinophil phenotypic changes may also play a role in the inflammatory processes of a subset of COPD patients.

The present work focused primarily on establishing and validating the methodological pipeline used to analyze eosinophil phenotypes in healthy subjects, asthma and COPD patients. For this reason, the clinical characterization of the cohort was intentionally limited to the confirmation of asthma and COPD diagnosis, which was sufficient for the preliminary validation steps described. A more comprehensive integration of clinical variables—such as allergic versus non-allergic asthma, total eosinophil counts, asthma severity, and the use of inhaled corticosteroids or biologic therapies—was beyond the scope of this methodological phase. However, these features are highly relevant for understanding the heterogeneity of eosinophil phenotypes in asthma.

In future studies the same analytical approach will be applied to a larger and clinically well-characterized cohort. This will allow us to investigate how specific clinical traits correlate with eosinophil subsets, whether particular phenotypes are enriched in certain patient subgroups, and how treatments may influence eosinophil distribution. Such analyses will be crucial to assess the translational value of the model and to explore its potential applications in patient stratification and personalized medicine.

A potential limitation of our study is that, although the use of an in vitro differentiated cell line offers significant advantages in terms of reproducibility and experimental control, it cannot fully capture the biological complexity and functional heterogeneity of eosinophils isolated from human blood. Similarly, stimulation with macrophages supernatants represents a simplification of the physio-pathological condition which, in this experimental setting, does not allow the effect of each cytokine and inflammatory element released by macrophages to be isolated. In addition, we could not exclude that IFNγ, LPS or IL-4, the stimuli used for macrophages polarization, are still present and active in the supernatants that were used for eosinophils stimulation. For this reason, future investigations will include quantitative assessment of cytokine carry-over as well as broader controls to distinguish macrophage-derived mediators from residual stimuli.

Conclusions and future perspectives

In conclusion, the results of this thesis indicate that treatment with butyric acid represents an effective experimental strategy for inducing the differentiation of the EoL-1 cell line. Treatment with butyric acid effectively induced morphological, phenotypic and molecular changes consistent with eosinophil maturation, associated with an increase expression of specific markers such as CCR3, IL5Rα and CD62L.

The stimulation with inflammatory stimuli (IL-5 and LPS) did not further increase the maturation of eosinophils in this experimental setting.

The integration of this treatment with specific inflammatory stimuli derived from differentiated macrophages (M0, M1, and M2) drove the cells toward a pro-inflammatory phenotype, as evidenced by increased CCR3 expression and decreased CD62L and IL5Rα, while also enabling the in vitro reproduction of conditions that more closely mimic the complexity of the tissue microenvironment in pathophysiological contexts.

Finally, our study demonstrated that asthmatic and COPD patients present higher levels of inflammatory eosinophils (iEos) in peripheral blood compared to healthy controls suggesting that these cells play a role in the inflammatory milieu in asthma and COPD.

Therefore, the findings of this thesis provide valuable insights into the cellular and molecular mechanisms regulating eosinophil function in chronic inflammatory lung diseases such as asthma and COPD, while also offering new opportunities for identifying potential therapeutic targets and developing innovative intervention strategies.

In light of these aspects, future studies should aim to further validate the proposed experimental model through the use of human eosinophils isolated from peripheral blood, in order to confirm the biological relevance of the results obtained and overcome the intrinsic limitations associated with the use of immortalized cell lines. This step is essential to ensure greater translationality of the data and to obtain a more realistic picture of eosinophil responses in physiological and pathological contexts.

At the same time, it will be essential to extend the analyses including a more in-depth functional characterization of the cells, through the integration of specific tests aimed at evaluating key parameters of eosinophil activity, such as degranulation assays, reactive oxygen species production and chemotactic migration.

Furthermore, it will be important to explore the effect of additional mediators of the inflammatory microenvironment, such as cytokines typically associated with the Th2

response, including IL-4 and IL-13, as well as specific chemokines involved in the recruitment and activation of eosinophils, such as Eotaxin-3. The addition of these stimuli may help to delineate more precisely the mechanisms that regulate the functional polarization of eosinophils in different pathological contexts.

Finally, it will be equally important to consider the dynamic interaction between eosinophils and other key cell populations present in the tissue microenvironment, such as T lymphocytes, which orchestrate the adaptive immune response, and epithelial cells, which are an important source of pro-inflammatory and regulatory signals. The integration of these components into the model will allow the development of a more complex and physiologically relevant system, useful for a complete understanding of the role of eosinophils in chronic inflammatory processes and associated pathologies.

Other studies

During the PhD, I collaborated to other studies that aim to investigate the molecular mechanisms that lead two chronic lung diseases: Chronic Obstructive Pulmonary Disease (COPD) and Idiopathic Pulmonary Fibrosis (IPF). Both of them are linked to chronic smoking exposure, which causes lung epithelial injury and a subsequent immune-inflammatory response. This initial epithelial damage determines the release of intracellular antigens usually not exposed to the immune system, and in predisposed patients this causes a chronic immune-inflammation against lung tissue. It is still unclear why, despite this common initial tissue damage, the two diseases evolve in a completely different pathogenetic and clinical way.

Study 1: "Macrophages-derived Factor XIII links coagulation to inflammation in COPD."

COPD is characterized by a smoke-induced lung tissue damage that leads to a chronic adaptive immune-inflammatory reaction, where macrophages, dendritic cells and T lymphocytes play a key role. CD8 T-lymphocytes, crucial adaptive immune cells, infiltrate bronchiolar airways and lung parenchyma, and it is well known that their levels correlate with the severity of the disease. Given the central role of the adaptive immune-inflammation, we focused our research on the coagulation system, particularly Factor XIII (FXIII), which is involved both 1) in the regulation of inflammation by modulating inflammatory cells activity, such as in macrophages and in dendritic cells which are pivotal in COPD, as well as 2) in the healing process after tissue damage by stabilizing fibrin deposits. These observations induced us to examine whether FXIIIA might also be expressed in alveolar macrophages and dendritic cells in COPD lungs, indicating a possible role in the adaptive immune response. What we found is that in patients with COPD there was an important expression of FXIIIA in alveolar macrophages (AM) and Dendritic Cells (DC-1) compared to controls. Furthermore, FXIII expression in AM increased with increasing airflow obstruction in COPD and positively related to the number of dendritic cells (DC-1) in the small airways. Moreover, both FXIIIA in AM and DC-1 numbers were positively correlated with the numbers and activity of T-cells in the lung parenchyma and with the severity of the disease. An important finding that needs to be underlined is that, for the first time in the literature in COPD, we documented the presence of FXIII expressing AM and DC-1 at confocal microscopy in the lung parenchyma

and in the small airways respectively, and we were also able to define its corpuscular cytoplasmic distribution in the cell. In our view these results are relevant since they define a mechanistic link between FXIII expression in immune inflammatory cells both in the small airways and in the lung parenchyma in COPD with the worsening of lung function. These results were published in the following peer reviewed journal:

Macrophages-derived Factor XIII links coagulation to inflammation in COPD. Bazzan E, Casara A, Radu CM, Tinè M, Biondini D, Faccioli E, Pezzuto F, Bernardinello N, ***Conti M****, Balestro E, Calabrese F, Simioni P, Rea F, Turato G, Spagnolo P, Cosio MG, Saetta M. Front Immunol. 2023 Apr 25;14:1131292. doi: 10.3389/fimmu.2023.1131292.*

<u>Study 2</u>: "Do Circulating Extracellular Vesicles Strictly Reflect Bronchoalveolar Lavage Extracellular Vesicles in COPD?"

The immune-inflammatory response elicited by the epithelial injury in COPD is modulated by inflammatory cells interact and reciprocally modulate their cell activity. This interaction takes place with many mechanisms, such as the release of cytokines, chemokines, physical contact, however mounting literature has proved that among these, the release of extracellular vesicles (EVs) represents a cutting-edge crucial mechanism of cell-cell communication. EVs, which are defined as lipid bilayer membrane-delimited particles that carry biomolecules such as miRNA and cytokines, play a significant role in modulating the inflammatory response. As a matter of fact, we have already proved with an analysis of EVs isolated from bronchoalveolar lavage in COPD patients, that EVs are directly involved in the modulation of the inflammatory response, and they represent a potential valuable clinical biomarker. To further develop this line of research, we investigated whether a potential isolation of EVs from a blood sample could give the same results as those from BAL, and hence could represent a valuable "liquid biopsy" in COPD. To do so, we conducted a study to assess whether EVs detected in blood would reliably mirror those identified in bronchoalveolar lavage (BAL) in COPD. Our study of EVs in both blood and BAL from smokers with and without COPD and non-smoking controls showed that there were profound differences in EVs levels between BAL and blood. Our results from EVs in BAL showed a higher number of macrophage-(CD14+), epithelial-(CD326+), and activated endothelial cell-derived EVs

(CD62E+) as compared to blood samples. Moreover, the expression of CD62E, a marker of activated endothelial cells expressing E-selectin, was higher in the BAL of smokers with COPD compared to control smokers and nonsmokers, results not reported from the analysis of plasma. Differently, non-activated endothelial cell-derived EVs (CD146+) were much more abundant in plasma than BAL but were similar in all groups.

These results, in our opinion have a significant clinical meaning, since it is crucial, in order to use the blood test as a "liquid biopsy", to have a correspondence between BAL and blood levels of EVs. In our study this is not the case, having reported significant differences in the levels of EVs among the two. We can conclude that, given the significant differences reported in the inflammatory lung, it is recommended to proceed with an analysis of BAL-derived EVs in the development of future clinical biomarkers.

These results were published in the following peer reviewed journal:

Do Circulating Extracellular Vesicles Strictly Reflect Bronchoalveolar Lavage Extracellular Vesicles in COPD? Tinè M, Neri T, Biondini D, Bernardinello N, Casara A, ***Conti M****, Minniti M, Cosio MG, Saetta M, Celi A, Nieri D, Bazzan E. Int J Mol Sci. 2023 Feb 3;24(3):2966. doi: 10.3390/ijms24032966.*

<u>Study 3</u>: "Suppressor of cytokine signaling-3 expression and its regulation in relation to inflammation in Chronic Obstructive Pulmonary Disease."

An important feature of extracellular vesicles consists in the ability of delivering a molecular cargo to target cells. This cargo can consist of different molecules, such as RNAs, proteins, but among these there is no doubt miRNAs play a fundamental role. miRNAs are small non-coding RNAs with an average 22 nucleotides in length and can regulate gene-expression post-transcriptionally by binding to the 3'-untranslated region on messenger RNA (mRNA), suppressing translation or inducing degradation of the target mRNA. Targeting specific mRNA allows for the ability to effectively modulate a specific biological effect, and it is now well established that inflammation can be regulated by miRNAs. Hence, we next decided to investigate whether EVs derived miRNAs could also be involved in the regulation of inflammation in COPD. In particular, we focused on the regulation of a specific family of proteins, SOCS3, that play a crucial role in negatively regulating the JAK-STAT signalling pathway which is involved in inflammation. It is reported

in the literature that many miRNAs are involved in the regulation of SOCS3, and among these we decided to concentrate on miRNA-19a-3p and miRNA-221-3p. In our study we isolated lung alveolar macrophages and BAL EVs from smokers with and without COPD and non-smokers' to investigate the presence of SOCS3, its relation to the severity of disease and lung inflammation, and whether its production could be linked to miRNA-19a-3p and miRNA-221-3p. Indeed, we reported that SOCS3 expression in tissue AM was higher in smokers with and without COPD than in non-smokers, while SOCS3 expression in BAL AM derived EVs (SOCS3+ CD14+ EVs) was higher in smokers with COPD than in those without COPD and in non-smokers. Furthermore, we investigated the levels of miRNA-19a and miRNA-221 in BAL macrophage EVs and showed that while smokers without COPD had high levels of both miRNA19 and miRNA221 – inhibiting EV SOCS3 production – smokers with COPD had a significantly lower miRNA expression, in the attempt to keep high levels of SOCS3.

These data revealed that the possible expression of SOCS3 in BAL macrophage-derived EVs might be useful to assess the degree of lung inflammation and possible progression of COPD, and miRNA-221 and miRNA-19a might be involved in the molecular regulation of SOCS3. Our research group had previously demonstrated that EVs play an important role in the modulation of the inflammation in COPD, and they can be used as clinical biomarkers. This new project adds further insight to this line of research as it indicates that, mechanistically, EVs-derived miRNAs in patients with COPD are directly involved in the regulation of proinflammatory pathways, and hence BAL EVs may be used both for the study of the disease and as clinical biomarkers.

These data were published in the following peer reviewed journal:

Suppressor of cytokine signaling-3 expression and its regulation in relation to inflammation in Chronic Obstructive Pulmonary Disease. Tinè M, Balestro E, Carpi S, Neri T, Biondini D, ***Conti M****, Casara A, Bernardinello N, Cocconcelli E, Turato G, Baraldo S, Celi A, Spagnolo P, Cosio MG, Saetta M, Bazzan E. Front Immunol. 2024 Mar 12;15:1320077. doi: 10.3389/fimmu.2024.1320077.*

<u>Study 4</u>: "Unveiling the Cutting-Edge Impact of Polarized Macrophage-Derived Extracellular Vesicles and MiRNA Signatures on TGF-β Regulation within Lung Fibroblasts."

The other smoking related lung disease, Idiopathic Pulmonary Fibrosis (IPF), despite sharing the same initial trigger of COPD evolves in a completely different manner. In the disease, the smoking-induced epithelial damage induces and immune-inflammatory response that determines a progressive replacement of normal lung tissue with extracellular matrix, leading to disruption of gas exchange and progressive respiratory failure. From a pathogenetic stand point, the attempt of repairing the epithelium damage evolves in a pathologic inflammatory reaction, characterized by macrophages recruitment in the lung and polarization into "pro-fibrotic" macrophages, either M1 or M2 according to the inflammatory milieu. Profibrotic macrophages cross-talk and eventually activate fibroblasts which secrete profibrotic cytokines, among which TGF-β is key, responsible for fibroblast proliferation and extracellular matrix deposition.

To investigate the mechanisms of the cross-talk between polarized macrophages and fibroblasts in pulmonary fibrosis, we hypothesized that polarized macrophages-derived EVs could play an important role. As a matter of fact, macrophages derived EVs can deliver molecules such as miRNAs, that can post transcriptionally regulate target gene expression. It is well known that specific miRNAs, such as miRNA-19a-3p and miRNA-425-5p, can selectively modulate the expression of TGF-β, targeting downstream effector SMADs, TGF-β receptors or TGF-β transcription. For these reasons we decided to investigate the possible effect of polarized macrophages-derived EVs (M0, M1 and M2 macrophages) and their miRNA-19a-3p and miRNA-425-5p cargo on the production of TGF-β by lung fibroblasts. We found that the production of TGF-β in lung fibroblast was significantly higher after stimulation with M0/M1 macrophages-derived EVs than with M2 derived EVs. Our molecular analysis of miRNAs levels within EVs demonstrated higher levels of miRNA-425-5p in M2 compared to both M1 and M0 derived EVs. Conversely, miRNA-425-5p expression levels were not significantly different in M1 compared to M0-derived EVs. In our opinion these results are relevant since miRNA-425-5p has an inhibitory effect on TGF-β production, and hence, it could explain why both M0 and M1, but not M2 macrophages-derived EVs induced a significant increase of TGF-β production in fibroblasts. No relevant findings were reported on the expression of miRNA-19a-3p. In conclusion, with this in vitro experimental model we gained new insight on the possible

molecular mechanisms of pulmonary fibrosis, highlighting how polarized macrophages derived EVs can be involved in delivering specific miRNAs that can modulate fibroblast TGF-β production, a crucial pathway in the progression of lung fibrosis.

Given the important role of polarized macrophages in the fibrotic process, we decided to move forward in the investigation developing an in vitro model of macrophages polarization, to better define the molecular signatures and the expression of specific markers of cell polarization. As a matter of fact, macrophages polarization is still debated in the literature, both in the in-vitro setting as well as in human disease. For these reasons, we applied a well known macrophages differentiation protocol to assess the mRNA expression of M1/M2 markers by Real-Time PCR. We found that the levels of mRNA of Alox15, which is a well known M2 differentiation marker, were much higher in M2 polarized macrophages compared to THP-1/M0/M1 cells. Moreover, mRNA levels of IRF1 and STAT1, M1 polarization markers, were much higher in M1 polarized macrophages compared to THP-1/M0/M2. These reported results on mRNA expression clearly highlighted an efficient molecular polarization of macrophages.

These data were published in the following peer reviewed journal:

Unveiling the Cutting-Edge Impact of Polarized Macrophage-Derived Extracellular Vesicles and MiRNA Signatures on TGF-β Regulation within Lung Fibroblasts. Casara A,* ***Conti M****, Bernardinello N, Tinè M, Baraldo S, Turato G, Semenzato U, Celi A, Spagnolo P, Saetta M, Cosio MG, Neri T, Biondini D, Bazzan E. Int J Mol Sci. 2024 Jul 8;25(13):7490. doi: 10.3390/ijms25137490. *These authors have contributed equally to the manuscript and share first authorship.*

Other publications:

1. **Conti M***, Casara A*, Turato G, Baraldo S, Tinè M, Semenzato U, Cocconcelli E, Biondini D, Damin M, Saetta M, Cosio MG, Balestro E, Spagnolo P, Bazzan E, Bernardinello N. What do we know about extracellular vesicles in patients with idiopathic pulmonary fibrosis? a scoping review. Front Immunol. 2025 Jul 2;16:1541645. *These authors have contributed equally to the manuscript and share first authorship.

2. Cenerini G, Chimera D, Pagnini M, Bazzan E, **Conti M**, Turato G, Celi A, Neri T. The Intricate Relationship Between Pulmonary Fibrosis and Thrombotic Pathology: A Narrative Review. Cells. 2024 Dec 18;13(24):2099.

3. Carpi S, Polini B, Nieri D, Doccini S, **Conti M**, Bazzan E, Pagnini M, Santorelli FM, Cecchini M, Nieri P, Celi A, Neri T. Extracellular Vesicles Induce Nuclear Factor-κB Activation and Interleukin-8 Synthesis through miRNA-191-5p Contributing to Inflammatory Processes: Potential Implications in the Pathogenesis of Chronic Obstructive Pulmonary Disease. Biomolecules. 2024 Aug 19;14(8):1030.

4. **Conti M***, Minniti M*, Tiné M, De Francesco M, Gaeta R, Nieri D, Semenzato U, Biondini D, Camera M, Cosio MG, Saetta M, Celi A, Bazzan E, Neri T. Extracellular Vesicles in Pulmonary Hypertension: A Dangerous Liaison? Biology (Basel). 2023 Aug 7;12(8):1099. *These authors have contributed equally to the manuscript and share first authorship.

5. Tinè M, Padrin Y, Bonato M, Semenzato U, Bazzan E, **Conti M**, Saetta M, Turato G, Baraldo S. Extracellular Vesicles (EVs) as Crucial Mediators of Cell-Cell Interaction in Asthma. Int J Mol Sci. 2023 Feb 28;24(5):4645.

Collaborations

During my PhD I had the opportunity to collaborate with Prof. Marina Camera (University of Milan and Centro Cardiologico Monzino IRCCS, Milano) on projects aimed to explore the relationship between hemostatic system and inflammation, in particular during acute phase of SARS-CoV-2 infection, in Long COVID syndrome or induce by COVID-19 vaccines. The prothrombotic phenotype of the patients and subjects enrolled was assessed in terms of platelet activation and extracellular vesicles (EVs) release. Here are the publications:

1. Brambilla M, Fumoso F, **Conti M**, Becchetti A, Bozzi S, Mencarini T, Agostoni P, Mancini ME, Cosentino N, Bonomi A, Nallio K, Galotta A, Pengo M, Tortorici E, Bosco M, Cernigliaro F, Pinna C, Andreini D, Camera M. Low-Grade Inflammation in Long COVID Syndrome Sustains a Persistent Platelet Activation Associated With Lung Impairment. JACC Basic Transl Sci. 2024 Nov 27;10(1):20-39.

2. Brambilla M, Becchetti A, Rovati GE, Cosentino N, **Conti M**, Canzano P, Giesen PLA, Loffreda A, Bonomi A, Cattaneo M, De Candia E, Podda GM, Trabattoni D, Werba PJ, Campodonico J, Pinna C, Marenzi G, Tremoli E, Camera M. Cell Surface Platelet Tissue Factor Expression: Regulation by P2Y12 and Link to Residual Platelet Reactivity. Arterioscler Thromb Vasc Biol. 2023 Oct;43(10):2042-2057.

3. Brambilla M, Frigerio R, Becchetti A, Gori A, Cretich M, **Conti M**, Mazza A, Pengo M, Camera M. Head-to-Head Comparison of Tissue Factor-Dependent Procoagulant Potential of Small and Large Extracellular Vesicles in Healthy Subjects and in Patients with SARS-CoV-2 Infection. Biology (Basel). 2023 Sep 13;12(9):1233.

4. Lammi C, Fassi EMA, Manenti M, Brambilla M, **Conti M**, Li J, Roda G, Camera M, Silvani A, Grazioso G. Computational Design, Synthesis, and Biological Evaluation of Diimidazole Analogues Endowed with Dual PCSK9/HMG-CoAR-Inhibiting Activity. J Med Chem. 2023 Jun 22;66(12):7943-7958.

5. Brambilla M, Canzano P, Valle PD, Becchetti A, **Conti M**, Alberti M, Galotta A, Biondi ML, Lonati PA, Veglia F, Bonomi A, Cosentino N, Meroni PL, Zuccotti GV, D'Angelo A, Camera M. Head-to-head comparison of four COVID-19 vaccines on platelet activation, coagulation and inflammation. The TREASURE study. Thromb Res. 2023 Mar;223:24-33.

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