

A TISSUE-ENGINEERED MODEL FOR THE STUDY
OF AN ALLERGIC INFLAMMATORY RESPONSE

By

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A TISSUE-ENGINEERED MODEL FOR THE STUDY
OF AN ALLERGIC INFLAMMATORY RESPONSE

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You gave me wings and made me fly

To my parents

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Abstract:

Allergy occurs when the immune system overreacts to allergens. Worldwide, up to 40% of the population suffers from different types of allergies. In severe cases, allergic reactions can lead to death. Therefore, determining the factors that are involved in the development of an allergic immune response is critical. Mast cells (MCs) are the foremost inducers of allergic responses, but their role in the initiation of the response is not well understood. Due to the effect of the extracellular matrix components on the tissue morphogenesis and cell behavior, utilizing a tissue model that closely mimics MC microenvironmental conditions *in vivo* has greater relevance for *in vitro* studies. Therefore, our goal was to develop a tissue-engineered model to determine the role of MCs in orchestrating an immune response by investigating the interaction between MCs and monocytes in response to an allergen, and the corresponding effect on lymphocyte function in an IgE-independent manner. The tissue-engineered model is comprised of two separate modules that together recapitulate an allergic response. In the first module, MCs were generated from progenitor cells in the presence of fibroblasts and endothelial cells. Endothelial cells are involved in leukocyte recruitment during inflammation and, along with fibroblasts, support the development of MCs. Generated cells within the tissue model exhibited MC morphology, immunophenotype, and released histamine in IgE-mediated reactions, showing typical MC functional phenotype in immediate-type allergenic responses. Monocytes, in response to the allergen-activated model, differentiated into CD1c⁺ cells, displaying immunophenotypic characteristics of dendritic cells. However, MCs mitigated the effect of the allergen on the development of CD1c⁺ cells and marginally influenced the expression of the molecules required for T cell activation. In the second module, monocyte-derived cells triggered the proliferation of T and B cells. MCs indirectly induced the emergence of IgE-producing plasma cells indicating that MCs can play a role in the initiation of an allergic response. In addition to investigating the mechanism of an allergic response, the tissue-engineered model can be used as a tool for personalized diagnosis of allergies, testing the effect of drugs for allergy treatment, and for studying other inflammatory disorders, such as asthma and angiogenesis.

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CHAPTER 1

Introduction

1.1. Background and significance

Allergic disorders are among the most common chronic diseases. Worldwide, environmental factors causes sensitization in 40% of the population [1]. In the U.S., more than 50 million people (more than 20% of the population) suffer from allergy, which placed it as the sixth leading cause of chronic diseases, costing \$18 billion annually for health care [2]. Furthermore, 10.3% and 12.1% of children under age 18 suffer from respiratory and skin allergies, respectively [3]. In severe cases, such as in food-related anaphylaxis and asthma, allergic reactions can cause unconsciousness and at least hundred death annually [4, 5]. Skin and blood tests are widely used for allergy diagnosis but their usefulness is limited by the possibility of severe reactions and lack of sufficient accuracy and reliability. The common occurrence, morbidity, and health care costs associated with allergic reactions signify the importance of determining the key factors that regulate an allergic response, which can lead to better diagnostic and therapeutic strategies.

Reaction of the immune system to certain substances known as allergens results in the activation of specific white blood cells and an inflammatory response. The overreaction or hypersensitivity of the immune system is called allergy, which causes symptoms, such as coughing, sneezing, runny nose, and in severe cases hives, dermatitis, and asthma.

Major classes of allergens include a variety of food and non-food proteins, such as peanuts, wheat, latex; and environmental factors, such as dust or pollen [6]. In addition, genetic factors may make a person more susceptible to allergy. Previous genome screens showed linkages of genes on several chromosomes to atopic diseases [7]. The candidate genes on these chromosomes play roles in the release of mediators and cell surface receptors that are related to high IgE levels. For example, the genes related to interleukin (IL)-4 release are present on chromosome 5; while the genes for the FcεRI β chain receptor are present on chromosome 11 [8].

Currently, skin and blood tests are the only widely diagnostic tests used for evaluating allergies. A skin test can be performed in two ways: percutaneous (skin prick) and intracutaneous (intradermal). In skin prick testing, the potential allergen is seeped into the skin surface (epidermis) by using needles or skin scratchers. In the intradermal method, the allergen is injected deeper into the dermis. The skin reaction is measured by wheals and flares, and the intensity is compared to the controls [9, 10]. In blood tests, the total and/or specific IgE titers against a specific allergen is measured [11, 12].

The existing diagnostic tests carry significant disadvantages. Skin tests are prone to false results due to the effect of existing medications, quality and concentration of the allergen being tested, season of the year, site of the test on the body (variations between the back and forearm), and interpretation of the results [13]. In addition, it carries a serious possibility of anaphylaxis [14]. Blood tests are less sensitive, prone to false results and are more expensive [15, 16]. In addition, blood tests merely detect the presence of an allergen-specific antibody, which may not coincide with an actual clinical allergic response from a skin test.

Other tests for evaluating allergy are histamine release and basophil activation tests, which are not widely available. In these tests, the release of histamine or the upregulation of a cell surface activation marker by basophils (isolated from leukocyte preparations of the patient) is measured after challenge with an allergen [17, 18]. These tests are prone to false results

associated with the lack of sensitivity of the isolated basophils, are not predictive, and are also limited by the low viability and function of the basophils [19].

Table.1.1. Differences between human and animal mast cell (MC) function [20].

Feature	Murine MCs	Human MCs
Protease content	Several tryptase and chymases with different specificities (such as mouse MCP1-MCP14) and β -hexosaminidase	Three tryptases (α , β and γ); one chymase
Functional IL-3 receptor expression	High	Not detected from long cells
IL-4 production	Spontaneously released and to a greater extent after stimulation	Not produced spontaneously
IL-5 production	Low or has not been studied quantitatively	High
TNF production	High	Mucosal subtype produce low levels
Constitutive Fc γ RI expression	High	Low or needs activation
CD14 expression	Moderate positive expression	Not detected
Functional TLR expression	High	Not detected or low expression depending on the cell source and culture condition

Fc γ RI: high affinity receptor for IgG; IL: interleukin; MCP: mast-cell protease; TLR: Toll-like receptor; TNF: tumor-necrosis factor.

In order to have better strategies for allergy diagnosis and treatment, understanding the mechanism of development of the disease is necessary. Several *in vivo* animal models have been used to evaluate the dermal responses to an allergen or IgE levels in serum. Valuable information can be obtained from animal models about the mechanism of the disease. However, the reaction of animal models is not always identical to the human response. For instance, low concentrations of some antigens (chemicals) do not cause sensitization in some animals (like the guinea pig) as is the case for humans [21]. Also, the kinetics of the response might differ between human and animal bodies. For example, mouse models do not show spontaneous airway hyperresponsiveness and do not respond to histamine [22]. Furthermore, there are significant functional differences between human and animal cells, i.e. mast cells (MCs), as is shown in Table.1.1 [20]. For example, cell phenotype, cytokine release, degranulation, and response to mediators in murine models do not mimic human cell function [23].

Understanding of the difficulties associated with studying allergic inflammation in human are facilitated by using *in vitro* tissue models. Currently, there are no tissue-equivalent type model with the complexity of features, such as a blood endothelium, activation of MCs, monocyte differentiation to antigen presenting cells, and antigen presentation to lymphocytes that results in antibody production. Therefore, the goal of this project was to create a tissue model to mimic the main events of an allergic response by using human samples. The model is comprised of a collagen gel to mimic a layer of connective tissue and the cells that reside in connective tissue and play a role in the development of an allergic response (Fig. 1.1). Analyzing the cellular interactions in such a complex co-culture system is an improvement in studying the cellular behavior during allergic inflammation.

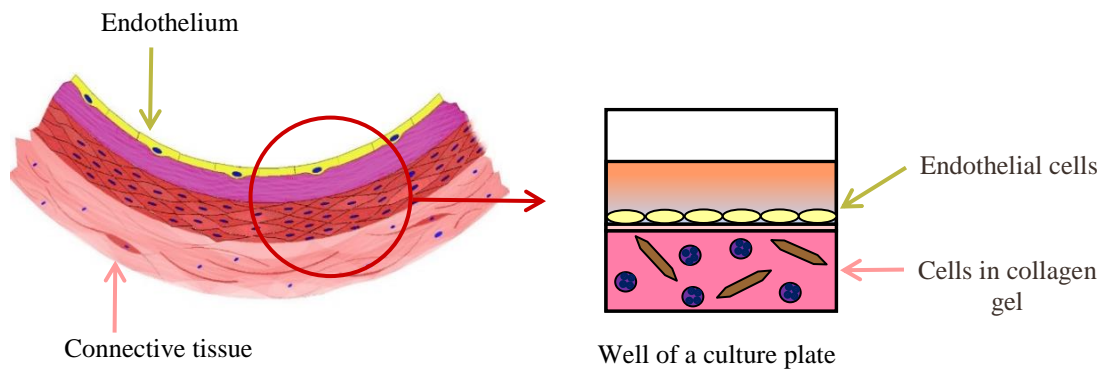


Fig. 1.1. Connective tissue-equivalent model. The model is comprised of collagen gel and the cells that play a role in the development of an allergic response.

1.1.1. Allergic inflammatory response development

After first contact with an allergen, activated lymphocytes produce a specific antibody that recognizes a unique part of the foreign target, called an antigen. Hence, immunity is acquired by establishing immunological memory, which causes a more rapid and effective response after the subsequent exposures to the same allergen. The subsequent exposure causes the release of proinflammatory mediators by immune cells, leading to the cell recruitment to the site of

challenge and ultimately redness, itchiness, swelling of the body, and other symptoms of an allergic inflammatory response [24].

Multiple cell types are involved in the formation of an adaptive immune response. Activated endothelial cells (ECs) express chemoattractants and adhesion molecules that cause the migration of the immune cells to the site of challenge. Dendritic cells (DCs) process and present antigens to T cells that carry specific receptors for each antigen. B cells produce IgE antibody after interaction with the antigen and activated T cells. MCs have receptors for the IgE antibody and release the content of their granules after activation, which affects the function of more immune cells [25].

The IgE-mediated reaction is called type-I hypersensitivity, which is divided into three major phases. During the sensitization phase, after the initial contact of the antigen, the IgE antibody associates with high-affinity IgE receptors that are expressed by MCs. In the activation phase, the second contact of the antigen results in cross-linking of the antigen with the IgE receptors, the degranulation and release of mediators, such as histamine from MCs. The response of MCs forms the activation phase that results in an inflammatory response in the effector phase, in which complex responses are caused by other cell types. Since the reaction occurs within a short time after the challenge, type I hypersensitivity is also called immediate hypersensitivity [25]. In this research, the role of MCs in the sensitization phase and in response to an allergen in the activation phase was studied.

1.1.2. Mast Cells (MCs) as central effector cells of an allergic immune response

MCs are important cell type in pathogenesis of allergy, as these cells are among the foremost inducers of an allergic response [26]. MCs are derived after their progenitors, which are circulating in blood, migrate into the tissue, differentiate and become mature [27]. MCs are abundant near surfaces exposed to the external environment, including the skin, gastrointestinal

tracts, respiratory organs and also present in secondary lymphoid organs and adjacent to blood vessels and the nervous system [28, 29]. Two MC subtypes; MC_T, which contains tryptase cytoplasmic granules and MC_{TC}, which contains tryptase and chymase cytoplasmic granules; reside in intestinal mucosa, skin, and submucosa [30]. Accordingly, MCs are positioned to be among the first cells (like DCs and macrophages) that interact with invading pathogens or allergens. As shown in Fig. 1.2, activated MCs secrete diverse mediators, such as histamine, cytokines, and proteases, which cause rapid vascular permeability, leading to leukocyte recruitment to the site of challenge [20]. Although MCs are known to play a role in the formation of type I hypersensitivity, their role in the initiation of an adaptive immune response is not well understood.

In conventional models, MC activation is dependent on IgE production, which occurs after an adaptive immune response is initiated. After the contact of DCs with an allergen, T cells are primed by antigen presentation of DCs in the lymph nodes through expression of MHC-II, co-stimulatory and adhesion molecules, and upregulation or downregulation of mediators (IL-10 and IL-12), inducing T cell proliferation and differentiation to the Th2 cell subtype [25, 31, 32]. Th2 cells release specific cytokines (IL-4, IL-5, IL-13) and express receptors (CD40L), which promote B cells to undergo antibody class switching to produce the IgE, eventually resulting in humoral immunity [25, 33, 34]. Later, the IgE binds with FcεRI receptors expressed by MCs and develop a stable antigen receptor, which causes MC sensitization. The subsequent exposure to the same allergen triggers the antigen receptor and MC activation. However, several other studies suggest that MCs can cooperate in the initiation of the adaptive immune response by affecting DCs, T or B cells, as shown in Fig. 1.3 [26]. By releasing mediators and expressing diverse receptors and co-stimulatory molecules, MCs can amplify and sustain the specific-IgE synthesis, directly through influencing T and B cells in the lymph node, or indirectly by exerting an effect on DCs in the connective tissue.

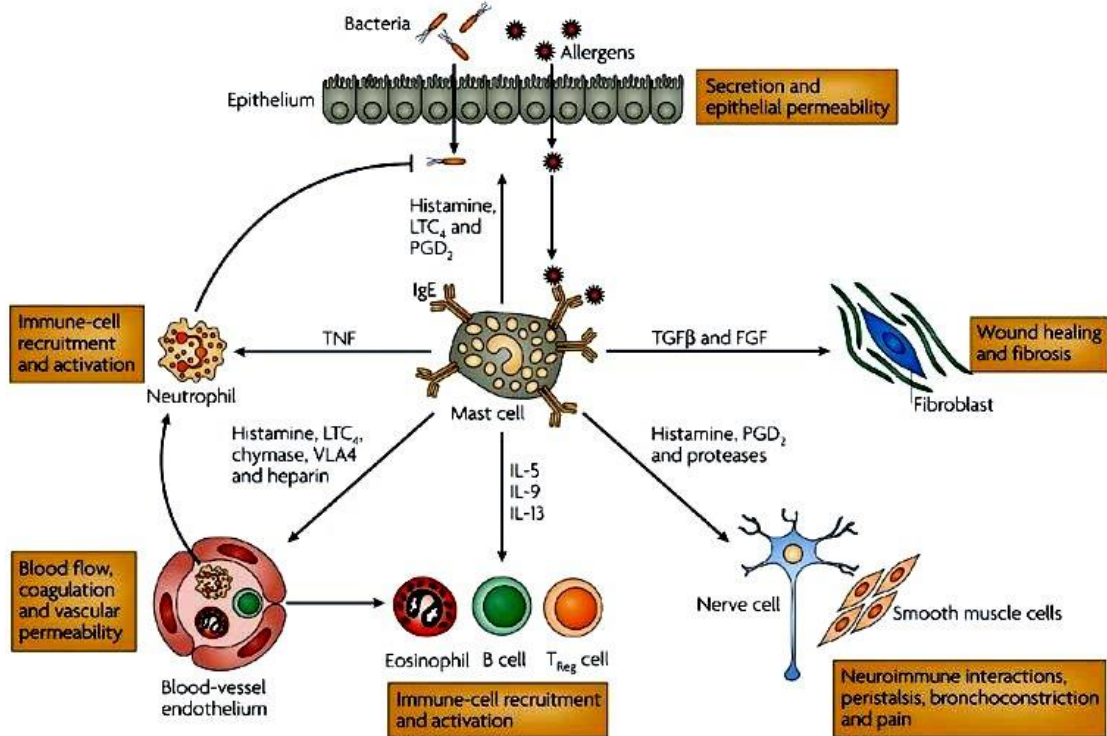


Fig. 1.2. Mast cell (MC) mediators can affect the function of various cell types [20]. FGF: fibroblast growth factor; IL: Interleukin; LTC₄: Leukotriene C₄; PGD₂: Prostaglandin D₂; TGFβ: transforming growth factor-β; TNF: tumor necrosis factor; T_{Reg} cell: CD4⁺ CD25⁺ regulatory T cell; VLA4: very late antigen 4.

Several MC mediators, such as TNF, PGD₂, IL-4, and mainly histamine can influence IL-10 and IL-12 production by DCs, enhance expression of MHC-II and co-stimulatory molecules on DCs, favor DC migration to the lymph node, and affect T cell mediator release and polarization to Th1 (cell-mediate immunity) or Th2 type (humoral immunity) [31, 32, 34-40]. By expressing MHC-II, co-stimulatory and adhesion molecules, MCs can act like DCs, migrate to lymph node, and present antigens to T cells [41-43]. MCs by expression of CD40L and secretion of IL-4 and IL-13 can induce B cell class switching to IgE production in the absence of T cells [33, 44]. However, for initiating the immune response and exerting an influence on other immune cells and lymphocytes, MCs should become activated by an allergen before IgE production. It is

hypothesized that allergens can activate MCs in an IgE-independent manner. In this work, by determining the role of MCs in orchestrating the adaptive immune response, the validity of the hypothesis was evaluated.

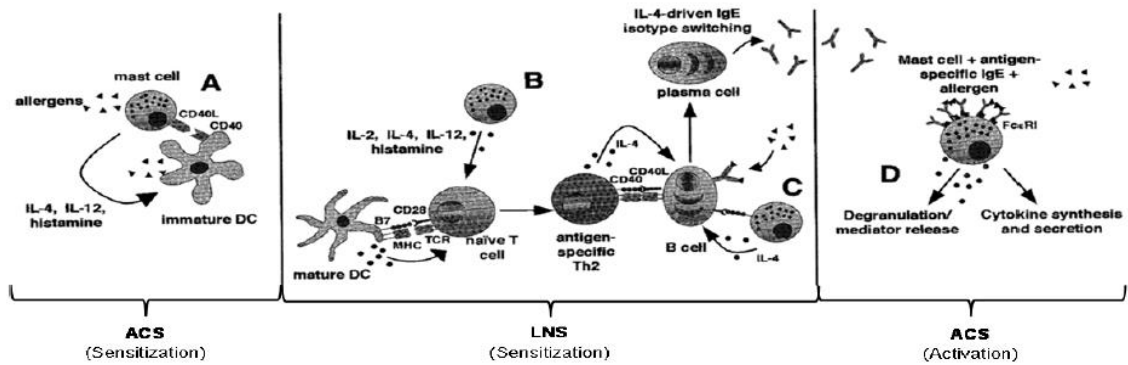


Fig. 1.3. Mast cells (MCs) can act at multiple sites to regulate adaptive immune response [26]. (A) MCs can be activated by an allergen and alter DC maturation and function. (B) In the lymph node, MCs can directly or indirectly modulate T cell fate. (C) Activated Th2 cells, in conjunction with the allergen, can stimulate allergen-specific B cells and promote B cell isotype switching to IgE. (D) Allergen specific IgE binds to MC receptors and causes MC degranulation after the subsequent exposure to the allergen.

In most of the mentioned studies, the effect of MC mediators was determined after addition of the mediators to the immune cells in the absence of MCs. The T cell-MC and B cell-MC interactions were studied by using *in vivo* animal models or *in vitro* two-dimensional (2D) cell systems, which mostly use animal cell lines. Although valuable information is obtained from studying MCs in 2D culture systems, neither of the previous models mimicked the human body. The 2D system is adequate for showing the cell behavior under various environmental factors; however, the 2D system cannot be used to examine multiple cell interactions and migration that are observed *in vivo*. A 3D tissue model consisting of human cells and a collagen matrix provides the added dimension for the control of many cellular mechanisms, such as MC and DC

interaction in the connective tissue. The investigation of human MCs within a 3D environment in this project, which is more similar to human physiology, expands the knowledge in MC function and role.

1.1.3. Mast cell (MC) origin and development

The addition of MCs to the tissue model is key to studying the allergic immune response. MCs are difficult to isolate from *in vivo* and maintain *in vitro*. Since MCs do not circulate in blood, there is no good source for fully mature MCs. MC precursors migrate through the blood system to the tissue and differentiate into the MCs. MCs can be obtained from tissue sections or intestinal mucosa provided from patients, but the isolated cells are low in number, do not survive in long-term culture systems, might undergo transformation, and do not function normally [45, 46]. In the presence of appropriate growth factors, MCs can be differentiated from their progenitors *in vitro*. MC progenitors can be isolated from various sources, such as bone marrow and peripheral blood [47-49]. Previous studies have investigated different cell types, such as mesenchymal cells, plasma cells, leukocytes, muscle cells, and T-lymphocytes as MC precursors [50-52]. Later, it was demonstrated that MCs can be derived from hematopoietic progenitors in bone marrow by developing MCs from transplanted bone marrow cells in mice [53]. Kirshenbaum et al. showed that CD34⁺/c-kit⁺ cells are precursors of human MCs [54]. Several other studies confirmed that MCs can be generated from progenitor cells in the presence of growth factors derived from T lymphocytes (IL-3, IL-6, IL-4, IL-9, and IL-10) and fibroblasts (SCF) [47, 48]. MC progenitors can also be obtained from various sources, such as bone marrow, fetal liver cells, umbilical cord blood, and peripheral blood. Irani et al. derived immature MCs after culturing fetal liver and cord blood cells with murine fibroblasts for four weeks in a lymphocyte conditioned media [55]. In the absence of fibroblast or lymphocyte conditioned media MCs cannot be developed.

CD34⁺ and CD133⁺ progenitor cells are chief sources for generating MCs *in vitro*. Saito et al. developed MCs from CD34⁺ cells isolated from cord blood and studied the effect of cytokines, such as SCF, IL-6, and IL-3 on MC proliferation for 8-10 weeks [56]. In the presence of SCF, IL-6 and PGE₂, MCs were mature, contained histamine and tryptase, and released histamine in challenge with IgE and anti-IgE. Mature MCs can also be developed from CD34⁺ cells isolated from peripheral blood in the presence of several growth factors added to the culture media at different time points [57]. The development of MCs from CD133⁺ cells, which include the CD34⁺ subset of progenitors, was performed by several researchers through isolating CD133⁺ cells from cord blood or peripheral blood [58, 59]. Comparison of the MC generation from CD133⁺ cells obtained from cord blood and peripheral blood shows that cells derived from peripheral blood were more mature and active because of higher histamine content and release after activation [60]. Several studies determined the effect of co-culture systems on the MC behavior and function. It was shown that direct interaction of adhesion molecules on ECs and c-kit receptor on MCs regulates MC development [61]. Fibroblasts can contribute to MC survival by releasing SCF and also through interaction of membrane SCF on fibroblasts and c-kit ligand on MCs [62-64]. Based on the mentioned studies, for this research, MCs were generated from CD133⁺ cells isolated from peripheral blood mononuclear cells cultured for seven weeks in the presence of cytokines, fibroblast and ECs that contribute to MC development.

1.1.4. Goal and specific aims

In this project, the goal was to develop a tissue-engineered model to determine the role of MCs in orchestrating an immune response. The first objective was to develop a tissue model that mimics a layer of human connective tissue. The model comprised of diverse cell types, such as ECs, fibroblasts, and immune cells, which are required for investigating the factors that regulate inflammation. The second objectives was to determine the role of MCs in development of the immune response by interacting with monocyte-derived cells. The tissue model used in this

research, consisted of the following two separate modules that together mimic an immune response to an allergen: the allergen contact site (ACS) and the lymph node site (LNS). As shown in Fig. 1.4, after activation by an allergen in the sensitization phase of the ACS, MCs release mediators and interact with DCs. DCs take up the antigen and migrate to the lymph node. In the LNS, MCs can indirectly influence T cell response type. Activated Th2 cells interact with B cells and promote isotype switching to IgE. The allergen-specific IgE binds to the FcεRI receptor on MCs in the activation phase of the ACS. After subsequent exposure to the allergen and antigen receptor trigger, MCs degranulate and release proinflammatory mediators.

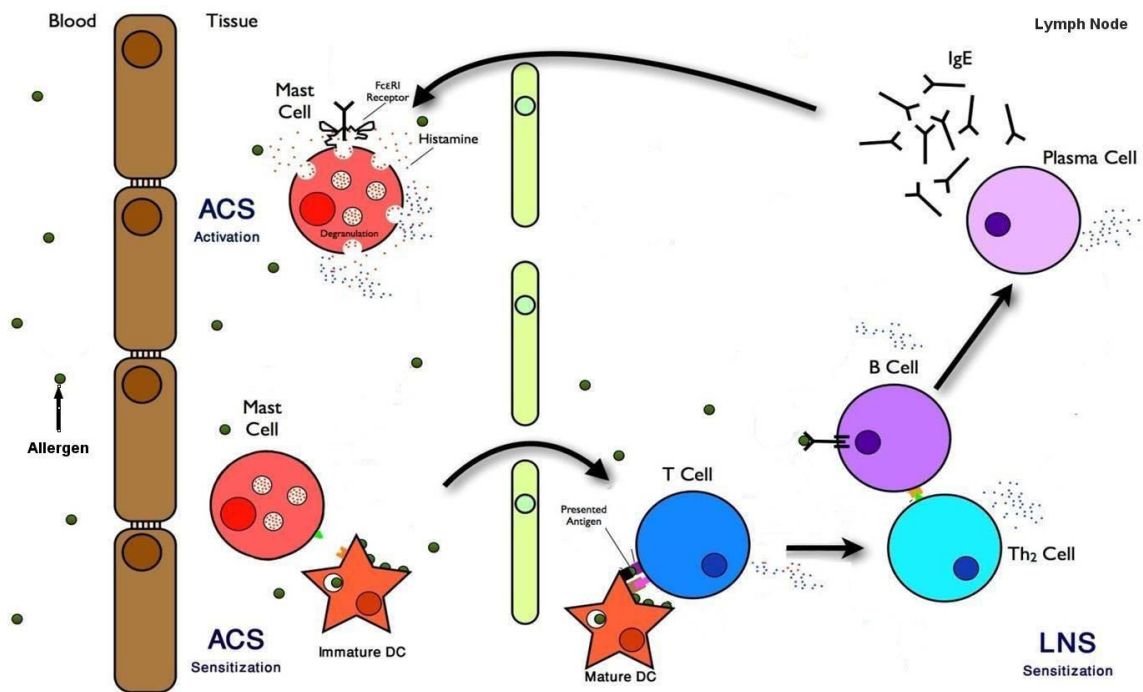


Fig. 1.4. Advanced allergy tissue model development. Mast cells (MCs) in the allergen contact site (ACS) are exposed to the allergen. In the sensitization phase of the ACS, allergen-activated MCs interact with dendritic cells (DCs) that induce lymphocyte proliferation and polarization in the lymph node site (LNS). Figure adapted from Sabban, 2011 [65].

To achieve the goal of this research, the ACS and the LNS were created and characterized to measure the response of the model to an allergen. Therefore, the following aims were completed:

1. Create the allergen contact site (ACS) and characterize the activation phase of the ACS.
 - 1.1. Create the ACS: The type of media that supported MC development and ancillary cells was determined. Then, MC progenitors were cultured in a 3D matrix along with fibroblast and ECs. The samples were incubated for seven weeks in a supplemented media until analysis.
 - 1.2. Characterize the ACS under non-inflammatory conditions: To confirm the development of MCs, the morphology and immunophenotype of the generated cells were determined under normal conditions and compared with characteristics of in vivo MCs.
 - 1.3. Characterize the activation phase of the ACS: The MC function was examined after challenge with human IgE and anti-IgE, which resembles the response of the model to an allergen. In order to investigate the response of the model to an allergen, the ACS was examined after exposure to dust mite allergen in an IgE-dependent manner. Serum of an allergic patient was used to passively sensitize the generated MCs.
2. Characterize the sensitization phase of the allergen contact site (ACS). The monocytes were added to the ACS and the immunophenotype of the monocyte-derived cells was determined. The model was used to test the hypothesis that an allergen can induce MC activation in an IgE-independent manner and MCs via interaction with monocyte-derived cells can play a role in the initiation of the response.
3. Create and characterize the lymph node site (LNS) in response to monocyte-derived cells from the allergen contact site (ACS). T and B cells were cultured in a 3D matrix along with monocyte-derived cells from the ACS. The response of the model was determined by characterizing T cells and measuring the IgE production by B cells. The response of

the LNS to monocyte-derived cells defined the role of MCs in orchestrating the immune response.

1.1.5. Significance and broader impact

This study complements previous human and animal models by creating a complex 3D *in vitro* system that included multiple cell types for mimicking connective tissue. The development of MCs in such a complex system advances the study of MC behavior *in vitro* specifically in allergic responses. The model can be applied to determining the mechanism of initiation and development of allergic responses at a molecular level, as well as studying other inflammatory or inflammatory-related diseases, such as asthma, fibrosis, and angiogenesis that involve the connective tissue-resident cell types. Furthermore, due to including the immune cells that are responsive in the skin test and specific antibodies that are assessed in the blood test, the developed tissue model is a combination of the allergy skin test and blood test. Since the model is comprised of immune cells of blood samples from the patient, it can be optimized and considered as an improved patient-specific allergy test. In addition, the model has the potential to change the way pharmaceutical companies test and develop therapies against allergens.

CHAPTER 2

Development of Human Mast Cells from Hematopoietic Stem Cells within a 3D Collagen Matrix: Effect of Stem Cell Media on Mast Cell Generation

2.1. Introduction

Release of preformed mediators and expression of diverse molecules have placed mast cells (MCs) among the foremost inducers of allergic responses and regulators of innate and adaptive immunity [66, 67]. MCs are abundant in tissue near surfaces exposed to the external environment, and their number and distribution change markedly during immune responses [68-70]. During immunoglobulin E (IgE)-dependent responses, crosslinking of the FcεRI/IgE complexes leads to MC activation and degranulation of a wide range of bioactive products, including histamine [67]. The mediators increase vascular permeability and recruit leukocytes to the site of insult, resulting in hyperemia and edema, the cardinal symptoms of acute inflammation [20]. MC mediators can exert multiple effects, such as extracellular matrix remodeling in fibrosis or degradation during early events of angiogenesis, giving them the potential to be involved in the pathogenesis of variety of disorders [71-73].

Although MCs are not normally present in circulation, they can be obtained from progenitor cells in the presence of T cell-derived cytokines and fibroblast-derived stem cell factor (SCF) [47-49]. CD133 and CD34 antigens are markers for primitive progenitor and hematopoietic stem cell (HSC) populations [74, 75]. Previous studies have shown that upon treatment with SCF,

interleukin (IL)-3, and IL-6, CD133⁺ HSCs isolated from various sources, including cord blood and peripheral blood can differentiate into MCs under two-dimensional (2D) culture condition [59, 60]. The growth, phenotype, and function of the generated MCs can be altered by the culture media supplements [27, 76, 77]. As an instance, addition of serum to the media from the beginning of culture can result in a low number of mature MCs with reduced FcεRI expression, while its addition at later weeks of the culture period promotes the expression of FcεRI and histamine release upon activation [58, 78]. Although generation of MCs under 2D culture conditions provided a source for human MC studies, they have been considered as immature MCs or “incomplete representatives of mature MCs” due to the lack of an *in vivo* microenvironmental conditions that may affect MC phenotypic and functional characteristics [66, 79]. Since MCs mature and interact with other cells within tissue, providing a condition that better mimics the *in vivo* three-dimensional (3D) milieu would be of greater relevance for studying MC responses and immunoregulatory roles. In fact, interaction between MC and extracellular matrix components can affect MC behavior and influence their biological functions [80]. Therefore, the first objective of this study was to demonstrate the generation of MCs within a 3D collagen matrix, which provides the conditions for investigating the cellular interactions that is not possible to examine within a conventional 2D culture system.

MCs are located near blood or lymphatic vessels in proximity to fibroblasts that are principal cellular component of tissue [79]. Previous studies have shown that the cross-talk between MCs, fibroblasts, and endothelial cells (ECs) mediates various physiological and pathological processes [81, 82]. Besides the release of growth factors that are essential for MC survival and maturity, direct interaction between fibroblasts and ECs can regulate MC development [55, 61, 64]. Therefore, incorporation of fibroblasts and ECs into the 3D tissue model allows the transmission of similar signaling molecules that HSCs may receive during differentiation into MCs from neighboring cells *in vivo*. However, for creation of such a tissue-engineered model, there is no

universal media that support the overall growth of MC precursors and the above-mentioned ancillary cells. In fact, selecting appropriate culture media is determinative to the success of a co-culture system. Since each cell type has specific growth requirements, a suitable media that regulates their survival needs to be determined. Previous studies have shown the expansion of CD34⁺ HSCs or their differentiation into MCs by using cytokine-supplemented and serum-free media [57, 83-85]. However, most studies with fibroblasts and ECs use media with serum and not specific for HSC growth and differentiation [86-88]. Therefore, the second objective of the current study was to determine a media that would support the generation of functional MCs from HSCs, as well as normal characteristics of fibroblasts and ECs. Having an understanding of the effect of culture media on each individual cell type is an important first step needed before the development of a co-culture model with multiple cell interactions.

2.2. Material and methods

2.2.1. Antibodies and reagents

M199 and StemPro[®]-34 SFM culture media were purchased from Life Technologies (Carlsbad, CA). HPGM[™] and StemSpan[™] SFEM cell culture media were purchased from Lonza (Walkersville, MD) and STEMCELL Technologies (Vancouver, Canada), respectively. Human SCF, IL-6, and IL-3 were purchased from ProSpec (Rehovot, Israel) or PeproTech (Rocky Hill, NJ). Defined HyClone fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences (Logan, UT). Human fibroblasts treated with mitomycin C were purchased from Merck Millipore (Billerica, MA), while human umbilical vein ECs were purchased from PromoCell (Heidelberg, Germany). Anti-human fluorochrome-conjugated CD117/c-kit (clone 104D2), FcεRI (clone CRA-1), CD31 (clone WM59), and their isotype controls, Ms IgG1 (clone MOPC-21), Ms IgG2b (clone MPC-11), were purchased from BioLegend (San Diego, CA). Anti-human fluorochrome-conjugated CD90 (clone 5E10), its isotype, Ms IgG1 (clone MOPC-21), and mouse anti-human tryptase (clone AA1) were purchased from Abcam (Cambridge, MA). Mouse anti-

human chymase (clone B7) and the secondary antibody goat anti-mouse IgG1 were from Chemicon (Temecula, CA) and Santa Cruz Biotechnology (Dallas, TX), respectively.

2.2.2. Cell Culture

2.2.2.1. Hematopoietic stem cell (HSC) culture

CD133⁺ cells were obtained from human peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from fresh leukocyte preparations (obtained from the Oklahoma Blood Institute; Oklahoma City, OK) by the Ficoll-Paque density separation method (GE Healthcare; Pittsburgh, PA). CD133⁺ cells were isolated from PBMCs using a magnetic separation kit (MACS Miltenyi Biotec; Bergisch Gladbach, Germany).

CD133⁺ cells were cultured and differentiated within a 3D collagen gel. For this, 2 mg/ml collagen solution was prepared by modification of a previous protocol [89] using 64.5 vol% of 3.1 mg/ml type-1 bovine collagen (Advanced BioMatrix, Carlsbad, CA), 8.1 vol% 10X M199, 13.3 vol% 0.1 N NaOH, and 14.1 vol% PBS. CD133⁺ cells were mixed with the collagen solution (5.1×10^5 cells/ml) and added to the cell culture plates. Following gel formation (45 min), media were added to the samples and the samples were incubated for seven weeks with media changes once a week. Media were supplemented with human SCF (100 ng/ml), IL-6 (50 ng/ml), and IL-3 (1 ng/ml) for the first three weeks of culture. The CD133⁺ cells were monitored weekly and characterized on the seventh week of culture. In order to study the effect of serum on the growth and differentiation of CD133⁺ cells, FBS (10%, v/v) was added at three different time points: (i) from the day of seeding until the end of the seventh week (Ser1-7), (ii) on the seventh week only (Ser7), and (iii) the first, second, and seventh week (Ser1,2,7). In all cases, media was changed once a week.

2.2.2.2. Fibroblast and endothelial cell (EC) culture

Cell culture plates (24-well, area 1.9 cm², Greiner BioOne, Monroe, NC) were coated with fibronectin (25 µg/ml in phosphate-buffered saline, PBS) for 2 h before cell seeding. Fibroblasts and ECs were cultured separately in serum-free StemSpan, StemPro, and HPGM. M199 containing 1 vol% PSG (penicillin, streptomycin, and L-glutamine) was used as the “standard media” [86, 87, 90]. ECs were cultured in media with and without the addition of FBS. Fibroblast and ECs were cultured at 35,000 cell/cm² and 12,000 cell/cm² density respectively, at 37°C, 5% CO₂ (defined here as “standard conditions”) with media changes on every other day until confluent and ready for testing.

2.2.3. Characterization of mast cells (MCs)

2.2.3.1. Yield and granule formation

In order to determine the number of generated viable cells, the collagen matrix was digested after incubation with 2 mg/ml of collagenase D (Roche Applied Science; Indianapolis, IN). The harvested cells in the digested solution were counted by a hemocytometer and the number of viable cells was determined by trypan blue exclusion. The cell yield was calculated as the ratio of the number of viable cells harvested to the number of cells seeded. Cytosolic granule formation was determined by Wright-Giemsa staining using an automated stainer (Ames HemaTek Stainer).

2.2.3.2. Expression of phenotypic markers

Expression of c-kit and FcεRI was assessed by flow cytometry. After seven weeks of culture, the expression of FcεRI was stabilized by incubating the cells for 24 h with myeloma IgE (2 µg/ml, Merck Millipore). Following collagenase D digestion of the matrix, the cells were collected, stained using anti-c-kit and anti-FcεRI antibodies or relevant isotype controls, and analyzed by flow cytometry. Dead cells were excluded by PI staining.

For immunocytochemical staining of tryptase and chymase granules, the cells were collected from the matrix and fixed by using fixation/permeabilization solution kit (BD Biosciences; CA).

After incubation with a blocking solution containing 10% goat serum (v/v%, Gibco; CA) for 1 h, the cells were incubated with primary antibodies against tryptase or chymase or isotype control. Following this, secondary antibody was added, and incubated for 30 min at room temperature. The cells were incubated for at least 1 h in the staining buffer containing 0.2% bovine serum albumin (BSA), prior to staining with anti-c-kit antibody and analysis by flow cytometry.

2.2.3.3. Activation and histamine release

At seven weeks post seeding, the function of the generated cells was examined by crosslinking the FcεRI receptors by IgE and anti-IgE antibodies. Activation was performed for cells within the matrix and for cells removed from the matrix. Cells were sensitized with 15 µg/ml myeloma IgE (Athens Research & Technology; Athens, GA) in complete media for 24 h and rinsed three times prior to activation with various concentrations of anti-IgE antibody (Chemicon International; Temecula, CA) in Tyrode's solution (Boston BioProducts; Ashland, MA) supplemented with SCF and IL-6 for 1 h. For measuring the cellular histamine, cells were lysed by freeze-thaw cycles in water and sonicated for 5 min. Histamine was quantified using a commercial ELISA kit (Labor Diagnostika Nord; Nordhorn, Germany). The percentage of histamine release is determined by taking the ratio of the total amount of histamine released by the cells to the total histamine content initially in the cells. In addition, the spontaneous amount of histamine released by the cells under normal conditions was subtracted from the total amount released. For the samples activated within the matrix, the total amount of histamine released by the cells is determined by measuring the amount of histamine in the media and the gel solution.

2.2.4. Characterization of fibroblast and endothelial cells (ECs)

2.2.4.1. Proliferation

To measure cell proliferation, fibroblasts and ECs were fluorescently labeled with CellTrace or CellTracker (Life Technologies) prior to culture. The stained cells were harvested by

trypsinization, and the fluorescent intensities were measured by flow cytometry. As cells divide, the fluorescent probe is split evenly between the daughter cells and the mean fluorescent intensity (MFI) per cell decreases. As a control for non-dividing cells, fibroblasts were stained with CellTrace and analyzed before seeding. Dead cells were stained with propidium iodide (PI, Life Technologies) [91]. All the cell dyes were used following the manufacturer's protocols.

2.2.4.2. Expression of surface receptors and secretion of mediators

Expression of CD90 by fibroblasts and CD31 by ECs was determined by flow cytometry. Trypsinized cells were collected and stained with anti-CD90 or anti-CD31 antibodies or their isotype controls (45 min, 4°C). Dead cells were excluded by PI staining. To determine the secretion of SCF and IL-6 by fibroblasts and ECs, culture supernatants were collected and analyzed by commercial enzyme-linked immunosorbent assay (ELISA) kits (PeproTech).

2.2.5. Statistical analysis

Experimental results are expressed as mean \pm SD of triplicate samples of one representative experiment. One-way analysis of variance (ANOVA) was selected to determine significant differences between groups. Tukey or Student's *t*-Test was used for pairwise comparison of groups or between two groups, respectively. A value of $p < 0.05$ was considered significant.

2.2.6. Possible hazard and safety precautions

All animal- and human-derived materials were purchased from commercial vendors that prescreen the products for biological hazards. The personnel conducted the research completed a training program for handling the biohazardous materials and working in a biosafety level 2 (BSL-2) laboratory. Prior to the experiments, all biohazards were identified and biosafety plans were prepared and followed. All procedures associated with the work followed "Universal Precautions". Biohazardous materials were disposed according to the guidelines for each waste category.

2.3. Results and discussion

2.3.1. Effect of culture media on the generation of mast cells (MCs) from CD133⁺ hematopoietic stem cells (HSCs)

M199, our standard media EC culture that was also used for fibroblasts, either with serum added from the beginning or in the last week of culture, did not support MC generation and survival, as verified by microscopy, viability, and flow cytometry analyses. From the first week, most cells in all the media, except for HPGM (Ser7), formed colonies as a sign of cell generation.

During differentiation, the morphology of MC progenitors sequentially change, until they mature into MCs. Initially, progenitor cells (blasts) have a high nuclear to cytoplasm ratio, and then gradually acquire granules that can be stained to form metachromatic blasts. The atypical type II MCs (called the promastocytes) have bi- or poly-lobed nuclei, which are oval or eccentrically located, and often possess hypogranulated cytoplasm. At the end of the developmental stage, the mature, typical MCs are formed, which are round or oval with granulated cytoplasm, low nuclear to cytoplasm ratio, and a centrally-positioned, round nucleus [92-94]. As shown in Fig. 2.1A, in the seventh week of culture for all the test media, the cells were mostly round or oval. Except for a few larger cells in the StemPro (Ser1-7) media, the size of the generated cells in all the test media were in range of *in vivo* MCs (8 – 20 μm) [80, 95]. As shown in Fig. 2.1B, the generated cells exhibited metachromatic cytoplasmic granules following Wright-Giemsa staining, which is a morphological characteristic of MCs [59]. In StemPro (Ser1-7), 30% of the cells were hypo-granulated, as shown by the black arrow in Fig. 2.1B. The results indicate that the cells generated from the MC precursors in the collagen matrix had the morphology of typical, mature and immature MCs, with distinct promastocytic characteristics.

As shown in Fig. 2.1C, there was no significant difference in cell yields for the media tested with serum from the first week of culture. For the media with serum added in the last week, there was a significantly greater cell yield in StemSpan compared to StemPro (3.1 ± 0.8 -fold, $p < 0.05$).

For StemSpan (Ser7), the number of cells at termination of culture were 2.2 ± 0.1 -fold higher than that of CD133⁺ cells initially seeded in the collagen matrix, which is similar to a 2D culture system that used the same culture medium and generated 3.2 ± 1 -fold that of the seeded cells [59].

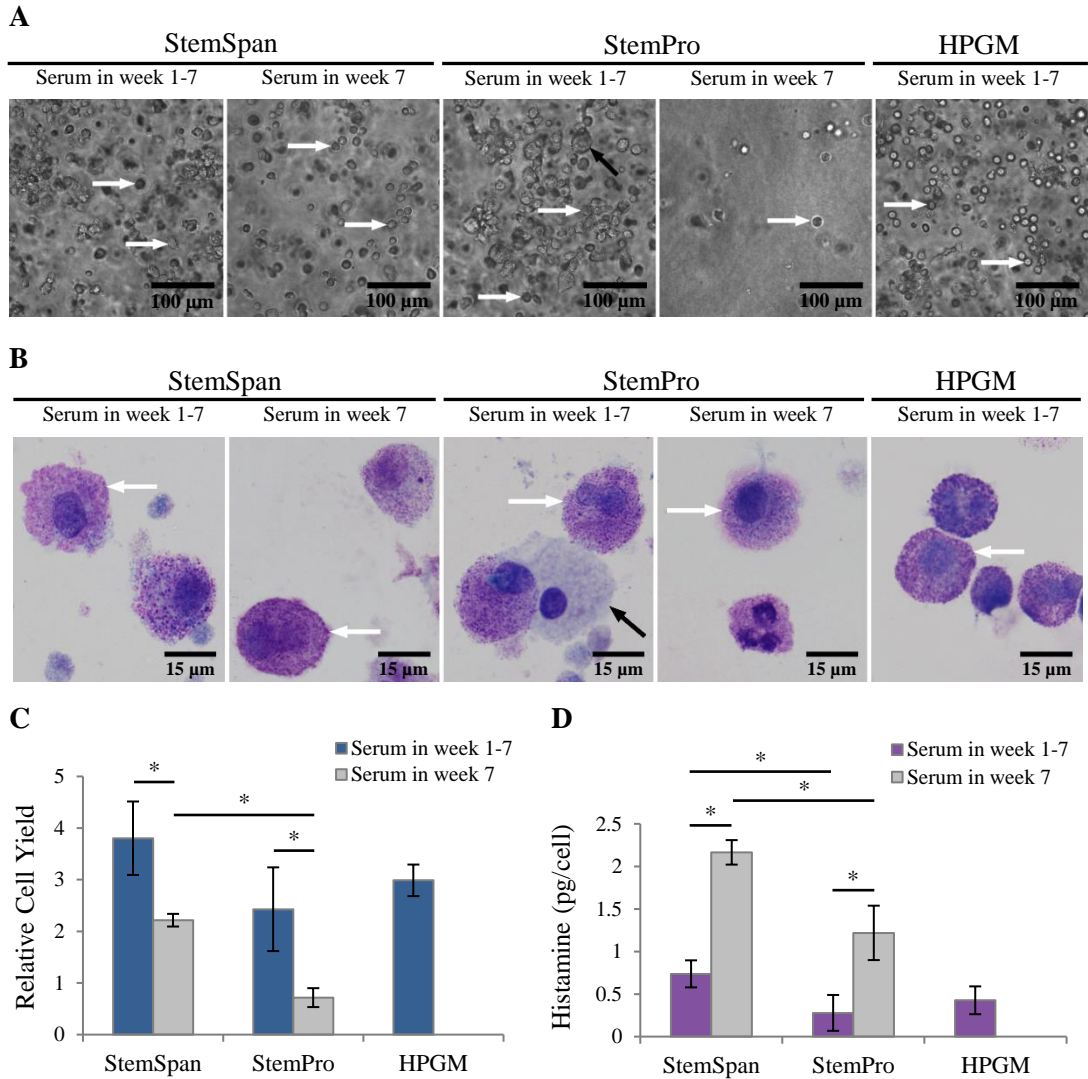


Fig. 2.1. Effect of culture media on the morphology, cell yield and granule formation of the cells generated from CD133⁺ hematopoietic stem cells (HSCs) in a matrix after seven weeks in culture. A) Micrographs showing the morphology of generated cells in the test media. White arrows highlight typical MCs. Black arrow highlights the larger sized MCs found in StemPro

(Ser1-7). B) Metachromatic staining of cytoplasmic granules in the generated cells. A few typical MCs are highlighted by white arrows. In StemPro, some cells were hypo-granulated, as shown by the black arrow. C) Cell yields in the culture media. Cell yield is defined as the ratio of the cells collected at the end of the culture period to the seeded cells. D) Histamine granule formation in the generated cells. Data are represented as mean \pm SD; n=3. * indicates $p < 0.05$.

The histamine content of MCs *in vivo* depends on their anatomic location and subtype. The histamine level in MCs varies from 0.8 – 12.5 pg/cell to 0.8 – 5 pg/cell in lung and skin, respectively [95-97]. In this work, the differentiated cells in all but StemPro (Ser1-7) and HPGM media had similar histamine content to that of *in vivo* MCs, and varied between 0.6 to 2.3 pg/cell. Cells cultured in StemPro (Ser1-7) had lower histamine content (0.28 ± 0.21 pg/cell), as verified by the presence of some hypogranulated cells in Wright-Giemsa stained sample. MCs generated in StemSpan (Ser7) had significantly greater histamine content compared to all other test media (Fig. 2.1D, $p < 0.05$). The histamine content of the MCs generated in a 2D culture system using StemSpan medium was 15.5 ± 5.3 pg/cell [59], which is higher than the histamine content observed in the *in vivo* MCs. However, this was not the case for the MCs generated within the collagen matrix using StemSpan medium in this study, with histamine content within the range of *in vivo* observations.

In addition to morphology and granule formation, immunophenotypical markers are used to distinguish MCs from other cell types. MCs, basophils, eosinophils, dendritic cells, macrophages, and ECs are known to have common precursor cells [98-101], but these cell types vary in their immunophenotype and expression of other markers. MCs, progenitor and HSCs, and ECs all express c-kit [102]. HSCs, ECs, eosinophils and cells of monocytic lineages lack histamine granules and possess significantly different morphology [103]. Basophils express histamine and Fc ϵ RI, but previous studies have shown that they do not express c-kit [104, 105]. Out of these cell

populations, only mature MCs possess histamine granules and express both FcεRI and c-kit receptors, making these the markers useful to define the MC phenotype.

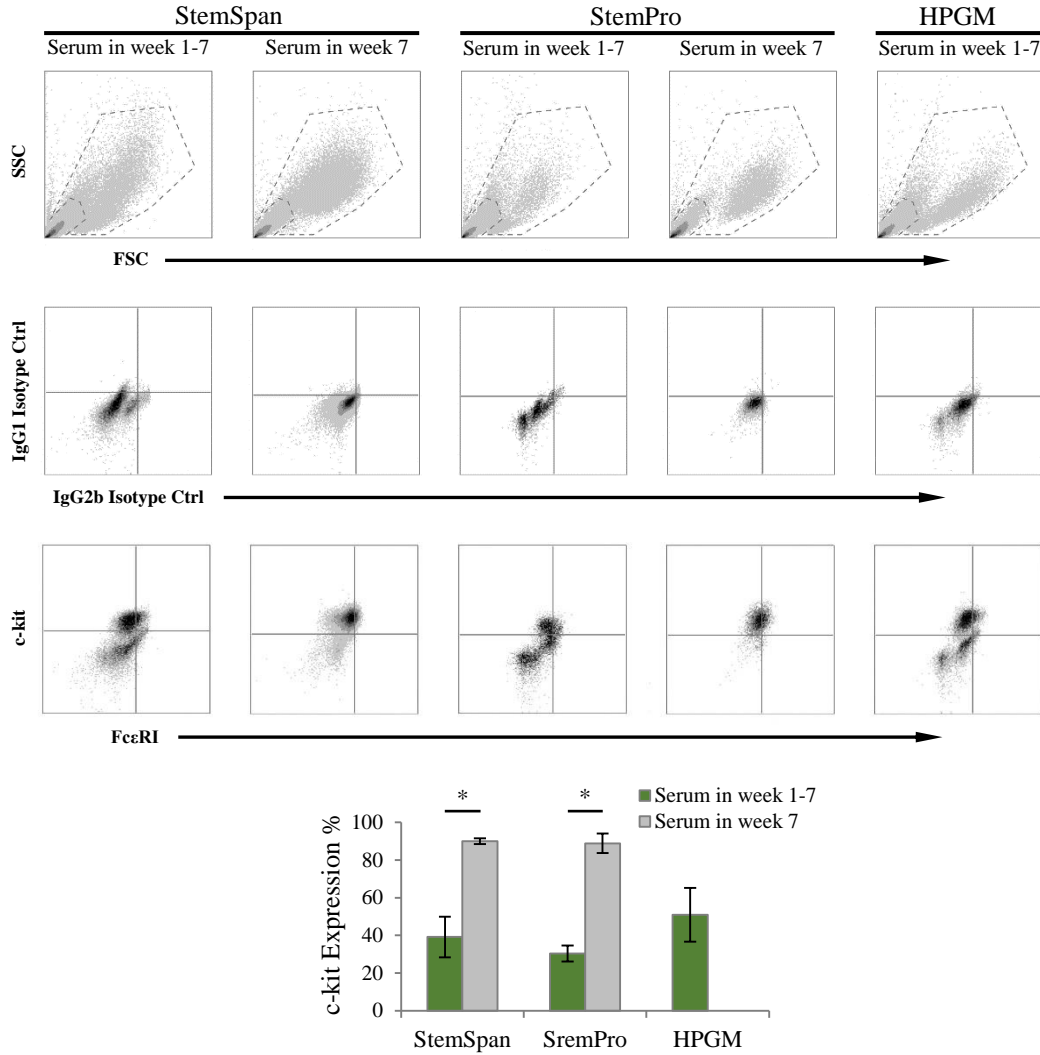


Fig. 2.2. Expression of c-kit and FcεRI by generated cells from CD133⁺ hematopoietic stem cells (HSCs) in a collagen matrix after seven weeks in culture. The figure shows the gating scheme (top panel) and representative density plots of the isotype control and expression of phenotypic markers (middle and bottom panel, respectively). More than 90% of the cells are gated in the bottom left corner of the isotype density plots. The bar graphs show the percentage of c-kit expression. Data are represented as mean \pm SD; n=3. * indicates $p < 0.05$.

As shown in Fig. 2.2, in the media where serum was added at the first week of culture, there was no significant difference in c-kit expression; however there was a 1.6 ± 0.1 - and 1.8 ± 0.1 -fold increase in c-kit density (MFI, $p < 0.05$) for StemSpan and HPGM compared to StemPro, respectively. Similarly, for media with serum added in the last week of culture, there was no significant difference in c-kit expression, with 1.3 ± 0.2 times more c-kit density (MFI, $p < 0.05$) in StemSpan compared to StemPro. A 2D culture method that used StemSpan media also showed a high percentage of c-kit positive cells ($88.3 \pm 2.2\%$) [59]. The expression of FcεRI was similar for all the test media (10 – 23% on average); however, the FcεRI density (MFI) was 1.4 – 2.3 times higher ($p < 0.01$) in StemSpan (Ser1-7) than for the other media. The expression of FcεRI was lower in comparison with 2D culture systems that used StemSpan or StemPro media [59, 106]. Other reports have shown that incubation with IgE antibody modulates the expression of FcεRI and can contribute to its detection [107, 108]. For all the test media, IgE was added to the cells in the collagen matrix, prior to collecting the cells and measuring FcεRI expression. The lower expression could be due to the binding of IgE with the matrix, resulting in less IgE available to interact with the matrix-embedded cells, compared to the other system with cells in suspension.

2.3.2. Effect of serum on mast cell (MC) development

Our results shows that the addition of serum to the culture conditions affected various characteristics of the HSC-derived cells, such as morphology, proliferation of HSCs, cell surface marker expression, and histamine content of the generated cells (Fig. 2.1 – 2.3). Other studies have shown that MCs in serum-supplemented media had multi-lobed nuclei or macrophage-like morphology, in contrast with the cells in serum-free media [58, 109]. The results from our work also indicate that the timing of adding serum to the culture media can affect the morphology and internal structure of the generated cells, which could be related to the stage of cellular development. When serum was added in the first two and last weeks of culture, more cells had bi-

(30%) or multi-nucleated (10%) morphology in comparison to when serum was added only in the last week of culture, with 8% and 2% bi- and multi-nucleated cells, respectively (Fig. 2.3A). Furthermore, similar to other serum-supplemented cell cultures [110, 111], the proliferation of progenitor cells increased (by 72% for StemSpan media) when serum was added from the beginning of culture, compared to the last week, for a seven-week culture period (Fig. 2.3B, $p < 0.05$). The results indicate that although serum induces cell proliferation, the addition of serum in the beginning of culture can delay the morphologic change from promastocytes to mature MCs.

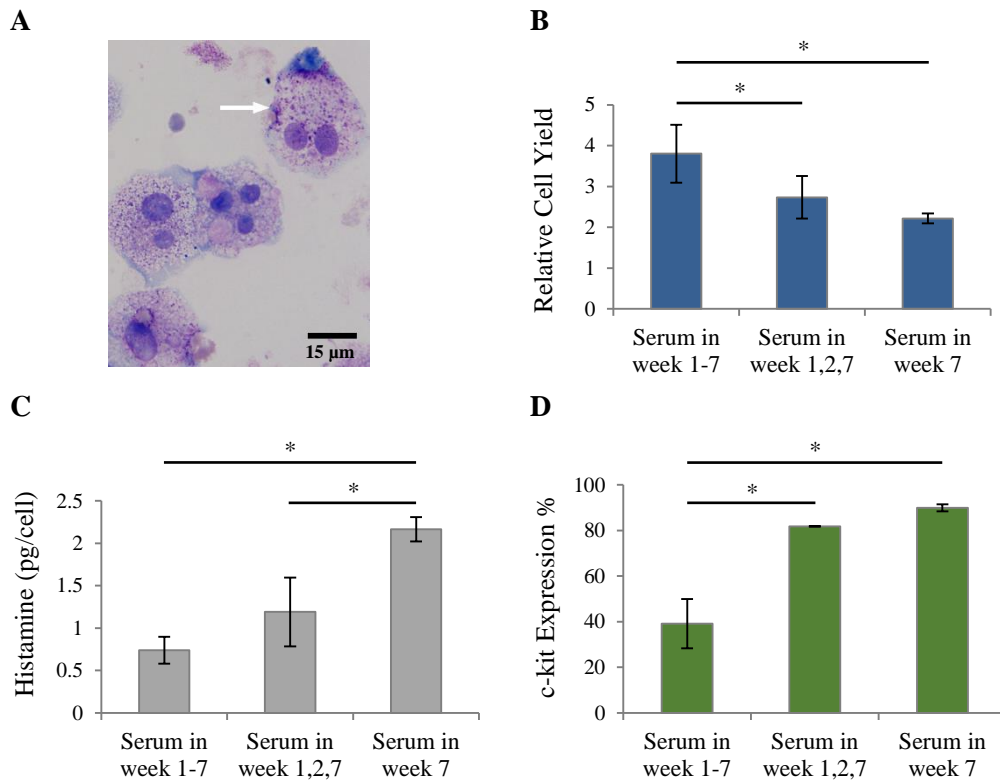


Fig. 2.3. Effect of serum on cell yield, granule formation, and marker expression of the generated MCs in the matrix after seven weeks of culture. A) Metachromatic staining of cytoplasmic granules in the cells generated with serum added in the first two and last weeks of culture. Some of the generated cells were bi- or multi-nucleated, as highlighted by a white arrow. B) Cell yield of generated cells with serum added at different time points. The ratio of cells

collected at the end of the culture period to the cells seeded at the beginning of culture was reported as relative cell yield. C) Histamine granule formation in the generated cells. D) Expression of c-kit by generated cells. In all cases, StemSpan medium was used. Data are represented as mean \pm SD; n=3. * indicates $p < 0.05$ between the media tested.

Moreover, serum appeared to have an inhibitory effect on histamine content. For MCs generated in StemSpan and StemPro, the histamine content was more than two-fold higher when serum was added in the last week of culture, compared to the beginning of culture ($p < 0.05$, Fig. 2.1C). As shown in Fig. 2.3C, when serum was excluded from the culture media after the second week, the inhibitory effect of the serum on the formation of histamine granules was mitigated.

As shown in Fig. 2.3D, compared to samples that contained serum throughout culture, excluding serum after the second week enhanced the c-kit expression, verifying that serum suppressed MC development. Although the effect of serum on Fc ϵ RI expression was not significant ($p > 0.05$), there was a significant increase in the receptor density (1.6 ± 0.2 -fold, $p < 0.05$) on the surface of cells cultured in StemSpan (Ser1,2,7) compared to StemSpan (Ser1-7). These results show that not only does serum affect the cellular immunophenotype, but the timing of its addition can also alter the expression and the density of the cell surface receptors. Previous studies have shown that the addition of serum to the media from the beginning of culture reduces the expression or the density of c-kit, while increasing the expression of myeloid markers (such as, CD14, CD11b, and CD13) [58, 109]. These studies suggested that serum factors can induce the generation of other cell types and delay the development of MCs. However, a serum-free media could also result in lower Fc ϵ RI expression and abolish MC activation [58].

2.3.3. Effect of culture media on fibroblasts

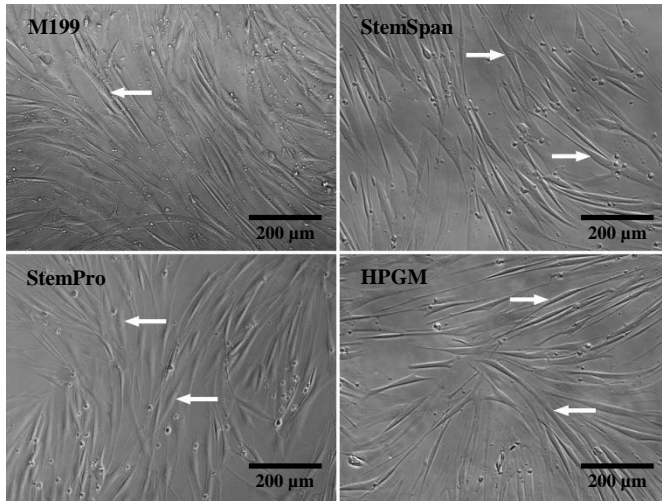
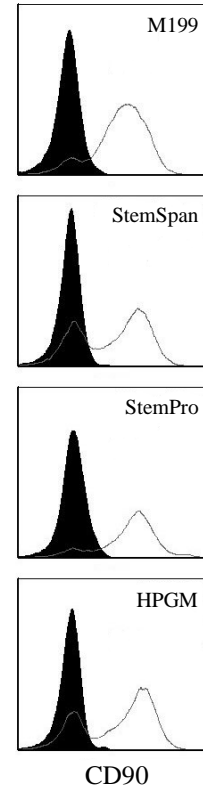
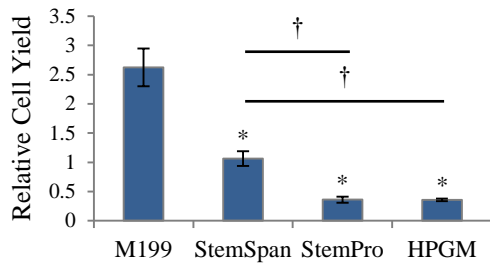
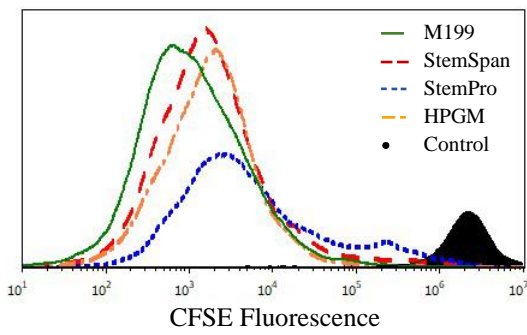
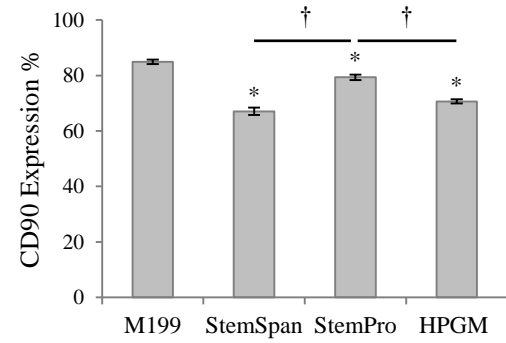
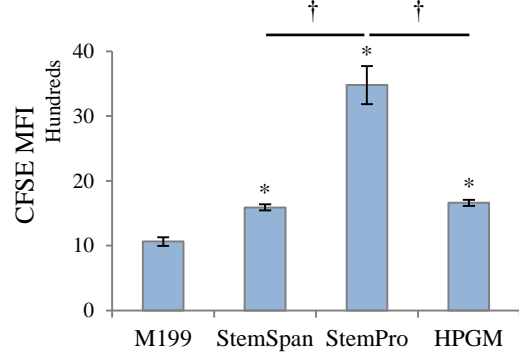
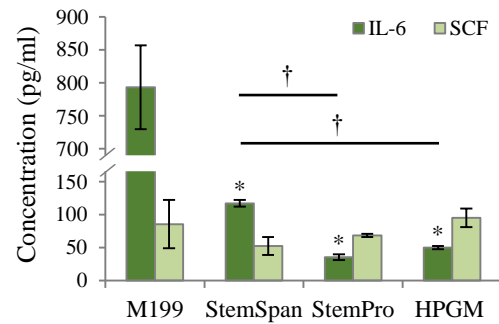
A**D(i)****B****C(i)****D(ii)****C(ii)****E**

Fig. 2.4. Effect of serum-free media on fibroblast growth and characteristics. A) Morphology of fibroblasts at six days post-seeding in serum-free media. Arrows show some of the typical fibroblasts. The rest of the experiments were performed ten days post-seeding. B) Fibroblast yield in the culture media. The ratio of the number of collected cells to the number of seeded cells is shown as relative cell yield. C(i) and (ii)) Proliferation of fibroblasts in the culture media. Solid histogram shows the cells analyzed on the day of seeding (non-dividing cells). D(i) and (ii)) Expression of CD90 by fibroblasts in the culture media. Expression of CD90 (open histogram) was compared to an isotype control (solid histogram). E) Release of SCF and IL-6 by fibroblasts. In all cases, serum-supplemented M199 was taken as the standard cell culture medium. Data are represented as mean \pm SD; n=3. * indicates $p < 0.05$ between M199 and the test media. † indicates $p < 0.05$ between the test culture media.

As shown in Fig. 2.4A, fibroblasts displayed normal, elongated morphology in all the test media. The test media are typically not used to culture fibroblasts, but our results indicate that the cells show normal morphology in all the media.

The cell yields varied across the test media (Fig. 2.4B). StemSpan showed a higher cell yield (more than two-fold) than StemPro and HPGM ($p < 0.05$, Fig. 2.4B). As shown in Fig. 2.4C(i), cells in all the media divided at least once in comparison with the control, non-dividing cells. The MFI of the CellTrace CFSE-stained cells in the test media were higher than M199 ($p < 0.05$), showing lower proliferation. StemPro did not support the proliferation of fibroblasts as much as the other test media, as specified by the higher MFI of stained cells ($p < 0.05$, Fig. 2.4C(ii)). Therefore, fibroblasts were more proliferative and higher in number in StemSpan than other media tested.

Fibroblasts cultured in all the test media expressed CD90 (a phenotypic marker of fibroblasts [112]), as shown in Fig. 2.4D(i) and (ii). CD90 expression was higher for fibroblasts in StemPro

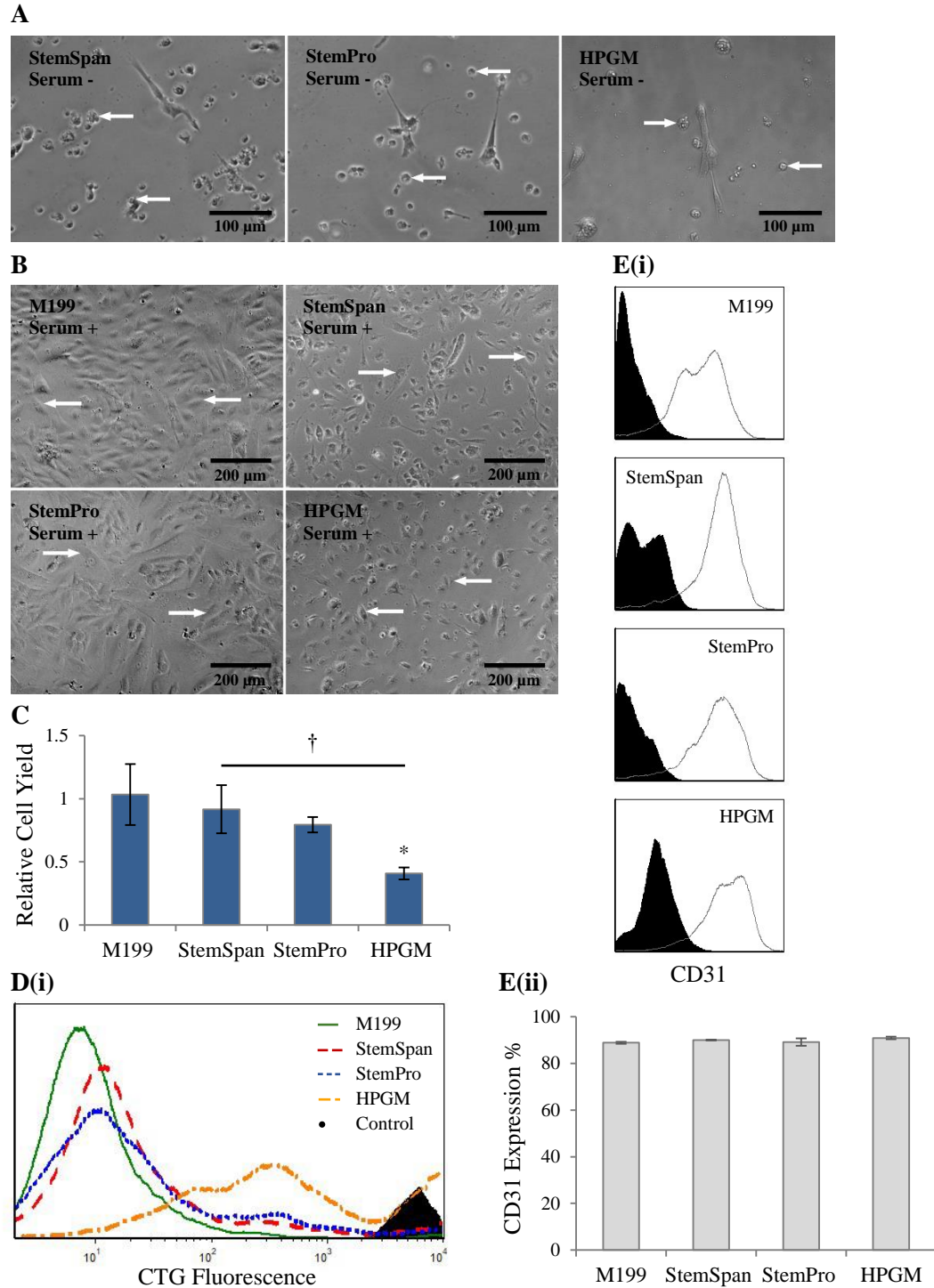
compared to StemSpan and HPGM ($p < 0.05$). Before seeding, almost all fibroblasts were positive for CD90. The lower expression after culture shows that the culture media affected CD90 expression, which has been shown to be related to their phenotypic heterogeneity [113].

The release of the cytokines IL-6 and SCF, which are involved in the differentiation of HSCs to MCs, was measured for fibroblasts. As shown in Fig. 2.4E, the fibroblasts released IL-6 in a media-dependent manner, with more than two-fold higher release for the cells cultured in StemSpan, compared to the release from fibroblasts cultured in StemPro and HPGM ($p < 0.05$). The expression of SCF gene by fibroblasts has been shown in previous studies [114] and in this work, SCF was released similarly by fibroblasts in all the test media (Fig. 2.4E). These data show that fibroblasts can release growth factors, which are necessary for the development and survival of MCs. The concentrations of IL-6 and SCF in the fibroblast culture media were not as high as the concentrations in the HSC media used for MC generation. However, the co-culture of fibroblasts with MCs has been shown to upregulate the IL-6 release [115] and may have the same effect on SCF secretion. Besides the effect of soluble factors in the medium, the direct interaction of fibroblasts with MCs was reported to be necessary for MC maturity, inhibition of apoptosis, and generation of MC subtypes specifically found in the connective tissue [116, 117]. Overall, considering the results from testing all of the media, StemSpan was superior for fibroblasts culture in a stem cell media.

2.3.4. Effect of culture media on endothelial cells (ECs)

In contrast with the fibroblasts, ECs failed to survive beyond three days in any of the serum-free media (Fig. 2.5A). As shown in Fig 5B, for the serum-supplemented media, ECs showed their characteristic, cobblestone-like morphology. The cell yield of ECs was the lowest in HPGM among the all media tested, shown in Fig. 2.5C. When measuring cell proliferation, the ECs divided at least once in all the culture media in comparison with the control, non-dividing cells (solid histogram, Fig. 2.5D(i)). ECs cultured in HPGM showed the highest MFI among the other

test media, indicating the lowest cell proliferation, ($p < 0.05$, Fig. 2.5D(ii)). Therefore, HPGM did not support the survival and proliferation of ECs to the extent seen with the other culture media.



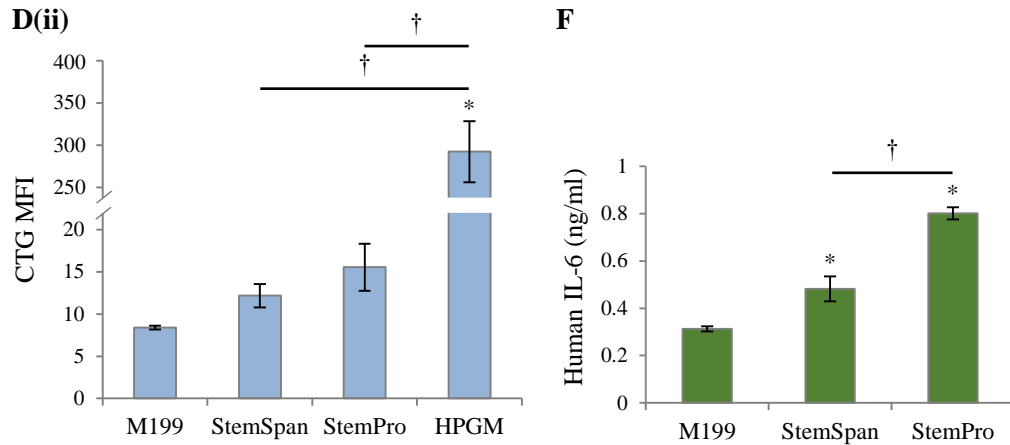


Fig. 2.5. Effect of culture media on endothelial cell (EC) growth and characteristics. A) Micrographs depicting poor survival of ECs at three days post-seeding in serum-free media. Typical detached or dead ECs are highlighted by arrows. B) Micrographs depicting survival of ECs at six days post-seeding in serum-supplemented media. Typical cells are highlighted by arrows. The rest of the experiments were performed 12 days post-seeding. C) EC yields in the serum-supplemented media. Ratio of the number of collected cells to the number of seeded cells is shown as relative cell yield. D(i) and (ii)) Proliferation of ECs in the serum-supplemented media. Solid histogram shows the non-dividing cells. E(i) and (ii)) Expression of CD31 by ECs in the serum-supplemented media. The expression of CD31 (open histogram) was compared to an isotype control (solid histogram). F) Release of IL-6 by ECs. In all cases, serum-supplemented M199 was taken as the standard cell culture medium. Data are represented as mean \pm SD; $n=3$. * indicates $p < 0.05$ between M199 and test media. † indicates $p < 0.05$ between test culture media.

The EC phenotypic marker, CD31 [118], was highly expressed for all cells in all the media tested (Fig. 2.5E(i) and (ii)), indicating that culture media did not affect the typical EC phenotype. The release of IL-6 and SCF was also measured for ECs. The release of IL-6 from ECs cultured in StemPro was higher than ECs cultured in StemSpan ($p < 0.05$, Fig. 2.5F) and it was not detected

from ECs cultured in HPGM (with the ELISA detection limit of 24 pg/ml). Higher levels of IL-6 in StemSpan and StemPro compared to the control media (M199) might be the effect of the media contents or supplements. IL-6 is a cytokine that facilitates the survival and maturation of MCs [56, 119]. In addition to the effect of soluble factors, the direct interaction of adhesion molecules on ECs and c-kit receptors on MCs have been shown to regulate MC survival and the development of connective tissue-type MCs [61]. Although, previous studies have reported the expression of the SCF gene by ECs [120], SCF was not detected in any of the media tested (with the ELISA detection limit of 16 pg/ml). Other studies have shown that SCF was either not released from ECs, or was released at low levels (e.g. 24.5 ± 1.5 pg/ml) [61, 121]. Taken together, our data suggests that both StemSpan and StemPro supplemented with serum supported EC proliferation and characteristic phenotype.

2.3.5. Mast cell (MC) morphological phenotype and function

Considering all the media tested for this study, and specifically focusing on histamine content and immunophenotype of the generated cells, we believe that StemSpan with serum added in the last week of culture is the most suitable media for MC development from HSCs. Also, StemSpan supported the ancillary cells. Therefore, the morphological phenotype and function of the cells generated within the matrix in this media after seven weeks in culture were examined. Human MCs are heterogeneous and on the basis of the expression of serine proteases have been classified to tryptase-positive (MC_T), chymase-positive (MC_C), and tryptase and chymase-positive (MC_{TC}) MCs [66]. As shown in Fig. 2.6, almost all the generated c-kit positive cells were expressing tryptase ($99.5 \pm 0.2\%$) and chymase ($97.1 \pm 0.9\%$) granules, exhibiting the MC_{TC} phenotype predominant in the skin and small intestinal submucosa [80]. However, when StemSpan was used to generate MCs from $CD133^+$ HSCs in a 2D culture system, MC_T subtype was observed [59]. This highlights the influence of the microenvironments or culture conditions on the MC phenotype.

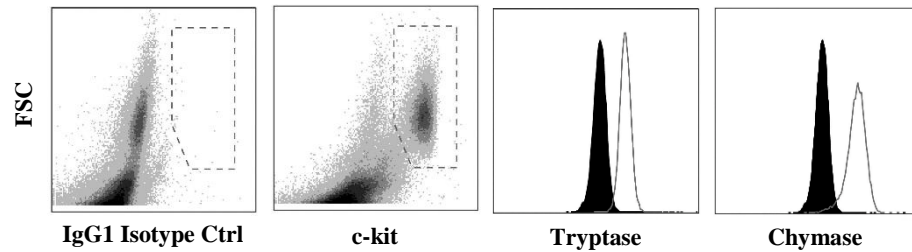


Fig. 2.6. Expression of tryptase and chymase by generated cells from CD133⁺ hematopoietic stem cells (HSCs) in a collagen matrix after seven weeks in culture. StemSpan with serum in the seventh week of culture was used as culture media. Representative density plots and histograms of the marker expression are shown. Expression of tryptase and chymase by c-kit positive cells (gray histogram) is compared with the isotype control (black histogram).

Histamine is one of the vasoactive amines in MC granules released upon activation. Sensitization with IgE and activation of the cells with various concentrations of anti-IgE antibody induced substantial degranulation of the cells upon FcεRI crosslinking (Fig. 2.7). The activation of cells removed from the matrix and cells in the matrix resulted in the release of up to $45 \pm 3\%$ and $35 \pm 3\%$ of the histamine content, respectively, which was about 19-fold higher than the spontaneous histamine release ($p < 0.05$). Also, Wright-Giemsa staining showed that cells partially or completely lost the metachromatic granules after activation (Fig. 2.7).

In vivo MCs can release less than 10% to more than 40% of total histamine content in response to anti-IgE antibody [122-124]. In this work, the generated cells released up to 48% of histamine content in an anti-IgE concentration-dependent manner, which is comparable to the histamine release from generated MCs in a 2D culture system [60].

The response from the generated cells that were activated either after the removal from the matrix or within the matrix was different with respect to anti-IgE concentration. For the cells that were activated after removal from the matrix, the maximum response was measured for 8 μg/ml

anti-IgE, and any further increase of the anti-IgE concentration resulted in a decreased response. For the cells that were activated within the matrix, increasing the anti-IgE concentration up to 200 $\mu\text{g/ml}$ resulted in increased responses, but never reached a maximum response as seen for the cells removed from the matrix. The lower response from the cells within the matrix may be due to the matrix interfering with the binding of IgE and/or anti-IgE with the cells, either due to transport limitations and/or nonspecific binding. In fact, previous studies have shown that other proteins, like monocyte chemoattractant protein-1 (MCP-1), can bind with the matrix and establish a concentration gradient [125, 126]. Therefore, higher concentration of the IgE antibody was needed to overcome any loss due to the matrix. Nevertheless, the results show that MCs generated within the matrix are functional and release histamine in an IgE-mediated reaction.

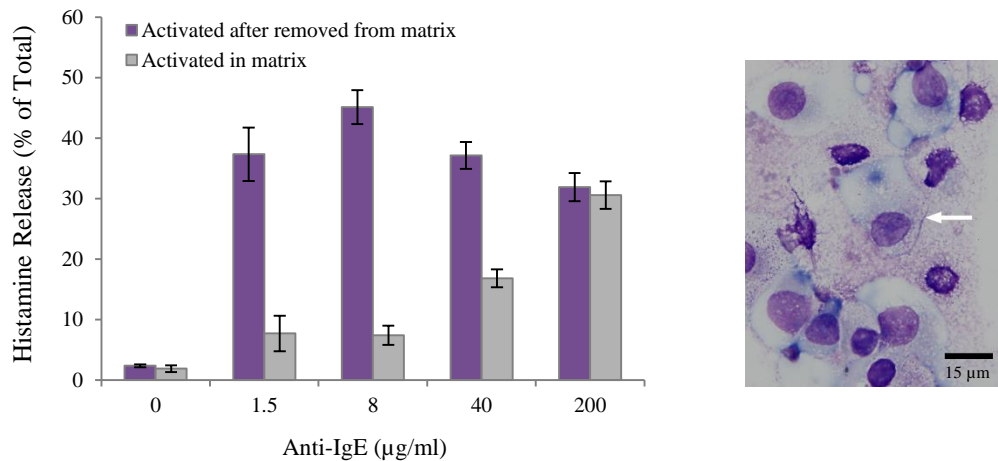


Fig. 2.7. Histamine release by the generated MCs after seven weeks in culture. The cells were activated either within the matrix or after removed from the matrix. The micrograph shows a few of the degranulated cells activated within the matrix. The white arrow highlights a degranulated MC. In all cases, StemSpan medium with serum in the seventh week of culture was used. Data are represented as mean \pm SD; $n=3$. All the data are significantly higher than the non-activated samples ($p < 0.05$).

2.4. Conclusion

In this work, we have established that MCs can be generated from HSCs isolated from peripheral blood within a 3D collagen matrix, based on the morphology of the CD133⁺-derived cells, the formation of cytoplasmic granules (histamine), and the expression of MC phenotypic markers (especially c-kit). In addition, according to the same criteria mentioned above, StemSpan with serum added in the last week of culture was the best media to generate functional MCs. StemSpan was also suitable for fibroblast and EC culture. Therefore, we established StemSpan as the ideal media, since it supports the differentiation of HSCs to MCs and phenotypic characteristics of ancillary cells. Furthermore, we determined that serum was not required for fibroblasts, but was required for EC survival and MC maturation during the last week of culture. As a result, to develop the 3D tissue model with all three cells types, fibroblasts and CD133⁺ cells would be seeded in the collagen matrix for six weeks using the serum-free media, then ECs would be added to the apical surface of the matrix in a serum-supplemented media during the seventh week of culture. This work demonstrates the possibility of creating the tissue model that could be used to study the effect of the microenvironmental factors with ancillary cells on MC development and function. However, this study shows that under the influence of microenvironmental factors the morphological and functional characteristics of the cells generated in 3D culture conditions can be altered, as evidenced by differences in their subtype and response to an activating agent when compared with a 2D culture system. The possibility of studying the effect of microenvironmental factors can be considered as the main advantage of utilizing the 3D matrix-embedded cells in elucidating MC ontogeny, biological profile, and immunoregulatory roles.

CHAPTER 3

Development of Human Mast Cells from Hematopoietic Stem Cells within a Connective Tissue- Equivalent Model

3.1. Introduction

Since the discovery of mast cells (MCs) by Paul Ehrlich in 1878 [127], researchers have attempted to understand the biological and functional characteristics of MCs to elucidate their role in the pathogenesis of innate and adaptive immune responses [67]. MCs are localized in tissues exposed to the external environment, accordingly positioned to be among the first immune cells that interact with invading pathogens and allergens [79]. Upon activation, MCs can selectively release the contents of their secretory granules and vesicles, including preformed mediators, such as histamine and neutral proteases, lipid mediators, and an array of *de novo* synthesized cytokines and chemokines [128]. Through the expression of such diverse molecules and receptors, MCs exert their effector and immunomodulatory functions on other immune and non-immune cells, such as fibroblasts and endothelial cells (ECs). For example, the MC histamine, tryptase, and leukotrienes can induce vascular permeability, collagen synthesis, and the influx of effector cells to the inflamed areas [129]. However, the local microenvironmental factors and the signals they receive from neighboring cells and molecules within the extracellular matrix (ECM) can alter their secretory profile and magnitude. For example, the epithelial cell-derived thymic stromal lymphopoietin, only in synergy with interleukin (IL)-1 and

tumor necrosis factor, induces the release of Th2 cytokines by MCs [130, 131]. Therefore, to fully understand the multifaceted roles of MC, they should be examined within the context of microenvironmental conditions that closely recapitulates the *in vivo* physiology.

MCs are derived from circulating stem and progenitor cells that migrate to tissues, differentiate, and mature into MCs under the regulation of the specific tissue milieu [80, 132]. Based on their protease contents, human MCs have been traditionally classified into two main subtypes: MC_{TC}, which contains both tryptase and chymase, and MC_T containing only tryptase [67, 80]. Altered site-specificity of MC subpopulations when infiltrated during inflammatory reactions along with their intrinsic differences in cytokine content and response to secretagogues and pharmacological agents indicate their distinct pathological roles [133, 134]. Although it is not clear whether certain factors direct the heterogeneous commitment of MC precursors, it is postulated that local and systemic environmental factors define MC phenotype and specialize them for particular biological and pathological functions [135, 136]. Furthermore, MCs express certain integrins and receptors for extracellular components of connective tissue, including laminin, fibronectin, and vitronectin that can regulate their localization, distribution, and proliferation in specific tissues [80]. Consequently, establishing a tissue-equivalent matrix has greater relevance for studying MC ontogeny and plasticity *in vitro*.

Due to the pivotal roles of *in vivo* microenvironmental factors, it has been challenging to define a method or approach to study MCs. Although murine models provide valuable information, fundamental differences between human and animal anatomy, cellular biology, and functions limit the applicability of the findings from murine models to human [20]. For example, murine MCs constitutively express Fc γ RI, while its expression by human MCs requires interferon- γ preincubation [137]. Also, in contrast to rodents, human MCs are plentiful in the alveolar parenchyma [138]. Due to difficulties in isolating human MCs *ex-vivo*, researchers have shown that the synergy of essential growth factors, including stem cell factor (SCF) and Th2-

derived cytokines or the presence of a layer of feeder cells, such as fibroblasts render human stem and progenitor cell differentiation into MC lineage *in vitro* [27, 47, 77]. However, due to the phenotypic and functional plasticity of MCs governed by tissue milieu, the generalization of the findings from a particular population that has been examined under conventional conditions in suspension (2D models) can be misleading and requires further investigation. Therefore, in this study, the objective was to establish a new culture condition for MC precursors that mimics their microlocalization within the connective tissue. MC precursors reside in proximity to fibroblasts and ECs in tissue, where they receive signaling molecules that contribute to their maturation and emergence of their distinct phenotype [55, 61]. Consequently, their incorporation into the tissue model would be a step toward replicating the MC *in vivo* microenvironment. Utilizing such a tissue model for MC characterization *in vitro* would shed more light on MC ontogeny, and physiological and pathological roles.

3.2. Material and methods

3.2.1. Antibodies and reagents

StemSpan media was purchased from STEMCELL Technologies (Vancouver, Canada). Human recombinant SCF, IL-6, and IL-3 were from PeproTech (Rocky Hill, NJ). Defined HyClone fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences (Logan, UT). Human dermal fibroblasts treated with mitomycin-C was purchased from Merck Millipore (Billerica, MA), while human umbilical vein ECs were from Promocell (Heidelberg, Germany). Anti-human fluorochrome-conjugated CD117/c-kit (clone 104D2), FcεRI (clone CRA-1), CD31 (clone WM59), CD90 (clone 5E10) and the isotype controls, Ms IgG1 (clone MOPC-21), Ms IgG2b (clone MPC-11) were from BioLegend (San Diego, CA). Anti-human fluorochrome-conjugated CD133/2 (clone 293C3) was purchased from MACS Miltenyi Biotec (San Diego, CA). For immunocytochemical staining, mouse anti-human tryptase (clone AA1) was purchased from Abcam (Cambridge, MA), mouse anti-human chymase (clone B7) was from Chemicon

International (Temecula, CA), and the secondary antibody goat anti-mouse IgG1 (polyclonal) was from Santa Cruz Biotechnology (Dallas, TX).

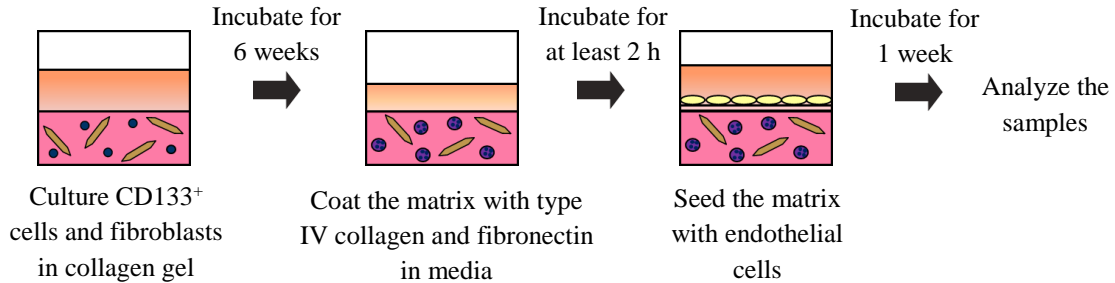


Fig. 3.1. Generation of mast cells (MCs) from stem cells within a connective tissue-equivalent matrix.

3.2.2. Cell culture

Preparation of samples is shown in Fig. 3.1. Briefly, MC progenitors, CD133⁺ cells, were obtained from peripheral blood mononuclear cells (PBMCs) using density gradient centrifugation method and magnetic separation technology (MACS Miltenyi Biotec). A type I collagen (Type-1 bovine, Advanced BioMatrix, CA) was used to prepare the gel solution [139]. Isolated cells from peripheral blood (70,000-75,000 cell/ml) and fibroblasts (40,000-70,000 cell/ml, mitomycin-C treated to prevent proliferation), were mixed with the collagen solution and added to the wells of a 48-solid well plate (Greiner Bio-One; NC) at 220 $\mu\text{l}/\text{cm}^2$. After 45 min incubation at 37°C, 5% CO₂ (defined as “standard conditions”) for the collagen to gel, StemSpan media (300 $\mu\text{l}/\text{well}$) was added to the top of the matrix. Media was supplemented with 100 ng/ml SCF, 50 ng/ml IL-6, and 1 ng/ml IL-3 for the first 3 weeks of culture. Samples were incubated for seven weeks to allow for the cell differentiation, proliferation, and maturation. Six weeks post seeding, spent media was aspirated and the gel was coated with 200 $\mu\text{l}/\text{cm}^2$ mixture of type IV collagen (Advanced BioMatrix; CA) and fibronectin (Alfa Aesar; MA) with final concentrations of 50 and 20 $\mu\text{g}/\text{ml}$, respectively, in media. After at least 2 h incubation and aspiration of the fibronectin solution, 45,000-55,000

cell/cm² of ECs were mixed with the described medium supplemented with FBS (20%, v/v) and seeded on top of the matrix. From day 1 of seeding, media was changed weekly and the morphology of the cells was monitored by microscopy.

3.2.3. Cell characterization

3.2.3.1. Cytology, histology, and immunohistochemistry

Metachromatic staining of MC cytoplasmic granules was evaluated cytologically following Giemsa stain using an automated stainer (Ames HemaTek 2000 Stainer). Histological evaluation for spatial observations of the tissue model was performed following careful removal of the 3D matrix from the well and fixation in 10% neutral buffered formalin. Following 24 h fixation, the matrix were routinely processed, paraffin-embedded and sectioned at 4 μ m. Sections were either stained with hematoxylin:eosin or submitted of immunohistochemistry for identification of CD117 and CD31. Immunostains were performed on a fee-for-service basis by the Histology Laboratory at North Carolina State University, College of Veterinary Medicine (Raleigh; NC).

3.2.3.2. Immunophenotype and proliferation

Expression of CD117/c-kit and Fc ϵ RI by the generated MCs was determined by immunofluorescence staining and flow cytometry (BD Accuri C6, BD Biosciences; CA). Cells were collected from the matrix after gel digestion by using 2.2 mg/ml collagenase D (Roche Applied Science; IN) and stained with appropriate antibodies or isotype controls. To sensitize MCs, 15 μ g/ml human myeloma IgE (Athens Research & Technology; Athens, GA) was added to the culture media for 24 h prior to assessing Fc ϵ RI expression. In order to identify MCs, fibroblasts and ECs or progenitor cells that did not differentiate were excluded by using their phenotypic markers, CD90 and CD31, respectively [139]. Cell numbers were determined after applying gates to the cell scatters based on the expression of phenotypic markers.

3.2.3.3. Immunocytochemistry

Intracellular tryptase and chymase were identified by immunocytochemical staining and flow cytometry. The cells were collected from the collagen matrix after gel digestion, added to a V-bottom 96-well plate (Greiner Bio-One; NC) and fixed/permeabilized using a commercial kit (BD Bioscience; CA). Cells were blocked with a buffer containing 1% bovine serum albumin (BSA, Sigma-Aldrich; MO) and 10% goat serum (v/v, Gibco; CA) in permeabilization solution for 1 h. Then, the samples were incubated with the primary antibodies against tryptase and chymase prior to the secondary antibody all 10 µg/ml in the blocking buffer for 30 min at room temperature. For microscopy, the cells were added to a glass slide and labeled with DAPI (Life Technologies; CA). After 1 h incubation in staining buffer containing 0.2% BSA and 0.09% sodium azide in phosphate-buffered saline (PBS), the cells were stained with anti-CD117, anti-CD90, and anti-CD31 antibodies or appropriate isotype controls for 45 min at 4°C and analyzed by the flow cytometer.

3.2.3.4. Cell activation

Generated cells were sensitized with 15 µg/ml human myeloma IgE for 24 h at 37°C. The free IgE was removed after three rinses with warm Tyrode's solution (Boston BioProducts; MA) supplemented with 100 µg/ml SCF and 50 µg/ml IL-6 prior to addition of 40 µg/ml monoclonal anti-human IgE (clone G7-18, BD Biosciences; CA) in Tyrode's supplemented solution for 1 h at 37°C. The Tyrode's solution consisted of 10 mM Hepes, 135 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 12 mM NaHCO₃, 0.4 mM NaH₂PO₄, 5.5 mM Glucose, and 0.25% BSA. The supernatant and the digested collagen gel were collected, and cellular histamine was measured after cell lysis in water by three freeze and thaw cycles and sonication for 5 min. Commercial ELISA kit (Labor Diagnostika Nord, Nordhorn; Germany) was used for quantifying histamine. Identical samples, but without IgE and anti-IgE was used as negative control. Release percentage was calculated as the ratio of histamine in the media and the digested gel to the total histamine content. In addition, the spontaneous amount of histamine released by the cells in the control group was subtracted from the total amount released.

To investigate the response of the generated MCs to an antigen, *Dermatophagoides pteronyssinus* (Der p) was selected. Non-allergic and allergic patients with a positive skin prick or intradermal test to Der p extract were included in this part of the study. A wheal of 3 mm or more in diameter developed within 15 min was considered positive with saline and 1% histamine as negative and positive control, respectively. The study protocol was approved by Oklahoma State University Institutional Review Board and written informed consent was obtained from each subject. MCs were generated by using the leukocyte preparations of the patients according to the protocol mentioned above. The cells were passively sensitized with 20% (v/v) serum of allergic (Phadia; MI) or non-allergic individuals (PlasmaLab International; WA) for 2 h at 37°C. The specific IgE in the allergic and non-allergic serum was 18-35 and 0.021 kU/l, respectively, validated by ImmunoCAP analysis done by the vendors. The same procedure as mentioned above for activation of the cells with anti-IgE was followed afterward, except that the Tyrode's solution used for rinses was not supplemented with cytokines and the Der p extract of mite (HollisterStier; WA) at various concentrations was added to the supplemented Tyrode's solution to induce cell activation. The concentrations and incubation times of the serum and allergen were selected based on the studies done on passive sensitization of MCs and leukocytes shown in Table 3.1 - 3.3 [140, 141]. A quantitative histamine plate kit (RefLab ApS, Copenhagen N; Denmark) was used to determine the concentration of histamine in the samples. Group of samples sensitized with the serum, but not activated with the allergen served as a negative control.

3.2.4. Statistical analysis

Experimental results are expressed as mean \pm SD of triplicate samples from three independent experiments unless otherwise stated. Student's *t*-test or Tukey-Kramer method was applied to determine significant difference among the groups. A value of $p < 0.05$ was considered significant. GraphPad Prism (GraphPad Software; CA) was used for nonlinear regression (curve fit) for histamine measurements and statistical analyses.

Table 3.1. Protocols for mast cell (MC) passive sensitization and activation.

	Source	Cell type	Stimuli	Passive sensitization	Stimuli incubation time	Allergen concentration	Ref.
1	Human	Intestinal MC	Der p, Grass pollen	30% plasma, 2 h	1 h	0.1, 100, 1000 U/ml	[141]
2	Human	Lung MC	DMA	2 h	30 min	0.1,1,10,100 µg/ml	[142]
3	Human	Lung fragment	Der p	12 h, 25°C	-	10,000 U/ml	[143]
4	Human	Lung fragment	Grass pollen	5% or 10%, 16-18 h, 20°C	15 min	1 µg/ml	[144]
5	Human	Lung fragment	Grass pollen	1%, over night at room T and 1 h at 37°C	15 min	100 µg/ml	[145]
6	Human	Lung fragment	Ragweed	40%, 90 min	13 min	100 PNU/ml	[146]
7	Human	Lung MC or fragment	Anti-IgE	2%, 2 h	10 min	-	[147]
8	Human	Skin fragment, basophils	Anti-IgE or Anti-FcεRI	50%, 20 min for skin fragments, 40 min for leukocytes	-	-	[148]
9	Human	Lung MC	Anti-IgE	Undiluted, overnight, room T	-	-	[149]
10	Human	Skin fragment	Anti-IgE	10%, 2 h	15 min	-	[150]
11	Human	Skin MC	Anti-IgE	10%, overnight, 23 or 37°C?	30 min	-	[151]
12	Human	Skin MC	Anti-IgE	10%, 16 h, 22°C	15 min	-	[97]
13	Human	Lung fragment	Anti-IgE	50%, 18 h, 4°C	45 min	-	[152]
14	Human	Bronchoalveolar lavage	Der p	-	30 min	0.03, 0.3, 3 BU/ml	[153]
15	Mice	BMMC	Der f	-	1-48 h	0.1-1 mg/ml	[154]
16	Mice, human	Mouse BMMC, human lung fragments	Grass	~11%, overnight	4 h	1:100, 1:20, 1:10, 1:2	[155]

BMMC: Bone marrow-derived mast cell; Der f: *Dermatophagoides farinae*; Der p: *Dermatophagoides pteronyssinus*; DMA: Dust mite allergen; MC: Mast cell. For passive sensitization and activation, the temperature is 37°C unless otherwise mentioned.

Table 3.2. Protocols for leukocyte (basophil) passive sensitization and histamine release test (HRT).

	Source	Cell type	Stimuli	Passive sensitization	Stimuli incubation time	Allergen concentration	Ref
1	Human	Leukocyte	Der p, cat, grass	50% EDTA plasma, 2 h	1 h	1, 100, 1000 BU/ml	[156]
2	Human	Leukocyte	HDM, cat, dog	50%, 2 h	1 h	0.05, 0.5, 5 HEP/ml	[157]
3	Human	Leukocyte	HDM	-	1 h	1, 10, 100, 1000 U/ml	[158]
4	Human	Leukocyte	Der p, cat, dog	-	1 h	500-0.5 BU/ml	[159]
5	Human	Leukocyte	Der p	-	-	Following [160]	[161]
6	Human	Leukocyte	Der p	-	15 min	2822-0.28 UBE/ml	[162]
7	Human	Basophil	Der p	-	15 min	0.16-16 µg/ml	[163]
8	Human	Basophil	Der p	-	1 h	0.8, 8, 80, 800 ng/ml	[164]
9	Human	Leukocyte	Der p	-	40 min	1.4 and 0.35 mg/ml	[165]
10	Human	Leukocyte	Der p	-	40 min	2, 20 ng/ml	[166]
11	Human	Leukocyte	Der p	-	40 min	0.01-1 µg/ml	[167]
12	Human	Basophil	HDM	20%, 2 h	45 min	-	[168]
13	Human	Leukocyte	Ragweed	-	10-60 min	10 ⁻⁵ -10 µg/ml	[160]
14	Human	Leukocyte	Ragweed	10%	5-60 min	0.1-2.5 ng/ml	[169]
15	Human	Leukocyte	Ragweed	10%	60 min	10 ⁻⁵ -1 µg/ml	[170]
16	Human	Leukocyte	Ragweed	5-20%, 1-4 h	1 h	0.025-100 ng/ml	[171]
17	Human	Leukocyte	Ragweed	<1-10%, 15-240 min	1 h	1-100 ng/ml	[140]
18	Human	Leukocyte	Ragweed	1.25-5%, 1-2 h	1 h	10 or 15 ng/ml	[172]
19	Human	Leukocyte	Ragweed	16 or 10%, 1 or 2 h	30-45 min	To cause 50% release	[173]
20	Human	Leukocyte	Ragweed	50-10%, 60-90 min	40 min	0.1 µg/ml	[174]
21	Human	Leukocyte	Ragweed	~16%, 90 min	40 min	Following [160]	[175]
22	Human	Leukocyte	Grass pollen	-	1 h	0.2, 2, 20, 200 PNU/ml	[176]
23	Human	Leukocyte	Grass pollen	9%, 1 h	1 h	1:1 M-1:10000	[177]
24	Human	Basophil	Birch pollen profilin	-	-	0.01-1000 ng/ml	[178]
25	Human	Leukocyte	-	50%, 40 min	-	-	[179]
26	Human	Leukocyte	Peanut	-	20 min	0.01-100 µg/ml	[180]
27	Rat	RBL	Egg	1%, 16 h	3 h	10-1000 ng/ml	[181]
28	Rat	RBL	Der p, pollen	2%, 48 h	1 h for anti-IgE	10 ⁻² -10 ⁻⁶ dilution	[182]
29	Rat	RBL	Pollen	5-2.5%, overnight	-	1:100-1:10 ⁻⁹	[183]

Der p: *Dermatophagoides pteronyssinus*; *Der f*: *Dermatophagoides farinae*; HDM: House dust mite; RBL: Rat basophilic leukemia cell line.

Table 3.3. Protocols for passive sensitization and cell activation using purified allergens.

	Source	Cell type	Stimuli	Passive sensitization	Stimuli incubation time	Allergen concentration	Ref
1	Human	Lung and skin MC, MC line, Basophils	rDer p1, HDM medium, Bee venom, Der p 3	-	2 h	10 µg/ml	[184]
2	Human	Nasal MC	rDer f 2	-	48 h	10 µg/ml	[44]
3	Human	CD133-derived MC	rDer p 2	80 U/ml rIgE	30 min	0.0001-100 ng/ml	[185]
4	Human	PB-derived MC	rDer p 2	0.8-800 U/ml rIgE, 2 weeks	30 min	0.0001-100 ng/ml	[186]
5	Human	Basophil	rDer f 1, rDer p 1	-	1 h?	0.1-100 ng/ml	[187]
6	Human	Basophil	Der p 1	-	-	0.01, 0.1, 1 µg/ml	[188]
7	Human	Basophil	r or nDer p 1 r and nDer f 1	-	1 h?	0.18, 1.8, 18, 180 ng/ml	[189]
8	Human	Basophil	r and nDer p 1	-	30 min	0.001-1000 ng/ml	[190]
9	Human	Leukocytes	Der p 1	Not mentioned	-	0.1 ng/ml-10 µg/ml	[191]
10	Human	Leukocytes	Der p 1, 2, Lol p 1, 5, Fel d 1	-	45 min	Following [160]	[192]
11	Human	Leukocytes	Der f 1	1:3 dilution, 90 min	-	10 ⁻¹⁰ -10 ⁻⁵ g/ml	[193]

Der f: *Dermatophagoides farinae*; Der p: *Dermatophagoides pteronyssinus*; Fel d: *Felis domesticus*; Lol p: *Lolium perenne*; MC: Mast cell; n: Natural; PB: Peripheral blood; r: Recombinant.

3.2.5. Possible hazard and safety precautions

All animal- and human-derived materials were purchased from commercial vendors that prescreen the products for biological hazards. The personnel conducted the research completed a training program for handling the biohazardous materials and working in a biosafety level 2 (BSL-2) laboratory. Prior to the experiments, all biohazards were identified and biosafety plans were prepared and followed. All procedures associated with the work followed “Universal Precautions”. Biohazardous materials were disposed according to the guidelines for each waste category.

3.3. Results

3.3.1. Morphology of the generated cells within the connective tissue-equivalent model

After seeding, the cells were evenly distributed within the matrix, while the MC precursors formed colonies as early as the first week of culture (Fig. 3.2A). The generated cells remained in colonies and spread within the matrix as the cell proliferation progressed. The cells were round or oval as well as tailed, showing their mobility within the matrix, as highlighted in Fig 3.2A, week 1. Seven weeks post seeding, around 80% of the round cells were 9 – 15 μm in diameter, which is in the range of *in vivo* MC size (data from four independent experiments, n = 230 cells) [95, 97]. Furthermore, the ECs formed a confluent layer on the matrix within 36 h post seeding (Fig. 3.2B). The histologic analysis of the collagen matrix confirmed a connective tissue-equivalent matrix (CTEM), with a monolayer of CD31⁺ ECs on the apical surface and the CD117⁺ cells along with the fibroblasts spread within the subendothelium (Fig. 3.2C-E). Although progenitor cells are not granular, the generated cells exhibited granular morphology with 70 – 90% of the granular cells mononucleated, as is the normal morphology of mature MCs (Fig. 3.2F, data from two independent experiments, n = 700 cells).

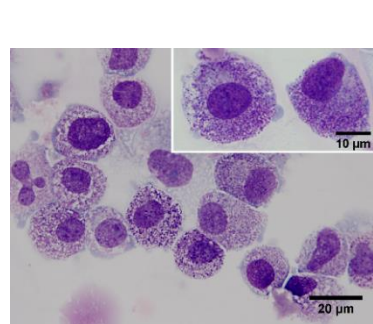
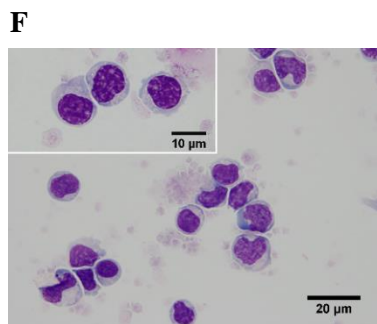
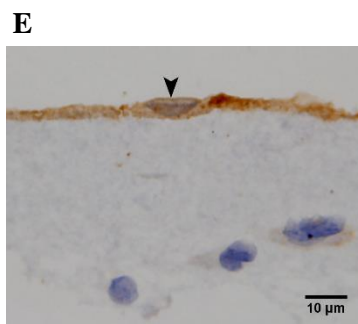
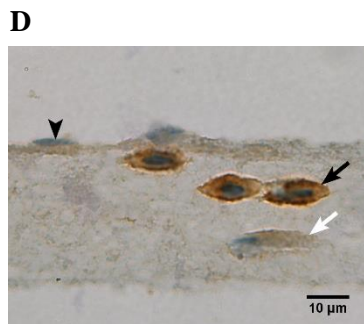
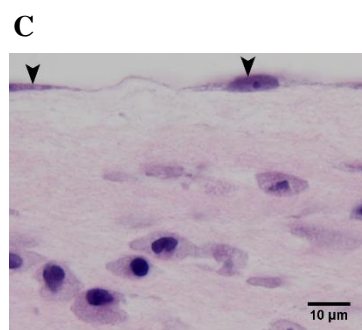
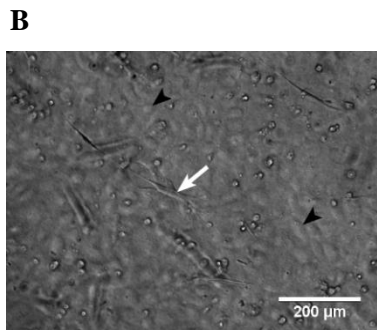
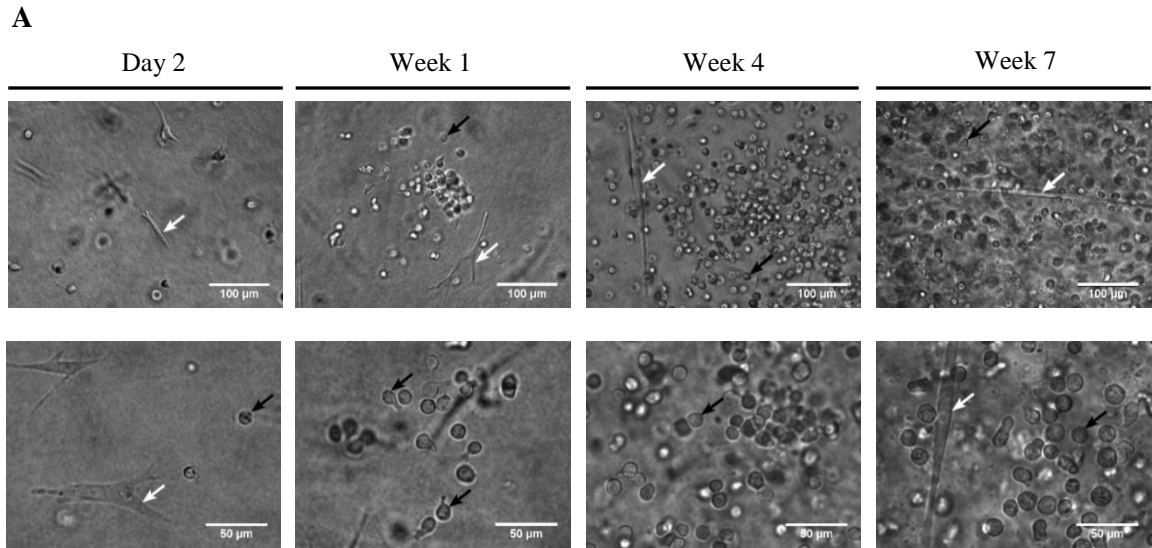


Fig. 3.2. Morphology of the seeded cells within the connective tissue-equivalent matrix. (A) The generated cells and fibroblasts 2 days and 1-7 weeks post seeding within the matrix. (B) The seeded matrix with ECs. Histologic analysis of the matrix stained with (C) hematoxylin and eosin (D) anti-CD117/c-kit (E) anti-CD31 antibodies. (F) Wright-Giemsa staining of (left) freshly isolated cells from peripheral blood and (right) generated cells after seven weeks in co-culture. Some of the MC precursors and fibroblasts are highlighted by black and white arrows, respectively and ECs are highlighted by the arrowheads.

3.3.2. Immunophenotype of the generated cells within the connective tissue-equivalent model

CD133 antigen has been used as a marker of hematopoietic progenitor and stem cells [194]. The frequency of isolated cells from human PBMCs was $0.7 \pm 0.4\%$ (from 20 buffy coats) and was comparable with other studies, as shown in Table 3.4. The purity of CD133⁺ cells within the magnetically isolated cells was less than 50%. As shown in Fig. 3.3A, the CD133⁺ cells before culture were more than 80% and around 40% positive for CD31 and CD117, respectively, while only less than 10% were expressing FcεRI. As shown in Table 3.5, the expression levels were in agreement with other studies. For better use of Table 3.5, the expression of CD34 by CD133⁺ cells are shown in Table 3.6. After 7 weeks in co-culture, the majority of the seeded progenitor cells lost the CD31 and expressed CD117 receptors. After excluding fibroblasts and ECs or non-differentiated progenitor expressing CD31 (Fig. 3.3B), the CD117⁺ CD31⁻ CD90⁻ cells were 3.2 ± 1.3 to 8.4 ± 0.1 folds higher in number than the seeded cells. Also, the expression of FcεRI was enhanced after 24 h incubation with the IgE antibody with 5.8 ± 1.3 -folds higher mean fluorescence intensity (MFI, $p < 0.01$), which reflects the upregulation of the surface receptor density. In fact, IgE incubation resulted in the expression of FcεRI by $31.9 \pm 3.6\%$ of CD117⁺ cells (Fig. 3.3B, data selected from three independent experiments, $n = 3$). Furthermore, almost

all the generated CD117⁺ cells were stained for tryptase and chymase granules ($98.7 \pm 0.8\%$ and $96.7 \pm 2.6\%$, respectively), exhibiting MC_{TC} phenotype (Fig. 3B).

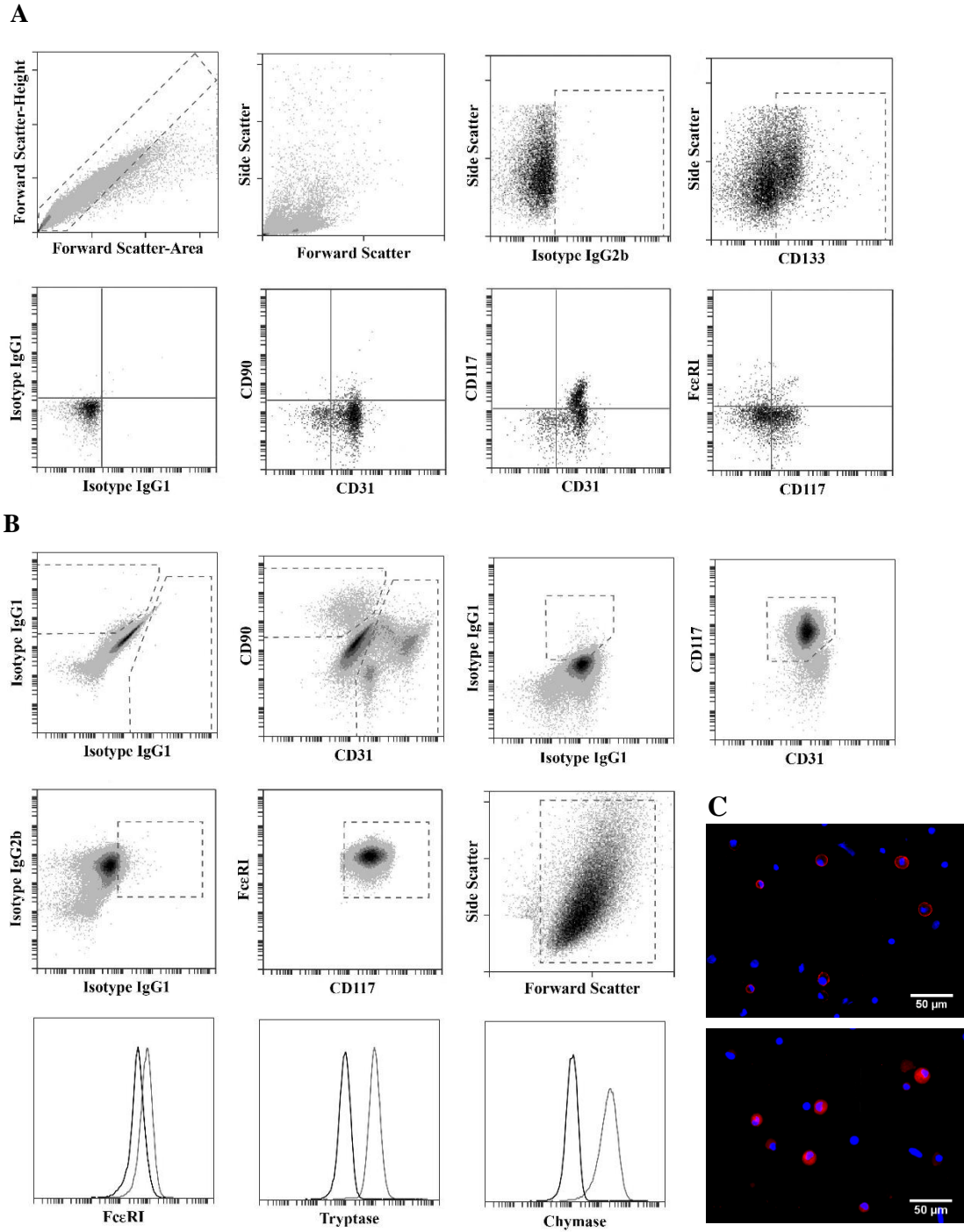


Fig. 3.3. Expression of mast cell (MC) phenotypic markers by the generated cells within the connective tissue-equivalent matrix. (A) Representative density plots of the expression of the markers used to identify MCs by CD133⁺ cells isolated from peripheral blood before co-culture. (B) Gating scheme and representative density plots of marker expressions by the generated cells after co-culture. In order to identify the CD117 expressing cells, anti-CD90 and CD31 antibodies were used to gate out fibroblasts and ECs or progenitor cells, respectively. The expression of FcεRI, tryptase, and chymase (gray histogram) by the CD117 positive cells is compared with the fluorescence minus one (FMO) control (black histogram). (B) Expression of (top) CD117 and (bottom) tryptase determined by immunofluorescence staining. Cells were stained with anti-tryptase or CD117 and PE-secondary antibody. Nuclei were stained with DAPI.

3.3.3. Activation of the generated cells within the connective tissue-equivalent model in an IgE-mediated challenge

After seven weeks in co-culture, the histamine content of the generated cells was 4.4 ± 0.8 pg/cell and their histamine spontaneous release was 2.3 ± 0.9 % of their total histamine content. Function of the generated MCs was examined after challenge with IgE and anti-IgE, which is known to cause histamine release upon FcεRI trigger [30]. Upon sensitization and activation with IgE and anti-IgE, the cells released 31.2 ± 2.0 % of their total histamine content, which was 13.3 ± 5.3 -folds higher than the spontaneous release of histamine ($p < 0.001$). Around 80-90% of the released histamine was detected within the matrix. When passively sensitized with allergic serum, the cells dose-dependently released histamine in response to Der p allergen (Fig. 3.4). Since the extract of the allergen contained glycerol as a preservative, identical concentrations of the glycerol were also tested, but did not induce histamine release (data not shown). The histamine release in response to 15 AU/ml of the allergen, which showed the highest average, was 18.09 ± 4.10 -folds higher than the control group that was not activated with the allergen ($p < 0.001$). In

contrast, the cells did not respond to the allergen when serum from a non-allergic individual that did not contain specific IgE to Der p allergen was used.

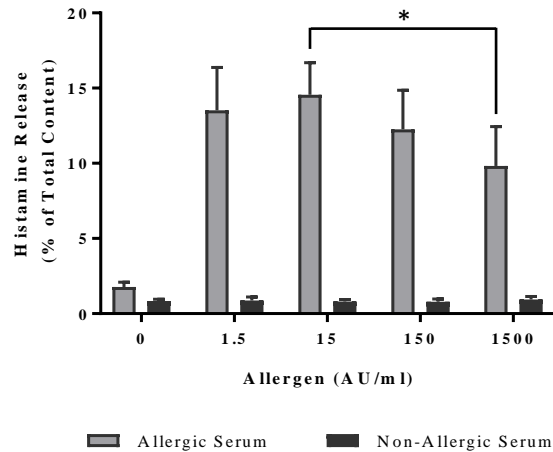


Fig. 3.4. Histamine release by the generated mast cells (MCs) in response to Der p allergen within the connective tissue-equivalent matrix. The cells were passively sensitized with human serum prior to activation with the allergen. All the data from allergic serum are significantly higher than the non-activated samples ($p < 0.01$). Data are mean \pm SD. * indicates $p < 0.05$.

3.4. Discussion

CD34⁺ and CD133⁺ stem cells or mononuclear cells have been previously used to generate MCs under 2D culture conditions for *in vitro* studies [60, 195, 196]. Depending on the cell source and culture conditions, specifically the culture media supplements, the characteristics of the obtained MCs varied [27, 60, 77], e. g., in contrast to IL-10, IL-4 enhanced IgE receptor expression and cell function [60, 197]. Given that the cells within a matrix are surrounded with the ECM components that can alter the distribution, morphological, and physiological characteristics of the cells [198-200], 2D models may lack the needed geometry and components to mimic the *in vivo*. A 3D matrix can regulate commitment of stem cells to a specific lineage

Table 3.4. CD34⁺ and CD133⁺ cell isolation yields.

	Source	Donor	Isolated	MNC number/volume	% of MNC	Ref
1	PB	H	CD133		0.035±0.011 (0.023-0.057)	[201]
2	PB	H	CD34		0.057±0.029 (0.031-0.133)	[201]
3	PB	H and UH	CD34	10 ⁹	0.05	[202]
4	mPB	H	CD133	1.3(0.7-3.8)×10 ¹⁰	0.75 (0.39-2.03)	[203]
5	mPB	UH	CD133		1.37±0.27	[204]
6	mPB	UH	CD34		1.75±0.31	[204]
7	mPB	H	CD34		0.8±0.5 (0.4-1.6)	[205]
8	mPB	UH	CD34		1.2±0.3-3.7±0.8	[206]
9	mPB	UH	CD34	17.05(3.86-67)×10 ⁹ /L		[207]
10	BM	H	CD133		0.52±0.11	[204]
11	BM	H	CD34		1.47±0.23	[204]
12	BM	UH	CD34		1.43 (0.26-4.11)	[207]
13	mBM	UH	CD34		1.06 (0.15-5.89)	[207]
14	FBM	H	CD34		10.5±3.2 (6.6-17.2)	[205]
15	ABM	H	CD34		1.7±1.2 (0.5-3.6)	[205]
16	CB	H	CD133	208.2 (99-294)×10 ⁶ /45±4 ml	~0.19, [0.4 (0.2-1.18)×10 ⁶]	[208]
17	CB	H	CD133		0.71 (0.3-2.4)	[209]
18	CB	H	CD133		0.16±0.03	[204]
19	CB	H	CD34	244 (77.5-700)×10 ⁶ /46±3 ml	~0.29, [0.7 (0.14-4.8)×10 ⁶]	[208]
20	CB	H	CD34	6 (4-10)×10 ⁸ /75 ml	0.4±0.1	[210]
21	CB	H	CD34	(66-90)×10 ⁶ /(30-32) ml	0.78 (0.4-2.7)	[209]
22	CB	H	CD34		0.8±0.7 (0-2.6)	[205]
23	CB	H	CD34		0.8-2.9	[211]
24	CB	H	CD34		0.32±0.06	[204]
25	FL	H	CD34		11.4±7.5 (2.3-25.8)	[205]
26	PB	H	CD133	1190 (440-2500)×10 ⁶ /46 (37-52)ml	0.73±0.43 (0.26-1.55)	Our work†

ABM: Adult Bone Marrow; BM: Bone Marrow; CB: Cord Blood; FBM: Fetal Bone Marrow; FL: Fetal Liver; H: Healthy; mBM: Mobilized Bone Marrow; MNC: Mononuclear cells; mPB: Mobilized Peripheral Blood; PB: Peripheral Blood; UH: Unhealthy. † Data from 20 buffy coats. The purity of the cells for every batch was not evaluated.

Table 3.5. Expression of CD90, CD117/c-kit, CD31, and FcεRI by CD133⁺ and CD34⁺ stem and progenitor cells.

	Source	Donor	Marker	PB	mPB	FBM	BM	ABM	mBM	CB	FL	Ref
1	CD34 ⁺ CD38 ⁻ Lin ⁻ flt-3 ⁻	H	CD90				46.1					[212]
2	CD34 ⁺	UH	CD90		Pos							[213]
3	CD34 ⁺	UH	CD90		Pos							[214]
4	CD34 ⁺ CD38 ⁻	H	CD90		1.2±0.4	3.1-2.3		0.8±0.6		3.2±1.8	19.7±13.4	[205]
5	CD34 ⁺ CD38 ⁺	H	CD90		~40	~10		~20		~30	~15	[205]
6	CD34 ⁺	H	CD90							Pos		[215]
7	CD34 ⁺	UH	CD90		18.1±1.1 (4.7-29.1)							[206]
8	CD34 ⁺	UH	CD90				16.9-48.5					[216]
9	CD34 ⁺	UH	CD90		8.2±4							[217]
10	CD34 ⁺	UH	CD90			5-25						[218]
11	CD34 ⁺	UH	CD90				~11(3-27)		3-41			[207]
12	CD34 ⁺ CD45RA ⁻ CD71 ⁻	H	CD90							5		[211]
13	CD34 ⁺ Lin ⁻	H	CD90					5-20				[219]
14	CD34 ⁺	H	CD90				25 (12-41)			32 (10-54)	30 (18-38)	[220]
15	MNC	H	CD90	0.2-1, 1.5 FPB		1.4-2		<0.1-0.5			<0.1-0.2	[221]
16	CD34 ⁺	H	c-kit				75±30					[222]
17	CD133 ⁺	H	c-kit							93.3±1.1		[223]
18	CD34 ⁺	H	c-kit					Pos				[224]
19	CD133 ⁺ and CD34 ⁺	H	c-kit				Pos					[225]
20	CD34 ⁺	H PB, UH BM	c-kit	Pos			Pos					[106]
21	CD34 ⁺	UH	c-kit		20.6±7.5							[226]
22	CD34 ⁺	UH	c-kit				~71(44-90)		0-88			[207]
23	CD34 ⁺	H and UH	c-kit	50								[202]
24	CD34 ⁺	UH mPB and H BM	c-kit	45±8 (34-60)	10±3-35±17		46±5 (35-69)					[227]
25	CD34 ⁺	H	c-kit				Pos			Pos	Pos	[220]
26	CD34 ⁺	UH	c-kit	12.6±3.2	4.6±1.2		32.2±3.3					[228]
27	CD34 ⁺	H	c-kit				71±5					[229]
28	CD34 ⁺	H	c-kit				77±12					[230]
29	CD133 ⁺	H	CD31	Pos								[231]
30	CD133 ⁺	H	CD31	Pos								[232]
31	CD45 ⁻ CD34 ⁻ CD133 ^{low}	H	CD31				Neg					[233]
32	CD34 ⁺	H and UH	FcεRI	Neg								[202]

ABM: Adult Bone Marrow; BM: Bone Marrow; CB: Cord Blood; FBM: Fetal Bone Marrow; FL: Fetal Liver; FPB: Fetal Peripheral Blood; H: Healthy; Lin: Lineage; MNC: Mononuclear cells; mPB: Mobilized Peripheral Blood; Neg: Negative; PB: Peripheral Blood; Pos: Positive; UH: Unhealthy.

Table 3.6. Expression of CD34 and CD133 by isolated CD133⁺ and CD34⁺ stem and progenitor cells, respectively.

	Donor	Isolated population	Marker expression	PB	mPB	BM	ABM	CB	FL	Ref
1	H	CD133 ⁺	CD34	69±3						[231]
2	H	CD133 ⁺	CD34					90.8±5.3		[222]
3	H	CD133 ⁺	CD34		100 (90% of donors)					[203]
4	H	CD133 ⁺	CD34	27.6						[232]
5	H	CD133 ⁺	CD34					78.8±3.9		[208]
6	H	CD133 ⁺	CD34	99						[201]
7	UH	CD133 ⁺	CD34		99.08					[101]
8	H	CD133 ⁺	CD34					100		[209]
9	H	CD34 ⁺	CD133	28.6						[232]
10	H	CD34 ⁺	CD133					26.5±11.5		[208]
11	H	CD34 ⁺	CD133	55±9 (41-73)	89	48		83±9 (68-95)		[201]
12	H	CD34 ⁺	CD133					78.7 (56.3-88.1)		[209]
13	UH	CD34 ⁺	CD133					60-80		[225]
14	H	CD34 ⁺	CD133					50		[210]
15	H	CD34 ⁺	CD133		75±1	36±2		51±2		[204]
16	H	CD34 ⁺	CD133		54.9	31.6	70	35.16	29.88	[74]

ABM: Adult Bone Marrow; BM: Bone Marrow; CB: Cord Blood; FL: Fetal Liver; H: Healthy; mPB: Mobilized Peripheral Blood; PB: Peripheral Blood; UH: Unhealthy.

and enhance their maturity [234, 235]. Therefore, in this study, we have established a novel culture method for the generation of functional MCs from peripheral blood progenitor cells within a CTEM. We have used type I collagen, which is the most predominant fibrillar component of the ECM found in the dermis and interstitial tissue, to create a matrix [236, 237]. The matrix was coated with type IV collagen and fibronectin in order to mimic the basement membrane, enhancing the EC growth and attachment to the matrix [238-240]. The microarchitecture of the matrix allowed the cell motility within the collagen matrix, as evidenced by colony formation and tailed-like cells (Fig. 3.2A). We have previously determined that StemSpan medium supports the generation of MCs and growth of fibroblasts and ECs [139]. Serum was a prerequisite for EC survival, while its addition to the media from the beginning of culture suppressed MC development [139]. When added in the seventh or eighth week of culture, serum induced FcεRI expression and histamine release by the generated cells [58, 59]. Therefore for the co-culture model in this work, ECs were added in the seventh week, which was the same time that media was supplemented with serum.

MCs are distributed near blood or lymphatic vessels and fibroblasts that are abundant in connective tissue. Both fibroblasts and ECs release growth factors, such as SCF and IL-6 that support MC survival and growth [49, 115, 139]. When 3T3 fibroblasts were in co-culture with human cord blood and human or mice bone marrow-derived MCs the differentiation and proliferation of MC precursors were enhanced [63, 241, 242]. In addition, ECs promoted MC proliferation more effectively than SCF-supplemented media through interaction between CD117 and very late antigen (VLA)-4 on MCs and membrane form of SCF (mSCF) and vascular cell adhesion protein-1 (VECAM-1) on ECs, respectively [61]. When compared with the 3D tissue model without ancillary cells [139], even with seven times lower progenitor cell seeding concentration, in co-culture with fibroblasts and ECs not only the cell yield was significantly higher, but the histamine content was also augmented ($p < 0.01$). The same has been measured for

mice bone marrow-derived MCs when co-cultured with 3T3 fibroblasts in a culture medium containing IL-3 [241]. Although fibroblasts were shown to support the survival of MCs, even when separated, the direct cellular interactions induced the maturity and regulated the effector functions of MCs [64, 132, 243, 244]. As an example, activated MCs isolated from the human lung in co-culture with fibroblasts released elevated levels of histamine in a cell contact-dependent manner [244]. The results in this work were similar to the previous findings as the generated cells in co-culture released approximately two-fold higher histamine compared with the 3D model in the absence of ancillary cells [139] in response to anti-IgE ($p < 0.001$). Soluble SCF in synergy with mSCF expressed by fibroblasts and other fibroblast-derived proteins, such as IL-33 may modulate MC maturity and function [64, 132, 245]. The mSCF also induce MC attachment to fibroblasts, ECs, and basement membrane proteins and may play a role in MC migration and localization of progenitor cells bearing its agonist, CD117, in connective tissue [246-248]. Furthermore, when in direct contact with fibroblasts, MCs were responsive to eosinophil major basic protein and increased eotaxin, an eosinophil chemotactic factor, all of which indicating the importance of cellular interactions in leukocyte infiltration during inflammatory responses and utilizing co-culture models for *in vitro* studies [243, 244].

In vitro studies have shown that stem cells can differentiate into MC_T or MC_{TC} phenotype based on the chymase expression. When human or mice cord blood- and mice fetal liver-derived MCs were in co-culture with fibroblasts they appeared as MC_{TC} and MC_T, respectively [55, 66, 249], suggesting that progenitor cells might be committed to a specific MC phenotype. However, human cord blood and peripheral blood CD34⁺ cells in the presence of SCF, IL-6, and IL-3 was shown to differentiate into both subtypes [57, 250, 251], while the presence of fibroblasts and IL-4 favored the generation of MC_{TC} [49, 116, 252], indicating that cell microenvironment and specific growth factors can influence the phenotype of the generated MCs. Therefore, the ultimate MC phenotype *in vivo* may be regulated under the influence of local tissue milieu that specializes

the generated MCs to perform certain functions. For example, the proliferation of mucosal MCs in mice, which are mainly chymase negative (MMC) caused by helminth infection suggests their role in defense against parasites [253] or appearance of MC_T in cutaneous lesions of patients with atopic dermatitis implies their role in skin inflammation [133]. In our previous work, in the absence of ancillary cells, the developed MCs within the 3D matrix appeared as MC_{TC} subtype [139]. In this work, in co-culture with fibroblasts and ECs, their phenotype was preserved, indicating that factors besides the cellular interactions, such as ECM proteins can determine the fate of MC phenotype. In addition, previous findings suggest that human MC subtypes are not restricted to a specific anatomical location and may be developed, recruited, or dynamically change as a result of inflammation, infection, interaction with neighboring cells, and released cytokines [66]. As an example, MCs in the respiratory system are site-specifically heterogeneous [254] and in asthmatic patients recruitment of MCs particularly of chymase positive phenotype to airway smooth muscle and small airway has been observed [69, 255]. Furthermore, human MCs generated from cord blood after co-culture with epithelial cells was shown to undergo a transition from MC_{TC} to MC_T type [256], while isolated intestine MCs in co-culture with ECs upregulated the expression of chymase, indicating the development of MC_{TC} [61]. This transition matches with the population of MCs located in proximity to epithelial cells in alveoli and bronchi (MC_T) and around blood vessels (MC_{TC}) [257]. The result of our study is also in line with these findings as the morphological phenotype of the developed cells within the CTEM matched with their counterparts in skin and submucosa (MC_{TC}) [67]. This has also been confirmed *in vivo* where MMCs generated from bone marrow were transferred into the peritoneal cavity of mice and acquired the features of resident connective tissue MCs. Also, when intravenously injected, MMCs could develop into both subtypes in MC-deficient mice in accordance with their anatomical location [258]. All of which signifies the complexity of MC ontogeny and plasticity that requires to be clarified within the context of *in vivo* tissue-equivalent models.

In order to compare the characteristics of the cells obtained in this study with previous protocols, a 2D culture method that generated MCs in a more similar condition than other studies (Table 3.7) to our 3D model was selected [59, 60]. In this study, considering that the cells were not expanded and maintained within a matrix, by using more than seven times lower concentration of progenitors, the yield for generation of CD117⁺ MCs (4.6 ± 2.4) was higher in average than the selected 2D method with yield of 3.2 ± 1 for granular cells and CD117 expression of $88.3 \pm 2.2\%$. The size and the histamine content of MCs developed within the CTEM were within the range of *in vivo* MCs isolated from human skin, intestine or lung with around 2-5 pg histamine/cell (Table 3.8) [97, 124, 259-261], while it was higher for the selected 2D culture method (23.3 ± 3.3 pg/cell). This indicates that the cells in 3D culture condition can be morphologically different from cells in 2D and a more representative of *in vivo* cells. Although FcεRI has been considered as a phenotypic marker of MCs, human MC subtypes can express IgE receptor at different levels [254]. Given that IgE antibody upregulates the expression of FcεRI, as has also been observed in this work, it can facilitate the detection of IgE receptors [107, 108]. Due to the possible binding of IgE antibodies to the matrix or transport limitations, a higher concentration of it than what has been typically used for the cells in 2D culture [57, 59, 262] was needed to mitigate the effect of the collagen matrix. Furthermore, because of the matrix volume, the actual concentration of the antibodies within the matrix is lower than the concentration in the media before addition to the samples. Regardless of whether the concentration of IgE antibody used for stabilization of the receptors was optimal or not, the expression of FcεRI for the generated cells within the CTEM was comparable with the selected 2D model. In order to examine the function of the generated cells, the release of histamine in an anti-IgE-mediated reaction was measured. As shown in previous studies (Table 3.8 and 3.9), the response of MCs depends on the concentration of anti-IgE used for their activation and the activation protocol [58-60, 142, 262, 263]. For this study, the response of the generated cells to a selected concentration of anti-IgE (40 μg/ml) is shown. According to our previous observation [139], the selected

concentration of anti-IgE is not high enough to induce the maximum histamine they can release within a matrix. Therefore, the histamine released ($31.2 \pm 2.0\%$) seems to be lower than the selected 2D model ($52.9 \pm 2.5\%$) that utilized an optimal concentration of anti-IgE to obtain the maximum histamine release. It is of great importance to highlight that when we examined the function of the cells that were generated within the matrix but removed from it prior to activation, the histamine degranulation was comparable to the cells developed and activated under 2D culture conditions [139]. This indicates that the reduced histamine release in the matrix was not because the generated cells were functionally impaired but rather the effect of anti-IgE was attenuated by the cell microenvironment. Reduced levels of histamine release in response to anti-IgE for MCs in lung fragments compared with dispersed cells from the same tissue has also been observed [261, 264]. Furthermore, the response varies with the tissue type that MCs were isolated from possibly due to their functional heterogeneity [124, 265, 266]. Therefore one cannot expect the same level of histamine release from MCs tested *ex vivo* or generated *in vitro*, besides the discrepancies in the activation protocols (Table 3.8 and 3.9). Nevertheless, our main objective was to demonstrate that the generated cells can also degranulate in an immediate hypersensitivity reaction. In contrast with serum of a non-allergic patient, the MCs, regardless of whether they were generated from peripheral blood of allergic or non-allergic individuals, were passively sensitized with allergic serum and released histamine in response to Der p allergen. The response curve is analogous to the response of passively sensitized MCs isolated from human tissue to dust mite or grass pollen allergen [142, 267]. With increasing the concentration of the antigen the histamine release reaches its maximum level and afterward due to the saturation of antibody-binding sites or formation of clusters of crosslinked receptors swept to the tail of the cells (patching and capping) decreases, therefore forming a bell-shaped response curve [268, 269]. It is noteworthy that in the control group that was also sensitized with the serum but not activated with the allergen when the allergic serum was used the histamine release was marginally higher than non-allergic serum ($p < 0.01$). In fact, previous studies have shown that some type of IgEs called

cytokinergic IgEs can induce histamine and cytokine release [270-272] and the results of this study indicate that human allergic serum can activate MCs in the absence of an antigen.

3.5. Conclusion

MCs can play a role in the pathogenesis of IgE and non-IgE mediated immune responses, as well as host defense against parasites, bacteria, and viruses [66, 67]. Here, we have created a CTEM with monolayer of ECs that supported the development of MCs from their precursors. The generated cells were granular and exhibited MC phenotypic markers. In addition, the stem cell-derived MCs within the CTEM were able to respond in IgE-mediated reactions, specifically to an allergen, therefore may serve as a novel tool in clinical settings for diagnosis of allergic diseases [273] or pharmacological studies. Indeed, previous studies on immunotherapy had shown that cells in 3D culture conditions exhibit a more similar resistance to drugs seen in human than 2D culture [274]. Therefore, 3D models may be widely used in clinical trials due to a higher potential in predicting *in vivo* cellular responses. Furthermore, the developed tissue model can be administered in researches on the mechanism of allergic diseases and other inflammatory disorders. As an example, MC histamine and tumor necrosis factor- α (TNF- α) can stimulate ECs to release proinflammatory cytokines and express adhesion molecules [275-277], suggesting the role of MCs in the late phase of inflammatory reactions, which can be demonstrated using the developed tissue model in this work. Furthermore, the release of fibrogenic, fibrolytic, and proangiogenic factors, such as tryptase, transforming growth factor- β (TGF- β), heparin, basic fibroblastic growth factor (bFGF), and vascular endothelial growth factor (VEGF) suggest MC role in fibrosis, tissue remodeling, and vascular diseases [81, 278]. MC co-culture with fibroblasts was shown to induce collagen synthesis as well as collagen contraction mediated by the expression of matrix metalloproteinases (MMPs), which may modulate the infiltration of inflammatory cells in asthmatic airways and fibrotic tissues [115, 279-281]. ECs alone or in co-culture with fibroblasts can also induce collagen contraction [282, 283]. Likewise, we have

observed the contraction of the collagen matrix depending on the number of seeded fibroblasts and generated MCs, especially after the gel was seeded with ECs. Therefore, the co-culture of all three cell types may be required for a more comprehensive study of the mechanism of tissue remodeling and wound healing. Many of the findings in this area have not been demonstrated by using primary human cells or direct cellular interactions, which occurs continuously in the human body. Therefore, with the advent of 3D culture method, which provides the condition for cell motility and migration, interaction with multiple cells types and ECM proteins, numerous hypothesis that remained unanswered or controversial can be examined.

Table 3.7. Characteristics of progenitor-derived mast cells (MCs).

	Cell	Source	Yield (fold)	Histamine content (pg/cell)	Tryptase/chymase content (pg/cell)	Tryptase %/ chymase %	c-kit %	FcεRI %	Media cytokine	Culture time (weeks)	Ref.
1	CD34	PB	32±29	-	-	Pos/Pos	55-90	55-90	SCF, IL-3, LDL	3	[284]
2	CD133	PB, CB	3.2±1 PB, 101±20 CB	23.3±3.3 PB, 5.9±1 CB	-	75/ND	88.3±2.2 PB 91.5±1.1 CB	31.4±3.3 PB 33.9±6.6 CB	SCF, IL-3, 6, FCS last week, tested: IL-4	7	[60]
3	CD133	PB	3.3±0.5	15.5±5.3	-	80±5/Neg	88.3±2.2	31.4±3.3	SCF, IL-6, 3, FCS on week 6	7-11	[59]
4	CD34	PB	40±2.2	7.6±1.8	16.1±1.2/ND	Pos/95	~100	92	SCF, IL-3, 4, 9, LDL	9	[57]
5	CD34	PB	0.06±0.03	4.3±0.7	-	Pos/20-30	-	-	SCF, IL-6, 3	12-14	[251]
6	MNC w/o Lym	PB	-	4.5±0.3	-	Pos/10	-	-	SCF, IL-6, 3, FCS	12	[262]
7	CD133	CB	>80, week 8	3.8±1.1	-	97±2/32±6 week 12	Pos	33.8±10.9	SCF, IL6, FCS on week 8	8-12	[58]
8	MNC	CB	-	-	2/ND	>95/<20	-	-	SCF, IL-6, PGE2, FBS	6	[285]
9	CD34	BM, CB	11.1±4.5 week 8, 6.8±1.8 week 12	3.1±1.9 BM, 4.5±4 CB week 8	5.1±1.3 BM & 9.5±13.5 CB/ 3.8±1.4 BM & 1.6±0.7 CB week 8	Pos/Pos	93.2 BM week 12	82.2 BM week 12	SCF, IL-6, 3	8-12	[286]
10	CD34	PB, CB	100 CB cells gave 9.6±2.4 colonies, each 9580±179 cells	13.8±8.4 CB, ~9-30 CB week 10	8-50/0.7-21 week 10	>96/11-47 or /4-98 week 10-11	-	-	SCF, IL-6, tested: IL-4	8-13 CB, 10-12 PB	[250]
11	MNC	CB	-	-	-	98/12	-	-	SCF, IL-6, FBS	12	[287]
12	MNC, CD34 CB	PB, CB	807±115 PB, 15440±3000 CB per colony on week 5-6	CB 8.6±0.7, PB 9.3±2.2 week 12-14	-	Pos/Pos mRNA	-	Pos PB, Neg w/o IgE CB	SCF, IL-6, 3, FCS on week 6	6-25	[196]
13	Lin ⁻ MNC	PB	~0.13	-	-	>95/ND	-	-	SCF, IL-6, 3, tested: IL-4	6	[288]

14	MNC, CD34	CB	1.9±0.2 MNC week 4	5.62±1.88 week >10	3.46±0.89/ND week >10	99/18	-	-	SCF, FCS, tested: IL-6, 3 PGE2, GM-CSF	4-12	[56]
15	MNC	CB	-	-	-	92/80	80	6	SCF, FCS	10	[289]
16	MNC	CB	-	3.6	3.5±0.9/ND	99/18	-	-	SCF, IL-6, PGE2, FBS	10	[290]
17	CD34	PB, BM	8 PB	5±0.45	-	Pos/3	Pos	Pos	SCF, IL-3, Human serum	6	[202]
18	MNC	FL, CB	-	0.9±0.5 FL, 1.1±1 CB	1.7±0.4 FL, 1.9±1.2 CB/ND	Pos/9-84 CB, Pos/Neg FL	-	-	FCS, Lym cultured media or fibroblasts	4	[55]
19	MNC	BM, PB	-	Po	2-70/ND week 6	Pos/ND	-	-	SCF, tested: IL-3, 4, M-CSF NGF, FCS	8	[48]
20	MNC	CB	-	1.8-2 week 11	-	Pos/84-94 in T cell media	-	-	FBS or T cell media	7-14	[291]
21	CD133	PB	4.6±2.4	4.4 ± 0.8	-	99±1/97±3	Pos	31.9±3.6	SCF, IL-6, 3, FBS on week 6	7	Our work

BM: Bone marrow; CB: Cord Blood; FBS: Fetal bovine serum; FCS: Fetal calf serum; FL: Fetal liver; GM-CSF: Granulocyte-macrophage colony-stimulating factor; IL: Interleukin; LDL: Low-density lipoprotein; Lin: Lineage; Lym: Lymphocyte; M-CSF: Macrophage colony-stimulating factor; MNC: Mononuclear cells; ND: Not determined; Neg: Negative; NGF: Nerve growth factor; PB: Peripheral Blood; PGE2: Prostaglandin E2; Pos: Positive; SCF: Stem cell factor.

Table 3.8. Activation of mast cells (MCs) isolated from human tissue.

	Source	Sensitization (µg/ml), time	Anti-IgE (µg/ml), time	Histamine content (pg/cell)	Min histamine release %	Max histamine release %	Ref
1	Carcinoma lung	1, 1 h	1:10-10000, 10 min	-	~9, ~5 in co-culture	46 fresh isolated w/o sensitization, 43±2.4 after 1 week in co-culture	[62]
2	Lung surgery	200, 4 h	10, 30 min	-	~33 w/o SCF	~64 w/SCF 100 ng/ml	[142]
	Foreskin	NM	1, 4 h	-	~10 w/o SCF	~63 w/SCF 3days culture	
3	Lung and uterus	2, 3-6 h	1-10, 30 min	-	~6 Spon, ~16±4	~22±5	[48]
4	Carcinoma kidney	1, 3 h	0.1-10, 30 min	0.9±0.7	1.5-10 Spon, ~37±10	~54±5	[292]
5	Children foreskin	10% atopic serum, 2 h	0.1-1% 15 min	-	2.4-9.4 Spon, 9.2±1.8	19.6±6.1	[150]
6	urticaria foreskin	2-fold serum dilution, 20 min	1:250, -	-	<3 Spon	19.8±13.1, ~2-28 donor dependent	[148]
7	Foreskin	2, 2 h	0.25-25, 30 min	-	4.8±1.1 Spon, 1.1±0.5	16.4±4.3	[293]
8	Foreskin	NM	10%, 5 min	-	5.6±1.3 Spon, 10.2±1.7	-	[294]
9	Foreskin	NM	25, -	-	-	13.9±2.2	[265]
	Lung		75, -	-	-	39.4±4.8	
	Colon mucosa		2.5, -	-	-	16.7±8.4	
	Colon muscle		2.5, -	-	-	16.6±5.7	
10	Breast skin	NM	1:300, 30 min	-	-	~21±3 w/o SCF, 27±3 w/SCF	[295]
11	Foreskin	10% atopic serum, 16 h, 22°C	<1-10%, 15 min	4.6±0.4	5±0.9 Spon, ~8.5±0.2	17.5±1.5	[97]
12	Foreskin	3 h	0.1-10, 30 min	-	0.8-2.8 Spon donor dep wo/SCF, 9.6-23.5 Spon w/SCF, ~10 w/o SCF, ~43 w/SCF	~36 w/o SCF, ~47 w/SCF, 20.3±8.8 to 1 µg/ml X-IgE w/o SCF	[296]
13	Foreskin	10% human atopic serum, 24 h	0.01-10, 30 min	3.5±0.5	~6±1	~20.5±1.5	[151]
	Breast skin		like foreskin w/ or w/o sensitization		~4	~32±7	
14	Breast skin	NM	3, 30 min	-	<10 Spon	~26±7	[297]
15	Breast skin	NM	3, 30 min	-	<10 Spon	~34±5 or ~15 donor dependent	[298]
16	Foreskin	IgE	2.5-250, 30 min	-	8.6-10.3	13.9±2.3 to 25 µg/ml X-IgE	[299]
17	Foreskin	NM	1:100, 30 min	-	<8 Spon	15-20	[300]
18	Carcinoma Lung	NM	1-10, 30 min	3.2±0.3	~4±1	~23±5	[301]
19	Lung	NM	1:10-1000, 15 min	-	~16±4	38±7.6, 30.4±8.4 to 1 mg/ml	[302]

20	Lung	NM	2 or 0.001-10, 45 min	-	-	25±9 to 2 µg/ml vs time, 44±19 vs dose	[263]
21	Carcinoma lung	NM	0.45-18, 20 min	2.7±0.3 - 4.8±0.7	<5 Spon, ~3±1	~31±7	[259]
22	Lung tumor	6	2, 5-45 min	-	<5 Spon	~40	[123]
23	Carcinoma lung dispersed cells and fragment	NM	0.03-10, 5-45 min	3.6±0.5	~2 Donor dependent	~50 donor dependent	[303]
24	Carcinoma lung dispersed cells and fragment	24 h	1:100-10000 of 36 µg/ml, 2-60 min or 30 min	-	1.1±1.6	22.8±8.2	[304]
25	Carcinoma lung	NM	0.001-10, 45 min	2.5±0.5 - 10.1±2.5 cell size dependent	~3	~50	[95]
26	Carcinoma lung	NM	0.3-3, 20 min	-	~3.5±1.5 Spon, ~22±10	32.1±8.5, ~37.5±9	[305]
27	Carcinoma lung	NM	1:100, 10 min or 2-60 min	-	6.7±1.6 Spon, 23±3.6	28.8±4.5 w/SCF	[306]
28	Carcinoma lung dispersed cells and fragment	4-6, 15 min	0.03-10, 45 min	2.7-12.4 cell size dependent	2-8 Spon, ~7±1	~34	[45]
29	Foreskin	10% atopic serum, 16 h, 22°C	0.1-10%, 15 min	4.6±0.4	4.3±0.6 Spon, ~4.5	17.5±1.5	[124]
	Breast skin			3.34	8.4±1.1 Spon, ~12	27.2±2.6	
	Lung			5.43±1.09	4±1.3 Spon, ~2	26.7±4.1	
	Adenoid				4±0.6 Spon, ~2	19.5±3.9	
Tonsil			6.9±1.3 Spon, ~20	30.6±6.8			
Carcinoma colon			2.81±0.22	5.5±1 Spon, ~6	15±1.1		
30	Uterine	NM	0.01-10, 30 min	2.1±0.2	~5	30±4	[307]
31	Bronchoalveolar lavage of asthmatic patients	NM	1:7000-1:30, 15 min or 1-30 min	1±0.53	~11±1.5	~25±4	[308]
32	Tonsillar	3 h	0.1-10, 30 min	2.2±0.7	<10 Spon, ~45±12	~57±22, ~82±4 w/SCF	[309]
33	Carcinoma or inflam bowel disease intestinal mucosal	NM	0.001-10, 40 min or 1-20 min	2.8±0.2	7±3.8 Spon, ~2±1	30±2.8	[310]
34	Carcinoma lung	NM	20, 30 min	1-5.5	14 Non-sensitized fragments, 33 dispersed cells	31 from sensitized fragments, 38 from dispersed cells	[264]

35	Cardiac	10, 3 h	0.1-1, 30 min	4.6±1.4	<6% Spon, ~10±4	52, ~29±5 w/SCF	[311]
	Skin			1.2±0.3		13.2±1.3	
36	Uterus	200 IU/ml atopic serum	NM	1.8±0.5	<6.7 Spon	21.1±3.5	[266]
	Lung			2.6±0.1		27.7±1.9	
	Carcinoma mucosal of large intestine						
37	Carcinoma muscle intestine	NM	0.03-10, 15 min or <1-15 min	2.81±0.22	5±0.83 Spon, ~2±0.5	15.1±1.1	[260]
				3.46±0.36	4.99±1.21 Spon, ~5±1	16.5±4.4	
38	Lung	Undiluted serum or IgE, over night	0.2-6, -	-	<1.5 Spon, 3.7±1.1	22.4±20	[149]
39	Lung fragment and dispersed cells	1:50 serum, 2 h	1:50-5000, 10 min	4.23±0.49	6.8±1 Spon, ~6±3	~46±7	[261]
40	Lung fragment	50% allergic serum, 18 h, 4°C	1:100-10000, 45 min	-	1.8±0.4 Spon, ~4±1	21±1.6	[152]

NM: Not mentioned or not clear; SCF: Stem cell factor; Spon: Spontaneous; X-IgE: anti-IgE.

Table 3.9. Activation of human progenitor-derived mast cells (MCs).

	Cell	Source	IgE ($\mu\text{g/ml}$), time (h)	Anti-IgE ($\mu\text{g/ml}$), time (min)	Histamine Content (pg/cell)	Spontaneous histamine release %	Histamine release %	Culture time (weeks)	Ref
1	CD133	PB, CB	1-16, 24	5, 30	23.3 \pm 3.3 PB, 5.9 \pm 1 CB	1.3 \pm 0.1 PB, 2.9 \pm 0.7 CB,	~42 - 52.9 \pm 2.5 PB, ~12 - 17.5 \pm 2.1 CB	7	[60]
2	CD34	PB	1, 24	2, 30	7.6 \pm 1.8		~36 \pm 5	9	[57]
3	CD133	CB	2, 24	100-4287, 30	3.8 \pm 1.1	~3 \pm 2	34.6 \pm 8.9 - ~26 \pm 4	8-12	[58]
4	CD133	PB	2, 24	1-7, 30	15.5 \pm 5.3	1.3 \pm 0.1	53.5 \pm 3 or 8.9 \pm 1.4 pg/cell	7-11	[59]
5	CD34	PB	1, 2	1:10 ⁵ -1:100, 30	4.3 \pm 0.7	8 \pm 1.2	~10 \pm 3 - 50 \pm 5	12-14	[262]
6	MNC	CB	1, 1	10, ~ 4	-	-	40.4 \pm 7.8	12	[287]
7	MNC w/o Lym	PB	0.1, 1	1:10 ⁵ -1:100, 30	4.5 \pm 0.3	6.9 \pm 1.1	~9 \pm 3 - 48 \pm 4.7	12	[251]
8	MNC, CD34 CB	CB, PB	1, 48	1.5, 30	CB 8.6 \pm 0.7, PB 9.3 \pm 2.2 12-14 week	-	1.7 \pm 0.6 CB, 9.5 \pm 3.3 (w/IL-4) CB, 16.1 \pm 2.4 PB, 38.9 \pm 5.2 (w/IL-4) PB 12-14 week	6-25	[196]
9	Lin ⁻ MNC	PB	5, 48	1.5, 30	-	-	15.9 \pm 2.8, 38.8 \pm 6.3 (w/IL-4)	6	[288]
10	MNC, CD34	CB	1, 24	0.15-15, 30	5.62 \pm 1.88 >10 week	3.70	~27 \pm 5-52.9, 10>week	4-12	[56]
11	MNC	CB	1, 2	1, 30	3.6	3.7 \pm 1.2	58.5 \pm 10.4	10	[290]
12	CD34	PB	1, 24	0.1 & 1, 60	-	-	~25 \pm 3 - ~38 \pm 4	3	[284]
13	CD133	PB	15, 24	40, 60	4.4 \pm 0.8	2.3 \pm 0.9	31.2 \pm 2.0	7	Our work

CB: Cord Blood; PB: Peripheral Blood; Lin: Lineage; Lym: Lymphocyte; MNC: Mononuclear cells.

CHAPTER 4

Monocyte Trafficking and Differentiation in Response to Allergen-Activated Peripheral Blood-Derived Mast Cells within an Allergy Tissue Model

4.1. Introduction

Since the early 1960s, the prevalence of allergic and atopic disorders has risen rapidly, which has placed allergy as one of the leading causes of chronic diseases, costing billions of dollars in healthcare per year [1, 312]. In the U.S., approximately 50 million people suffer from allergies, with skin allergy affecting more than eight million children [313]. Almost 50% of the allergic patients are sensitive to house dust mite (HDM) [314] and nearly 50% of the total immunoglobulin E (IgE) accounts for specific IgE to group 1 and 2 of the *Dermatophagoides pteronyssinus* (Der p) of HDM [315]. Der p can rupture the epithelial layer tight junctions, induce leukocyte infiltration, and IgE production [316]. An IgE-mediated response includes a concerted effort of multiple cell types. Dendritic cells (DCs) process the allergen, migrate to the lymph node (LN), and present them to T cells. The T cells, upon stimulation, proliferate, polarize into helper cells (Th), and induce the immunoglobulin (Ig) class switching to specific IgE by B cells. Subsequent exposure to the allergen crosslinks the IgE receptors expressed by mast cells (MCs) and release of an array of *de novo* synthesized cytokines, lipid and preformed mediators that lead to the responses in the effector phase [136]. In the early phase of the reaction, happening

within minutes after the MC activation, MCs release factors, such as histamine, leukotriene C₄, prostaglandin D₂, tumor necrosis factor (TNF), which immediately causes bronchoconstriction, vascular permeability, vasodilation, mucus production, and neuron signals [66]. The late phase of the reaction, which peaks after a few hours, is a consequence of the slow-release of mediators, such as leukotriene B₄ (LTB₄), interleukin (IL)-8, TNF, and chemokines that initiates the infiltration of leukocytes including, monocytes, eosinophils, neutrophils, and Th2 cells [317]. Activated endothelial cells (ECs) express chemoattractants and adhesion molecules and elicit the migration of immune cells to the site of challenge. The recruitment of the immune cells along with the dysregulation of the tissue cells results in the symptoms of allergy [20]. Therefore, MCs have been considered as the central effector cells of an allergic inflammatory response.

Although the role of MCs in the effector phase of the response is well established, their ability to directly interact with a pathogen, DCs, and lymphocytes suggests that they may regulate the response prior to IgE production in the sensitization phase [79, 136]. MCs express various pattern recognition receptors (PRRs) and other molecules that may directly or indirectly associate with a pathogen. This includes Toll-like receptor (TLR)4, TLR2, Dectin-1, Fc receptors, and protease-activated receptor 2 (PAR-2) [136]. ECs, eosinophils, DCs, and other immune cells can interact with HDM or the allergen-derived peptides, through e.g. PAR-2 or TLR4 and it is possible that MCs can also respond to an allergen directly in the absence of specific IgE [318-320]. MCs by expressing integrins and adhesion molecules can communicate with other cells and transfer the endocytosed antigens to DCs, as well as their granules to e.g. fibroblasts and ECs in a process termed transgranulation [321, 322]. Not only that MC cellular interactions and mediators can influence DC co-stimulatory molecule and cytokine expression but they also can present antigens to T cells and induce IgE production by B cells [31, 33, 40, 41]. Therefore, MCs can uptake an antigen and similar to antigen presenting cells (APCs) shape the immune response. Previous studies on MC-DC interactions were dependent on MC activation in IgE-mediated

responses [323, 324]. Although the mechanism of direct interaction of MCs with an allergen is currently undefined, in this work the goal was to investigate the interaction between MC and monocytes-derived cells in response to an allergen in the sensitization phase of the response. The antigen uptake or presentation capability of MCs can be regulated by the tissue milieu where they interact with other cell types and released cytokines. For example, Fc γ RI expression that modulates MC activation requires interferon (IFN)- γ treatment [136]. Therefore, we utilized a tissue-engineered model that mimics MC microenvironmental conditions in connective tissue to determine their role in the initiation of an allergic inflammatory response.

DCs are a heterogeneous type of APCs. The ontogeny of different human DC subsets are still under investigation. Granulocyte-macrophage progenitors (GMP) and multi-lymphoid progenitors (MLP) originated from bone marrow can give rise to DCs [325]. Monocytes, which are also a progeny of GMP can be precursors of DCs *in vivo* and differentiate into DCs *in vitro* [326-328]. Immature or quiescent DCs similar to macrophages (M Φ) interact with peripheral cues, take up, and process the antigen. When DCs present the fragment of the processed antigen on major histocompatibility complex (MHC) molecules, they migrate to draining lymph nodes (dLN), lose their antigen processing capability while becoming immunogenic. Functionally mature DCs present the antigen to antigen-specific T cells causing substantial clonal expansion and polarization of T cells into effector cells, e.g. Th1, Th2, and Th17. Phenotypically mature DCs express high levels of MHC, co-stimulatory (CD80, CD86, and CD83), and adhesion molecules, such as integrin lymphocyte function-associated antigen 1 (LFA 1) [329]. Several factors can affect the development of an immune response, such as the density and affinity of MHC molecules, level of co-stimulatory molecule expression, the ratio of DC to T cells, duration of contact, and, more importantly cytokines released in the microenvironment [330]. Therefore, multiple factors can play a role in priming a specific class of immune response. In this work, monocytes were used as a source for developing DCs, while unlike other *in vitro* two-

dimensional (2D) models for DC development, no exogenous factors, such as IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) were added to the tissue model [331, 332]. Consequently, this work demonstrates the influence of allergen-activated model on monocyte trafficking and differentiation to DCs. Within only 48 h, the monocytes in the model differentiated to MΦ-like and dermal DCs (dDCs), with different characteristics, while the presence of MCs in the model influenced monocyte differentiation and promoted the phenotypic maturity of monocyte-derived cells. The monocyte-derived cells were capable of priming T cells and inducing IgE production specifically when MCs were included in the model. The findings of this study not only can be applied to clarify the ontogeny of different DC subsets that may be found under inflammatory conditions *in vivo* but can also elucidate the capability of MCs in orchestrating non-IgE mediated responses.

4.2. Materials and methods

4.2.1. Antibodies and reagents

StemSpan media was from STEMCELL Technologies (Vancouver, Canada). Human recombinant stem cell factor (SCF), IL-6 and IL-3 were purchased from PeproTech (Rocky Hill, NJ). Defined HyClone fetal bovine serum was from GE Healthcare Life Sciences (Logan, UT). Human dermal fibroblasts treated with mitomycin C and human umbilical vein ECs were from Merck Millipore (Billerica, MA) and Promocell (Heidelberg, Germany), respectively. The following antibodies for the flow cytometry analysis were from BioLegend (San Diego, CA): anti-human Brilliant Violet (BV)785 human leukocyte antigen (HLA)-DR (clone L243), PE/Dazzle 594 CD1c (clone L161), BV421 CD206 (clone 15-2), APC/Fire 750 CD14 (clone 63D3), PerCP/Cy5.5 CD64/FcγRI (clone 10.1), APC CD83 (clone HB15e), BV605 CD86 (clone IT2.2), PE CD252/OX40L (clone 11C3.1), BV711 CD16/FcγRIII (clone 3G8), PE/Cy7 thymic stromal lymphopoietin receptor (TSLPR) (clone 1B4), APC CD117/c-kit (clone 104D2), FITC CD31 (clone WM59), Alexa Fluor 700 CD45 (clone HI30), and the isotype controls,

fluorochrome-conjugated Ms IgG1 (clone MOPC-21), Ms IgG2a (clone MOPC-173), and Ms IgG2b (clone MPC-11). Zombie Aqua fixable viability kit and human TruStain FcX (Fc receptor blocking solution) were also from BioLegend. BB515 anti-human CD197/CCR7 (clone 3D12), its isotype control Rat IgG2a (clone R35-95), Brilliant Stain Buffer, and CompBead compensation particle set were purchased from BD Biosciences (San Jose, CA).

4.2.2. Cell culture

Leukocyte preparations were obtained from Oklahoma Blood Institute (Oklahoma City, OK). MC progenitors, CD133⁺ cells, and autologous CD14⁺ monocytes were positively isolated from human peripheral blood mononuclear cells by magnetic cell separation technology following the manufacturer's protocols (MACS Miltenyi Biotec; San Diego, CA). MCs were generated within a collagen matrix in co-culture with fibroblasts and ECs, as explained before in the Methods section of chapter 3. Briefly, the CD133⁺ cells (70,000 cells/ml) and fibroblasts (40,000 cells/ml) mixed with a type I collagen gel solution were seeded in 48-solid well plates (Greiner Bio-One; NC) and incubated for six weeks. The StemSpan culture media supplemented with SCF, IL-6, and IL-3 (first three weeks) was refreshed weekly. At the end of the sixth week, following the coating of the gel with a fibronectin solution, the top of the matrix was seeded with ECs (50,000 cells/cm²) in the described media supplemented with serum (20%, v/v). After total seven weeks, Der p extract of mite (HollisterStier; WA) at 300 AU/ml in the described serum-supplemented media was added to the samples and incubated for 24 h, as shown in Fig. 4.1. The concentration and incubation time of the allergen was selected based on studies on leukocyte activation (Table 4.1) [333, 334]. Then, the top media was removed and monocytes at 1.5×10^5 cells/cm² in 0.3 ml/well complete media were added to the endothelium layer, which formed a confluent layer on top of the collagen gel and incubated for 3 h. The monocyte concentration was according to a transendothelial model and our previous unpublished studies [335]. Furthermore, previous studies done on monocyte migration in the absence of MCs and fibroblasts have shown that monocytes

adhere and extravasate to the subendothelial layer in around 2 h [89, 336]. The top layer was gently rinsed three times with warm phosphate buffer saline (PBS) to remove any non-adherent cells. The adherent cells were incubated for 48 h to allow monocyte migration and differentiation to DCs that may reverse-transmigrate and traverse the endothelium monolayer, representing their migration from tissue to lymphatic vessels [337]. It was previously shown that after 48 h no significant reverse-transmigration occurs [336]. Cells that were loosely adherent or transmigrated back to the apical surface were collected by three gentle rinses with warm PBS, counted by a hemocytometer and trypan blue exclusion. The remaining cells within the matrix were also collected after digesting the gel using 2.2 mg/ml collagenase D (Roche Applied Science; IN). The test group was referred to as group AM and identical samples without MC progenitors (group A) or allergen (group M) served as controls for determining the influence of allergen-activated MCs and allergen on the phenotype of the collected cells, respectively.

4.2.3. Immunophenotyping and flow cytometry

The phenotype of the collected cells was identified by immunofluorescence staining and multi-color flow cytometry for the expression of the cell surface markers. The cells were stained with the viability dye prior to incubation with the Fc receptor blocking solution. The cells collected from the apical and subendothelial layer were stained separately using different antibody cocktails. For the cells collected from the apical layer, the CCR7 antibody or its isotype control in a staining buffer containing 0.2% bovine serum albumin (BSA; Sigma-Aldrich; MO) was added to the cells and incubated for 30 min at 37°C. After rinsing, the cells were stained with the cocktail of antibodies or isotype controls in the staining buffer containing 0.2% BSA, 0.09% sodium azide, and Brilliant Stain Buffer in PBS for 45 min at 4°C. Data were acquired on a BD LSR II flow cytometer using the acquisition software BD FACSDiva (BD Biosciences) and analyzed using FlowJo software (Ashland, OR). Spectral overlap was calculated using single-color compensation beads.

Table 4.1. Protocols for cell activation using extract or purified allergens.

	Source	Cell type	Stimuli	Allergen incubation time	Allergen concentration	Ref.
1	Human	T cell	Der p	48-72 h or 8-11 days	200 AU/ml	[338]
2	Human	T and B cell	Der p 1	24 h	10 µg/ml	[339]
3	Human	PBMC (T cell)	rDer p 1	6, 24, 48, 72 h	0.2, 1, 5 µg/ml	[340]
5	Human	T cell	rDer p 1	1 h or 6-72 h	0.6, 2.5, 5, 10 µg/ml	[341]
6	Human	PBMC	Der p, Der p 1, Der p 2	7 days	0.1, 1, 10 µg/ml	[342]
7	Human	PBMC	Der p 1	5 days	10 µg/ml	[35]
8	Human	PBMC	Der p	6-7 days	10 µg/ml	[343]
9	Human	PBMC	Der p	6 days	100 µg/ml	[344]
10	Human	PBMC	Der p	3 days-? Weeks	250 AU/ml†	[333]
11	Human	Spleen cells	rDer p 1	72 h	20 µg/ml	[345]
12	Human	B cell line	Der p 1	1 h	5 µg/ml	[346]
13	Human	DC	Der p 1	24 h	0.2, 1, 5 µg/ml	[339]
14	Human	DC	Der p	48 h	10 µg/ml	[347]
15	Human	Mon-derived DC	Der p 1	6, 24 h	10, 100, 1000 ng/ml	[348]
16	Human	Mon-derived DC	Der p 1	24 h	10, 100 ng/ml	[334]
17	Human	Eosinophil	Der p	19-60 h	100 µg/ml	[349]
18	Human	Eosinophil	Der f 1	4 h	25, 100 µg/ml	[319]
19	Human	Epithelial	Der p 1	3-24 h	0.2-2000 ng/ml	[350]
20	Human	Epithelial cells or cell line	Der p 1, Der p 9	24 h, or 2,4,8,24 h	0.01-4 µg/ml	[351]
21	Human	Keratinocytes	rDer f 1, rDer p 1	3-48 h	0.25 and 2.5 µg/ml	[352]
22	Mice	Spleen T cell	rDer p 1	1 h	10 µg/ml	[353]

DC: Dendritic cell; Der f: *Dermatophagoides farinae*; Der p: *Dermatophagoides pteronyssinus*; Mon-derived: Monocyte-derived; PBMC: Peripheral blood mononuclear cells; r: Recombinant.

† The allergen is purchased from Hollister-Stier.

4.2.4. Statistical analysis

Experimental results are from five independent experiments (donors). Statistical analyses were performed using GraphPad Prism (GraphPad Software; San Diego, CA). Box plots present the minimum to maximum of the data. Student's *t*-test with Welch's correction was applied to determine significant difference between two groups. A *p*-value < 0.05 was considered significant.

4.2.5. Possible hazard and safety precautions

All animal- and human-derived materials were purchased from commercial vendors that prescreen the products for biological hazards. The personnel conducted the research completed a training program for handling the biohazardous materials and working in a biosafety level 2 (BSL-2) laboratory. Prior to the experiments, all biohazards were identified and biosafety plans were prepared and followed. All procedures associated with the work followed "Universal Precautions". Biohazardous materials were disposed according to the guidelines for each waste category.

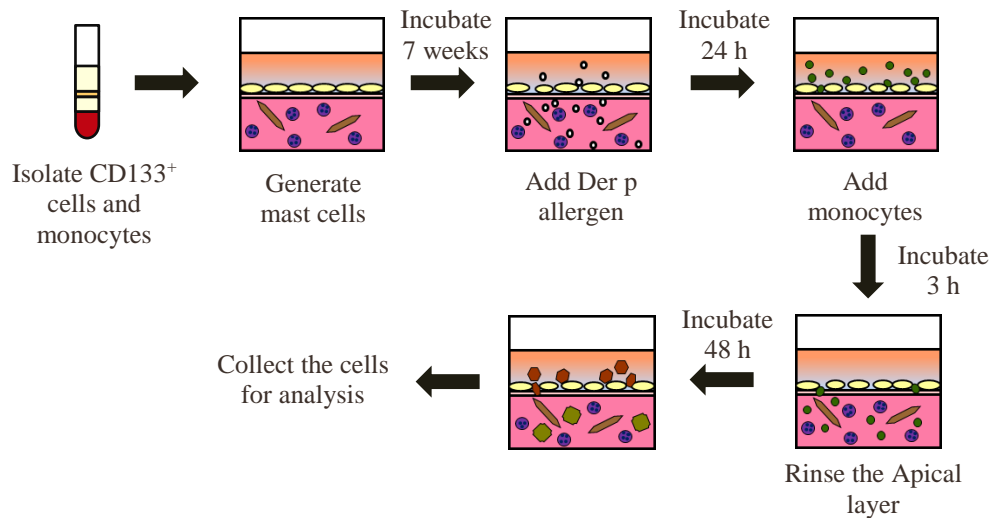


Fig. 4.1. Development of monocyte-derived cells within the connective tissue-equivalent model.

4.3. Results and discussion

MCs and other APCs are co-localized in the dermis in proximity to fibroblasts, where they interact with invading pathogens. A substantial information can be found in literature about the phenotypic and functional characteristics of different human DC subsets being tested *ex vivo* or *in vitro*. However, not much is known about the interaction of DCs with human MCs, while both are considered as sentinels of the immune system. The direct interaction of DCs with MCs was only discussed subsequent to the MC sensitization with IgE antibody [323, 324]. It is unclear how non-IgE-dependent MC activation that may be modulated by the tissue milieu affect the DC function and ultimately the immune response. Here in this work, we examined the influence of MCs on monocyte transendothelial migration and differentiation to DCs in response to an allergen in an IgE-independent manner. We have used a three-dimensional (3D) tissue model that supported the development of mature MCs in co-culture with fibroblasts and ECs within a connective tissue-equivalent matrix. The features of the model allow investigating cell migration and cellular interactions, which is not possible to examine in a 2D system. After addition of the monocytes to the model following activation with Der p extract of mite, the collected cells from the apical and subendothelial layer were analyzed and compared with the control groups (A or M).

4.3.1. Monocytes differentiated into dendritic and macrophage-like cells in the connective tissue-equivalent model

After excluding doublets and dead cells, the collected cells from the apical layer, regardless of the addition of MC progenitors or allergen to the model, were highly HLA-DR positive (Fig. 4.2A). As shown in Fig. 4.3A, for the cells collected from the digested gel, ECs and MCs were excluded by using their phenotypic markers, CD31 and CD117, respectively [139]. CD45, which is a leukocytes marker was used to exclude fibroblasts [354]. Monocyte-derived cells from the apical layer in all the groups and also from the subendothelial layer from group A were all CD206⁺ (Fig. 4.2A and 4.3A). Therefore, for the subendothelial cells in group AM and M, the

MC precursors that were CD45⁺ but not expressing CD31 or CD117 were excluded by gating out CD206⁻ cells (Fig. 4.3A). The monocyte-derived cells in the subendothelial layer were also highly expressing HLA-DR (Fig. 4.3A). CD1c, also known as blood DC antigen (BDCA)-1, which is a human tissue and blood DC marker [355] was absent on monocytes, while it was expressed by the monocyte-derived cells at variable levels (Fig. 4.2B and 4.3B). Interestingly, the cells upregulated the density of the expression of CD14 in comparison with the monocytes (Fig. 4.4 and 4.5). One possibility might be that some of the CD14 surface receptors were saturated by the isolation beads on peripheral blood-isolated CD14⁺ monocytes and they were not detectable. Immature monocyte-derived-DCs (moDCs) developed *in vitro* under the influence of differentiating cytokines, such as IL-4 and GM-CSF were mainly CD14^{low/-} [331, 356]. While, the reverse-transmigrated cells developed within a transendothelial model created by Randolph et al. were CD14⁺ under steady-state condition and upregulated HLA-DR compared with monocytes [336], similar to our connective tissue-equivalent model shown in Fig. 4.4 and 4.5. However, when stimuli, such as lipopolysaccharide (LPS) or zymosan were incorporated into the collagen matrix, CD14 expression was highly downregulated by the reverse-transmigrated cells [336], which is not the case in our work that an allergen was used for stimulation. Besides monocytes, CD14 is expressed by a subset of dDCs and interstitial MΦ [357, 358]. As shown in Fig. 4.2A and 4.3A, the cells were highly expressing macrophage mannose receptor (MMR/CD206), characteristic of moDCs, dDCs, inflammatory dendritic epithelial cells (IDECs), and MΦ, but not monocytes, blood DCs, and Langerhans cells (LCs) [359, 360]. CD206 provides the ability to capture mannoseylated proteins in a receptor-mediated endocytosis [360]. It was shown that CD206 can modulate the allergen uptake and the development of a Th2 response [361, 362]. The expression of both CD1c and CD206 by the monocyte-derived cells, which are uncharacteristic of monocytes along with the upregulation of HLA-DR indicated that the monocytes have differentiated.

The collected cells were gated based on the expression of CD1c and CD14 into three populations: CD1c⁻ CD14⁺, CD1c⁺ CD14⁺, and CD1c⁺ CD14⁻ cells (Fig. 4.2B and 4.3B), as these two markers can be helpful in identifying different DC subsets and MΦ. The CD1c vs CD14 distribution of the human HLA-DR⁺ cutaneous cells is similar to the results of our study except with the existence of a population of CD1c⁺ CD14⁻ cells that includes CD141⁺ DCs [327]. Therefore, the developed cells in our model might be homologous to dDCs. The cells were also expressing CD64 and CD16 IgG receptors, at variable levels, as shown in Fig. 4.4 and 4.5. Expression of CD64 is characteristic of monocytes and MΦ, but also detected on steady-state moDCs developed in the transendothelial model and dDCs [336, 358]. In mice, CD64 has been considered as a marker of moDCs to distinguish them from conventional DCs [363], however, this may not be applied to human moDCs, specifically to lung residents [328]. CD16, which is not expressed by classical monocytes was also shown to be absent on human moDCs developed *in vitro* in IL-4 and GM-CSF-supplemented media [332, 364]. This receptor is neither expressed by CD1c⁺ blood DCs nor dDCs and LCs, whereas a population of blood DCs (SlaDCs) and MΦ are CD16 positive [358, 365]. In our model, the highest expression of CD16 belonged to group M that was not activated with the allergen with a higher expression level on CD1c⁻ cells ($p < 0.01$, Fig. 4.4 – 4.7). Analyzing the HLA-DR CD11c-expressing cells collected from two inflammatory fluids from human samples demonstrated two populations of CD1c⁺ CD16^{low/-} and CD1c⁻ CD16⁺ cells [366]. Both populations were expressing CD14 and CD206, but the CD1c⁺ cells had dendrites, whereas the CD1c⁻ cells were exhibiting MΦ-like morphology and were poor stimulators of CD4⁺ T cells. Interestingly, the gene signature analysis suggested that the CD1c⁺ CD14⁺ cells were derived from monocytes. Similar CD1c⁺ CD14⁺ CD206⁺ DCs were also found in the spleen of patients with gastric cancer associated with chronic inflammation indicating that CD1c⁺ CD14⁺ cells can appear under inflammatory conditions [366]. Therefore, the CD1c⁺ CD14⁺ cells were referred to as inflammatory (inf) DCs [366]. Indeed, in our model, the percentage of CD1c⁺ CD14⁺ cells were higher in group A and AM that were stimulated with the

allergen than group M ($p < 0.0001$, Fig. 4.2B and 4.3B). Therefore, comparison of the characteristics of the developed cells in our connective tissue-equivalent model with human DC subsets and M Φ mentioned above implies that the CD1c⁺ CD14⁺ cells could be counterparts of a subset of *in vivo* dDCs or infDCs, while the CD1c⁻ CD14⁺ cells with higher density of expression of CD14, CD64, and CD16 but lower HLA-DR might be M Φ -like cells or dermal dendritic-appearing M Φ ($p < 0.01$, Fig. 4.6 and 4.7). Further evidence is a study that demonstrated monocytes as precursors of CD1c^{low/-} CD14⁺ cells in the skin with similarities in gene expressions to tissue M Φ [327]. The CD14⁺ dermal cells were not as potent as CD1c⁺ CD14⁻ cells in stimulating allogenic naïve T cells, consequently functioning more like M Φ than efficient antigen-presenting DCs [327]. However, this does not rule out the possibility of the development of CD1c⁻ dDCs homologous to CD1c⁻ CD14⁺ dDCs described by Klechevsky et al. [357], specifically in group A and AM with lower CD16 expression. More importantly, the contour plot of expression of CD14 vs CD1c in Fig. 4.2B and 4.3B, clearly shows that the CD1c⁺ CD14⁺ cells were being developed from the CD1c⁻ CD14⁺ M Φ -like population. In turn, under the influence of the allergen, the CD1c⁺ CD14⁺ cells were in transition to CD1c⁺ CD14⁻ cells, meaning that CD1c⁺ CD14⁺ cells were not terminally differentiated cells. This transition became more evident in another experiment that samples were stimulated for the second time with the allergen and a higher percentage of them were CD1c⁺ CD14⁻ (Fig. A.2B). This is not in agreement with Randolph et al.'s conclusion assuming that DCs arise from reverse-transmigrating monocytes [336]. The CD14⁺ dDCs in the skin express CD1c at a very low level, whereas CD1a⁺ dDCs or LCs that are almost negative for the expression of CD14 are CD1c⁺ [357]. Considering that the CD14⁺ cells in our model were in transition to CD14⁻ cells under inflammatory condition, it is possible that with further incubation or stimulation they differentiate into another subset like LCs. Indeed, dermal CD14⁺ cells can be LC precursors [367]. On the other hand, Lin⁻ CD1c⁺ IDECs also being referred to as myeloid inflammatory DCs that unlike LCs are Langerin⁻ CD206⁺ cells appear in the lesional skin of patients with atopic dermatitis to amplify the allergic inflammation

and are among the cell types that can be differentiated from monocytes and dDCs [358, 368, 369]. Each subset of DCs may have a specific role in the initiation, development, and even suppression of a humoral response. More investigation is required to fully define the phenotypic and functional characteristics of the monocyte-derived cells developed in the connective tissue-equivalent model in this study to elucidate their mission in an allergic response.

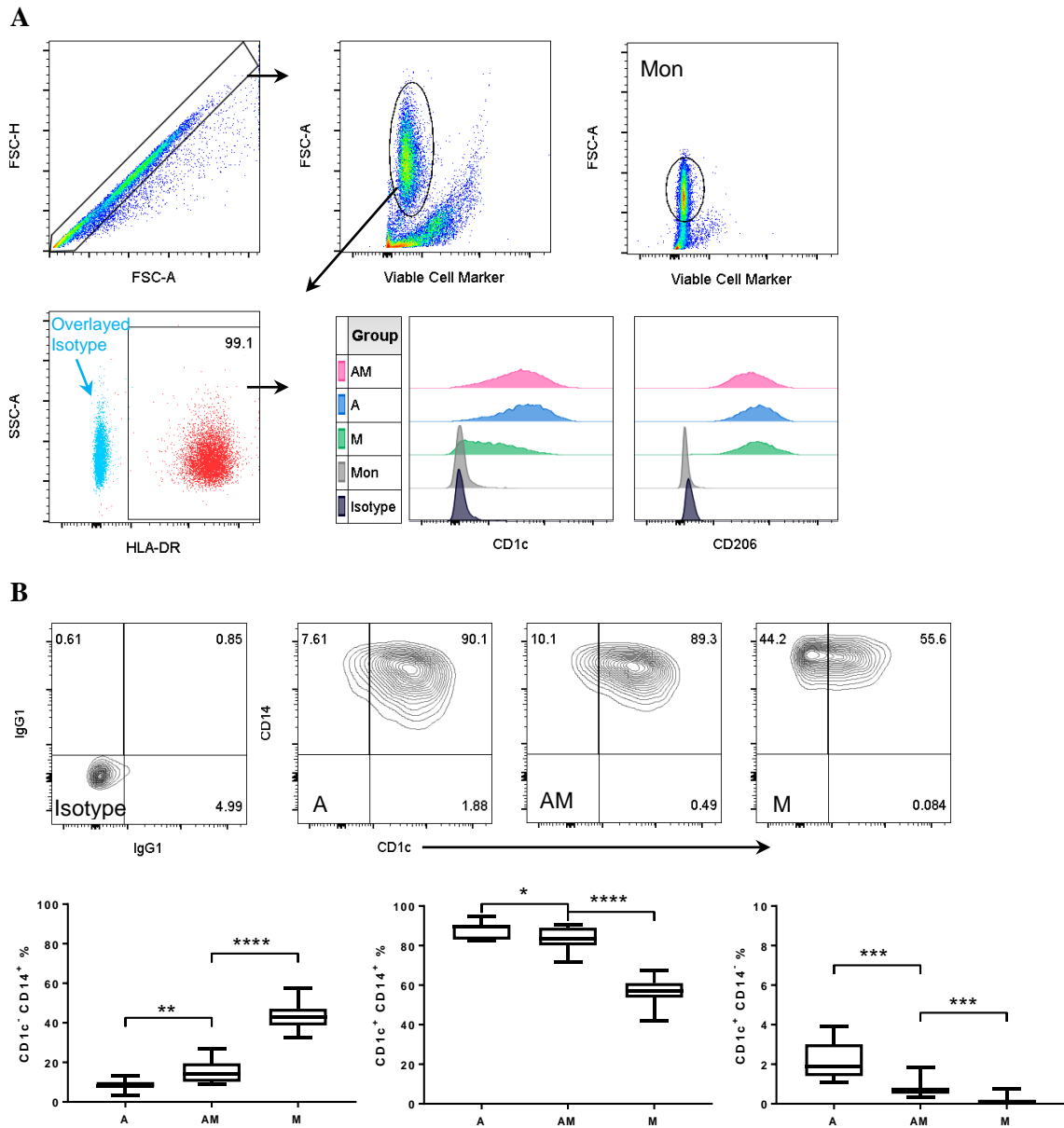
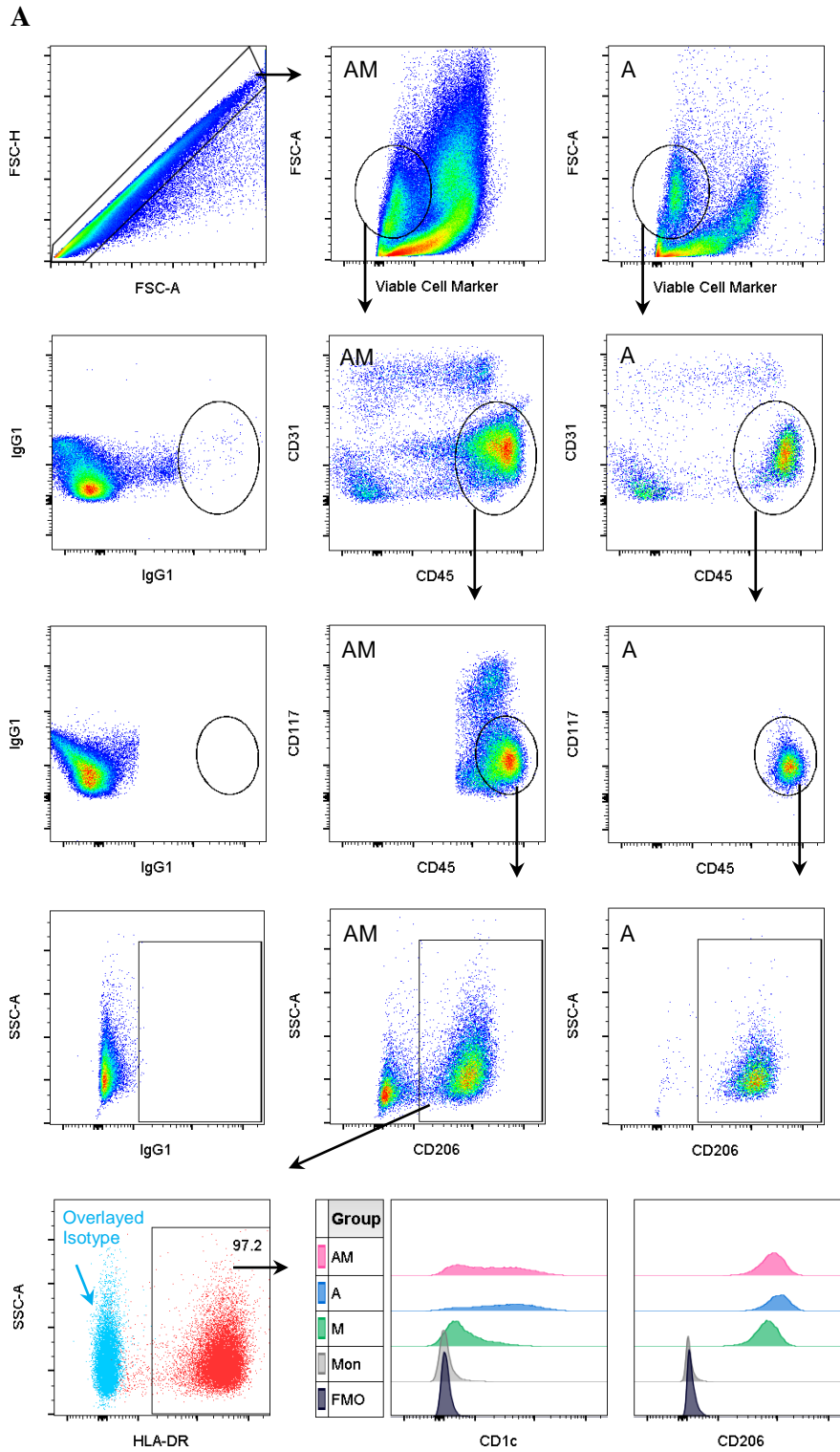


Fig. 4.2. Characterizing the monocyte-derived cells collected after 48 h from the apical layer of

the connective tissue-equivalent model. (A) Gating scheme and representative pseudocolor plot and histogram overlays of the expression of the markers used to define dendritic cells (DCs) in the test (AM) and control groups (A and M). Monocytes (Mon) before culture are also shown for comparison. (B) Representative contour plot of CD14 vs CD1c across the groups. HLA-DR⁺ cells were gated based on the expression of CD1c and CD14 and the percentage of the cells in each population in group AM was compared with group A and M. Data are from 5 independent experiments; n = 2 - 3. *, **, ***, and **** indicate $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

4.3.2. Allergen promoted the development of CD1c⁺ dendritic cells (DCs)

To compare the influence of the allergen on the characteristics of the monocyte-derived cells, group AM was compared with group M that was not activated with the allergen (Fig. 4.5 and 4.6). In group M, CD1c⁻ MΦ-like cells could spontaneously differentiate into CD1c⁺ CD14⁺ cells (Fig. 4.2B and 4.3B). As described in the previous section, under the inflammatory condition, a higher percentage of CD1c⁺ CD14⁺ and CD1c⁺ CD14⁻ cells were developed in group AM than group M ($p < 0.0001$, Fig. 4.2 and 4.3). Addition of the allergen to group AM did not allow the upregulation of CD64, CD16, and CD14 to the extent seen on the cells in group M ($p < 0.0001$, Fig. 4.4 and 4.5). Higher expression of CD64 has been implicated as a more efficient uptake of an antigen, which is mainly a MΦ phenotypic characteristic [370]. CD16 signals following IgG-immune complex ligation was shown to induce the upregulation of IL-33 by APCs and contribution to the development of a Th2 response in a mice model of airway inflammation [371]. However, this may not be applied to human dDCs as they appear not to express CD16 and indeed, may be a good marker to discriminate human dDCs from MΦ [359]. In Fig. 4.4, CD16 expression on the apical CD1c⁻ cells in group AM, as well as A, was also low, suggesting that these cells may be phenotypically different from CD1c⁻ MΦ-like cells in group M, and possibly counterparts of CD1c⁻ CD14⁺ dDCs described by Klechevsky et al. [357].



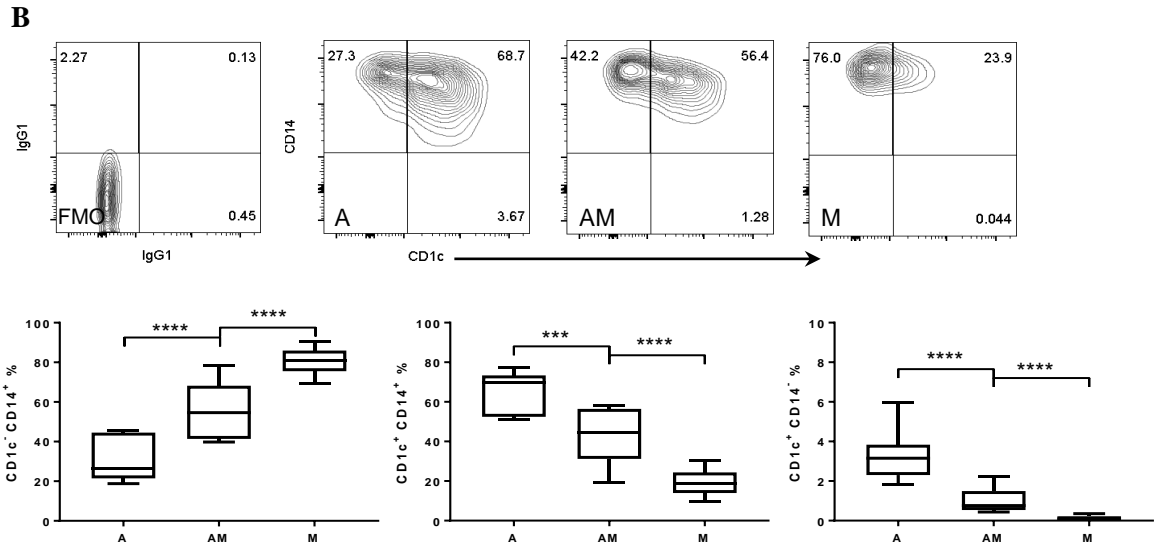
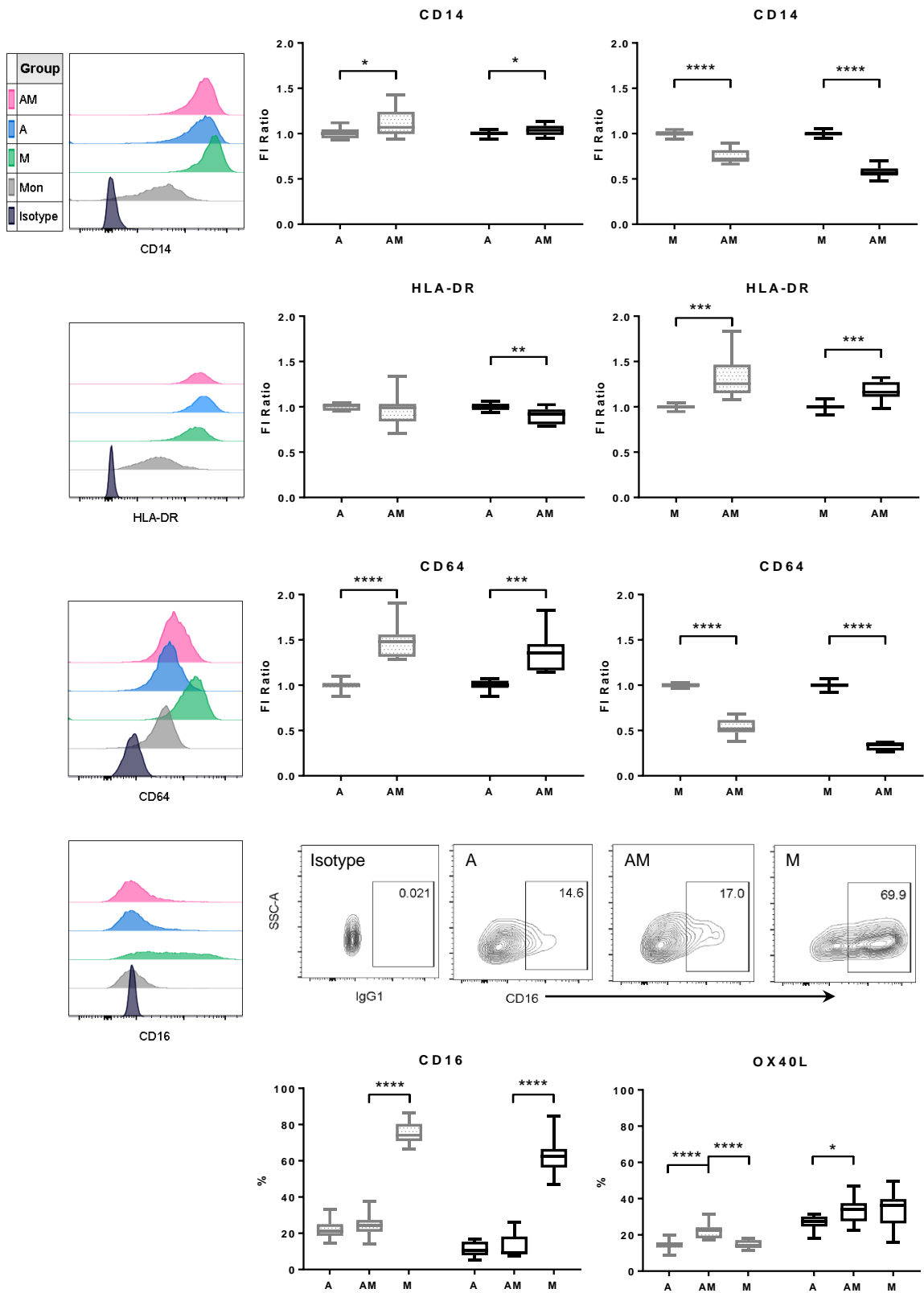


Fig. 4.3. Characterizing the monocyte-derived cells collected after 48 h from the subendothelial layer of the connective tissue-equivalent model. (A) Gating scheme and representative pseudocolor plot and histogram overlays of the expression of the markers used to define dendritic cells (DCs) in the test (AM) and control groups (A and M). Endothelial cells (ECs) and mast cells (MCs) were excluded by gating out CD31 and CD117 positive cells, respectively. CD45 was also used to gate out any contaminating fibroblasts. MC precursors that were not expressing CD31 nor CD117 were excluded by using CD206 in group AM and M. Monocytes (Mon) before culture are also shown for comparison in the histograms. (B) Representative contour plot of CD14 vs CD1c across the groups. HLA-DR⁺ cells were gated based on the expression of CD1c and CD14 and the percentage of the cells in each population in group AM was compared with group A and M. Data are from 5 independent experiments; n = 2-3. *** and **** indicate $p < 0.001$ and 0.0001 , respectively.

We have also determined the expression of TSLPR and OX40L, linked to Th2 responses. The monocyte-derived cells collected from both the apical and subendothelial layer were positive for the expression of both TSLPR and OX40L, which was not the case for the monocytes before addition to the model (Fig. 4.4 and 4.5). In atopic lesions, high expression of TSLPR mRNA has

been reported [372] and blockade of TSLPR could lead to impaired maturation and migration of airway DCs and diminished Th2 response [373]. In addition, in asthmatic airways, TSLP release was correlated with the disease severity [374] and TSLP-stimulated myeloid DCs induced the production of Th2 cytokines by CD4⁺ naïve T cells [375]. However, the expression of a receptor can be affected by many factors, including the cytokines being released in the microenvironment and signals from neighboring cells. In our model, in response to the allergen stimulation, TSLPR density was not as high as group M on both apical and subendothelial cells ($p < 0.05$, Fig. 4.4 and 4.5). One explanation can be a higher production of TSLP in response to the allergen, possibly by fibroblasts and MCs [374] and internalization of TSLPR following the interaction with its agonist. Furthermore, TSLP can upregulate the expression of MHC-II and co-stimulatory molecules, as well as OX40L, by myeloid DCs [375, 376]. OX40L expression was shown to be required for naïve T cells to produce the Th2 cytokines. However, in the presence of IL-12, it can lead to polarization of Th1 cells by enhancing the production of TNF- α and IFN- γ but not IL-10 [375]. Therefore, OX40L expression is not per se an indicator of the development of a Th2 response. Under the influence of the allergen, the expression of OX40L was not necessarily upregulated in both CD1c⁺ and CD1c⁻ populations (Fig. 4.4 and 4.5). Indeed, in the subendothelial layer, fewer number of CD1c⁺ cells in group AM were expressing OX40L than group M ($p < 0.05$, Fig. 4.5). With this data, we cannot link the expression level of TSLPR to OX40L by assuming that endogenous TSLP could have internalized the TSLPR, but upregulated OX40L. OX40L expression may not be modulated only through TSLP and expression of other factors may have led to the downregulation of OX40L. Moreover, on migratory LCs, TSLP treatment did not induce the upregulation of OX40L implying that different DC subsets may have different mechanisms to promote a T helper response [377]. Nevertheless, the expression level of OX40L by the developed cells was not high in agreement with the response of mice moDCs from lungs after dust mite sensitization [363]. Regardless of the expression level, OX40L may be required for the development of Tfh cells and their migration to B cell follicular zones [378].



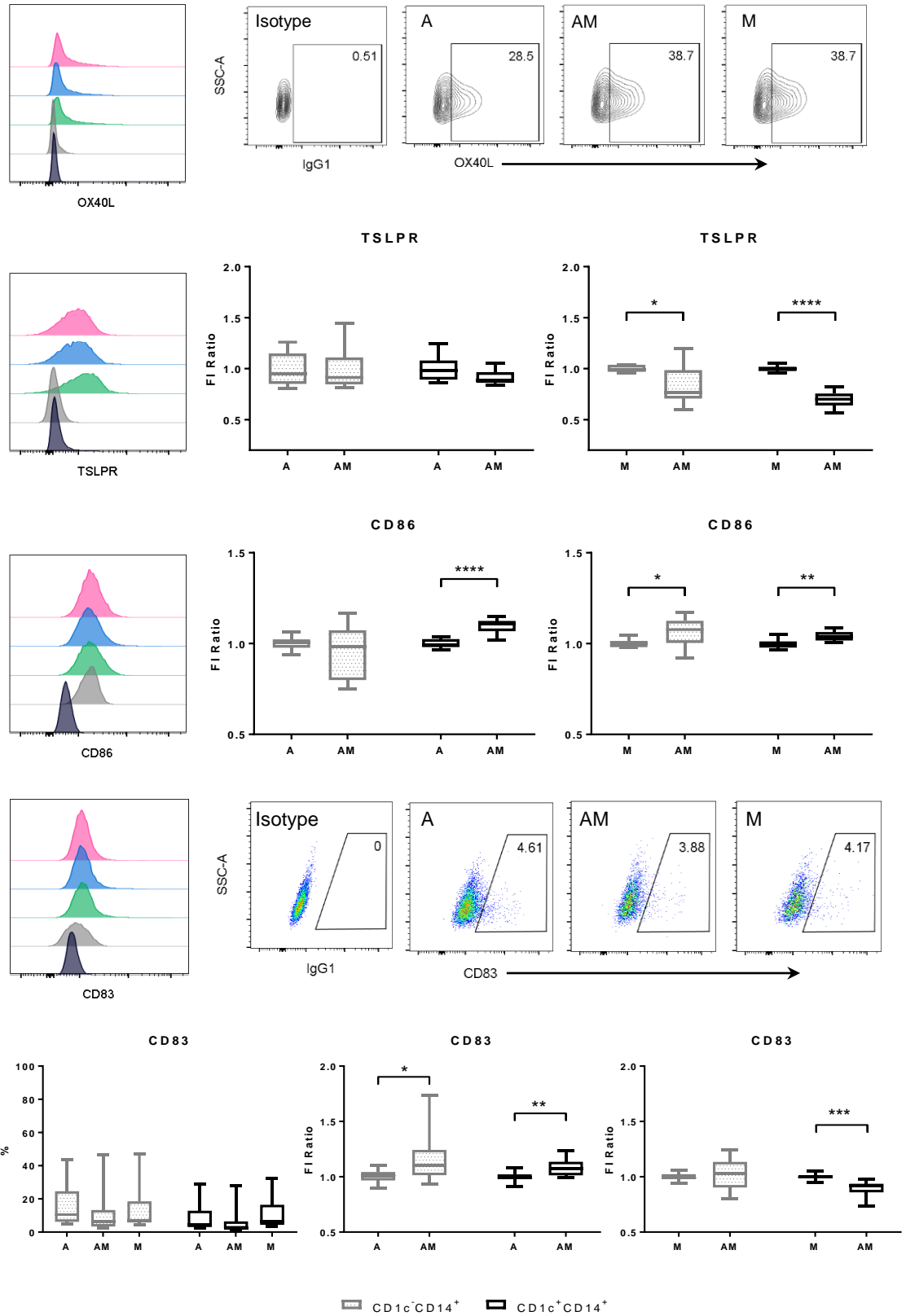
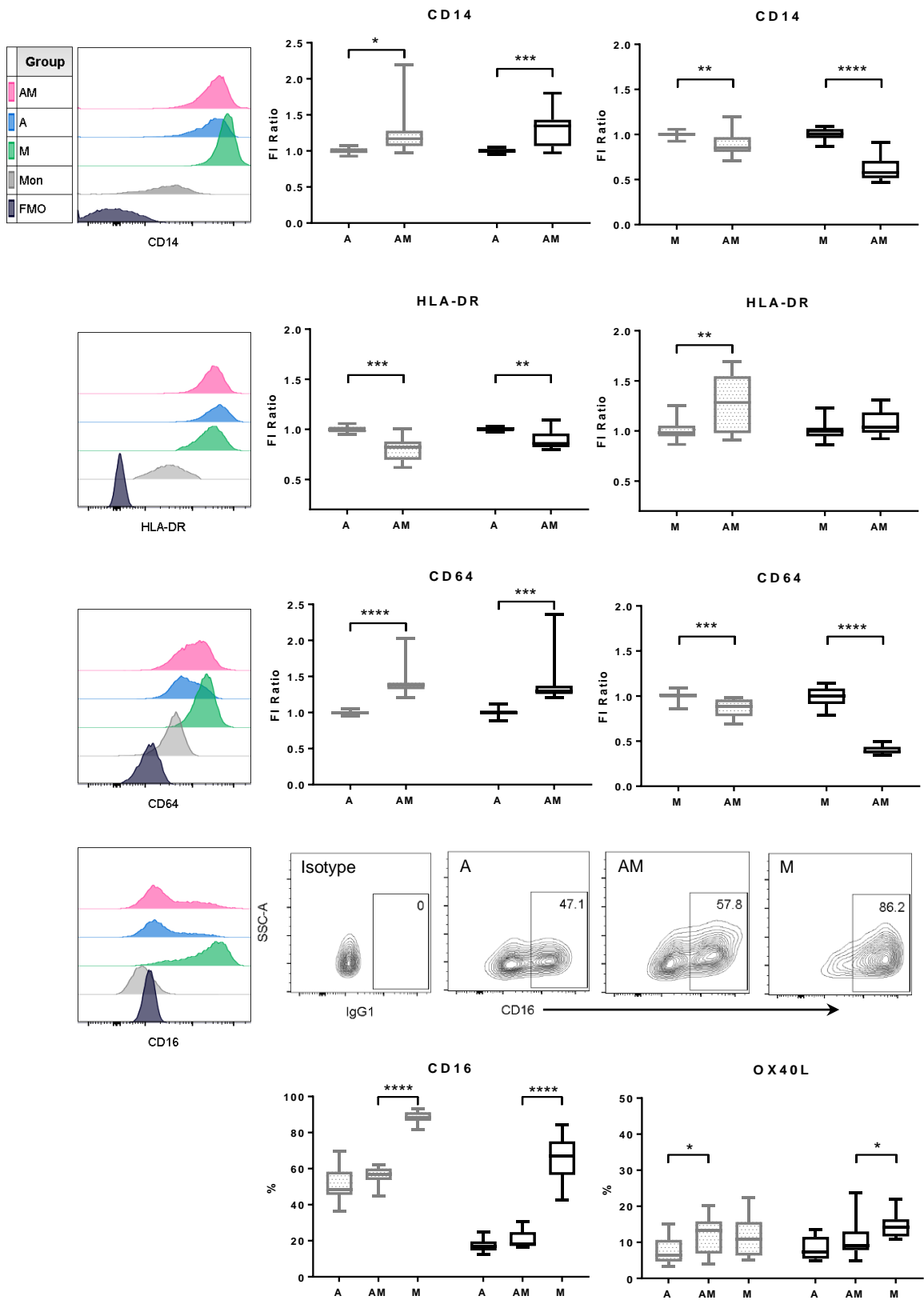


Fig. 4.4. Effect of the allergen and mast cells (MCs) on the characteristics of the monocyte-derived cells collected after 48 h from the apical layer of the connective tissue-equivalent model. Representative histogram overlays of the expression of the markers used to define dendritic cells (DCs) in the test (AM), control groups (A and M), and monocytes (Mon) before culture are shown for comparison. Gating scheme and representative contour or pseudocolor plot of CD16, OX40L, and CD83 expression are also shown. For comparing group AM with the control group A or M, the fluorescence intensity (FI) of the marker was normalized to the control group in each experiment. Data are from 5 independent experiments; $n = 2-3$. *, **, ***, and **** indicate $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

Under the influence of the allergen, HLA-DR and CD86 co-stimulatory molecule were only marginally upregulated by the monocyte-derived cells collected from the apical layer ($p < 0.001$ and 0.05, respectively, Fig. 4.4), while this was not the case for CD86 expression on the subendothelial cells (Fig. 4.5). Activation with the allergen also did not have a major effect on the CD83 expression level by the apical-collected cells and surprisingly induced a lower density of it on CD1c⁺ cells ($p < 0.001$, Fig. 4.4). We have not determined the expression of CD83 by the subendothelial cells. This is not in agreement with Randolph et al.'s transendothelial model with upregulation of both CD86 and CD83 on the reverse-transmigrated cells upon stimulation [336]. However, CD86 was downregulated on the subendothelial cells and CD83 was not expressed [336]. GM-CSF and IL-4, DC differentiating factors, were shown to keep the cells in the mature state, while IL-10 can suppress their maturation and render loss of CD83 [370]. Transforming growth factor beta (TGF- β) can also downregulate CD86 and CD83 [379]. Therefore, the balance of proinflammatory and immunosuppressive agents produced by the cells can have an effect on the phenotypic maturity of DCs and ultimately the type of response. Furthermore, the allergen was removed from the model prior to the addition of the monocytes, keeping in mind that it could have diffused into the subendothelial layer and remained in the matrix. In a mice model of

allergic inflammation, the response of different DC subsets was dependent on the dose of the dust mite allergen [363]. In the absence of sufficient stimulating factors, DCs may remain immature or semi-mature and induce tolerance [380]. Although the tolerogenic DCs can present an antigen to T cells, they are not able to deliver enough co-stimulatory signals for T cell activation. On the other hand, a weak T cell receptor signal may promote a Th2 response [381]. MΦ have a poor T cell antigen-presenting capability and are low on co-stimulatory molecules, whereas only a subset of dDCs (CD1a⁺ cells) express CD83 [382, 383]. Notably, in the presence of DC maturation cytokines, such as IL-1β, IL-6, and TNF-α dermal CD1c⁺ cells isolated enzymatically from human skin did not upregulate CD83 receptors [383]. Even stimulating CD14⁺ dDCs through TLR ligands did not induce the upregulation of co-stimulatory molecules [384]. However, when LPS was added to our model, both CD86 and CD83 were upregulated, which is in contrast to the direct stimulation of the monocyte-derived cells with the allergen (Fig. A.3). This means that the moDCs in our model under certain circumstances can exhibit a mature phenotype. Our data indicate that the remaining allergen in the model or the indirect effect of the allergen through other cell types in the model, along with the cytokines in the microenvironment, did not induce the upregulation of CD83 on CD14⁺ cells. Whether this is due to the dose, the incubation time of the allergen, the cell types in the model, or an intrinsic property of the allergen, CD14⁺ cells, or the connective tissue-equivalent model, requires further investigation. In fact, skin APCs are more resistant to maturation in response to intradermal vaccine administration in comparison with *in vitro* cells, suggesting that tissue milieu may maintain tolerance [384, 385]. In conclusion, regardless of the maturation status of the monocyte-derived cells, the cells within the model responded to the allergen and influenced the characteristics of the monocyte-derived cells.



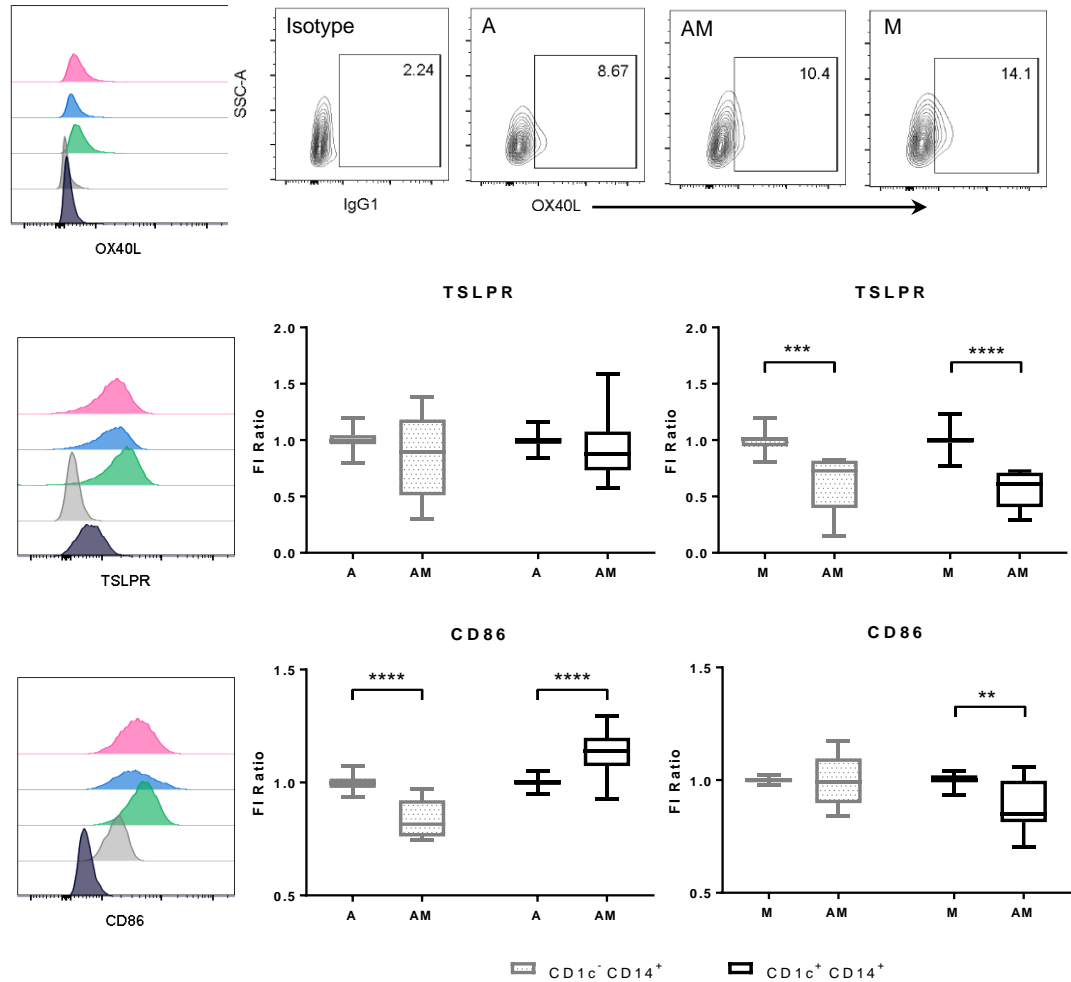


Fig. 4.5. Effect of the allergen and mast cells (MCs) on the characteristics of the monocyte-derived cells collected after 48 h from the subendothelial layer of the connective tissue-equivalent model. Representative histogram overlays of the expression of the markers used to define dendritic cells (DCs) in the test (AM), control groups (A and M), and monocytes (Mon) before culture are shown for comparison. Gating scheme and representative contour or pseudocolor plot of CD16 and OX40L expression are also shown. For comparing group AM with the control group A or M, the fluorescence intensity (FI) of the marker was normalized to the control group in each experiment. Data are from 5 independent experiments; $n = 2-3$. *, **, ***, and **** indicate $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

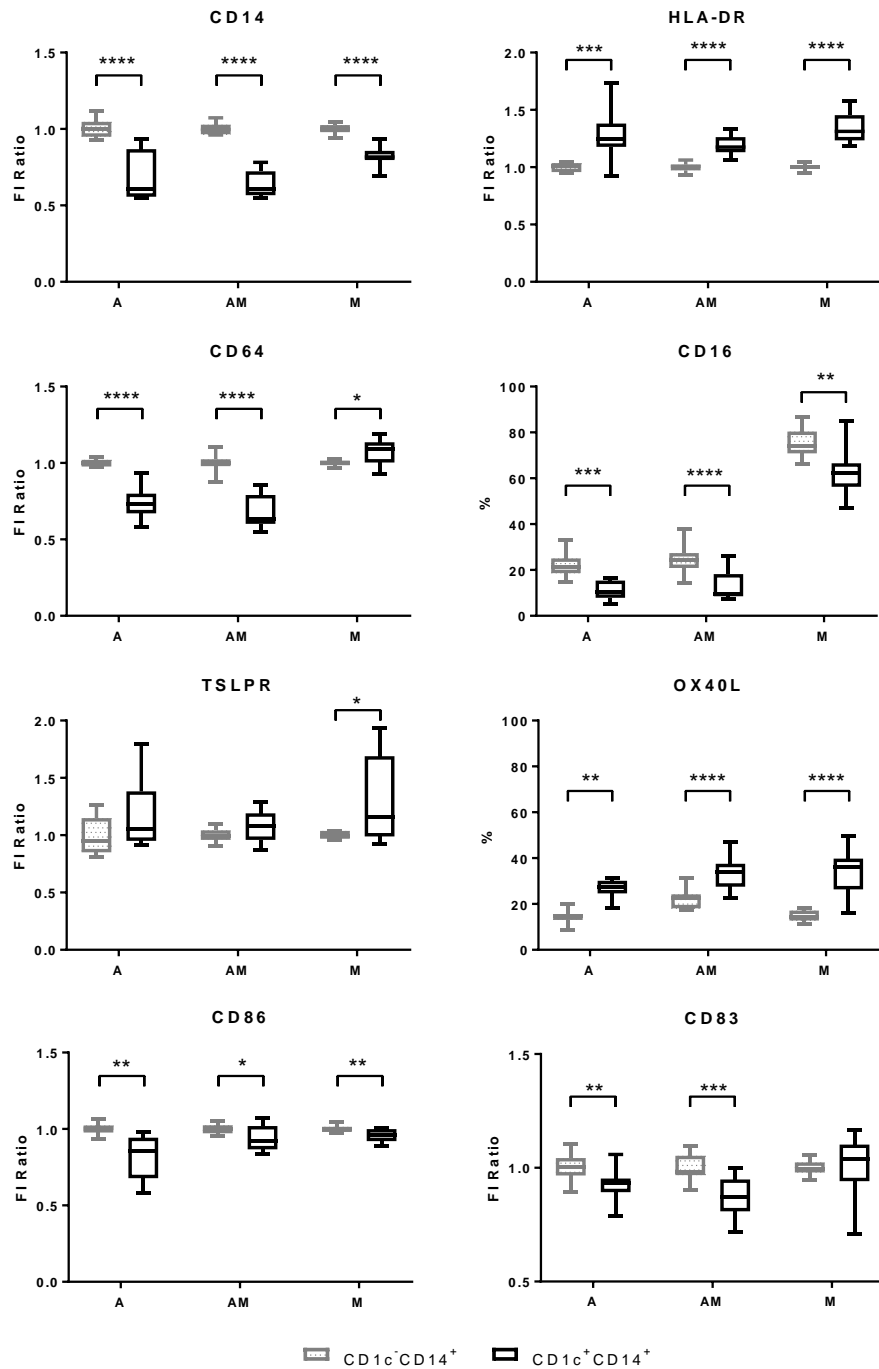


Fig. 4.6. Comparison between CD1c⁺ CD14⁺ and CD1c⁻ CD14⁺ monocyte-derived cells collected after 48 h from the apical layer of the connective tissue-equivalent model. For comparison, the fluorescence intensity (FI) of the marker on CD1c⁺ population was normalized to the CD1c⁻

population in each group from each experiment. Data are from 5 independent experiments; $n = 2-$

3. *, **, ***, and **** indicate $p < 0.05$, 0.01, 0.001, and 0.0001, respectively.

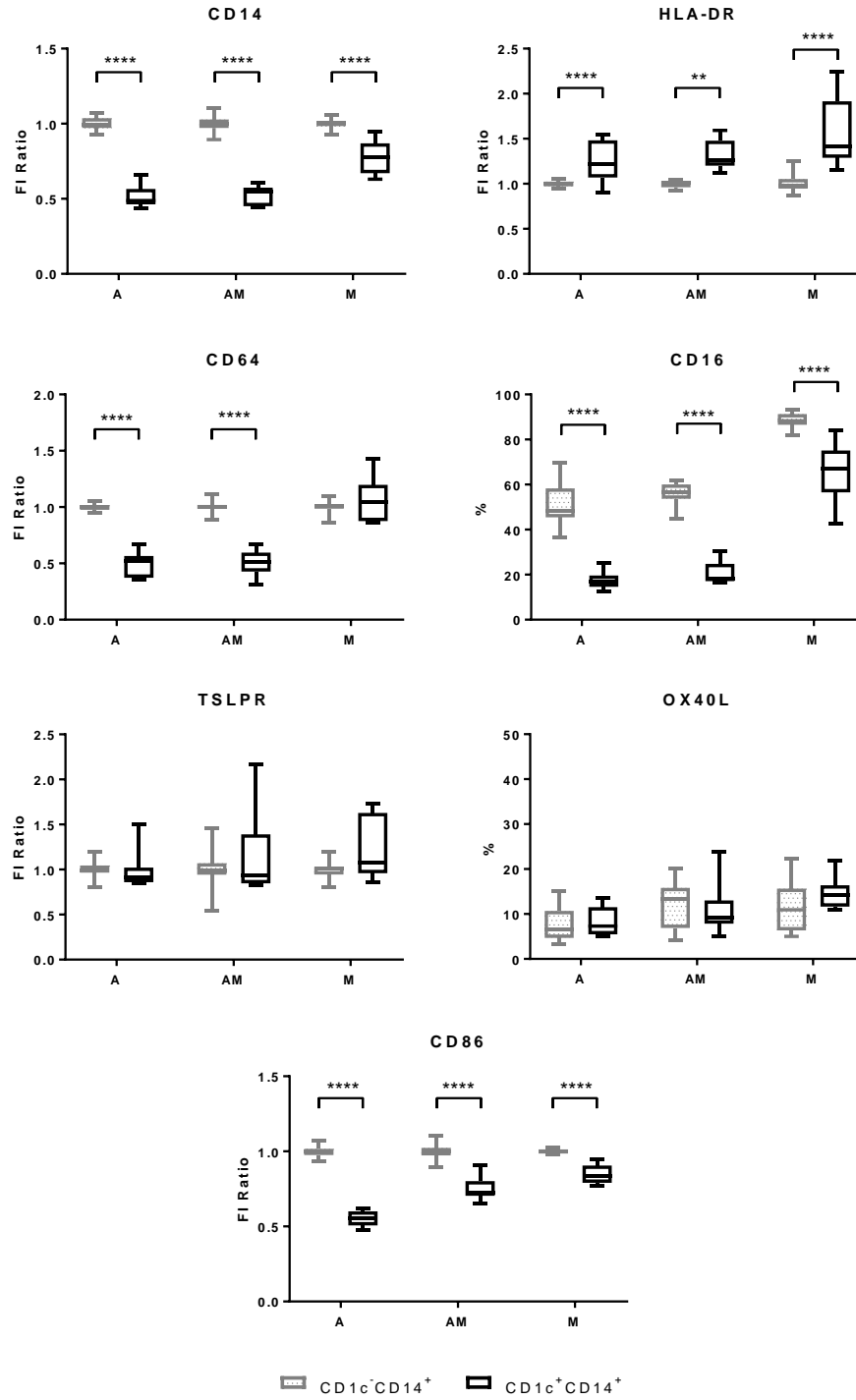


Fig. 4.7. Comparison between CD1c⁺ CD14⁺ and CD1c⁻ CD14⁺ monocyte-derived cells collected after 48 h from the subendothelial layer of the connective tissue-equivalent model. For comparison, the fluorescence intensity (FI) of the marker on CD1c⁺ population was normalized to the CD1c⁻ population in each group from each experiment. Data are from 5 independent experiments; n = 2-3. ** and **** indicate $p < 0.01$ and 0.0001 , respectively.

4.3.3. Mast cells (MCs) mitigated the effect of the allergen on the development of CD1c⁺ dendritic cells (DCs)

To determine the influence of MCs on the characteristics of monocyte-derived cells, group AM was compared with group A, which did not contain MCs in the matrix. As shown in Fig. 4.2B and 4.3B, the percentage of the cells expressing both CD1c and CD14 was lower in group AM than group A, indicating that the presence of MCs in the matrix mitigated the effect of the allergen on the development of CD1c⁺ cells ($p < 0.05$ for the apical and <0.001 for the subendothelial layer). The difference is more intense for the cells within the subendothelial layer, where MCs resided (Fig. 4.3B). Main growth factors for the development of different DC subsets are IL-4, GM-CSF, TGF- β , and TNF- α , all among MC mediators [79, 386, 387]. However, MCs can also produce M-CSF and IL-6, which are M Φ -inducing agents [387, 388]. Histamine was also shown to promote the development of CD14⁺ DCs [389]. This does not rule out the indirect effect of MCs through fibroblasts and ECs on attenuating the allergen-induced CD1c⁺ DC development. The overall characteristics of the developed cells in the presence of MCs in the model shown in Fig. 4.4 and 4.5, such as higher expression of CD14 and CD64 but lower HLA-DR ($p < 0.05$), implies that the cells have exhibited slightly more M Φ -like phenotype than group A, meaning that the cells might have been more efficient in antigen uptake, even though the changes on the apical cells were marginal. Assuming that in response to the allergen the CD1c⁻ CD14⁺ cells were in transition to CD1c⁺ CD14⁻ cells with a higher HLA-DR and lower CD64

expression, the characteristics of the cells in group A were more similar to the CD14⁻ cells than group AM. This suggests that MCs may have alleviated the effect of the allergen on this transition or influenced the kinetics of the transition. Importantly, CD1c^{low/-} CD14⁺ dDCs were shown to favor the humoral immunity in contrast to LCs or CD1a⁺ dDCs, which are CD1c⁺ CD14⁻ cells, by inducing the production of immunoglobulins by B cells [357]. MCs, by attenuating the development of CD14⁻ DCs, may play a role in the initiation of a humoral response. This is confirmed by our preliminary data on the production of IgE by B cells in a mixed leukocyte reaction (Fig. A.6). Therefore, the CD14⁺ dDCs (Fig. 4.4 and 4.5) might be involved in the polarization of naïve T cells to Th2 or Tfh cells that enhance the development of antibody-producing plasma cells.

CD16 and TSLPR expression remained unchanged when MCs were incorporated into the model (Fig. 4.4 and 4.5, AM comparison with A). Even though it is not clear whether TSLPR expression is correlated with TSLP production in the system, MCs express TSLP mRNA and release it upon FcεRI aggregation in the presence of IL-4 [376, 390]. TSLP release in response to the allergen could have led to a higher percentage of OX40L⁺ cells in the model with MCs ($p < 0.05$, Fig. 4.4 and 4.5), as TSLP-stimulated DCs were shown to upregulate OX40L [375]. Interestingly, CD86 density was also upregulated on CD1c⁺ cells collected from the apical and subendothelial layer ($p < 0.0001$, Fig. 4.4 and 4.5). In addition, although the number of CD83⁺ cells did not significantly change, the density of its expression was also marginally upregulated by monocyte-derived cells in group AM than A ($p < 0.05$, Fig. 4.4). MCs activated in IgE-mediated reactions in co-culture with DCs were shown to promote the co-stimulatory molecule and HLA-DR expression and alter the cytokine profile of DCs [323, 324]. In our work, we have shown that MCs can influence DC development and their characteristics in an IgE-independent manner. MC crosstalk with DCs or other cell types within our model in response to the allergen led to upregulation of co-stimulatory molecules and OX40L by the monocyte-derived cells.

Expression of these molecules may have equipped the DCs in group AM to favor a different type of T cell response than group A. A higher expression of CD14 and CD64, which could be implicated as a more antigen uptake ability and a higher co-stimulatory molecule expression, which suggests a more mature phenotype may not necessarily be contradictory. This may occur because when immature DCs begin to become more immunogenic, the antigen processing functions increase transiently [329]. Activation of human lung and skin MCs with an allergen in the absence of allergen-specific IgE resulted in histamine and IL-4 release [184]. MC histamine could abolish IL-12p70 synthesis by DCs leading to a Th2 response [31]. The same response was obtained when MC-derived PGD₂-treated DCs were injected into a mouse [32]. However, the direct interaction of activated peritoneal MCs from mice with bone marrow-derived DCs favored a Th1 and Th17 response [323]. In contrast, activated human bone marrow-derived MCs in co-culture with moDCs promoted IL-4-producing T cells in response to LPS/IFN- γ stimulation [324]. MCs can release an array of stimulatory as well as suppressive cytokines. The balance of these mediators may provide a microenvironment that can, indirectly through DCs, contribute to the development of Th1, Th2, or another type of response [136]. Overall, the allergen directly or indirectly by stimulating other cells types in the model, e. g. ECs, altered MCs and induced the development of cells from monocytes with different characteristics in an IgE-independent manner.

4.3.4. CD1c⁺ CD14⁺ cells in the tissue model were not phenotypically more mature than CD1c⁻ CD14⁺ cells

As shown in Fig. 4.6 and 4.7, the CD1c⁺ cells that were mainly developed in response to the allergen, downregulated the expression level or the density of CD14, CD64, and CD16, while HLA-DR was upregulated ($p < 0.05$, with an exception for CD64 expression in group M). The differences in the expression level of the above-mentioned markers between CD1c⁺ and CD1c⁻ cells were greater on the subendothelial cells (Fig. 4.7). The changes highlight the previous

conclusion that CD1c⁻ CD14⁺ cells may phenotypically be more similar to MΦ in terms of the expression of molecules related to the uptake an antigen. CD83 expression remained the same, but CD86 was surprisingly downregulated on CD1c⁺ cells on both the apical and subendothelial cells ($p < 0.05$, Fig. 4.6 and 4.7). One explanation can be that the CD1c⁺ CD14⁺ cells were in transition to another subset and during this transition the co-stimulatory molecules were downregulated. This may be tied to the phenotypic characteristics of the population that they were differentiating to, besides the influence of the cytokines in the microenvironment on the expression of co-stimulatory molecules. For example, LC precursors migrate as immature cells and similar to the regulatory DCs release IL-10 and TGF-β that can sustain an anti-inflammatory microenvironment and prevent their maturation [330, 370, 379]. Unlike the subendothelial cells with a variable expression of TSLPR and OX40L (Fig. 4.6), the OX40L⁺ cells collected from the apical layer were more in the CD1c⁺ population than the CD1c⁻ population ($p < 0.01$, Fig. 4.6). This suggests that OX40L expression may involve signaling pathways independent of co-stimulatory molecules. Overall, regardless of the addition of the allergen or MCs to the model, the CD1c⁺ cells were not phenotypically more mature than CD1c⁻ cells based on the expression of co-stimulatory molecules.

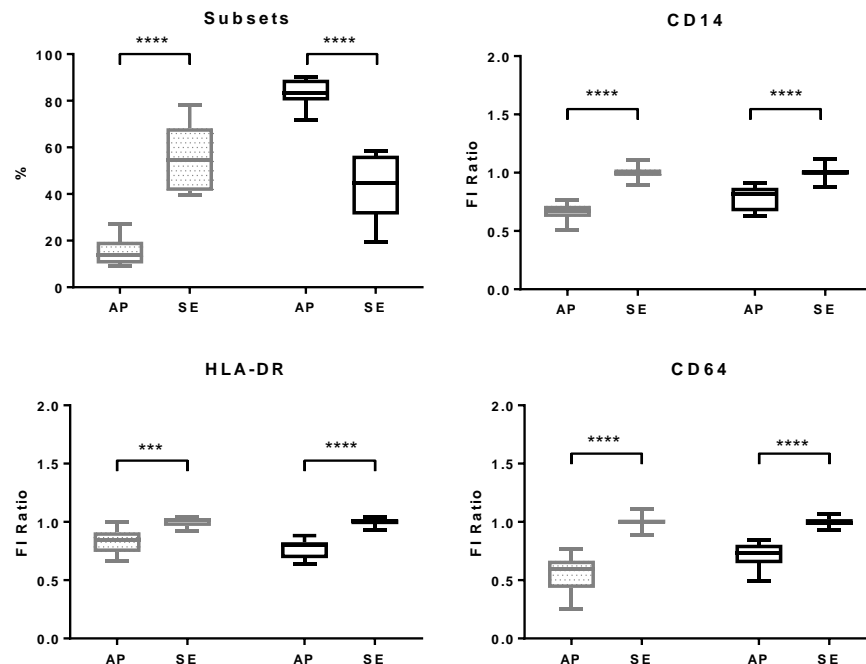
4.3.5. Monocyte-derived cells may not be ready for migration to lymphatics after 48 h incubation

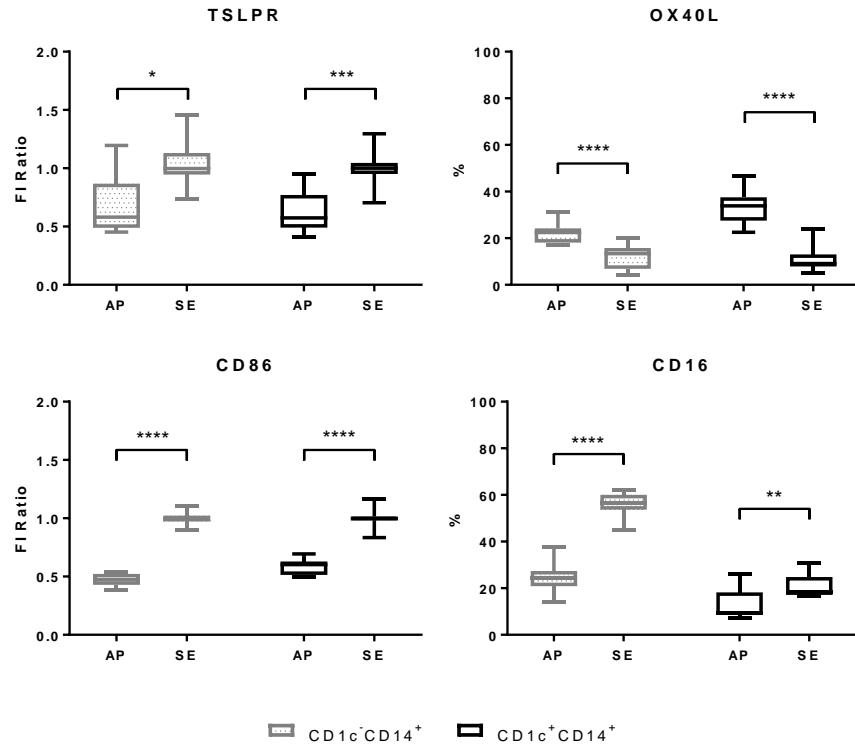
To compare the characteristics of the cells collected from the apical layer with the cells collected from the subendothelial layer, group AM with the allergen and MCs in the model is selected. The percentage of CD1c⁻ CD14⁺ cells collected from the apical layer was significantly lower than the subendothelial cells ($p < 0.0001$, Fig. 4.8A). On the contrary, the percentage of CD1c⁺ cells was higher in the collected cells from the apical layer ($p < 0.0001$). In accordance with Randolph et al.'s transendothelial model [336], which has not incorporated MCs and fibroblasts in the matrix, after 3 h incubation of the monocytes, around half of the monocytes

attached to or diapedesed into the subendothelial layer. This occurred regardless of whether the cells were activated or not (Fig. 4.8B). In contrast, after 48 h incubation, only a very low percentage of the cells were loosely attached to the endothelium and were collected from the apical layer (Fig. 4.8B). This indicates that the majority of the cells were not migratory, confirmed by no expression of CCR7. These findings suggest that some of the monocytes migrated across the endothelium, while a few of them remained attached to the ECs. The cells remaining on the apical layer differentiated into the CD1c⁺ cells at a faster pace than the subendothelial monocytes. In general, the cells in 3D culture conditions are slower in proliferation and differentiation than 2D systems and we have also observed a slower differentiation of MCs in the matrix [274]. This may be due to the intrinsic property of the connective tissue in sustaining homeostasis. Consequently, after 48 h, the collected cells that were, at that time, loosely attached to the endothelium were more CD1c⁺ than the subendothelial cells ($p < 0.0001$, Fig. 4.8A). These cells never migrated into the subendothelial layer and were in direct contact with the ECs the whole time. Therefore, their characteristics were slightly different from the subendothelial cells (Fig. 4.8A). Monocytes in co-culture with human umbilical vein ECs were shown to acquire the morphological characteristics of CD14⁺ skin cells, whereas with dermal fibroblasts, monocytes exhibited a slightly higher autofluorescence and expression of CD16, indeed, a more MΦ-like phenotype [327]. This indicates that the direct contact with ECs and fibroblasts may favor the development of DCs with different characteristics that may lead to differences in the phenotype of the cells collected from the apical and subendothelial layers in our model (Fig. 4.8A). Furthermore, dermal fibroblasts were shown to promote the maturation of human moDCs by upregulating the expression of co-stimulatory molecules and HLA-DR in TNF- α and ICAM-1-dependent manner [391]. In our model, the monocytes that migrated across the endothelial layer first differentiated into CD1c⁻ CD16⁺ MΦ-like cells and then, into CD1c⁺ CD16^{low} cells. Possibly, due to the interaction with other cell types in the subendothelial layer, they expressed a different level of co-stimulatory molecules, as well as TSLPR and OX40L (Fig.

4.8A). After 48 h, monocyte-derived cells either from the apical or subendothelial layers that were in transition to CD1c⁺ CD14⁺ and then to CD1c⁺ CD14⁻ cells were not set for migration to lymphatic vessels. The kinetics of migration of different dDC subsets may not be the same [392]. When injected subcutaneously into a mouse, migration of DCs to the dLN peaked after two days [393], whereas LCs arrive in the LN around 72-98 h after stimulation [394]. In addition, moDCs may require a stronger stimulation than conventional DCs to become capable of migration [395]. Therefore, the allergen dose may not have been sufficient to induce full maturation and migration of the cells, considering that CD14⁺ moDCs in our model may not be a migratory type of DCs. Indeed, although moDCs could efficiently uptake the dust mite allergen and were the main population in the lungs of sensitized mice, they were the smallest population in the LN, implying that they are poor migratory cells [363]. Furthermore, MΦ, even upon stimulation, do not express CCR7 and remain fixed in the tissues, whereas CCR7⁻ CD14⁺ MΦ-like cells could migrate spontaneously without entering afferent lymphatic vessels [327, 382]. Therefore, CD14⁺ cells in our model may not be migratory type or not in a state to migrate to the LN.

A





B

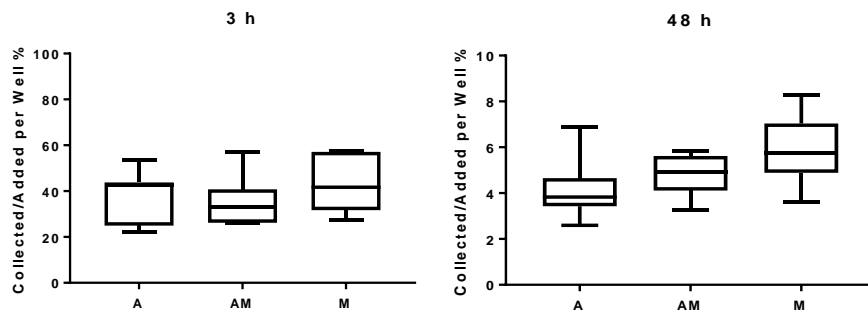


Fig. 4.8. Comparison between the cells collected after 48 h from the apical (AP) and subendothelial (SE) layers of the connective tissue-equivalent model from group AM. (A) For comparison, the fluorescence intensity (FI) of the marker on the AP cells was normalized to the SE cells in each experiment. Data are from 5 independent experiments; $n = 2-3$. ** and **** indicate $p < 0.01$ and 0.0001 , respectively. (B) The percentage of the monocytes and monocyte-derived cells collected from the apical layer after 3 and 48 h, respectively. Monocytes were added at 150,000 cells/well. Data are from 7 and 5 independent experiments for 3 and 48 h, respectively.

4.3.6. Mast cells (MCs) indirectly promoted the development of a humoral response

Due to a low expression of CD83 by the monocyte-derived cells and a low number of migrating cells, for the mixed leukocyte reaction (MLR), the samples were stimulated with the allergen for the second time. Even though the second stimulation promoted the development of CD1c⁺ CD14⁻ cells, it did not induce the upregulation of CD83 expression by CD1c⁺ cells in group AM (Fig. A.3). The second stimulation of the model also diminished the number of cells collected from the apical layer, confirming that the cells in our model were either not migratory or not ready for migration, even after a direct stimulation with the allergen. Previous studies have incubated B cells for 12-14 days for to produce immunoglobulins, however, this timing may not be ideal for characterizing T cells [347, 357]. Also, after the addition of IL-2, the media of the samples with APCs remained yellowish in color the entire time, indicating that the lymphocytes were proliferating. In the control group without APCs, the change in the color of the media happened after the seventh day of culture, meaning that the addition of IL-2 ultimately induced the proliferation of the cells in the control group and a background signal. In fact, IL-2 can act as a T and B cell growth factor and induce B cell proliferation and activation [396]. After 12 days, T cells in all the groups highly proliferated, as evidenced by the downregulation of the CFSE signal and formation of at least six generations (Fig. A.4B). T cells in group M were marginally less proliferating ($p < 0.001$, Fig. A.4B), possibly because of higher CD1c⁻ CD14⁺ MΦ-like cells. B cells also proliferated in all the groups with lower proliferating cells in group AM (no statistical analysis is available for the preliminary data). Due to the differences in the proliferation of the cells in the control group without APCs and the test groups, the APCs collected from all three A, AM, and M groups induced T and B cell proliferation (Fig. A.4B). Twelve days post seeding, more than 90% of the CD4⁺ T cells were CD45RO⁺ memory cells (Fig. A.5). Interestingly, as shown in Fig. A.5, FOXP3⁺ population in the CD45RO⁺ CD4⁺ T cells were more in the absence of MCs in the ACS (group A, $p < 0.05$). The percentage of the cells that were expressing IFN- γ

but not IL-4 and IL-13 was also higher in group A ($p < 0.01$, Fig. A.5), suggesting that the APCs collected from group A favored the development of Treg and IFN- γ -producing T cells (Th1 or Th1 Th17 cells). As shown in Fig. A.2B, since the percentage of the CD1c⁺ CD14⁻ cells in group A was higher than group AM, the data implies that CD1c⁺ CD14⁻ cells may have characteristics of regulatory DCs or Th1-inducing DCs (DC1). A higher percentage of Th1 and Treg cells in group A resulted in no IgE production by B cells (was below 1.7 ng/ml the sensitivity of the assay, Fig. A.6). On the other hand, T cells in group M in both GATA3⁺ and GATA3⁻ populations were more IL-4 and IL-13-producing cells than group AM ($p < 0.01$, Fig. A.5). Since the percentage of CD1c⁻ CD14⁺ cells were more in group M than group AM, the CD1c⁻ CD14⁺ cells may be required for Th2 polarization (Fig. A.2B). Notably, the total IgE measurements showed that the cells in group AM have promoted the development of IgE-producing plasma cells compared with group A (Fig. A.6, $p < 0.01$). In fact, Th2 cells can elicit isotype switching of B cells to IgE and IgG1, whereas IFN- γ -producing T cells can promote IgG2a production [357]. Therefore, a higher level of Th2 cytokines and lower IFN- γ in the microenvironment may have favored the production of IgE in group AM. The background in the control group without APCs might have been caused by the exogenous IL-2. Nevertheless, as shown in the previous sections, the characteristics of the CD1c⁺ and CD1c⁻ cells were not the same across the groups. Therefore, when MCs were included in the model, the balance between different DC populations and their cytokine profile, along with the cytokines produced by T cells, led to the emergence of IgE-producing plasma cells.

4.4. Conclusion

In this work, we have used a 3D tissue model to study the effect of allergen-activated MCs on monocyte migration and differentiation in an IgE-independent manner. The monocytes within the model with MCs spontaneously differentiated into CD1c⁺ cells. Addition of the allergen to the model promoted the development of the CD1c⁺ cells. Overall, the characteristics of the CD1c⁺

cells were not the same as CD1c⁻ cells and they were not phenotypically more mature. Furthermore, allergen-activated MCs attenuated the effect of the allergen on development of CD1c⁺ cells. Ultimately, the monocyte-derived cells from the ACS with MCs in co-culture with lymphocytes could elicit the isotype class switching to IgE. This implies that CD1c⁻ CD14⁺ monocyte-derived cells can regulate humoral immunity. Considering that the findings from our preliminary study on the T and B cell response need to be confirmed, our preliminary data suggest that MCs can play a role in the development of a humoral response through influencing the characteristics of the monocyte-derived cells in an IgE-independent manner. Therefore, MCs besides orchestrating the effector phase of an allergic response can contribute to the initiation of the response.

CHAPTER 5

Conclusion and Future Work

In this research, a novel 3D tissue-engineered model was developed, in order to study the main events of an allergic response. The cross-talk between the immune cells, such as T and B cells, and antigen presenting cells (APCs) regulates an immune response [397]. Mast cells (MCs), a key effector cell of inflammatory reactions, arise from hematopoietic stem cells that mature within vascularized tissues. Recent findings suggest that MCs may interact with an allergen and contribute to shaping the immune response [79]. The main objective of this study was to utilize the tissue-engineered model for testing the hypothesis that an allergen can activate MCs in an IgE-independent manner. Since MCs are tissue-resident cells, the development of a tissue-engineered model with ancillary cells that more closely mimics the 3D *in vivo* microenvironment would provide greater relevance for MC studies. The model comprised of a collagen matrix to mimic the connective tissue, and fibroblasts and endothelial cells (ECs) that play a role in the maturation of progenitor cells in the tissues.

To mimic an inflammatory response to an allergen, the tissue model was comprised of two separate modules. The first module, which was called an allergen contact site (ACS), was used to investigate two phases of an allergic immune response. In the sensitization phase of the ACS,

MCs that were developed from progenitor cells come into contact with an allergen and interact with monocyte-derived cells in an IgE-independent manner. In the activation phase of the ACS, IgE-sensitized MCs were exposed to the allergen. In the second module, which was called lymph node site (LNS), monocyte-derived cells from the ACS interacted with T and B cells to induce production of IgE.

Before the co-culture of the cell types included in the model, the type of media, which would support the growth of all the cell types was investigated. Media were selected based on the specificity for the growth and expansion of hematopoietic cells. Fibroblasts and ECs were cultured separately and characterized for cell proliferation, surface marker expression, and cytokine secretion. For MC generation, CD133⁺ progenitor cells were isolated from peripheral blood and cultured for seven weeks in a collagen matrix with different media. Differentiation of CD133⁺ cells to MCs was verified by metachromasia, histamine granule formation, and expression of MC phenotypic markers. The following are the findings from this study:

- Progenitor cells within the collagen matrix cultured in StemSpan media with serum added the last week yielded a greater number of cells exhibiting MC phenotypic markers and amount of histamine granules compared to other media tested.
- Timing of the addition of serum to the culture media influenced the cell yield and MC characteristics. Serum addition from the beginning of culture promoted cell proliferation, while exerting an inhibitory effect on MC development.
- The generated MCs were phenotypically from the type mainly found in human skin and submucosa.
- The developed cells released histamine in response to sensitization with IgE and activation with anti-IgE. The response of the cells that embedded in the matrix was different from when removed from the matrix, possibly due to the interaction of the antibodies with the matrix.

- Media supplemented with serum were necessary for EC survival, while fibroblasts survived irrespective of serum with a higher cell yield in StemSpan.
- Fibroblasts released interleukin (IL)-6 and stem cell factor (SCF), and ECs secreted IL-6, all of which are cytokines necessary for the survival and maturation of MCs.
- StemSpan medium with serum in the last week of culture was chosen for the co-culture of MC progenitors, fibroblasts, and ECs. Since serum was required for EC survival for the co-culture model, they were included in the in the last week of culture, which was the time that serum was added to the media.

For creating the ACS, CD133⁺ progenitors isolated from human peripheral blood were cultured with human primary dermal fibroblasts in the collagen matrix to represent the connective tissue. The matrix was coated with type IV collagen and fibronectin prior to seeding with primary human ECs, representing the capillary wall. Samples were incubated for seven weeks to allow the proliferation, differentiation, and maturation of MC precursors. To confirm the development of MCs, the expression of MC cytoplasmic granules and phenotypic markers were determined. The response of the developed MCs was investigated in IgE-mediated reactions by measuring the histamine release upon degranulation. To our knowledge, this was the first study that showed the development of human MCs within a matrix in co-culture. Creating the ACS and characterizing the activation phase of the ACS led to the following findings:

- Histological analysis confirmed the development of a connective tissue-equivalent matrix (CTEM) with the generated MCs and fibroblasts distributed in the subendothelial layer.
- The generated cells in the CTEM expressed MC phenotypic markers and granules. The size and histamine content of the cells were within the range of *in vivo* MCs. The cells exhibited the phenotype of the human MCs mainly found in skin and submucosa in accordance with their microenvironment in the CTEM.

- Crosslinking the IgE receptors on the developed MCs resulted in their degranulation. There was a significant amount of histamine released by the cells in response to Der p allergen after passive sensitization with serum of an allergic patient, showing the function of the cells in the activation phase of the ACS. The histamine release was not dependent on whether the MCs were derived from allergic or non-allergic individuals, but rather dependent on whether the serum used for passive sensitization contained specific IgE or not.
- The co-culture of MC progenitors with ancillary cells in the CTEM promoted cell proliferation, formation of histamine granules, as well as histamine release in response to IgE/anti-IgE. The phenotype of the cells in co-culture was similar to those in the model without ancillary cells.
- Together with the findings of the media study, the 3D tissue-engineered model supported the development of granular and functional MCs. MC histamine content and release were different in the CTEM than 2D culture methods and the model without ancillary cells, signifying the importance of using an *in vivo*-like microenvironment for *in vitro* studies.

The ACS was used to test the hypothesis that an allergen can stimulate MCs in an IgE-independent manner. Therefore, Der p allergen was added to the CTEM that was not sensitized with IgE. Autologous monocytes were added to the model after removal of the media containing the allergen. The cells were collected after 48 h from both the apical and subendothelial layer to define and characterize the monocyte-derived cells. The characteristic of the cells in the test group (AM) was compared to the following two control groups: one without allergen (M) and one without MCs (A), to determine the influence of the allergen and MC on the monocyte-derived cells, respectively. The following are the concluding remarks from the sensitization phase of the ACS:

- Monocytes that were CD1c⁻ CD14⁺ CD206⁻ differentiated into CD206⁺ HLA-DR^{high} cells that were CD1c⁻ CD14⁺, CD1c⁺ CD14⁺, and a few CD1c⁺ CD14⁻ in the CTEM. The CD1c⁻ population might be macrophage (MΦ)-like cells homologous to *in vivo* MΦ or human CD14⁺ dermal DCs (dDCs). The CD1c⁺ cells could be inflammatory DCs (infDCs).
- Monocytes first differentiated into CD1c⁻ CD14⁺ cells with higher expression of CD64 and CD16 in accordance with a MΦ-like phenotype and then into CD1c⁺ CD14⁺ cells. Under the influence of the allergen, a higher percentage of CD1c⁺ CD14⁺ cells were developed. These cells were also in transition to CD1c⁺ CD14⁻ cells. Therefore, under inflammatory conditions, monocytes can be the precursors of CD1c⁺ CD14⁻ cells.
- The CD1c⁺ cells that were developed in response to the allergen did not upregulate the co-stimulatory molecules CD83 and CD86 in comparison with CD1c⁻ cells, which might be due to the effect of cytokines produced by the cells in the model, the phenotype of the cells they were differentiating to, or a property of transient cells.
- The monocyte-derived cells expressed TSLPR, OX40L, and CD86 required for priming T cells at variable levels. The cells in response to the allergen-activated model only marginally upregulated CD86, while CD83 expression was not altered. This indicates that factors, such as the intrinsic property of the allergen and the balance between inflammatory and suppressive mediators produced in the model sustained the immature or semi-immature phenotype of the cells.
- Allergen-activated MCs mitigated the influence of the allergen on the differentiation of CD1c⁻ to CD1c⁺ cells. MCs induced a higher expression of CD14 and CD64 by the monocyte-derived cells, suggesting that they were more efficient in antigen uptake. MCs also promoted the maturity of the cells by upregulating the density of expression of co-stimulatory molecules as well as OX40L⁺ cells. Therefore, the allergen-activated MCs in

the model affected the characteristics of monocyte-derived cells in an IgE-independent manner.

- The cells collected from the apical layer were not migratory as they were not expressing CCR7, implicating that these cells never migrated into the matrix and remained loosely attached to the endothelium. The monocytes that migrated across the endothelial layer were exposed to a different microenvironment, and consequently were different from the cells collected from the apical layer. The cells remaining on the apical layer differentiated into CD1c⁺ cells at a faster pace than the subendothelial monocytes. Therefore, a higher percentage of the cells on the apical layer were CD1c⁺. Furthermore, HLA-DR and CD86 expression levels were higher on the subendothelial cells, suggesting that factors in the matrix promoted the maturity of the cells. Whereas, higher expression of CD14 and CD64 by the subendothelial cells indicate their capability for antigen uptake and along with CD16 expression aligned with a more MΦ-like phenotype.

For creating the LNS, the cells in the ACS were activated for a second time with the allergen, to allow the monocyte-derived cells to interact with the allergen. The cells from the apical layer were collected and co-cultured with autologous T and B cells for 12 days in a collagen matrix.

The following are the main findings from the preliminary study of the LNS:

- Monocyte-derived cells collected from the apical layer of the ACS could prime T and B cells to proliferate. When allergen was not added to the model, the proliferation of T cells was marginally lower, probably due to a higher percentage of CD1c⁻ CD14⁺ MΦ-like cells.
- Twelve days post seeding, more than 90% of the cells were CD45RO⁺ memory T cells.
- Allergen-primed APCs reduced the percentage of IL-4 and IL-13 producing T cells, indicating that the allergen stimulation did not promote a Th2 response. Furthermore, this

can be implicated as CD1c⁺ DCs that were generated in response to the allergen diminished the development of Th2 cells.

- When MCs were not included in the ACS, a higher percentage of cells were FOXP3⁺ and IFN- γ producing, implicating that the presence of MCs in the ACS diminished the development of APCs that may trigger the polarization of Treg and Th1 cells. Indeed, IL-4 and IL-13 producing T cells were more when APCs were collected from the model with MCs. The data suggest that MCs, by alleviating the influence of the allergen on the development of CD1c⁺ DCs, elevated the percentage of T cells that were producing Th2 cytokines.
- APCs collected from the ACS with MCs induced IgE production by B cells in the LNS, probably due to a lower percentage of FOXP3⁺ and IFN- γ producing T cells and higher Th2 cytokines in the microenvironment.

To answer the hypothesis, by utilizing a tissue-engineered model it was shown that MCs can be activated by an allergen in an IgE-independent manner and influence the development of APCs from monocytes that can facilitate the production of IgE by B cells to induce a humoral response. These findings are significant as they suggest that MCs, in addition to orchestrating the effector phase, can play a role in shaping the sensitization phase of an allergic inflammatory response.

In the preliminary work of this project, we have shown that a stem cell medium can support the growth of fibroblasts, ECs, and MCs. Therefore, this medium can be used for any other study that aims to co-culture the mentioned cell types. This research contributes to understanding the mechanism of development of allergic reactions. We have shown that MCs can play a role in the development of an allergic response even in the absence of IgE. Therefore, targeting MCs and neutralizing or blocking the factors that MCs release or express to interact with other cell types or an allergen might be a promising strategy for treatment and prevention of allergic responses. The

model can also be used for understanding the interactions that can lead to the reactions in the effector phase of an allergic inflammatory response. The developed model in this work can be applied to other research and used for understanding the mechanism of other inflammatory or inflammatory-related diseases, such as asthma, fibrosis, and angiogenesis that MCs also play a role in their development. Determining the factors that are involved in the development of the disease can lead to better therapeutic strategies. Furthermore, the model can be used as a tool for testing the effect of various drugs on the cell behavior for the treatment of the disease. The model also has the potential to be used for the diagnosis of allergies as an alternative to current clinical tests.

Still, further investigation is required to fully understand the role of each cell type in the model and the mechanisms modulating the immune response. Consequently, it is recommended that the future work should be directed toward addressing the following points:

- i. Determine the mechanisms underlying the influence of the matrix, fibroblasts, and ECs on MC development, immunophenotype, and function within the tissue-engineered model. Extracellular component of connective tissue can regulate the proliferation and commitment of stem cells [80, 235]. We have also observed that the function of MCs can be different in a matrix by an unknown mechanism. Furthermore, previous studies have shown that fibroblast-derived proteins and membrane molecules may modulate the maturity and function of MCs [64, 132]. ECs can also promote MC growth and influence their morphological phenotype [61]. However, it is not clear whether the interaction of MCs with the fibroblasts and ECs within the CTEM was through soluble proteins or cell-contact dependent, and which factors were playing a role in the enhanced cell proliferation and function that we have observed in the co-culture model. The interaction of MCs with the extracellular matrix proteins may also influence the cross-talk with other cell types and explain the differences between the characteristics of the cells in 2D and

3D cell culture models. The findings of this study can contribute to a better understanding of the molecular signals that regulate the hematopoietic stem cell differentiation in tissues. It can also be applied to studies on MC disorders, such as mastocytosis and MC activation syndrome, which is caused by the abnormal proliferation of MCs and an excessive release of MC mediators, respectively [398].

- ii. Investigate the mechanism of allergen interaction with the developed MCs within the tissue-engineered model. The findings of this study suggested that MCs interact with an allergen in an IgE-independent manner. ECs express pattern recognition receptors that can bind with an allergen [318] and it is possible that the allergen exerted its influence on MCs by stimulating ECs. However, MCs also express various pattern recognition receptors that an allergen may trigger directly or indirectly through complement receptors [136]. Blocking the pathway that an allergen interacts with MCs or the other cell types in the model can lead to better therapeutic strategies for allergic diseases.
- iii. Investigate the conditions under which a humoral response is developed and the mechanism of monocyte differentiation in the tissue-engineered model. In this research, the allergen was added to the model prior to the addition of the monocytes. It is not clear whether changing the dose, incubation time, timing of the addition of the allergen, or incubation time of the monocytes could affect monocyte migration, differentiation, characteristics of the monocyte-derived cells, and ultimately the type of response. MCs may contribute to the development of a humoral response only under certain circumstances and understanding those conditions may be important for the therapy of the disease. Furthermore, this study provided preliminary data on the interaction of APCs from the ACS in the LNS. Therefore, additional experiments need to be performed in order to confirm the results. In this work, the IgE produced in the LNS was not specific to the allergen. Therefore, priming the LNS with the allergen may be required for inducing a

specific response to the allergen. The ratio of the APCs to lymphocytes can also change the type of the response [399] and may affect the IgE production level, which needs to be further investigated. Studying the influence of the above mentioned factors may define the circumstances under which a humoral response is developed. Consequently, controlling the number of APCs in the lymph node and their characteristics may lead to better therapeutic strategies for allergic responses.

Cytokines, such as IL-4, GM-CSF, and TGF- β can induce monocyte differentiation in vitro [359, 387]; however, the source of these cytokines, other differentiating factors, and cellular interactions that may have been involved in this process in the CTEM are not known. In addition, the presence of MCs in the model affected the characteristics of the monocyte-derived cells. In order to gain a better insight into the role of MCs in the disease initiation, the mechanism of MC cross-talk with the cell types in the model needs to be investigated. Moreover, the monocyte-derived cells did not express the migration marker CCR7 after 48 h. It is possible that each type of monocyte-derived cells has a specific time for migration and consequently a certain role in the initiation, exacerbation, or regulation of the immune response. Therefore a kinetic study is required to fully understand the mission of each type of APCs that were developed in the CTEM. Investigating the factors that modulate the monocyte differentiation and migration of the monocyte-derived cells can contribute to elucidating the pathophysiological mechanisms underlying the early stages of the development of an allergic inflammatory response.

- iv. Determine the direct effect of MCs on the response of the LNS. Previous studies suggest that MCs, by expressing MHC-II, co-stimulatory, and adhesion molecules similar to APCs, can present an antigen to T cells and release mediators to induce B cell isotype class switching [41, 44]. The response of the LNS to allergen-primed MCs in synergy with monocyte-derived cells from the ACS would show the direct influence of MCs on

leukocyte function and response type. The findings of this study will expand our understating of MC immunomodulatory functions in the development of an allergic inflammatory response.

v. Validate the model with clinical data and utilize the model as a tool for allergy diagnosis.

In this study, we have used samples from patients with positive and negative skin reactions to Der p allergen to develop the model. Serum samples with or without specific IgE to Der p were used for passive sensitization. When serum of an allergic patient was used, the cells degranulated in response to the allergen, regardless of whether the cells were generated from an allergic or non-allergic patient. Therefore, the activation phase of the ACS can be used as a tool for allergy diagnosis by testing a sample from the serum of the patient. If the serum of a patient contains specific IgE to the allergen of interest the cells would respond to the corresponding allergen. Furthermore, the LNS can also be used as a complementary allergy test. Since the blood sample of an allergic patient contains allergen-specific lymphocytes, it is expected that B cells produce specific IgE in response to the APCs. The histamine release level from the ACS and the produced specific IgE from the LNS can be used together for allergy diagnosis. However, the model requires optimization and validation with clinical data. The model can also be used for testing the response to allergens other than the dust mite.

vi. Use the tissue-engineered model for studying other inflammatory diseases. The tissue model includes the cell types that reside in connective tissue. Therefore, it can be used for studying the mechanism of skin inflammatory disorders, specifically with MCs as the modulator of the disease, such as fibrosis and angiogenesis. The components of the model, such as the collagen gel and the cells can be substituted with a different type to be applied to other diseases e.g. cells from asthmatics along with lung-resident cells for studying the pathophysiology of the disease.

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APPENDIX A

Create and Characterize the Lymph Node Site

A.1. Materials and methods

A.1.1. Antibodies and reagents

RPMI 1640 medium, penicillin-streptomycin, L-glutamine, MEM non-essential amino acids solution, sodium pyruvate, and 2-mercaptoethanol (2-ME) were all from Gibco (Carlsbad, CA). StemSpan medium was purchased from STEMCELL Technologies (Vancouver, Canada). Human recombinant SCF, IL-6, IL-3, and IL-2 were from PeproTech (Rocky Hill, NJ). Defined HyClone fetal bovine serum (FBS) was purchased from GE Healthcare Life Sciences (Logan, UT). Human dermal fibroblasts treated with mitomycin-C was purchased from Merck Millipore (Billerica, MA), while human umbilical vein ECs were from Promocell (Heidelberg, Germany). CellTrace CFSE cell proliferation kit was purchased from Life Technologies (Carlsbad, CA). The following antibodies for cell staining and flow cytometry were purchased from BioLegend (San Diego, CA): anti-human Alexa Fluor (AF)700 CD3 (clone UCHT1), PE CD19 (clone HIB19), APC/Fire 750 CD45RA (clone HI100), brilliant violet (BV)786 CD45RO (clone UCHL1), PerCP/Cy5.5 CD4 (clone OKT4), PE/Dazzle 594 FOXP3 (clone 206D), BV711 T-bet (clone 4B10), BV421 GATA3 (clone 16E10A23), PE IL-4 (clone 8D4-8), APC IL-13 (clone JES10-5A2), PE/Cy7 IL-10 (clone JES3-9D7), BV605 IFN- γ (clone 4S.B3), and the isotype controls, fluorochrome-

conjugated Ms IgG1(clone MOPC-21), Ms IgG2b (clone MPC-11), Ms IgG2a (clone MOPC-173), and Rat IgG1 (clone RTK2071). Zombie Aqua fixable viability kit and cell activation cocktail with Brefeldin A were also from BioLegend. Brilliant Stain Buffer and CompBead compensation particle set were purchased from BD Biosciences (San Jose, CA).

A.1.2. Cell culture

Leukocyte preparations were obtained from Oklahoma Blood Institute (Oklahoma City, OK). CD133⁺ cells, autologous CD14⁺ monocytes, CD3⁺ T cells, and CD19⁺ B cells were sequentially isolated from human peripheral blood mononuclear cells by magnetic cell separation technology following the manufacturer's protocols (MACS Miltenyi Biotec; CA). MCs were generated within the connective tissue-equivalent matrix, as explained in chapter 3. After seven weeks, Der p extract of mite (HollisterStier; WA) at 300 AU/ml was added to the samples and incubated for 24 h (Fig. A.1). Monocytes were added to the samples following the protocol explained in chapter 4. After 48 h, the samples were stimulated by 100 AU/ml of the allergen for the second time in the complete medium without IL-6. A group of samples that were stimulated with the allergen were primed with lipopolysaccharide (LPS; Sigma-Aldrich; MO) at 0.15 µg/ml (Table A.1) [324]. The samples were incubated for 24 h prior to three gentle rinses of the apical layer with warm phosphate buffer saline (PBS). The collected cells were counted by a hemocytometer and trypan blue exclusion. For immunophenotyping, the remaining cells within the matrix were also collected after digesting the gel using 2.2 mg/ml collagenase D (Roche Applied Science; IN). For determining the influence of the allergen-activated MCs and allergen on the phenotype of the collected cells, identical samples without MC progenitors (group A) or allergen (group M) served as controls, respectively. The samples that were considered for characterizing the monocyte-derived cells were stained for flow cytometry, according to the Methods section of chapter 4.

Table A.1. Protocols for activation of dendritic cells by lipopolysaccharide (LPS).

	Type	LPS strain	Concentration (µg/ml)	Incubation time (h)	Ref.
1	Human	Salmonella abortus equi	0.1	<1-48	[400]
2	Human	Escherichia coli serotype 0111:B4	1	48	[399]
3	Human	Escherichia coli O26:B6	0.5	15-20 min before adding fresh media and incubation for 3 days	[356]
4	Human	Escherichia coli serotype 0111:B4	0.5 for 1 M cell/ml	6 hr after stimulation with TNF for 2 days	[332]
5	Human		0.01	3 days	[336]
6	Human	Escherichia coli serotype 0111:B4	0.1	24	[324]
7	Human	Salmonella minnesota	0.1 for 1 M cell/ml	24	[31]
8	Human		0.2	24	[339]
9	Human		1	24	[334]
10	Mice		0.1	24	[323]
11	Mice	Escherichia coli O26:B6	1	24	[401]
12	Mice	Escherichia coli O26:B6	0.05	18	[402]
13	Human	Salmonella enterica	0.05 or 1	48	[403]
14	Mice	Escherichia coli serotype 0111:B4	1	24	[404]
15	Mice	Escherichia coli serotype 0111:B4	1	14	[405]
16	Human	Escherichia coli serotype 0111:B4	10	24	[406]
17	Human	Escherichia coli serotype 0111:B4		20	[407]
18	Human		3	24	[408]
19	Human		0.1	36	[389]
20	Human	Escherichia coli O26:B6	0.05	24-48	[409]

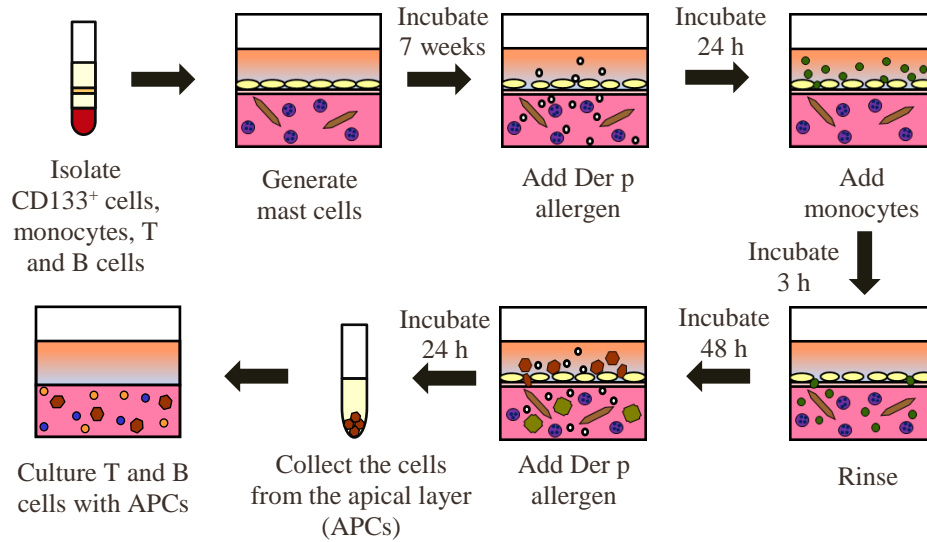


Fig. A.1. Preparation of the lymph node side (LNS) to study the indirect effect of mast cells (MCs) on lymphocyte response.

RPMI medium containing 1 vol% PSG, 1 vol% non-essential amino acids, 1 vol% sodium pyruvate, 0.1 vol% 2-ME, and FBS (10%, v/v) was used for preparation and culture of samples with lymphocytes (Table A.2). T and B cells at 5 M cells/ml were stained with working concentration of 5 μ M CFSE dye in PBS for 10 min at room temperature [410]. Collected antigen presenting cells (APCs) from the apical layer were mixed with 10 M cells/ml CFSE-stained T and B cells at the ratio 1: 50: 50 of APCs: T cells: B cells. The cells were mixed with the type I collagen gel solution and added to a 96-solid well plate (Greiner Bio-One; NC) at 50 μ l/well. After 45 min incubation at 37°C, 5% CO₂ for the collagen to gel, complete RPMI medium (130 μ l/well) was added to the top of the matrix. Three days post seeding, 20 μ l/well complete media supplemented with IL-2 with the final concentration of 10 ng/ml (assuming 150 μ l/well total media and 40 μ l/well of gel) was added to the samples to expand the T cells [324]. The samples were incubated for 12 days with media changes every other day. The samples were incubated based on the time required for the B cells to produce IgE [31, 33]. A Group of samples without APCs was considered as a control for the spontaneous response of T and B cells.

Table A.2. T cell culture media protocols.

	Cells	Serum	L-glutamine	Antibiotic (U/ml)	HEPES (mM)	Other supplements	Ref.
1	T cells	10% FCS	2 mM	Pen/Str	10	-	[324]
2	T cells	5% Heat inactivated human serum	2 mM	50 Pen/Str	-	1% Non-essential amino acids, 1% sodium pyruvate	[411]
3	PBMC	10% Human serum	-	100 Pen/Str	-	0.25 µg/ml Amphotericin	[412]
4	T cells	5% Human serum	2 mM	100 Pen/Str	-	-	[413]
5	T cells	10% FCS or 5% Human serum	2 mM	50 µg/ml kanamycin	-	1% Non-essential amino acids, 1% sodium pyruvate, 0.05 mM 2-ME	[400]
6	T cells	10% FBS	2 mM	100 Pen/Str	-	-	[414]
7	T cells	10% FCS	2 mM	100 Pen/Str	10	-	[399]
8	B cells	10% FCS	RPMI-Glutamax	Pen/Str	-	Non-essential amino acids, 1 mM sodium pyruvate, 0.025 mM 2-ME	[415]
9	PBMC	5% Human AB serum	Glutamax	Kanamycin	-	Non-essential amino acids, 1% sodium pyruvate, 0.1% 2-ME	[416]
10	PBMC	5% Human serum	1 mM glutamax	100 µg/ml Kanamycin	10	Non-essential amino acids, 1 mM sodium pyruvate	[417]
11	PBMC	10% FBS	-	PSG	10	2-ME	[418]
12	T cells	5% Human serum	1%	1% Pen/Str	25 for rinses	1% Non-essential amino acids, 1% sodium pyruvate, 0.1% 2-ME	[419]

Only protocols that used RPMI media and human cells are listed. FBS: Fetal bovine serum; FCS: Fetal calf serum; PBMC: Peripheral blood mononuclear cells; Pen/Str: Penicillin and streptomycin; PSG: Penicillin- streptomycin- glutamine; 2-ME: 2-Mercaptoethanol.

A.1.3. IgE quantification

On day 12, the media of the samples were collected for measuring the IgE produced in the samples. The matrix was also digested and combined with the corresponding top media after removal of the cells. The samples were stored at -80°C until analyzed. IgE-QBA assay (Indoor Biotechnologies, Inc.; VA) was used for measuring the total and specific IgE.

A.1.4. Immunophenotyping and flow cytometry

For intracellular detection of cytokines, the collected cells from the matrix at 1.5 M cells/ml were stimulated with an activation cocktail containing PMA, ionomycin, and Brefeldin A at 2 µl/ml for 5 h at 37°C in a 96-round-bottom well plate. The cells were stained with the viability dye prior to staining for surface markers. The eBioscience FOXP3/Transcription factor staining buffer set (Invitrogen; CA) was used for the intracellular staining of the cytokines and the transcription factors with the cocktail of antibodies or isotype controls following the manufacturer's protocol. Data were acquired on a BD LSR II flow cytometer using the acquisition software BD FACSDiva (BD Biosciences) and analyzed using FlowJo software (Ashland, OR). Spectral overlap was calculated using single-color compensation beads.

A.1.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software; San Diego, CA). One-way ANOVA and Tukey's multiple comparison test were applied to determine significant difference among the groups. A *p*-value < 0.05 was considered significant.

A.1.6. Possible hazard and safety precautions

All animal- and human-derived materials were purchased from commercial vendors that prescreen the products for biological hazards. All procedures associated with the work followed "Universal Precautions".

A.2. Results

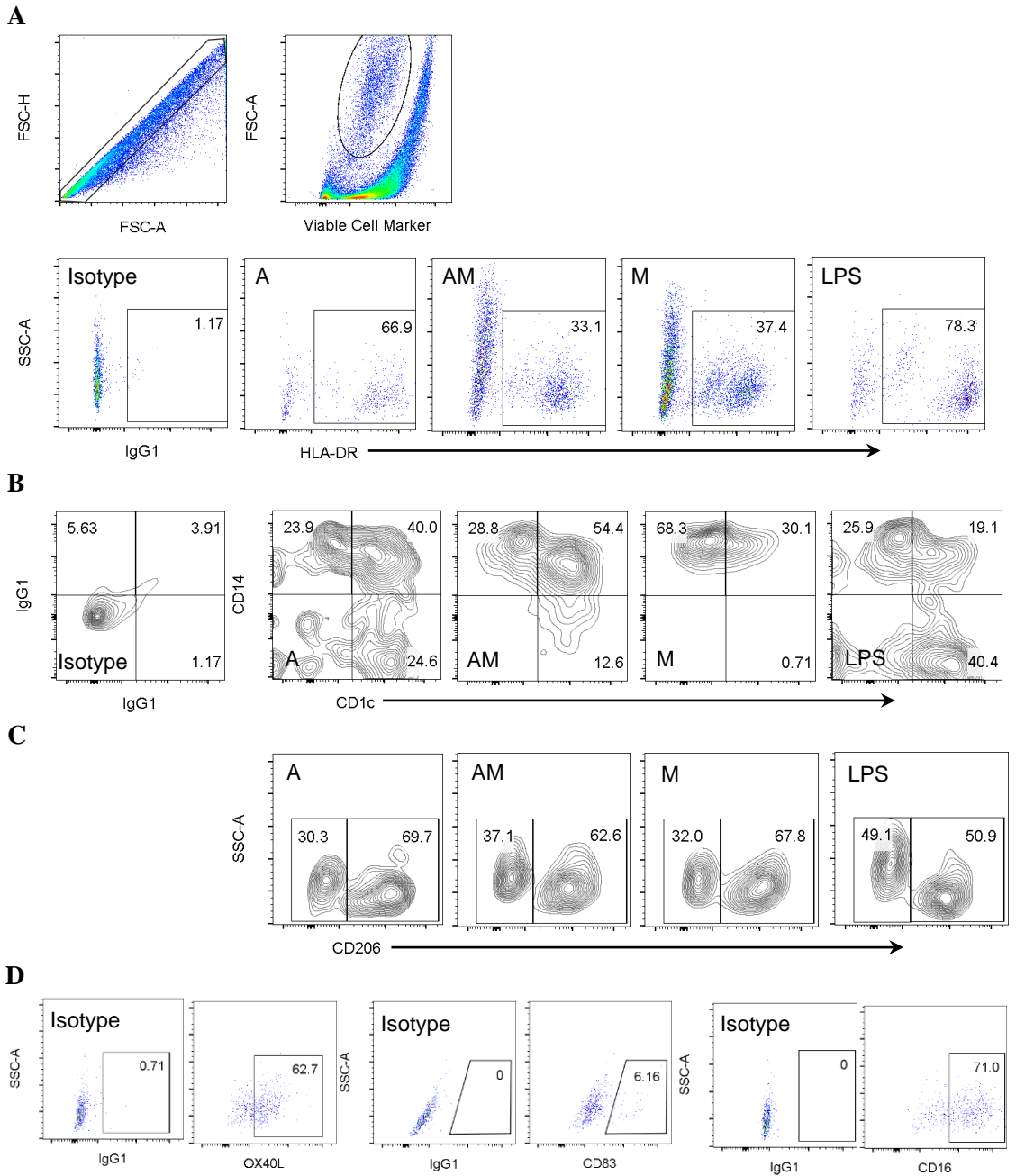


Fig. A.2. Characteristics of the cells collected from the apical layer of the connective tissue-equivalent model after the second stimulation with the allergen. (A) Gating scheme for excluding doublets and dead cells. HLA-DR⁺ cells on viable cells were gated. (B) The HLA-DR⁺ cells were gated into 3 main populations based on the expression of CD1c and CD14. (C) CD1c⁻ CD14⁺ cells were gated into CD206⁺ and CD206⁻ cells. (D) Gating scheme and representative pseudocolor plot of OX40L, CD83, and CD16 expression. A: with allergen without MC; AM: with allergen and MC; M: without allergen with MC; LPS: Lipopolysaccharide.

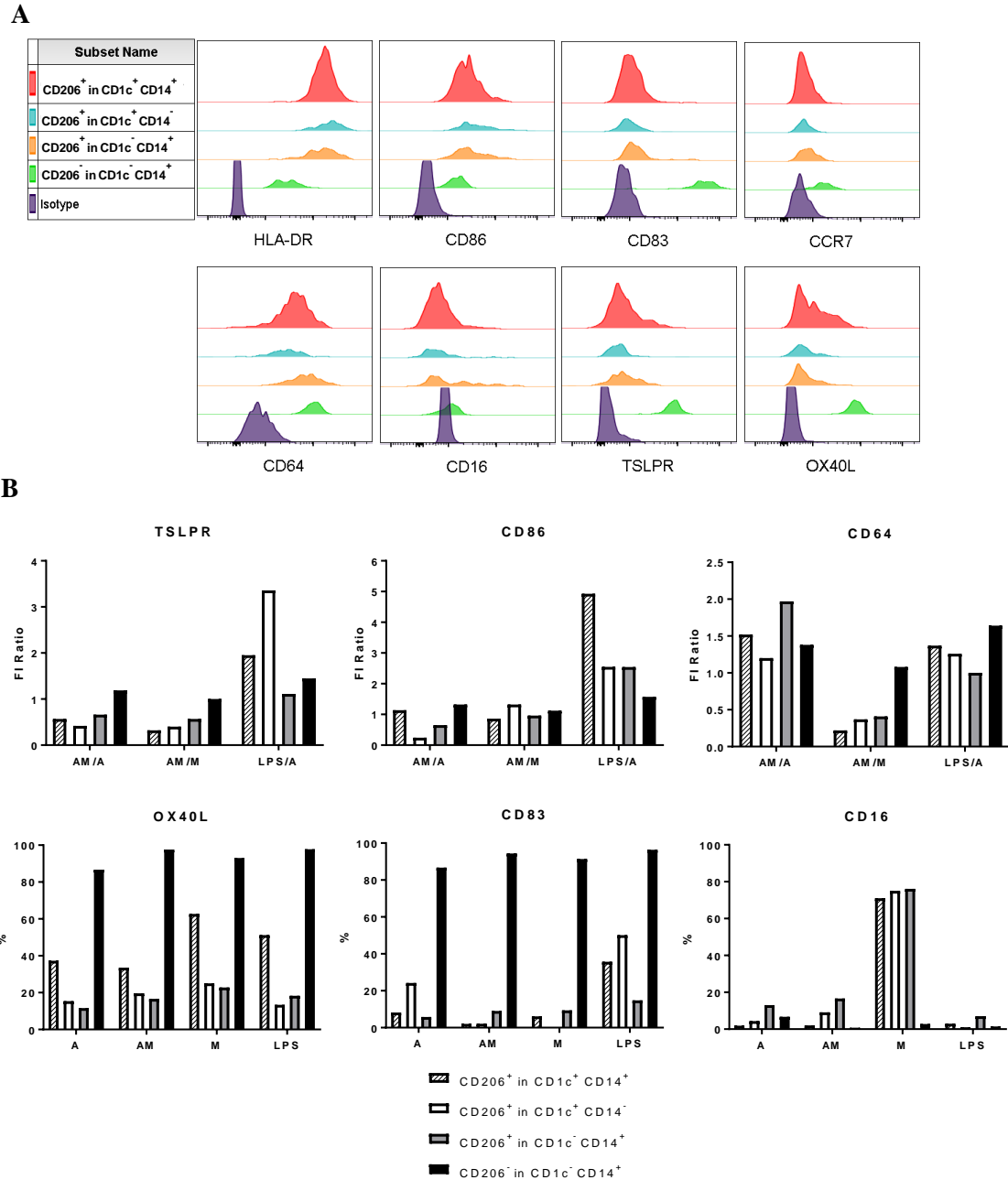


Fig. A.3. Characteristics of the monocyte-derived cells collected from the apical layer of the connective tissue-equivalent model after the second stimulation. (A) Histogram overlays and (B) expression of the markers used to define dendritic cells (DCs). For TSLPR, CD86, and CD64 expression, the fluorescence intensity (FI) of the marker in group AM was normalized to the control group (A or M). Samples activated with LPS were normalized to group A. Data are from one experiment; $n = 1$.

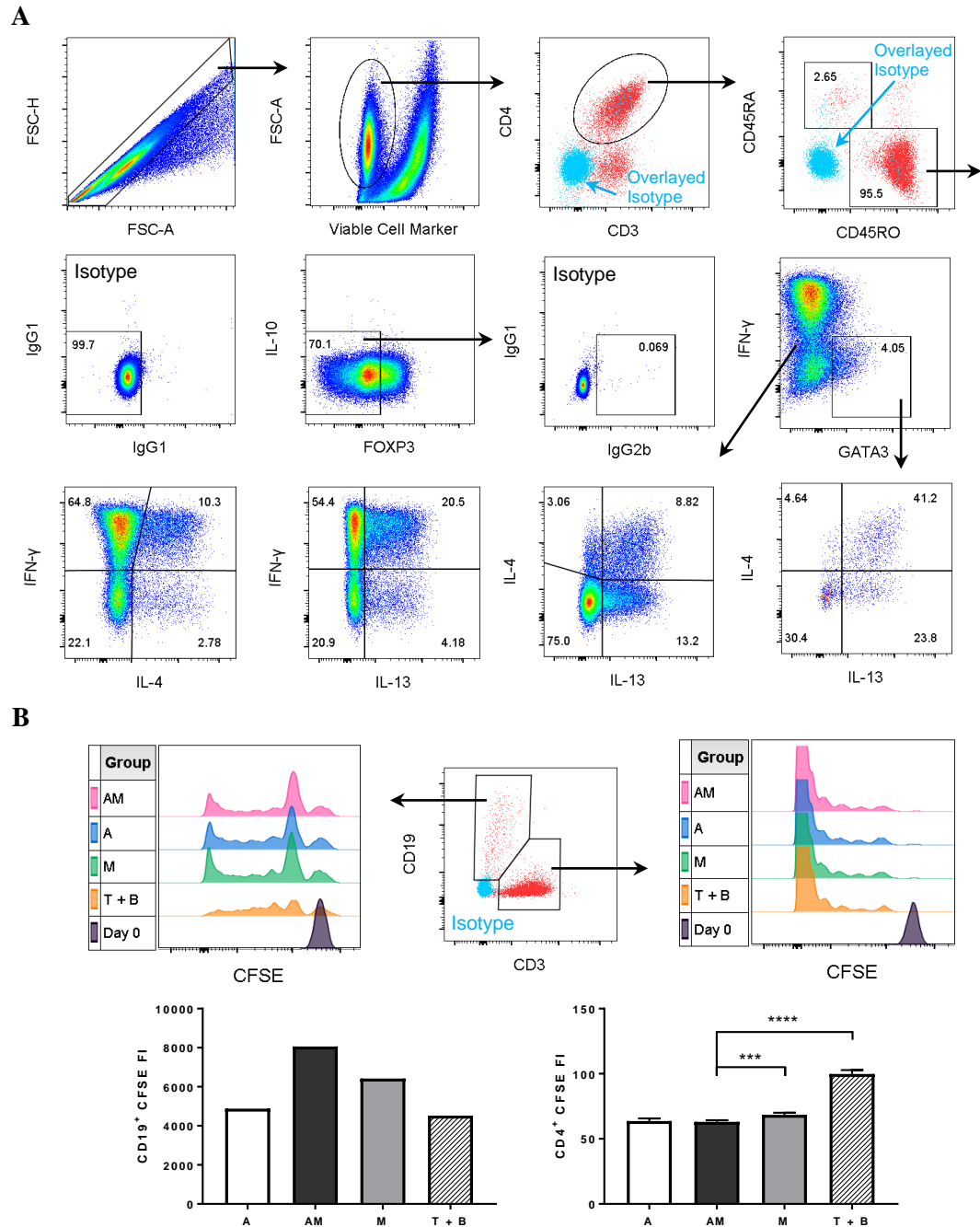


Fig. A.4. Analyses of the T and B cells after 12 days in co-culture with the collected cells from the apical layer of the connective tissue-equivalent model. (A) Gating scheme and representative pseudocolor plot of the expression of the markers used to characterize T cells. (B) Representative histogram overlay of the CFSE-stained CD3⁺ T cells and CD19⁺ B cells. The fluorescence intensity (FI) of the CFSE-stained cells is shown in the graph. Data are mean \pm SD from one experiment; $n = 3-4$ except CD19⁺ CFSE FI graph with $n = 1$. *** and **** indicate $p < 0.001$ and 0.0001 , respectively. A: with allergen without MC; AM: with allergen and MC; M: without allergen, with MC; T + B: T and B cells without antigen presenting cells (APCs).

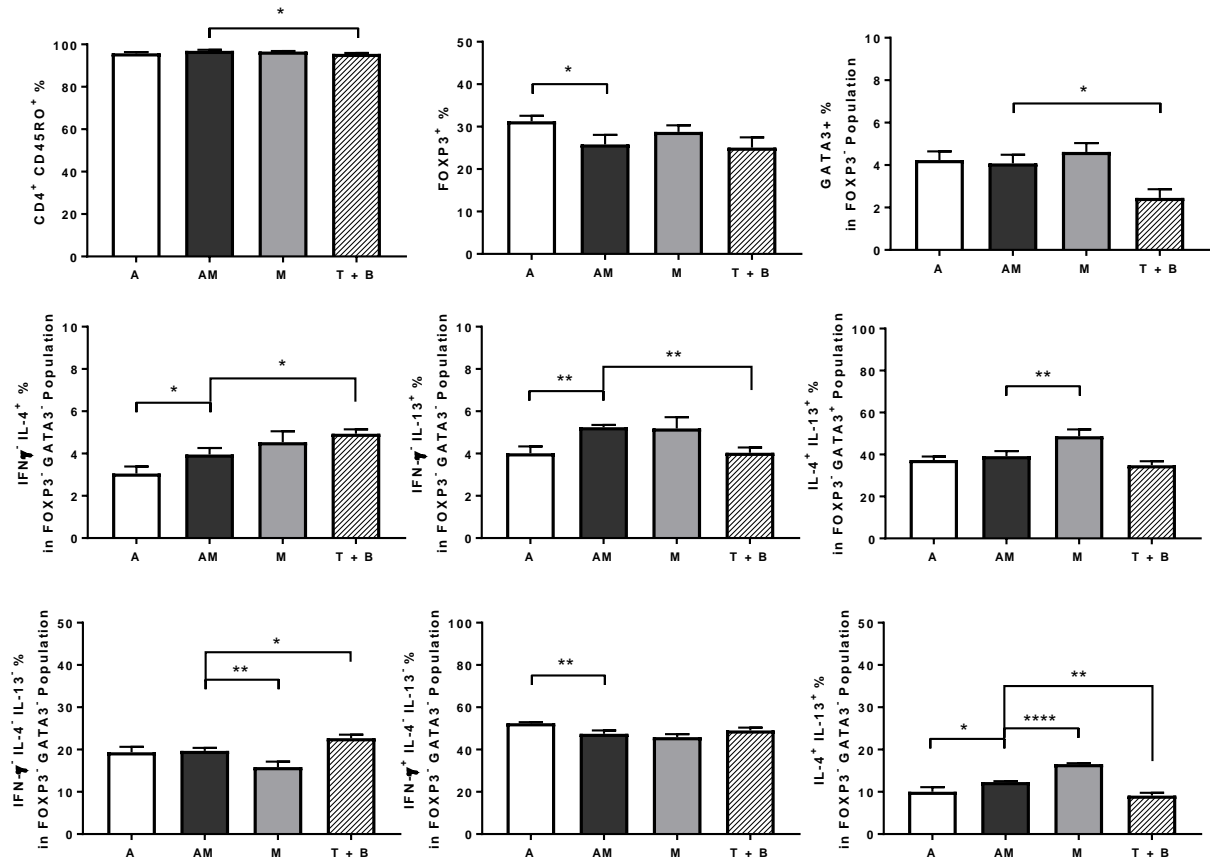


Fig. A.5. T cell characteristics after 12 days in co-culture with B cells and the collected cells from the apical layer of the connective tissue-equivalent model. Data are mean \pm SD from one experiment; $n = 3-4$. *, **, and **** indicate $p < 0.05$, 0.01 , and 0.0001 , respectively. A: with allergen without MC; AM: with allergen and MC; M: without allergen, with MC; T + B: T and B cells without antigen presenting cells (APCs).

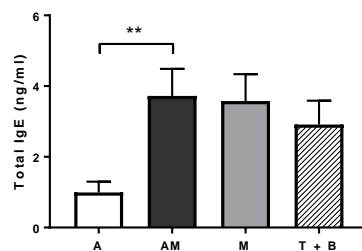


Fig. A.6. IgE production by B cells after 12 days in co-culture with T cells and the collected cells from the apical layer of the connective tissue-equivalent model. Data are mean \pm SD from one experiment; $n = 3-4$. ** indicates $p < 0.01$. A: with allergen without MC; AM: with allergen and MC; M: without allergen, with MC; T + B: T and B cells without antigen presenting cells (APCs).

APPENDIX B

Dust Mite Allergen Cysteine Activity Restore

B.1. Introduction

Nearly 50% of allergic patients are sensitive to house dust mite (HDM) allergen, which is a risk factor of asthma in children [314, 315]. *Dermatophagoides pteronyssinus* and *Farinae* of HDM are the most causative agents of allergy among indoor allergens [420, 421]. Group 1 and 2 are the main content of dust mite (DM) and patients are more frequently allergic to them [316, 422]. The prevalence is age and location dependent, for example, in adults, the prevalence to Der p 1 and 2 is lower than children and more people in France responded to rDerp 2 than Sweden [316]. Group 1 binds with specific IgE (sIgE) in sera of 80-100% of DM-allergic patients at high strength [423]. Der p 1 can affect more than 80% of DM-allergic patients and 70% of allergic patients with either bronchial asthma and/or atopic dermatitis [188, 316, 424]. Nearly 50% of IgE accounts for sIgE to Der p 1 and 2 [315, 425].

Group 1 allergens are papain-like proteins belonging to cysteine proteases [188, 421]. The allergenicity of the group 1 allergens is associated with its cysteine protease activity and play a role in pathogenesis of allergy [341]. Der p 1 cleaves the serine protease inhibitor, which protects the airway mucosa against proteolytic damage. Also, it increases the permeability of the epithelial cells and bronchial mucosa, consequently promoting the passage of macromolecules across the

mucosa [426, 427]. Der p 1 and 2 can induce the release of proinflammatory mediators from epithelial cells, which play a role in eosinophil infiltration and bronchial hyperresponsiveness as well as dendritic cell (DC) development, recruitment, and maturation [316, 351, 428]. Der p 1 can affect DC function to produce more interleukin (IL)-6 and IL-10, while reduces Th1-inducing cytokine IL-12 [316, 339], in addition to triggering the release of IL-4 by mast cells and basophils [184]. It can also modulate a Th2 response by inducing the release of IL-4 and decrease of interferon (IFN)- γ by T cells [316, 340]. Additionally, it can cleave CD23 receptors on B cells that mediates IgE production, therefore, promote a humoral response [316, 346]. Group 2 allergens can also prime T cells and affect their cytokine release [423].

HDM extracts are made from aqueous extraction of variable mixture of whole mites, nymphs, faecal pellets, eggs, and mite culture media and can contain more than 30 different proteins [342, 423]. Approximately 50 to 100% of allergenicity to DM extract is due to Der p allergen [342]. Although the main content of the extract is group 1 and 2, the concentration of each group of allergen is unknown and can consist of non-allergic proteins and low molecular weight substances [429]. The quality of the extract can be affected by the quality of raw material, extraction procedure, protease content, contamination by other allergens, and presence of proteolytic enzymes that might be allergenic or cause degradation and loss of potency [429]. Therefore, the use of purified and recombinant allergens has become prevalent. The reagents used in the procedure of allergen purification, such as benzamidine can suppress the enzymatic activity of the allergen [188]. Thiol is the functional group of amino acid cysteine that contains carbon-bonded sulfhydryl (R-SH group). During allergen purification, the thiol group becomes oxidized and results in the loss of the activity of the allergen [340]. Therefore, the allergen needs treatment to restore the enzymatic activity. The thiol group can be generated by reducing cysteine disulfides using dithiothreitol (DTT) or glutathione degradation, and L-cysteine, as shown in Table B.1-2.

In this work, the cysteine activity of the purified Der p 1 allergen was restored by using DTT and it was validated. After restoration of the activity, the purified allergen can be used for cell activation as well as in an assay for measurement of sIgE.

B.2. Materials and methods

To restore the cysteine activity of the allergen, the reaction buffer was made by using 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM sodium phosphate (pH = 7). Natural Der p 1 allergen (Indoor Biotechnologies, VA) was added to the DTT buffer with the final concentration of 600 µg/ml (25 µM) of the allergen in 1 mM DTT (Fisher Bioreagents, PA). The allergen was incubated for 20 min at 37°C [430, 431]. A sample without DTT was considered as a negative control.

For the allergen activity restore validation, Boc-Gln-Ala-Arg-AMC substrate (Enzo Life Sciences, Inc, NY) was used. The activated allergen and the control nonactivated allergen were added to the substrate to have 0.24 µg/ml (10 nM) of the allergen in 0.25 mM substrate in the reaction buffer and incubated at 37°C. The fluorescence was measured at Ex: 380 nm/ Em: 460 nm for 60 min. As a positive control, 7-amino-4-methylcoumarin-AMC (Alfa Aesar, MA) was diluted in the reaction buffer and the fluorescence was also measured. The difference between the fluorescence of the sample and the substrate in the reaction buffer was reported as the mean fluorescence intensity (MFI).

B.3. Results

As shown in Fig. B.1, the cysteine activity of the allergen was successfully restored. The allergen after reaction with DTT cleaved the substrate to a fluorescence product. The MFI of the sample was at every time point more than 25-folds higher than the nonactivated control group. However, further investigation is required to confirm that the cysteine activity of the allergen was fully restored. The reaction between the substrate and the allergen follows Michaelis-Menten

equation [426]. The positive control reagent can be used to make a standard curve for AMC concentration and by using the reaction equation, concentration of the allergen at each time point can be calculated.

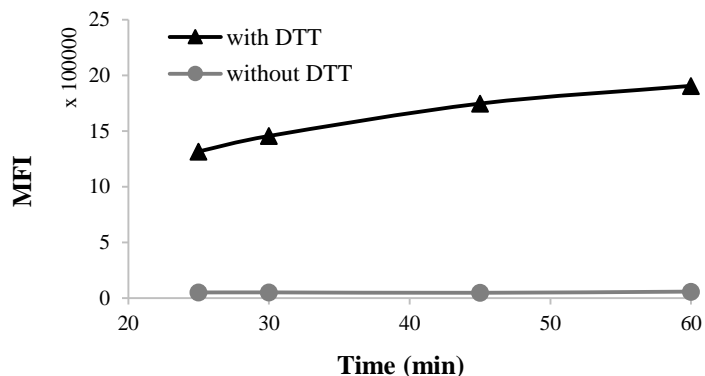


Fig. B.1. Validation of the cysteine activity restore of natural Der p 1 allergen after reaction with dithiothreitol (DTT). MFI is mean fluorescent intensity.

Table B.1. Cysteine proteases activity restore protocols.

	Reagent	Concentration	Time	Temperature (°C)	Ref
1	Acid or DTT	1 mM DTT	-	-	[432]
2	Acid, L-cysteine	5 mM cysteine	30 min	37	[187]
3	L-cysteine	5 mM	24 h	4	[342]
4	Sodium acetate pH=5.7	50 mM acid, 20 mM cysteine	-	-	[188]
5	DTT	1mM	5 min	37	[430]†
6	DTT, L-cysteine	1 mM DTT 2.5 mM cysteine	5 min DTT, 10 min cysteine	37	[422]†
7	L-cysteine	5 mM	30 min	37	[319]
8	Cysteine	5 mM	-	-	[350]
9	Sodium acetate pH=4, cysteine	100 mM acid, 20 mM cysteine	-	-	[190]
10	Sodium acetate pH=4, cysteine	50 mM acid, 20 mM cysteine	1.5 h	60	[191]
11	Sodium acetate pH=4	100 mM acid	3 h acid- 24-48 h	RT-4	[433]
12	Cysteine	5 mM	-	-	[339]
13	Acetate buffer pH=4	100 mM	48 h	4	[421]
14	Cysteine	5 mM	-	-	[340]

15	Sodium acetate pH=4, cysteine	50 mM acid, 20 mM cysteine	-	60	[434]
16	Sodium acetate pH=4.5	25 mM	5 ml/min	-	[435]
17	Cysteine	5 mM	-	-	[353]
18	DTT	1 mM	-	25	[426]
19	Ammonium bicarbonate	0.05 M	-	-	[436]
20	Cysteine	5 mM	1 min	-	[184]
21	Glutathione or DTT	0.5 mM or 1 mM	-	-	[427]
22	Glutathione	5 mM	-	-	[437]

† The natural allergen was purchased from Indoor Biotechnologies.

Table B.2. Cysteine proteases activity restore validation protocols.

	Method†	Substrate	Concentration (mM)	Time (min)	T (°C)	Inhibitor	Ref.
1	330/ 410	ADZ 50, 059	-	0-40	30	ADZ	[432]
2	365/ 465	Boc-Gln-Ala-Arg-AMC	0.1	-	-	E-64, 0.1 mM	[188]
3	Not mentioned	Boc-Gln-Ala-Arg-MCA	0.1	0-60	37	E-64 (50 µM), AEBSF (500 µM), 15 min	[430]
4	Sp for DTT, Abs (490) for cysteine	Boc-Gln-Ala-Arg-MCA for DTT, Azocoll for cysteine	0.1	10 for DTT	-	E-64, 15 min	[422]
5	Not mentioned	Azocoll	-	-	37	E-64, 10 µM	[350]
6	380/ 460	Boc-Gln-Ala-Arg-AMC	0-0.1	-	25	E-64, 10 µM	[190]
7	355/ 460	Boc-Gln-Ala-Arg-AMC	0.1	0-25	37	-	[433]
8	Not mentioned	Boc-Gln-Ala-Arg-AMC	-	-	-	E-64	[339]
9	380/ 460	Su-APAY-MCA or Boc-VLA-MCA	20	30	37	-	[421]
10	380/ 460	Boc-Gln-Ala-Arg-AMC	0-1	20-160 sec	25	E-64	[340]
11	380/ 460	Cbz-Phe-Arg-AMC and Boc-Gln-Ala-Arg-AMC	0.1	-	-	-	[434]
12	Not mentioned	Boc-Gln-Ala-Arg-AMC	-	0-160 sec	-	E-64	[353]
13	380/ 460	Boc-Gln-Ala-Arg-AMC	0-1	20-160 sec	25	-	[426]
14	520	Azocoll	-	-	-	E-64, 0.1-100 mM	[427]

† Spectrophotometry (Sp) Excitation/ Emission (nm) or absorbance (Abs) are mentioned.

VITA

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