Immunohistochemical Analysis of MCC, TARC and CD 104 Antigens in Human Lung Tissue

İnsan Akciğer Dokusunda MCC, TARC ve CD 104 Antijenlerinin İmmünohistokimyasal Analizi

Metin KORKMAZ, MD,[a]
Murat AKKUŞ,[b]
Ayşe YILDIRIM,[c]
Özlem PAMUKÇU BARAN,[b]
Ayfer AKTAŞ[b]

[a]Department of Histology and Embriology, Bursa State Hospital, Bursa
[b]Department of Histology and Embriology, Dicle University Faculty of Medicine, Diyarbakır
[c]Department of Histology and Embriology, Mustafa Kemal University Faculty of Medicine, Hatay

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Yazışma Adresi/*Correspondence:*
Dr.Özlem PAMUKÇU BARAN
Dicle University Faculty of Medicine, Department of Histology and Embriology, Diyarbakır,
TÜRKİYE/TURKEY
obaran@dicle.edu.tr

ABSTRACT Objective: The aim of this study was to investigate the the distribution of CMA1 protein (MCC), CCL17 protein (TARC) and Integrin beta 4 (CD 104) monoclonal antibodies in normal adult human lung tissue. **Material and Methods:** In this study we examined the lung biopsy specimens obtained from the adult patients who underwent operations for different reasons in the Department of Lung Surgery at Dicle University Hospital, Diyarbakır, Turkey. The tissue samples were immediately frozen in liquid nitrogen at -196º C. The samples were immunostained by indirect immune peroxidase technique. As primary antibodies, MCC, TARC and CD104 were used. As secondary antibodies, 1:200 rabbit anti - mouse IgG peroxidase diluted in PBS/BSA and 1:100 normal human serum solution were used. Negative control staining was performed using irrelevant mouse monoclonal antibodies omitting the primary antibody step. Sections were examined and photographed by Olympus BH2 light microscope. **Results:** We observed moderate reaction with vascular endothelial cells and with vascular smooth muscle cells with MCC which was aimed to show overall the presence of human lung mast cells. TARC, which was aimed to express the follicular dentritic cells, moderately reacted with follicular dentritic cells, bronchial smooth muscle cells and vascular endothelial cells. CD104, which is the integrin β4 subunit that plays an important role in the adhesion of epithelium to basement membranes, moderately reacted with vascular endothelial cells and mainly reacted with the follicular dentritic cells of bronchi. **Conclusion:** In our study, it was defined that the expressions of these molecules have important role in understanding the function of human lung tissue. We reached the conclusion that our study, by presenting the difference between the normal lung tissue and pathologic lung tissue with expression the related proteins immunohistochemically may constitute preliminary findings for excluding the diseases like interstisial lung disease or chronic obstructive lung disease for the aim of. Our preliminary findings may shed light on future studies.

Key Words: CMA1 protein, human; CCL17 protein, human; Integrin beta4

ÖZET Amaç: Bu çalışmada CMA1 protein (MCC), CCL17 protein (TARC) ve Integrin beta 4 (CD 104) monoklonal antikorlarının normal erişkin insan akciğer dokusundaki dağılımının incelenmesi amaçlanmıştır. **Gereç ve Yöntemler:** Bu çalışmada, Dicle Üniversitesi Hastanesi Göğüs Cerrahisi Bölümü'nde farklı sebeplerle opere edilen erişkin hastalardan elde edilen akciğer biyopsi örnekleri değerlendirildi. Doku örnekleri -196ºC'de sıvı nitrojen içerisinde hızlı bir şekilde dondurulduu. Doku örneklerine indirekt immünperoksidaz tekniğiyle immün boyama yapıldı. Primer antikorlar için MCC, TARC ve CD104 kullanıldı. Sekonder antikorlar için 1:200 PBS/BSA'da ve 1:100 normal insan serumunda dilüe edilmiş tavşan anti-fare IgG peroksidaz solüsyonu kullanıldı. Negatif kontrol boyama primer antikor basamağı atlanarak fare monoklonal antikorları kullanılarak uygulandı. Akciğer doku kesitleri incelendi ve Olympus BH2 ışık mikroskopu kullanılarak fotoğrafları çekildi. **Bulgular:** İnsan akciğer dokusundaki mast hücrelerini göstermek amacıyla kullanılan MCC'ın damar endotel hücreleri ve vasküler düz kas hücreleri ile orta derecede reaksiyon gösterdiğini; folliküler dendritik hücreleri eksprese etmek amacıyla kullandığımız TARC'in folliküler dendritik hücreler, bronş düz kas hücreleri ve vasküler endotelyum ile orta derecede reaksiyon gösterdiğini ve integrin β4 altgrubu olarak epitelin bazal membrana adezyonunda önemli rolü olan CD 104'ün ise vasküler endotel hücreleri ile orta dereceli ve esas olarak bronş bağımlı lenfoid dokunun folliküler dendritik hücreleri ile reaksiyon gösterdiğini gözlemledik. **Sonuç:** Çalışmamızda, bu moleküllerin ekspresyonlarının dokunun işlevini anlamamız için önemli katkıları bulunduğu belirlenmiştir. Normal akciğer dokusu ile patolojik akciğer dokusu arasındaki farkı ortaya koymak amacıyla immünohistokimyasal olarak ilgili proteinlerin ekspresyonu, interstisyel akciğer hastalığı ve kronik obstrüktif akciğer hastalığı gibi hastalıkların ekarte edilmesi yönündeki çalışmamızın ön bulgu olabileceği kanaatine varıldı. Bu ön bulgu niteliğindeki bazı gözlemlerimizin ileride yapılacak çalışmalara ışık tutacağı düşünüldü.

Anahtar Kelimeler: CMA1 proteini, insan; CMA1 proteini,insan; Integrin beta4

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The notion of lung as an immunological organ has developed in the past decade and it has been described in several reviews. The immunocompetent cells in the lung can be located in different compartments: the vascular pool, the interstitial pool, the bronchoalveolar space and the organized lymphoid tissue associated with the bronchi, the so-called bronchus-associated lymphoid tissue.[1]

The bronchus-associated lymphoid tissue constitutes organized lymphoid aggregates that are capable of both T- and B-cell responses to inhaled antigens. Bronchus-associated lymphoid tissue , located mostly at bifurcations of the bronchi in man, is present in the fetus and develops rapidly following birth, especially in the presence of antigens. Humoral immune responses elicited by bronchus-associated lymphoid tissue are primarily immunoglobulin A secretion both locally and by bronchus-associated lymphoid tissue-derived B cells that have trafficked to distant mucosal sites similarly located T-cell responses have been noted.[2]

The respiratory tract is protected by various immune mechanisms. These comprise a combination of local mucosal immunity, characteristic of the upper respiratory tract, and peripheral-type immune reactions which characterise the lower portion. The initiation of inflammatory events may occur in the lumen or in the tissues and to some extent the response is greatly modulated by the types of cells involved and their secretory products.[3]

The lung is furnished with a rich supply of lymphatics and lymphoid tissue. Lymphatic channels lay along bronchovascular structures and pulmonary veins, as well as in septa and pleura. Lymphatics do not extend into alveolar walls.[4]

Mast cells (MCs) are the key effector cells of the allergic response and when stimulated by specific allergen through the high-affinity IgE receptors or through other stimuli, they release a number of potent mediators of inflammation.[5] Among these are the serine proteases tryptase and chymase. In man, tryptase is the most abundant mediator stored in mast cells. Mast cell chymase (MCC) is present in more moderate amounts in a subpopulation of mast cells.[6]

Airway epithelial cells actively participate in the inflammatory response in asthma by secreting cytokines, reactive oxygen metabolites, and other mediators that regulate infiltrating inflammatory cells like lymphocytes, eosinophils, and MCs. MCs are dispersed widely throughout the body reaching largest numbers on mucosal surfaces. As primary effector cells in immediate-type hypersensitivity reactions, MCs play a critical role in anaphylaxis, allergic rhinitis and asthma.

In the lung, a constitutive baseline MC subpopulation resides in the perivascular connective tissue in the bronchial submucosa, whereas an immunohistochemically and functionally distinctive reactive MC subpopulation develops at mucosal surfaces in response to inflammation.[7-10]

Bronchial epithelial cells are major cell sources to produce thymus and activation-regulated chemokine (TARC) protein in allergic regions.[11] T cells that produce helper (Th)2-type immunoregulatory cytokines play an important role in orchestrating immune and inflammatory processes. The Th2 cell-attracting chemokine TARC is a ligand for the chemokine receptor CCR4. A number of cellular sources of TARC have been identified, including macrophages, dentritic cells, natural killer cells and bronchial epithelial cells. Recent studies report that TARC is a key chemokine in the migration of Th2 cells to inflammation sites related to allergic disorders. More recently, the bronchial epithelium of asthmatic patients was shown to express TARC protein, and CCR4+ CD4+ T cells were also found on bronchial epithelium.[12]

T cells that produce T helper (Th) 2-type immunoregulatory cytokines lead to immunoglobulin (Ig) E production, allergic inflammation and accompanying tissue injury. The number of T cells producing the Th2 cytokines interleukin (IL)-4 and IL-13 are increased in the respiratory mucosa of patients with allergic asthma , and are further increased after allergen challenge.[13,14] Th1 and Th2 cytokine-producing CD4 T cells express different chemokine receptors and this enables their selective recruitment. For example, Th2 cytokine-producing CD4 T cells preferentially express CCR4, the receptor for TARC.[15,16]

Bronchial epithelium lines the mucosal surface of the airways, forming a mechanical barrier that separates the external environment from the internal milieu. It has been long believed that the function of epithelial cells is limited to protecting against invading microorganisms and removing particulate matters by means of the mucociliary stairway. Recently, however, substantial evidence indicates that airway epithelial cells are able to liberate a number of chemokines fundamental to both inflammatory and immune responses has emerged.[17,18] Thus, through a paracrine mechanism, chemokines secreted by bronchial epithelial cells may be involved in the initiation and progression of inflammation of various types. Both IL-8 and monocyte chemoattractant protein (MCP)-1 liberated by airway epithelial cells have been implicated in the initiation and prolongation of acute airway inflammation. Epithelial cells are also considered to participate in amplifying respiratory allergic disorders.[17] Indeed, epithelial cells of patients with allergic respiratory diseases are more likely to be activated than those of normal individuals in terms of their liberation of inflammatory mediators. Recently, the pathogenic role of airway epithelial cells in airway allergic inflammations has become more clear since these cells have been identified as a potent source of eosinophil-specific chemokines such as eotaxin, and MCP-4.[17,19,20] Given the potential importance of bronchial epithelial cells in the pathogenesis of respiratory allergic diseases, it has been reported that bronchial epithelial cells represent an important cellular source of TARC, which is potentially responsible for the recruitment of Th2 cells in allergic airway disorders.[17,21]

Allergic airway inflammation is characterized by a local increase in cells secreting Th-2 type cytokines. Regulated TARC expression has recently been studied in the normal human bronchial epithelial cells and a human xenograft model. It was reported that TARC expression increased in normal human bronchial epithelial cells in response to tumor necrosis factor-alpha stimulation.[13,22]

Integrins are cell surface receptors mediating interactions with extracellular matrix (ECM) proteins.[23] They are transmembrane glycoproteins consisting of ab-heterodimers. There are 8 b- and 16 a- subunits known. Within a b-subfamily, the b-subunit can associate with various a-subunits. Each heterodimer selectively binds its own ligand, with many integrins binding more than one. Individual cells may vary for adhesive properties of their integrins. Thus each cell's response to a particular form of an extracellular matrix molecule will depend on the number and subunit composition of integrins expressed on its surface and their binding properties.[24-27]

Little information is available about the integrin expression in the developing lung.[26,28] The distribution of integrins has been studied in kidney and mammary glands where development is associated with tubular branched morphogenesis and more recently in fetal and adult human lungs. The researchers suggest that b1-integrins mediate a critical role for human epithelial cell-matrix interactions during tubular morphogenesis of human airways.[29,30]

The present study was undertaken to study the expression of MCC, TARC and CD104 in adult human lung tissues to determine the histology of the smooth muscle cells in order to bronchi and alveolar structures, epithelial cells, connective tissue structures, macrophages and lymphocytes.

MATERIAL AND METHODS

This study was based on the ethical principles of Helsinki Declaration, and Ethical Committee of Dicle University approved the experimental protocol. Additionally all patients signed their informed concents. Tissue samples from adult human lung (n= 15) were obtained from the clinically uninvolved parts of lungs removed for various reasons at operations performed in Department of Lung Surgery, and samples were stained immunohistochemically in Department of Histology and Embryology in University of Dicle, Faculty of Medicine, Diyarbakır, Turkey. The tissue samples were immediately frozen in liquid nitrogen at -196º C and were kept in a -30º C deep freeze until use. Using a cryostat, seven micrometer thick serial sections were cut and these sections were mounted on

gelatin covered microscopic slides. After drying at room temperature, the samples were kept in humidity-free containers with silica gel (Merck, 1.01925) until immunostaining has been performed. An indirect immune peroxidase technique, as previously described by Dijkstra et al. was used.[31] As primary antibodies, mast cell chymase mouse monoclonal antibody (NCL-MCC, Novocastra Laboratories Ltd., United Kingdom), thymus and activation-regulated chemokine mouse monoclonal antibody (NCL-TARC, Novocastra Laboratories Ltd., United Kingdom) and CD104 (β4) mouse monoclonal antibody. (NCL-CD104-511, Novocastra Laboratories Ltd., United Kingdom) were used. As secondary antibodies, 1:200 rabbit anti-mouse IgG peroxidase (Sigma, cat. No: A -9044, Saint Louis, USA) diluted in PBS/BSA, and 1:100 normal human serum solution were used. Control staining was performed using irrelevant mouse monoclonal antibodies, omitting the primary antibody step. Sections were examined and photographed by Olympus BH2 light microscope.

RESULTS

The findings of the adult lung tissue samples are summarized as follows:

(-) Controls: In the (-) control group which was immunostained by omission of primary antibody and no administration of endogen peroxidase blockade, there was no specific staining. Only some granulocytes reacted strongly with DAB because of endogen peroxidase activity (Figure 1).

MCC reactivity: The aim of immunostaining with MCC monoclonal antibody was to show overall presence of human lung mast cells. In the lung tissue sections which was immunostained with MCC antibody, the mast cells reacted with MCC but we did not observe any reaction in the bronchial epithelium (Figure 2).

TARC reactivity: We aimed to express the follicular dendritic cells in the area of bronchus- associated lymphoid tissue and the immunoreactivity of the vascular endothelium by immunostaining the lung tissue sections with TARC monoclonal antibody . We detected that follicular dendritic cells,

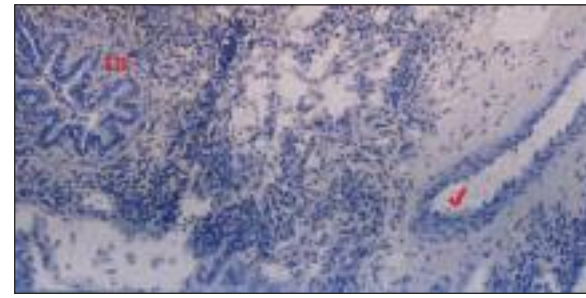

FIGURE 1: Adult human lung tissue of a control subject immunostained with the omission of the primary monoclonal antibody stage. **(TB)** Terminal bronchiolus, **(Arrow)** vascular endothelium and the other structures show normal histology. Indirect immunoperoxidase (IP) - hematoxylin counter stain (HCS) (Original magnification X16).

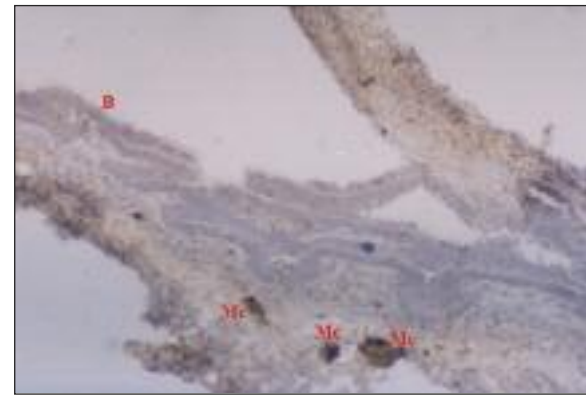

FIGURE 2: Adult human lung tissue stained with MCC. No reaction is seen in the **(B)** Bronchial epithelium. Note that **(Mc)** Chymase-containing mast cells are clear with MCC reaction. Indirect immunoperoxidase (IP)- hematoxylin counter stain (HCS) (Original magnification X41).

smooth muscle cells of bronchi and vascular endothelial cells reacted with TARC monoclonal antibody. The hyaline cartilage in the wall and the epithelium of bronchus did not react with this monoclonal antibody (Figure 3a).

In different human lung tissue sections immunostained with TARC monoclonal antibody, we detected this antibody in the vascular endothelial cells. In the large magnifications, we saw that the blood cells in the vascular lumen slightly nonspecifically immunostained, and we concluded that this reaction was due to the secondary antibody. However, some of them were reacted moderately. Some lymphocytes which invaded the vascular wall also reacted with TARC antibody (Figure 3b).

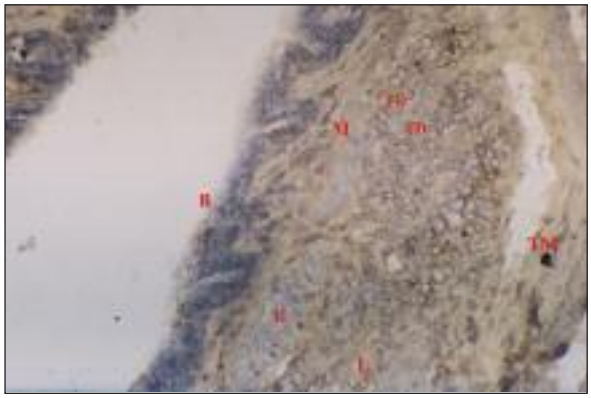

FIGURE 3A: Adult human lung tissue stained with TARC. Note that **(B)** bronchial epithelium is normal, no reaction is seen. The **(fd)** follicular dentritic cells show reaction, **(H)** hyaline cartilage is intact, (M) smooth muscle cells, **(PV)** pulmoner venule reacted with TARC. Indirect immunoperoxidase (IP) - hematoxylin counter stain (HCS) (Original magnification X82).

FIGURE 3B: Adult human lung tissue stained with TARC. Note that **(Arrowhead)** vascular endothelium and **(arrows)** lymphocytes expressed TARC antibody. Indirect immunoperoxidase (IP) - hematoxylin counter stain (HCS) (Original magnification X164).

CD104 reactivity: The monoclonal antibody of CD104 is the integrin β4 subunit that plays an important role in the adhesion of epithelium to basement membranes via interactions with laminin anchoring filaments. It is demonstrated on the hemidesmosomes of stratified epithelium and Schwann cells. When we inspected the human lung sections immunostained with CD104 monoclonal antibody, there was moderate reaction in the endothelial cells; therefore the reaction was slight in the vascular and bronchial smooth muscle cells. There was no reaction in the bronchial basement membrane and therefore no reaction in the bronchial epithelium (Figure 4a).

When we observed the areas of bronchus-associated lymphoid tissue in different human lung tissue sections which were immunostained with CD104 monoclonal antibody, moderate reaction of follicular dendritic cells seemed to be an interesting finding. In addition to this result, vascular endothelial cells slightly reacted but there was no reaction in the alveolar macrophages (Figure 4b).

DISCUSSION

Mast cells have been implicated in the expression of a wide variety of biological responses, including immediate hypersensitivity reactions, immunologi-

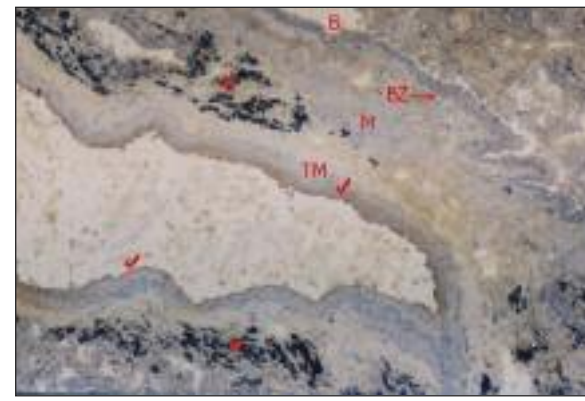

FIGURE 4A: Adult human lung tissue stained with CD104. Moderate reaction of the **(arrow)** vascular endothelium is seen, **(B)** bronchial epithelium and **(BZ)** bazal membrane did not stain Note that normal appearance, **(star)** alveolar macrophage cells, poor reaction is seen in the vascular **(TM)** tunica media. Indirect immunoperoxidase (IP) - hematoxylin counter stain (HCS) (Original magnification X41).

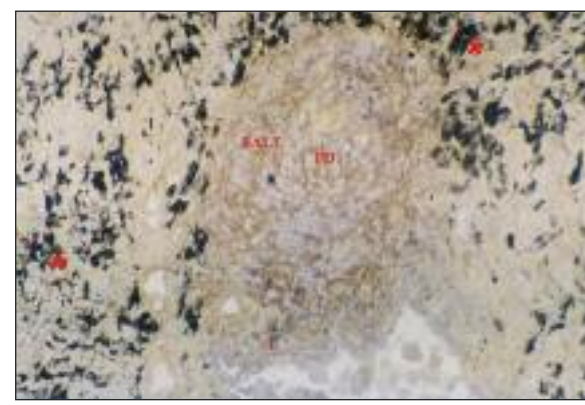

FIGURE 4B: Adult human lung tissue stained with CD104. No reaction in seen in the **(star)** alveolar macrophage cells, moderate reaction in the **(fd)** Follicular dentritic cells when observing the **(BALT)** bronchus associated lymphoid tissue Poor reaction is seen in **(Ve)** vascular endothelial cells. Indirect immunoperoxidase (IP) - hematoxylin counter stain (HCS) (Original magnification X82).

cally non-specific inflammatory and fibrotic conditions, angiogenesis and tissue remodeling and wound healing.[32] They may contain both tryptase and chymase in their secretory granules (MC_{TC} cells) or tryptase but not chymase (MC_T cells).[33] Two main types of human MC have been described based on the types of neutral serine proteases in their granules: MC which contain tryptase only (MC_T cells) and MC_S which contain both tryptase and chymase (MC_{TC} cells).[34] MC_{TC} cells are essentially the exclusive type of mast cell in normal skin but account for the minority of mast cells in normal lung.[35] We attempted to express the mast cells in the lung tissue sections by MCC. As a result of our immunostaining, we observed that the mast cells which belong to human lung reacted moderately. However, bronchial epithelium, alveolar machrophages and lymphocytes of connective tissue had no reaction whereas fibroblasts reacted slightly.The findings which had proposed before by Irani et al and Kitamura were similar to the results of our study in that the human lung tissue sections stained with MCC, moderately reacted due to the protease contained by the mast cells.[36,37]

Chymase has been reported to be the most efficient and specific angiotensin II forming enzyme in various human tissues, including skin, lung, heart, and blood vessels. In human vessels, it is reported that approximately 70% of total angiotensin II formation was due to chymase, whereas only 30% was due to angiotensin converting enzyme (ACE). Angiotensin II, formed by the action of ACE and chymase, has been shown to be an important mediator of vascular smooth muscle cell growth, and thus to affect vessel wall remodeling.[32,38,39] In recent studies it has been shown that chymase is found in mast cells as well as in endothelial cells and in some other cell types. We also showed moderate degree of immunostaining in the lung slides stained with MCC. This finding was similar to those of Urata et al and Baltau et al.[40,41]

The chemokine TARC is a ligand for the chemokine receptor CCR4 expressed on (Th)2-type CD4 T cells. Allergic airway inflammation is characterized by a local increase in cells secreting Th2-type cytokines.[13] It was reported that keratinocytes and bronchial epithelial cells were major sources to produce TARC protein in allergic regions. Maeda et al. studied the molecular cloning of canine TARC gene and its expression in various tissues.[11] Faffe et al. suggested that human airway smooth muscle cells might orchestrate and perpetuate airway inflammation in asthma.[22] However, there were no reports on the ability of human airway smooth muscle cells to express TARC.[22] We also studied the distribution of TARC protein in the human lung tissue in our research and we detected that bronchus-associted lymphoid tissue had slight expression in the lymphocytes, however alveolar macrophages located at the periphery of bronchus-associated lymphoid tissue and bronchial epithelium did not express this molecule. However, Sekiya et al. demonstrated that bronchial epithelium was an important source of a Th2- spesific chemokin, TARC. Because regulation of TARC generation has therapeutic potential for the treatment of allergic airway disorders, they investigated transcriptional regulation of the TARC promoter. Contrary to our findings, they observed strong immunoreactivity in bronchial epithelial cells in asthmatic patients. However in our study we demonstrated negative immunoreactivity in bronchial epithelium in normal human lung tissue.[17] On the other hand, we also observed that the vascular endothelial cells of lung tissue which was immunostained with TARC molecule reacted slightly and some blood cells inside the lumen of the vessel reacted moderately. We detected the presence of poor reaction in some lymphocytes which invaded the wall of the vessel.

Integrins are transmembrane glycoproteins that consist of α-β heterodimers.[26] Integrin expression has been identified by a variety of methods in essentially all cell types.[42] In addition, it has been repeatedly shown that integrin repertories differ in nonneoplastic cells of substantially different phenotypes such as epithelium, myoepithelium, or endothelium, and may differ even within the same phenotype, possibly reflecting functional specialization.[43,44] Damjanovich et al., suggested that the integrin subunits that bind to collagen and laminin and the alpha subunit, which can pair with beta1, beta3,

or 5 and promote fibronectin, fibrinogen, or vitronectin binding, were the predominant integrins expressed on the major cell types of the lung, i.e., bronchial epithelium, vascular endothelium, and smooth muscle.[45] In our study we also determined reaction in the vascular endothelial cells similar to the results of the aforementioned researchers. Although we observed slight reaction in the vascular and bronchial smooth muscles cells, there was no reaction in the bronchial epithelium.

In man, bronchus-associated lymphoid tissue differs in many aspects from typical gut associated lymphoid tissue, i.e. Peyer's patches: it is not present before birth and there is a clear link between the amount of bronchus-associated lymphoid tissue and antigenic stimuli, although causative agents have not been yet characterised. Other compartments of the lung also contain lymphocytes in large numbers and obviously are all connected to each other by migrating lymphocytes and partly dendritic cells. More information is required to define the role of dendritic cells and bronchus-associated lymphoid tissue in antigenic uptake and processing in the human lung. It is for example unclear whether there is a site specificity for a certain type of antigen, and whether bronchus-associated lymphoid tissue can compensate for a small number of dendritic cells.[46] In our study we observed expression of CD 104 by the dendritic cells in the area of bronchus-associated lymphoid tissue. In our view, these findings are consistent with the results of the previous recent studies.[47,48]

The distribution of fibronectin and laminin in normal pleura was demonstrated by Barth et al.[23]

In our study we also detected excessive expression of CD 104 in the pleura. This finding is similar to the results of Barth et al.

Expression of MCC, TARC and CD104 has been studied in pathologic human lung recently but the data in normal human lung tissue is lacking. In this respect, our findings will be valuable as preliminary data for future studies.

The studies in the future may perform a quantitative analysis on human lung tissue using MCC, TARC and CD 104 antibodies and present the difference between the normal human lung, and pathologic human lung such as comparing tissue of patients with asthma, interstitial lung disease and chronic obstructive pulmonary disease etc.

In our study, the human lung tissue was studied to present its detailed structure by using various monoclonal antibodies. The presence of vascular endothelial cells, bronchus and vascular smooth muscles, bronchial epithelium, pleura, mast cells and lymphocytes in the human lung were demonstrated by monoclonal antibodies. It has been emphasized that the expressions of these molecules have an important role for understanding the function of the tissues. Our preliminary findings may shed light of future studies.

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