Expression and potential functions of dipeptidyl peptidase 4 (CD26) in the asthmatic rat lung

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Zusammenfassung

Die Dipeptidyl Peptidase 4 (DP4, CD26) ist eine Serin-Protease, die auf einer Vielzahl von Zelltypen membranassoziiert als Dimer exprimiert wird und als lösliches Molekül vorkommt. DP4 und funktionshomologe Peptidasen wie DP8 und DP9 spalten N-terminal Dipeptide von Proteinen mit L-Prolin an vorletzter Stelle. Diese Spaltung, beispielsweise von inflammatorischen Mediatoren, führt zur Regulation verschiedener physiologischer Prozesse. Zusätzlich spielt DP4 während der Aktivierung von T-Zellen als kostimulatorisches Molekül, sowie bei Adhäsionsprozessen aufgrund seiner Bindungsstellen für Komponenten der extrazellulären Matrix eine potentielle immunregulatorische Rolle, jedoch sind eine Reihe funktionell-morphologischer Aspekte dabei unbekannt. In der vorliegenden Arbeit wurde daher an Wildtyp- und DP4-defizienten Ratten unter naiven und unter allergischen Bedingungen die Expression und Funktion von DP4 hinsichtlich folgender Fragestellungen untersucht: (1) Gibt es eine kompartiment-spezifische Verteilung von DP4 und von DP4-homologen Peptidasen in der Lunge? (2) Wird die Expression von DP4 und von DP4-homologen Peptidasen in der Lunge bei einer allergischen Entzündung reguliert? (3) Wie beeinflusst DP4 die Verteilung von T-Zellen in der Lunge nach der Induktion einer allergischen Entzündung? (4) Welche Rolle spielt DP4 bei der Adhäsion von T-Zellen in der gesunden und entzündeten Lunge?

Die Ergebnisse zeigen, dass es 1) kompartiment-spepzifische Expressionsmuster von DP4 und den DP4-homologen Peptidasen DP8, DP9 und DP10 in der Lunge gibt, dass es 2) nach der Induktion einer asthmoiden Entzündung zu einer gesteigerten Expression und Enzymaktivität von DP4 und seiner Funktionshomologa DP8 und DP9 in der Lunge kommt, dass dabei 3) peribronchial mehr T-Zellen in den Lungen der Wildtyp-Ratten nach Asthmainduktion gefunden werden und dass dies mit einer gesteigerten Produktion eines DP4-Substrats in diesem Kompartiment korreliert, und dass 4) eine DP4-vermittelte Adhäsion von T-Zellen in der Lunge durch einen Kreuztransfer von DP4-positiven und DP4-negativen T-Zellen in Wildtyp- und DP4-defiziente Ratten nicht belegt werden konnte. Weiterhin legen diese Ergebnisse nahe, dass die Funktionen der Dipeptidyl Peptidase 4 nicht isoliert von den Funktionen der DP4-homologen Peptidasen betrachtet werden sollten und dass bei Untersuchungen zur Verteilung verschiedener Zelltypen in der Lunge eine genauere Betrachtung aller Kompartimente sinnvoll ist.

Schlagwörter: Dipeptidyl Peptidase 4 – Asthma bronchiale – T-Zellen

Abstract

The dipeptidyl peptidase 4 (DP4, CD26) is a serine protease, which is expressed membrane-associated as a dimer on a variety of cells but also exists in a soluble form. DP4 and functional homologues such as DP8 and DP9 N-terminally cleave dipeptides from proteins with L-proline at the penultimate position. This N-terminal truncation, for example of inflammatory mediators, leads to a regulation of various physiological processes. In addition, DP4 plays a potential immunoregulatory role in T-cell activation as a costimulatory molecule and in adhesion processes due to its binding capacity for extracellular matrix components. So far functional-morphological aspects have not been examined in depth. Therefore, in this thesis, the expression and functions of DP4 were studied in naïve wild-type and DP4-deficient rats and in an animal model of an asthma-like inflammation with regard to the following questions: (1) Is there a compartment-specific distribution of DP4 and its homologues in the lungs? (2) Is the expression of DP4 and its homologues in the lungs regulated in an allergic inflammation? (3) How does DP4 influence the distribution of T cells in the lungs after the induction of an allergic inflammation? (4) Which role does DP4 play in the adhesion of T cells in healthy and inflamed lungs?

The results show 1) distinct compartment-specific expression patterns of DP4 and its homologues DP8, DP9, and DP10 in the lungs, 2) upregulation of expression and enzymatic activity of DP4 and its functional homologues DP8, and DP9 after induction of an asthma-like inflammation in the lungs, 3) selective recruitment of T cells at peribronchial sites in wild-type rats after the induction of asthma, which correlates with an elevated production of a DP4-substrate in this compartment, and 4) lack of a significant role for DP4 in T-cell adhesion in the lungs after cross-transfer of DP4-positive and DP4-negative T cells into wild-type and DP4-deficient rats. More generally, these findings also suggest that the functions of dipeptidyl peptidase 4 are closely related to those of homologous peptidases and that studies examining the distribution of different cell types in the lungs should include a detailed examination of all compartments of the lungs.

Keywords: dipeptidyl peptidase 4 – bronchial asthma – T cells

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List of abbreviations

aa	amino acids
ADA	adenosin deaminase
APC	antigen-presenting cell
Asp	aspartic acid
BAL	bronchoalveolar lavage
BALT	bronchus-associated lymphoid tissue
CARMA-1	caspase recruitment domain-containing membrane-associated guanylate kinase protein-1
CD	cluster of differentiation
CFSE	carboxyfluorescein diacetate succinimidyl ester
DASH	DP4 activity and/or structure homologues
DP	dipeptidyl peptidase
DPL	dipeptidyl peptidase like protein
ECM	extracellular matrix
ER	endoplasmic reticulum
F344	Fischer 344
FAP	fibroblast activation protein
GIP	glucose-dependent insulinotropic polypeptide
GLP	glucagon like peptide
Gly	glycine
HEV	high endothelial venule
His	histidine
HIV	human immunodeficiency virus
Ig	immunoglobulin

kDa	kilo Dalton
MCP-1	monocyte chemoattractant protein
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B cells
OVA	ovalbumin
$p75^{NTR}$	pan-neurotrophin receptor
qrtPCR	quantitative real-time polymerase chain reaction
SDF-1	stromal cell derived factor-1
Ser	serine
Trp	tryptophan
Tyr	tyrosine
X/Xaa	any amino acid

1 Introduction

1.1 Dipeptidyl peptidase 4

The dipeptidyl peptidase 4 (DP4, DPPIV, EC 3.4.14.5) is a multifunctional serine protease, which is equivalent to the cell surface glycoprotein CD26. It was first reported in 1966 as glycyl-prolyl-β-naphthylamidase [1] and belongs to the clan SC, family S9, according to the structure-based classification of peptides from Neil D. Rawlings and Alan J. Barrett [2]. DP4 exists in a membrane-bound and in a soluble form and is capable to regulate various physiological processes by N-terminal cleavage of dipeptides from peptides with L-proline or -alanine at the penultimate position [3]. It has a widespread distribution in tissues and is expressed on a variety of different cell types, for example on T cells, B cells, and natural killer cells, and also on endothelial and epithelial cells. In addition to its peptidase activity, DP4 is reported to be involved in T-cell activation as a costimulatory molecule [4], and in cell adhesion by binding of extracellular matrix (ECM) molecules [5, 6]. Moreover, DP4 is the archetypal member of the DP4 gene family [7] that consists of several DP4 activity and/or structure homologues (DASH).

1.1.1 Structure of DP4

The complete cDNA of human DP4 was published in 1992 [8] and encodes a protein of 766 amino acids (aa). Rat DP4 codes for 767 aa that exhibit about 85% identity to the human DP4 [9]. It can be divided in five different regions (Figure 1): a cytoplasmic region, a transmembrane region, a glycosylated region, a cysteine-rich region, and a catalytic region.

The N-terminus of DP4 contains six amino acids that form the hydrophilic cytoplasmic domain, which is followed by 22 aa of the hydrophobic transmembrane domain [10] that classifies it as a type II integral membrane protein. The remaining 739 aa form the extracellular part of DP4 with five of eight potential glycosylation-sites in the N-glycan-rich region, ten of twelve cysteine-residues in the cysteine-rich region, and the C-terminal catalytic region with the catalytic triad. The catalytic triad is composed of serine (Ser^{631}), aspartic acid (Asp^{709}), and histidine (His^{741}). The active site Ser^{631} of rat DP4 is located in the sequence Gly-Trp-Ser-Tyr-Gly, which corresponds to the motif Gly-X-Ser-X-Gly,

common to serine proteases. It is grouped into clan SC because of its secondary structure with an α/β-hydrolase fold that consists of a core α/β sheet of eight β strands connected by α helices and loops containing the three catalytic residues. A C-terminal catalytic domain is typical for all peptidases in the family S9. This catalytic triad is covered by the β-propeller, which controls substrate access to the active site [11]. The cysteine residues in the cysteine-rich region are highly conserved between members of the subfamily S9B. This region is potentially involved in the interaction of cells with the ECM, due to binding sites for collagen [5] and fibronectin [6]. In humans, this region also contains a binding site for adenosine deaminase (ADA), however, this is not the case for rat DP4 [12]. The biological role of DP4 N-glycosylation is still unclear, although it was suggested to be involved in protein trafficking and correct protein folding [13].

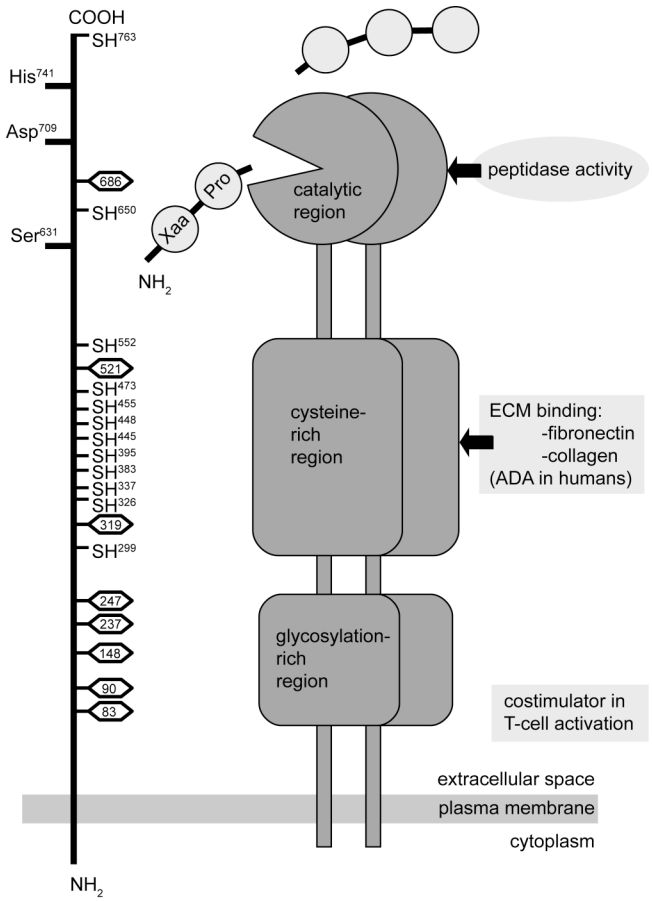

Figure 1: Schematic overview of the structure of rat dipeptidyl peptidase 4 and labelling of its functional properties.

1.1.2 Distribution of DP4

The membrane-bound form of DP4 is expressed in many organs such as kidney, lung, adrenal gland, intestine, liver, spleen, and testis, with the highest activity in the kidney [14], followed by the lung [15]. In these organs, it is constitutively expressed on a variety of tissues and cell types, such as endothelial and epithelial cells [16]. DP4 is also expressed on a variety of immune cells, such as B cells and T cells, where it is upregulated upon activation [17], as well as on natural killer cells [18], and on subsets of macrophages [19]. In addition, DP4 expression was found on a restricted subpopulation of dendritic cells in lymph nodes draining the skin and intestine [20].

Each monomer of the membrane-bound dimer-form has a molecular weight of about 110 kDa, but a 105 kDa soluble form of DP4 also exists [21], which lacks the intracellular part and the transmembrane region. The soluble form is found in body fluids [22] such as seminal fluid, plasma, and cerebrospinal fluid. The origin of soluble DP4 is still unknown, but it is thought to be shed from the cell surface by proteolytic processes.

1.1.3 Peptidase activity of DP4

The presence of proline near the N-terminus of polypeptides serves as a structural protection against non-specific proteolytic degradation. Only few peptidases exist that specifically or selectively attack proline bonds, one of them is DP4. Dimerization of DP4 is a prerequisite for its enzymatic activity [23]. In addition to homodimers, heterodimers of DP4 together with the fibroblast activation protein alpha (FAPα) [24] exist. After dimerization, DP4 is capable of cleaving a post-proline or post-alanine bond, with proline or alanine at the penultimate position. Naturally occurring substrates of DP4 are chemokines such as eotaxin (CCL11) and SDF-1 (stromal cell derived factor-1, CXCL12), neuropeptides such as neuropeptide Y and substance P, and hormones such as glucose-dependent insulinotropic polypeptide (GIP) and glucagon like peptide (GLP). By the cleavage of proline bonds, DP4 modulates receptor specificities and/or inactivates peptides, and therefore has the potential to regulate various physiological processes (see [15] for overview). For example, cleavage of eotaxin leads to its inactivation and therefore a lower attraction of eosinophils [25], and cleavage of GLP is involved in glucose metabolism and therefore important in type 2 diabetes [26].

1.1.4 Involvement of DP4 in T-cell activation

DP4 was originally described as a T-cell differentiation antigen [27], expressed on $CD4^+CD45RO^+$ memory T cells, and upregulated upon activation. Expression of DP4 responds to recall antigens [28], activates cytotoxic T cells [29], and also induces B cell immunoglobulin (Ig) G synthesis and immunoglobulin isotype switching [30].

In general, activation of T cells requires at least two signals, 1) stimulation of the T-cell receptor complex by a specific peptide-antigen or antibody, and 2) triggering of costimulatory surface molecules. Engagement of CD28 on T cells and CD80 or CD86 on antigen-presenting cells (APCs) provide a costimulatory signal and their interaction leads to T-cell proliferation, differentiation, and cytokine secretion [31]. Crosslinking of DP4 and CD3 also induces T-cell costimulation and enzymatic activity of DP4 is required for this costimulatory role [32]. Caveolin-1 is identified as a functional receptor for DP4 in APCs [33]. Its binding results in a signalling cascade that leads to the upregulation of CD86 on monocytes [34]. Therefore, the subsequent interaction of CD26 and CD28 on T cells is enhanced to induce antigen-specific T-cell proliferation and activation. Furthermore it was shown that the short cytoplasmic tail of DP4 is responsible for T-cell costimulation, and that the tail of dimeric DP4 is directly bound by CARMA1, resulting in signalling events that lead to NF-κB activation in T cells [35].

Binding of adenosine deaminase (ADA) to DP4 might also be involved in T-cell activation, because a lack of ADA causes an impairment of cellular and humoral immunity in humans (severe immunodeficiency syndrome). However, DP4 does not work as an ADA-binding protein in rats [12].

1.1.5 Adhesion properties of DP4

Because of its binding sites for collagen and fibronectin [5, 6], proteins that contribute to the structural integrity of ECM, DP4 is potentially involved in cell adhesion. Interaction of lung endothelial DP4 and fibronectin in adhesion and metastasis of rat breast cancer cells has been shown *in vitro* and *in vivo* [36]. Several groups have investigated the association between DP4 expression, tumour cell adhesion, and metastasis in different models [37, 38], but the role of DP4 in cell adhesion of non-tumour cells under physiological conditions has not been examined in depth. So far, only *in vitro* experiments have been performed

investigating its function in adhesion of T cells to endothelial cells [39]. These *in vitro* experiments do not confirm adhesion properties of DP4 on T cells and question its role as a classical adhesion receptor.

1.1.6 DP4 activity and/or structure homologues and the DP4 gene family

DP4 is the founding member of a family of homologues, namely the DP4 activity and/or structure homologues (DASH). Members of this family are DP4, FAPα, DP8, DP9, DP2, DP6, and DP10. DP4, FAPα, DP8, and DP9 are functionally homologous and therefore members of the S9b family of serine proteases. DP2 is functionally homologous and a member of the family S28. Family S9b and family S28 are both members of clan SC of serine proteases with similar enzymatic activities (Figure 2). DP6 and DP10 are functionally inactive, but they form the DP4 gene family together with DP4, FAPα, DP8, and DP9 [40]. While DP4 has been studied thoroughly during the last years, details about its homologues remain unknown.

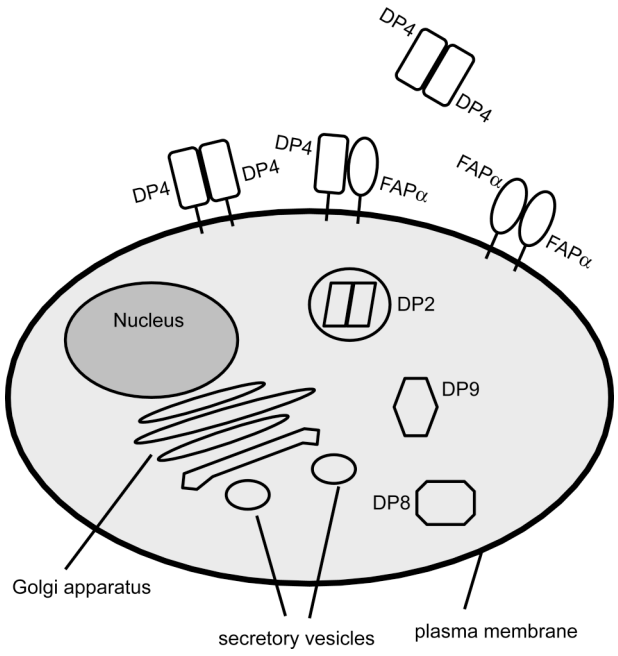

Figure 2: Schematic overview of the cellular localization of dipeptidyl peptidase 4 and its functional homologues.

DP2, alias quiescent cell proline dipeptidase, is a soluble serine protease, which is located in cellular vesicles and catalytically active as a homodimer. It is ubiquitously expressed and its peptidase activity is similar to DP4. In contrast to DP4, it has an acidic pH optimum of 5.5 [41].

FAPα, alias seprase, is active as a homodimer and also as a heterodimer together with DP4 [24]. It has a gelatinase activity and is expressed on reactive stromal fibroblasts of epithelial cancers and healing wounds, but not in normal tissues [42]. Therefore, it plays a suggested role in tissue remodelling and contributes to the invasiveness of certain cancers.

DP8 and DP9 are closely related peptidases and seem to be very similar proteins. Both are described as ubiquitously expressed cytoplasmic molecules [43, 44]. They have previously been reported to be monomeric, but recent data suggest a dimeric structure [45]. Activity of DP8 and DP9 is found in human lymphocytes and monocytes [46], and overexpression studies showed that both proteins enhance induced apoptosis, but they have different functions in cell adhesion and migration [47].

DP6 is also called DPX or dipeptidyl peptidase like protein (DPL) 1 [48] and DP10 is also called DPL2 [49]. Both are members of the DP4 gene family but they lack enzymatic activity, possibly due to the absence of the catalytic serine. They are type II membrane-bound glycoproteins that are predominantly expressed in the brain. DP6 and DP10 are associated with the pore-forming subunits of Kv4-mediated A-type potassium channels and modulate the cellular trafficking, membrane targeting, and functional properties of these channels [50, 51].

1.1.7 Role of DP4 in different diseases and potential use of DP4-inhibitors

DP4 truncates many bioactive peptides of medical importance due to its enzymatic activity. Its additional interactions with different molecules, for example in T-cell activation and in adhesion of cells, suggest an important function of DP4 in the immune system. It is potentially involved in allergic inflammations like bronchial asthma, but also in diseases such as Crohn's disease, chronic liver disorders, osteoporosis, multiple sclerosis, eating disorders, rheumatoid arthritis, cancer, HIV, and diabetes type 2.

In diabetes type 2, the physiological role of DP4 even provides a basis for a therapeutic approach in the treatment. DP4 degrades and thereby inactivates the incretin hormones

GLP-1 and GIP that are involved in glucose homeostasis. GLP-1 amplifies meal-induced insulin release and synthesis in a glucose-dependent manner. It suppresses the secretion of glucagon and is important for basal β-cell function [52]. GIP is synthesised and secreted by endocrine K cells of the proximal intestine in response to nutrients. It stimulates insulin release from pancreatic β-cells in the presence of elevated glucose levels. Inhibition of DP4 enhances the endogenous incretin hormone activity and improves the insulinotropic effects of GLP-1 and GIP. Therefore, the development of DP4-inhibitors has been proposed as being a therapeutic approach for lowering glucose levels in type 2 diabetes [53]. The existence of DASH proteins complicates the use of these inhibitors, because several inhibitors that were thought to be DP4-specific might turn out to be non-specific due to these various DP4 homologous proteins. Except for DP4, the functions of these enzymes are mostly unknown, resulting in unknown consequences after inhibiting them. Therefore, the degree of selectivity of inhibitors for treatment of diseases is very important. In addition, until now, it remains unclear how chronic DP4-inhibition will affect DP4-mediated immunological responses. Although DP4 knockout mice develop normally without obvious defects in immune functions [54], DP4-inhibition in humans might have other effects because of slightly different functions and binding sites, such as the ADA-binding site [12].

1.2 Bronchial asthma

Asthma is one of the most common chronic diseases in the world, with about 300 million people suffering from it [55]. It is an inflammatory disease of the airways and the alveolar areas in response to exogenous and endogenous stimuli that causes wheezing, breathlessness, chest tightness, and coughing [56]. Many cell types participate in the pathogenesis of bronchial asthma, for example T cells, neutrophils, eosinophils, and mast cells. The inflammation causes airflow obstruction, airway hyperresponsiveness, oedema, and hypersecretion (for review see [57]).

The allergic asthmatic reaction and acute inflammation can be divided into two consecutive reactions, namely the early-phase reaction (initiated very rapidly) and the late-phase reaction (6-9 hours after the allergen provocation). In allergic patients, inhaled allergen challenge leads to the activation of cells bearing allergen-specific IgE like

mast cells, proinflammatory mediators are released, and that causes oedema, airway obstruction, and mucus-hypersecretion. In the following late-phase reaction, eosinophils, T cells, basophils, neutrophils, and macrophages are recruited to the airways and alveolar areas and are activated. Subsequent release of proinflammatory mediators and cytokines leads to further recruitment of inflammatory cells into the airways and parenchyma of the lung.

The mortality rate for asthma is relatively low. Current treatment protocols recommend the inhalation of anti-inflammatory corticosteroids, which suppress the inflammation, but the allergic inflammation cannot be cured until now. The pathogenesis of asthma is not completely understood and seems to be caused by an interaction of environmental and genetic factors. One of the potential genes associated with asthma prevalence is the gene of DP10 [58].

1.3 Aims of the present thesis

Previous studies of our group revealed a largely blunted allergic-like responsiveness in DP4-deficient rats, thereby illustrating a critical involvement of DP4 in the lungs in experimental asthma. However, several morphological and functional aspects of this protective-like effects mediated by DP4-deficiency remained unknown. The overall aims of the present thesis were, therefore, to elucidate the distribution, expression, and function of DP4 in the lungs under naïve and asthmatic conditions using different rat substrains that either expressed DP4 on a normal level or were DP4-deficient. The following aims were targeted:

1) Functional-morphological characterization of DP4 expression sites. Therefore, a staining method should be established that complements common immunohistochemical stainings and allows the characterization of the sites of enzymatic activity and expression of DP4, DP8, and DP9 in the lungs. In addition, a potential regulation of these peptidases should be analysed using quantitative real-time PCR and an *in vitro* activity assay. The hypothesis was that different expression patterns of these peptidases might reveal their functional differences in the lungs and a regulated expression might reflect a potential role during an asthma-like inflammation.

2) Characterization of the T-cell distribution in the lungs. The precise anatomical site of a differential increase of T cells after the induction of an asthma-like inflammation in the different rat substrains should be defined following the hypothesis that the recruitment of T cells to a specific compartment in the lungs is triggered by the interaction of DP4 with surrounding structures or by the DP4-dependent turn-over of DP4-substrates directly at these sites. Therefore, different compartments of the lungs were screened using morphometrical methods and quantitative real-time PCR.

3) Characterization of the contribution of DP4 expression to T-cell adhesion *in vivo* in the lungs. Cross-transferred T cells should be monitored in wild-type and DP4-deficient recipients to illustrate a potential role of DP4-mediated adhesion in the naïve and asthmatic lungs. An effect similar to the pro-adhesive effects of DP4 in tumour adhesion was hypothesised under naïve and asthma-like conditions.

2 Regulation of Expression and Function of Dipeptidyl Peptidase 4 (DP4), DP8/9, and DP10 in Allergic Responses of the Lung in Rats

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Abstract

The expression of dipeptidyl peptidase 4 (DP4, CD26) affects T-cell recruitment to lungs in an experimental rat asthma model. Furthermore, the gene of the structural homologous DP10 represents a susceptibility locus for asthma in humans and the functional homologous DP8/9 are expressed in human leukocytes. Thus, although several mechanisms may account for a role of DP4-like peptidases in asthma, detailed information on their anatomical sites of expression and function in lungs is lacking. Therefore, bronchi and lung parenchyma were evaluated using immunohistochemistry and histochemical/enzymatic activity assays, as well as quantitative real-time PCR for this family of peptidases in naïve and asthmatic rat lungs derived from wild-type F344 and DP4-deficient F344 rat strains. Surprisingly, results show not only that the induction of experimental asthma increases DP4 enzymatic activity in the bronchoalveolar lavage fluid and parenchyma, but also that DP8/9 enzymatic activity is regulated and, as well as the expression of DP10, primarily found in the bronchial epithelium of the airways. This is the first report showing a differential and site-specific DP4-like expression and function in the lungs, suggesting a pathophysiologically significant role in asthma.

Keywords: dipeptidyl peptidase 4 - dipeptidyl peptidases 8/9 - dipeptidyl peptidase 10 - asthma - lung - bronchi - F344 rat substrains - DP4-like activity

2.1 Introduction

Asthma is a chronic inflammatory disease of the airways, which is characterized by bronchial hyper-responsiveness and airway obstruction and is accompanied by wheezing, coughing, and breathlessness (Busse and Lemanske 2001). Furthermore, an asthmatic response is characterized by an elevated production of IgE, cytokines and chemokines; mucus hyper-secretion, and eosinophilia. Many of these disease parameters are induced or modulated by T-cell recruitment and activation, cell adhesion, and chemokine metabolism. All of these disease-modulating processes are potentially further modulated by dipeptidyl peptidase 4 (DP4) (EC 3.4.14.5, CD26) expression and enzymatic activity (Boonacker and Van Noorden 2003). This might also be the case for the other members of the DP4-like gene family, including the structural homologous DP10 and the functional homologous DP8/9 (Chen et al. 2003). In addition, DP4 has been reported to be expressed in serosal submucosal glands of the human bronchus (van der Velden et al. 1998) and in human bronchoalveolar lavage (BAL) fluid (van der Velden et al. 1999).

In line with this concept, it was recently demonstrated that lack of DP4/CD26 expression in a deficient rat model remarkably reduces T-cell recruitment to the lungs during experimental asthma (Kruschinski et al. 2005) and that especially DP4-positive, activated T-cells are recruited to the lungs after induction of asthma in F344 rats (Skripuletz et al. 2007). In addition to these preclinical data indicating an important role of DP4 in asthma, large-scale genetic association screenings revealed that the gene of DP10, a structural homolog of DP4 (Qi et al. 2003), is a susceptibility marker of human asthma (Allen et al. 2003). The other functional homologs DP8 (Abbott et al. 2000) and DP9 (Ajami et al. 2004), show cytoplasmatic expression, but there is no direct evidence yet of a functional involvement during asthma except for their expression in human leukocytes (Maes et al. 2007).

For a better understanding of these important indications of the role of DP4-like peptidases in asthma, the present study investigates in detail their sites of expression in rat lungs with and without an allergic-like inflammation status.

2.2 Materials and Methods

Animals

Male wild-type F344/Ztm rats ($DP4^{pos}$) and male DP4 mutant rats [F344/Crl(Wiga)SvH-*Dpp4*^{m}] lacking DP4 activity as well as DP4 expression ($DP4^{neg}$) were used (Karl et al. 2003). All animals were housed at the Central Animal Facility of the Hannover Medical School, maintained in a separate minimal barrier-sustained facility, and microbiologically monitored according to Federation of European Laboratory Animal Science Associations recommendations (Rehbinder et al. 1996). The temperature was regulated at 21 ± 2C and a relative humidity of 60 ± 5% with an air change rate of 15 times per hour, under a 12-hr light 12-hr dark cycle (lights on at 06.00 am). Food and water were available ad libitum. All research and animal care procedures had been approved by the review board of the Landesamt fuer Verbraucherschutz und Lebensmittelsicherheit (Oldenburg, Germany) and were performed according to international guidelines on the use of laboratory animals.

Sensitization and Allergen Challenge

At the age of 12 weeks (260 ± 30 g), five DP4-positive and five DP4-negative rats in the asthma group were sensitized 14 and 7 days before challenge, as previously described (Skripuletz et al. 2007). In brief, sensitization was performed with 1 mg of ovalbumin (OVA; Sigma, Deisenhofen, Germany) and 200 mg of $Al(OH)_3$ (Sigma) in 1 ml 0.9% (sterile, pyrogen-free) NaCl applied subcutaneously in a hind limb. As a second adjuvant, concentrated preparations of 6×10^9 heat-killed Bordetella pertussis bacilli (kindly provided by Chiron Behring, Marburg, Germany) in 0.4 ml 0.9% NaCl were given intraperitoneally at the same time. Animals were challenged with 7.5% of aerosolized OVA using a Pari LC Star nebulizer (Pari, Starnberg, Germany). The control group consisted of five DP4-positive and five DP4-negative naïve rats that were neither sensitized nor challenged.

Dissection of animals

The animals were dissected under isoflurane anesthesia 22 ± 0.5 hr after challenge, as previously described (Skripuletz et al. 2007). Briefly, the animals were sacrificed by aortic

exsanguination, thereby collecting EDTA blood samples. For BAL isolation, a cannula was inserted into the trachea in situ and the lungs were lavaged four times with portions of 5 ml 0.9% NaCl solution. The recovery of fluid was over 90% in all animals. For further analysis, both lungs were excised from the thorax. The trachea, main bronchi, and hilar lymph nodes were removed. For PCR analyses, the inferior lobe of the right lung was frozen in liquid nitrogen. The left lungs were instilled with 3 ml of Tissue-Tek O.C.T. compound (Miles Inc., Elkhart, IN) mixed 1:4 with PBS and placed on aluminum foil on dry ice.

Synthesis of the Histochemical Substrate H-Gly-L-Pro-1-hydroxy-4-naphthylamide Hydrochloride

On the basis of a method described by Dikov et al. (1999), the synthesis of H-Gly-L-Pro-1-hydroxy-4-naphthylamide hydrochloride was modified according to a two-step procedure: First, the precursor of the substrate Boc-Gly-L-Pro-1-hydroxy-4-naphthylamide was synthesized from Boc-Gly-L-Pro-OH (Bachem; Bubendorf, Switzerland) and 1-Hydroxy-4-naphthylamine (Sigma-Aldrich Chemie GmbH; Taufkirchen, Germany) using the mixed-anhydride method composed of isobutyl chloroformate and *N*-methylmorpholine. After the usual workup, the crude product was purified by flash chromatography (gradient elution, $CH_3OH/CHCl_3$: 1/1 → 3/1 → CH_3OH) to give a pure compound with a yield of 88%. In a second step, the precursor compound Boc-Gly-L-Pro-1-hydroxy-4-naphthylamide was dissolved in a solution of hydrochloric acid in 1,4-dioxane (8 M) and stirred for 4 hr at room temperature. The deprotection solution was removed under reduced pressure and the resulting residue was purified by flash chromatography (gradient elution, $CH_3OH/CHCl_3$: 1/1 → 3/1 → CH_3OH) generating the substrate H-Gly-L-Pro-1-hydroxy-4-naphthylamide hydrochloride, with a yield of 41% and a purity of 100%. In addition to the usual HPLC analysis, the identity and purity of the compound was further characterized by proton nuclear magnetic resonance spectroscopy and mass-spectrometry electrospray ionization.

Histochemical Activity Assay

Lungs were cut on a cryotome, and resulting sections (10 μm) were mounted on poly-L-lysine-coated glass slides and fixed in acetone for 10 min at -20C. Sections were washed in

0.1 M phosphate buffer (pH 7.8) and then incubated for either 25 min or 20 hr at 37C in an incubation solution consisting of 0.25 mM H-Gly-L-Pro-1-hydroxy-4-naphthylamide hydrochloride (synthesized at Probiodrug, Halle/Saale, Germany), and 0.25 mM Nitro Blue Tetrazolium (NBT) (Sigma-Aldrich; Steinheim, Germany) dissolved in a minimal volume (less than 0.6%) dimethyl sulfoxide (DMSO) (Merck; Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.8). Control sections were incubated in 0.25 mM NBT dissolved in DMSO in 0.1 M phosphate buffer (pH 7.8). After incubation, the sections were washed in 0.1 M phosphate buffer (pH 7.0), fixed in 4% paraformaldehyde, and stained with methyl green nuclear counterstain (Vector Laboratories; Burlingame, CA). Subsequently, the sections were dehydrated in increasing concentrations of alcohol, cleared in xylene, and covered with Eukitt (O. Kindler GmbH & Co.; Freiburg, Germany). The above histochemical assay is specific for DP4, DP8, DP9, and, to a lesser extent, for DP2, owing to the slightly alkaline pH. However, the activity of DP2 is inhibited by NBT (Dikov et al. 2000). Cleavage of the substrate by these DPs forms a strong reducing agent, which reduces NBT to a diformazan. This diformazan precipitates at the sites of enzymatic activity and is visible as a blue staining. As a further control for specificity and in addition to the use of DP4-deficient animals, this histochemical activity assay was also performed using a DP4-specific inhibitor as previously described (Frerker et al. 2007), which was added to the incubation solution in a final concentration of 2 μM. The sections were incubated for 20 hr in this solution and treated further as described above. As a general rule, during each run of stainings, sections of all groups were incubated in the same cuvette and treated completely identically to ensure the comparability between stainings.

Light microscopy investigations were carried out on a Nikon Eclipse 80i microscope (Nikon GmbH; Duesseldorf, Germany), and representative pictures were taken with a MicroFire digital microscope camera (Optronics; Goleta, CA).

Immunohistochemistry

Two consecutive alkaline phosphatase antialkaline phosphatase (APAAP) stainings (Cordell et al. 1984) were performed on 40 μm acetone-fixed cryostat sections of DP4-positive whole left lungs with Fast Blue (Sigma) as the detection system for labeled T-cells and Fast Red (Sigma) as the detection system for labeled DP4-positive cells. In detail, the sections were incubated with the primary monoclonal antibody (mAb) against the

α/β T-cell receptor (mAb R73; Serotec, Duesseldorf, Germany; 1:5000) for 4 hr at room temperature. After washing with TBS-Tween (0.05% Tween 20; Serva, Heidelberg, Germany), the sections were incubated for 30 min with the bridging antibody (Dako, Hamburg, Germany; 1:50 in PBS, with 5% inactivated rat serum), washed again, and incubated with the APAAP complex (Dako; 1:50 in TBS-Tween) for 30 min. The incubations with the bridging antibody and the APAAP complex were repeated once for 15 min. After the addition of the substrate Fast Blue for 30 min, the incubation with the primary antibody against DP4 (mAb 5E8; Cell Sciences, Canton, MA) was performed overnight at 4C, followed by an identical staining procedure except that Fast Red was the substrate. Finally, the sections were covered with Mowiol (Hoechst AG; Frankfurt/Main, Germany). The DP4-negative lung sections were not stained with an anti-DP4 antibody. As in previous studies, no mAb binding in the knock-out-like model used in this study could be detected (Shingu et al. 2003).

Similarly, rabbit polyclonal antibodies against DP8 (Abcam; Cambridge, United Kingdom, 1:500), DP9 (Abcam; 1:1000), and DP10 (Abcam; 1:250) were incubated on 10 µm lung sections using a mouse anti-rabbit antibody (Dako; 1:50) for 30 min after the 30 min incubation with the primary antibody, and a hemalaun counterstaining (Merck; 1:5 in PBS) for 20 sec after the APAAP staining with Fast Red.

Enzymatic Activity Assay In Vitro

DP4-like enzymatic activity of the different rat strains was determined by incubating EDTA-plasma samples and BAL fluid samples with the substrate H-glycyl-prolyl-4-nitroaniline hydrochloride (H-Gly-Pro-pNA*HCl) (Bachem; Bubendorf, Switzerland) and measuring the release of paranitroaniline (pNA) by an increase in absorbance at 405 nm over time, as described before (Karl et al. 2003), using the PowerWave XS photometer (Bio-Tek Instruments; Bad Friedrichshall, Germany). The assay was composed of 20 µl of the samples in 40 mM HEPES buffer (pH 7.6) and 0.4 mM H-Gly-Pro-pNA*HCl. Prior to the enzymatic reaction, the samples were incubated with the HEPES buffer for 15 min at 37C. The reaction was initiated by adding the substrate, and the release of pNA was measured up to 10 min. One unit is defined as the amount of enzyme necessary to hydrolyze 1 µM of substrate per minute. The assay is selective for DP4-like activities; however, due to the alkaline pH the contribution of DP2 is negligible (Frerker et al. 2007).

Quantitative Real-time PCR

RNA was prepared from shock-frozen lobes of the right lungs, trachea, bronchi, parenchyma, and brain by using the RNeasy Mini Kit (Qiagen; Hilden, Germany) and cDNA was synthesized using M–MLV reverse transcriptase (Invitrogen; Karlsruhe, Germany) following the manufacturer's guidelines.

PCR was carried out on a thermal cycler (Eppendorf; Hamburg, Germany). The 50-µl reaction mixture was composed of 75 ng cDNA, 200 µM deoxynucleotide triphosphate, 0.1 µM primer (each), 1x PCR buffer (minus Mg), 1.5 mM $MgCl_2$, and 2.5 U Taq (Invitrogen). The protocol contained a 5-min initial denaturation step at 95C and 35 cycles of the following steps: 30-sec denaturation at 95C, 1-min primer annealing at a primer-specific temperature (Table 1), and 45 sec extension at 72C. A final extension step for 5 min at 72C was performed. To visualize the PCR products, gel electrophoresis (2% agarose in tris acetate EDTA buffer) was performed, and 15 µl of the final PCR product was applied to the gel. This protocol was used for all primers except DP10. Because of the low amounts of the DP10 PCR product, 90 ng cDNA was used in a 50-µl preparation, 40 cycles were performed, and 30 µl of the final PCR product was applied to the agarose gel.

Quantitative real-time PCR was carried out on an iCycler thermal cycler with the iQ5 real-time PCR detection system (Bio-Rad; Munich, Germany) using a SYBR green detection protocol (Qiagen) with 12.5 ng of each cDNA and a final concentration of 0.6 µM of each primer per preparation. A 15-min initial activation step at 95C was performed, followed by 45 cycles of 15-sec denaturation at 95C, annealing for 30 sec at a primer-specific temperature, and 30-sec extension at 72C. Primers for the detection of DP4, DP8, DP9, DP10, and the housekeeping gene ribosomal protein L13a (RPL13a) were designed based on sequences from the National Center for Biotechnology Information database (RPL13a: NM_173340; DP4: NM_012789; DP8: XM_236345; DP9: XM_217309; DP10: NM_001012205) using the Primer3 application (Rozen and Skaletsky 2000). Details are provided in Table 1. Mean normalized expression was calculated using the Q-Gene application (Simon 2003). The sequences of rat DP8 and rat DP9 have not been published until now. Accordingly, primers were designed using predicted sequences derived from genomic sequences by automated computational analyses. DP4-negative rats carry a mutation that causes a rapid intracellular degradation of DP4 without the mutant protein

being processed to the mature form (Tsuji et al. 1992). The primers used for DP4 detection are located outside of the mutated region of DP4 in the DP4-negative rats, explaining the positive PCR signals in these rats. A similarity between mutant and wild-type DP4 mRNA levels has been described by Thompson et al. (1991), although the protein of DP4 is functionally inactive and retained inside the cell in DP4-negative F344 rats.

Statistical analysis

Differences among groups were analyzed using two-way ANOVA. Treatment (control versus asthma) and genetic background ($DP4^{pos}$ versus $DP4^{neg}$) were the factors, followed by the Fisher protected least-significance test for post hoc comparisons, if appropriate. Statistically significant effects between the asthma group and the control group are indicated by asterisks (*p<0.05), and for comparison of $DP4^{neg}$ and $DP4^{pos}$ groups, by rhombs (###p<0.0001). All data are displayed as mean ± SEM.

2.3 Results

By means of the histochemical activity assay, the localization of sites exhibiting DP4, DP8, and DP9 enzymatic activity in rat lungs under naïve (control) and asthmatic conditions was documented in DP4-negative and DP4-positive rat lungs. Positive blue precipitates in lungs of DP4-negative rats or of those incubated with a DP4-specific inhibitor were interpreted as DP8/9 specific. Thus, the blue staining of lung sections obtained from wild-type Fischer rats represents the sum of the enzymatic activity of the three dipeptidyl peptidases DP4, DP8, and DP9, visible even after 25 min of incubation. No staining was detectable on control sections of DP4-positive lungs incubated for 20 hr in NBT without substrate (not shown).

Incubation periods of 25 min and 20 hr appeared well-suited to analyze the enzymatic activity on DP4-positive and DP4-negative lung sections. Although NBT inhibits the activity of DP2, the resulting staining on the lung sections of DP4-positive rats is caused by the cumulative activity of DP4, DP8, and DP9 (Figures 1A-1C, 1G-1I), and the staining on lung sections of DP4-negative rats (Figures 1D-1F, 1J-1L) is based only on DP8 and DP9 enzymatic activity.

The enzymatic reaction product of cumulative DP4-like peptidases in lung sections was much more pronounced compared with the DP8 and DP9 activity. This was clearly demonstrated by comparing DP4-positive and -negative lung sections after an incubation period of 20 hr (Figures 1G and 1J) and was even visible after 25 min in the lung parenchyma (Figures 1A and 1D). Although there was no remaining staining at all after 25 min on DP4-negative sections (Figure 1D), a slight blue staining was visible on DP4-positive sections (Figure 1A). After 20 hr the DP4-positive sections were intensively blue-colored (Figure 1G), whereas the DP4-negative sections were only faintly blue-stained (Figure 1J).

The comparison of DP4-positive and -negative sections also revealed differential DP4-like activities in different compartments of the lungs. Although the DP4 activity was very pronounced in lung parenchyma (Figure 1G), the activity of DP8 and DP9 was primarily located in the bronchi (Figure 1J) and to a lesser extent in the parenchyma. The bronchi of DP4-positive and -negative sections showed almost no difference after 20 hr (Figures 1H and 1K), which indicated that the activity in the bronchi was mainly derived from DP8 and

DP9. This finding was confirmed by the additional use of a DP4-specific inhibitor (Figures 1M and 1N). Incubation of a DP4-positive section in the presence of this inhibitor (Figure 1M) showed the same staining pattern and kinetics as incubation of a DP4-negative section in the solution (Figure 1N), as did DP4-negative sections in the solution without this inhibitor. Thus, the genetic model and pharmacological approach were cross-validated and strongly suggest specificity of findings.

In addition to the different compartmentalization of DP4 and DP8/9 activity, an upregulation of their activities after induction of asthma was observed (Figures 2A-2E). In addition to the enzymatic activity assay on lung sections, the enzymatic activity of both rat substrains was also measured in vitro. Plasma and BAL fluid samples from DP4-negative rats showed only a very low DP4-like enzymatic activity. The DP4-like activity of the plasma samples did not vary between the naïve control group and the asthma group (data not shown), whereas the DP4-like activity of BAL fluid samples showed a significant asthma-specific treatment effect (p=0.01), in addition to a significant effect of the genetic background (p<0.0001) (Figure 2A). In line with the findings in the BAL, the histochemical determination of DP4 enzymatic activity in the lung parenchyma of F344 wild-type rats of the asthma group (Figure 2C) appeared to be upregulated, compared with the control group (Figure 2B) after an incubation period of 25 min. In addition, the activity of DP8/9 appeared to be upregulated in the bronchi after asthma induction (Figure 2E) compared to the control group (Figure 2D) after an incubation period of 20 hr.

Immunohistochemical staining for DP4-like proteins further confirmed and complemented our findings based on enzymatic assays (Figures 2F-2Q). Staining of DP4-positive lungs from rats of the control group and the asthma group with a monoclonal antibody against DP4 also revealed no antibody binding in the bronchi (Figure 2F) and a more pronounced staining of lungs after asthma induction (Figure 2H) compared with control lungs (Figure 2G), which is indicative of an upregulation of DP4 protein expression and also in accordance with an increase of DP4 enzymatic activity on tissue and in vitro. Immunohistochemical characterization of DP8, DP9, and DP10 protein expression under naïve and asthmatic conditions also provided evidence of an upregulation of these proteins after induction of experimental asthma. In contrast to DP4, these three peptidases appeared to be strongly expressed in the bronchi and in some leukocytes, but were weakly expressed in the parenchyma. This was most noticeable in lungs that were not lavaged (Figures 2I,

2L, 2O), but was also evident in lungs after the BAL procedure (Figures 2J-2K, 2M-2N, 2P-2Q).

To obtain additional information regarding mRNA expression levels of DP4, DP8, DP9, and DP10, PCR and real-time PCR approaches were performed (Figure 3A). Taken together, the real-time PCR data of DP4, DP8, and DP9 revealed treatment effects between naïve and asthmatic lungs, whereas no differences were observed when comparing DP4-positive and DP4-negative substrains (Figures 3B-3D). Specifically, although the expression of DP4 did not differ after asthma induction (Figure 3B), the expressions of DP8 and DP9 (Figures 3C and 3D) were significantly upregulated in the asthma group compared with the control group (p=0.02 for DP8 and p=0.02 for DP9).

Highest mRNA coding for DP10 was found in the brain, followed by trachea and bronchi, but could not be amplified in samples from whole lung and from lung parenchyma (Figure 3E). Apparently, the expressions in tissues that were not lavaged (Lanes 3-5) were higher compared with tissues from rats after a BAL (Lanes 6-8), and this was in line with observations on immunohistochemical stainings with a DP10 antibody. In general, because the BAL procedure removes leukocytes and proteins from airways of the lungs, this reduced mRNA expression pattern and faint immunohistochemical-based protein detection suggest significant leukocyte-associated and soluble components of these DP4-like proteins during asthma.

2.4 Discussion

In this study, a site-specific and disease-associated expression of the dipeptidyl peptidases DP4, DP8, DP9, and DP10 in lungs was documented in an F344 rat model of bronchial asthma.

Regulation of DP4 expression during experimental asthma provides further evidence for its involvement in the regulation of inflammatory processes. Probably, DP4 is not only an inflammatory marker per se but also exhibits distinct functional properties within this process. For example, it has been shown recently that a genetically induced DP4 deficiency is associated with blunted T-cell recruitment to the lungs in asthma (Kruschinski et al. 2005). Therefore, we were interested in whether this effect was primarily due to a T-cell-specific functional role of DP4 or whether DP4 expression in lungs may also contribute to that observation. The findings presented here suggest not only that T-cell-specific DP4-related functions play a role but also that DP4 expression in the lungs might significantly be involved in this process. One potential mechanism could be that DP4 is also involved in cell adhesion; specifically, that DP4 mediates adhesion to extracellular matrix proteins such as collagen and fibronectin (Hanski et al. 1988; Piazza et al. 1989). In the course of an inflammatory process, such an upregulation of DP4 may be beneficial, because DP4-positive leukocytes are more easily recruited to the sites of inflammation. Because this has to be a rapid response, we found that DP4 mRNA expression at 22 hr after challenge was already in the downslope, whereas the corresponding protein expression and DP4 activity were upregulated in histochemical stainings and in an enzymatic activity assay in vitro. To date, however, such a more general role for DP4 via mediation of leukocyte adhesion has not been documented for leukocyte recruitment during inflammatory processes and has recently been attributed only to tumor cell adhesion (Shingu et al. 2003).

The documented upregulation of DP4-like activity in the BAL fluid of DP4-positive rats after asthma induction is contrary to observations by others (van der Velden et al. 1999). Van der Velden and colleagues did not observe any differences in the peptidase activities between allergic asthmatics and healthy nonsmokers. Because the DP4-like activities in the BAL fluid and the lung might at least partially be released by activated CD4-positive T-cells (Juillerat-Jeanneret et al. 1997), they explained their observations with an unaltered T-cell number in the allergic asthmatics (van der Velden et al. 2000). Corresponding to the above-documented upregulated DP4-like activities in the lung and BAL fluid of rats after

asthma induction, our previous studies showed an elevated number of activated T-cells after asthma induction in this rat model (Skripuletz et al. 2007).

The site-specific activity and expression of DP4 and its related peptidases DP8, DP9, and DP10 indicate their differential functional roles during allergic diseases such as bronchial asthma. The fact that asthma is a disease of the airways and the observation that DP10 and the activities of DP8 and DP9 are primarily located in the bronchi, strongly suggest an involvement of these peptidases during the clinical course of asthma. In humans, the DP10 gene has already been identified as a locus for asthma susceptibility (Allen et al. 2003), but until now, no information about the functional role of DP10 was available, apart from its association with the Kv4-mediated A-type potassium channels (Zagha et al. 2005). Similarly, until now, it was only known that DP8 and DP9 are intracellularly expressed proteins in leukocytes (Maes et al. 2007) and that they have ubiquitous expression patterns (Abbott et al. 2000; Ajami et al. 2004) with a DP4-like activity. No natural substrates were identified to date in vivo, although the hydrolysis of glucagon-like peptide 1 (GLP-1), GLP-2, and neuropeptide Y have already been demonstrated in vitro (Bjelke et al. 2006). In this regard, our study should motivate specific research activities to clarify their functional role. A strong DP10 mRNA expression was found in brain samples and a weaker mRNA expression in trachea from F344 rats, which corresponds to observations made by other groups (Allen et al. 2003; Takimoto et al. 2006). Likewise, we could not find any mRNA expression in samples from the whole lung (Chen et al. 2006), whereas in samples from the bronchi, the PCR signal and immunohistochemical protein detection were positive. The strong PCR signal for DP10 in the central nervous system-derived positive control might even suggest that the more precise site of expression of DP10 within the bronchi might be found in the bronchus-associated nervous system, which would then in turn strengthen the concept of an important nervous system-mediated component in the regulation of allergic responsiveness.

The observation that DP8 and DP9 mRNA levels are upregulated during asthma induction and the localization of their activity in the bronchi suggest that these intracellular peptidases specifically respond to the inflammatory stimulus. Elevated DP8 mRNA levels have also been documented in activated lymphocytes (Abbott et al. 2000; Gorrell 2005). Likewise, the contribution of DP8/9 to the overall DP4-like activity and their necessity for

T-cell proliferation have been demonstrated using selective inhibitors (Lankas et al. 2005; Maes et al. 2007).

In conclusion, because asthma is a disease of the airways, a site-specific expression and a regulation of DP4 and the DP4-like peptidases DP8, DP9, and DP10 in the whole lung and especially in the bronchi after asthma induction point to their potential role in asthma, which should be further investigated.

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2.7 Tables and Figures

Gene	Primer sequence (5'-3')	Annealing temperature (C)	Size PCR product (bp)
RPL13a	CCTCCACCCTATGACAAGGA (forward)	58	186
	TTCCGGTAATGCATCTTTGC (reverse)		
DP4	TCCCAACTCCAGAGGACAAC (forward)	57	152
	CAGGGCTTTGGAGATCTGAG (reverse)		
DP8	ACAGCAAACCCAAAGGTCAC (forward)	58	152
	TCTGGAGTCCATCCAGCTCT (reverse)		
DP9	AATGACTATGACTGGACGGA (forward)	60	196
	CGTAGAGGTGATGTTCCAGG (reverse)		
DP10	TCATTTCCAGCATTCAGCAG (forward)	53	176
	GCAGCACGGATACTTCTTCC (reverse)		

Table 1: Primers used for rat RPL13a, DP4, DP8, DP9, and DP10.

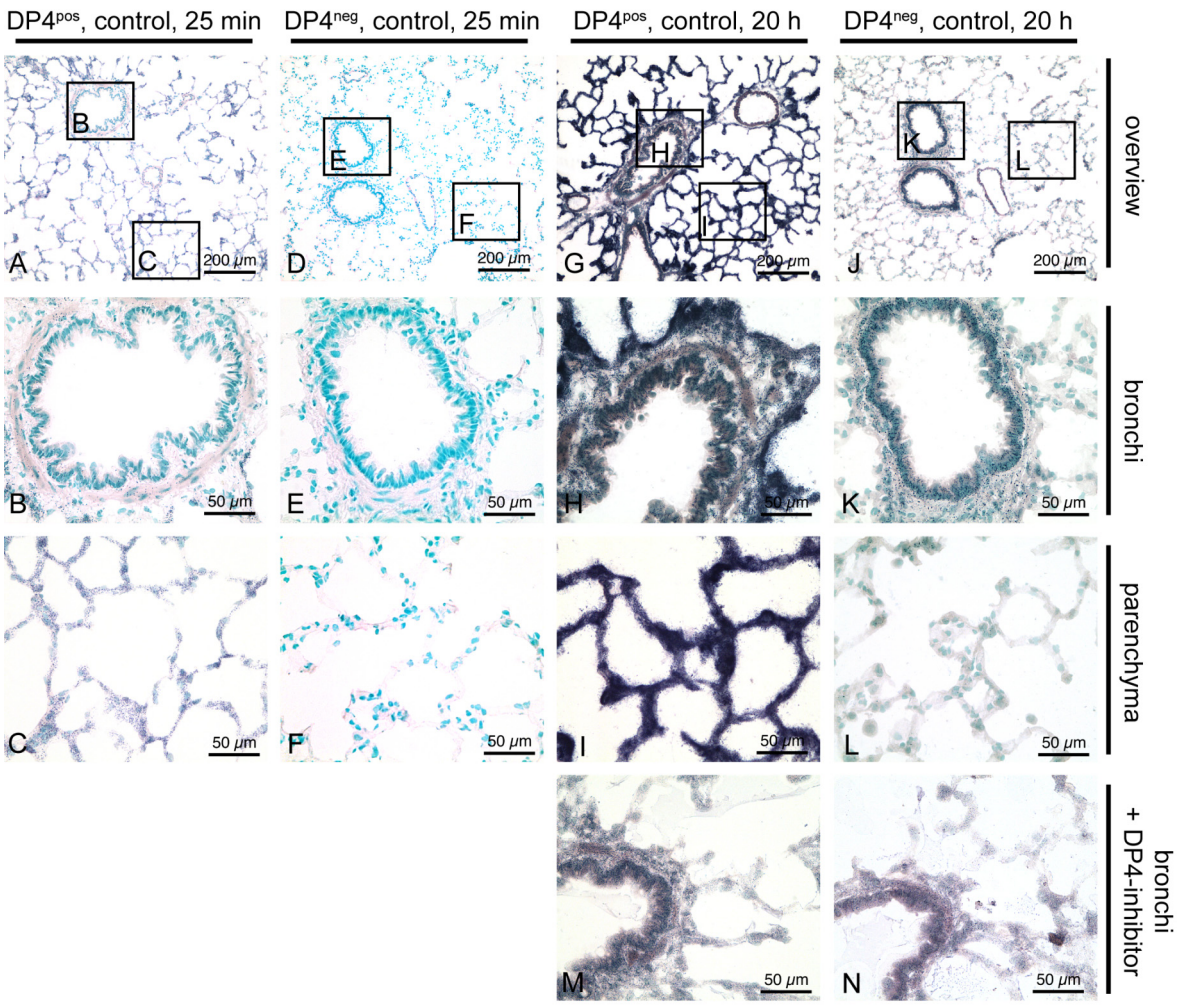

Figure 1 Detection of DP4-like activities with a histochemical activity assay. (**A-C**) Lung section of a DP4-positive control rat after an incubation period of 25 min with a slight staining of the lung parenchyma. (**D-F**) Lung section of a DP4-negative control rat after an incubation period of 25 min with no visible staining. (**G-I**) Lung section of a DP4-positive control rat after an incubation period of 20 hr with a strong staining of the lung parenchyma and a weaker staining of the bronchi. (**J-L**) Lung section of a DP4-negative control rat after an incubation period of 20 hr with hardly any staining of the lung parenchyma and a weak staining of the bronchi. (**M**) Lung section of a DP4-positive control rat after 20 hr incubation in the presence of a DP4-specific inhibitor. (**N**) Lung section of a DP4-negative control rat after 20 hr incubation in the DP4 inhibitor solution.

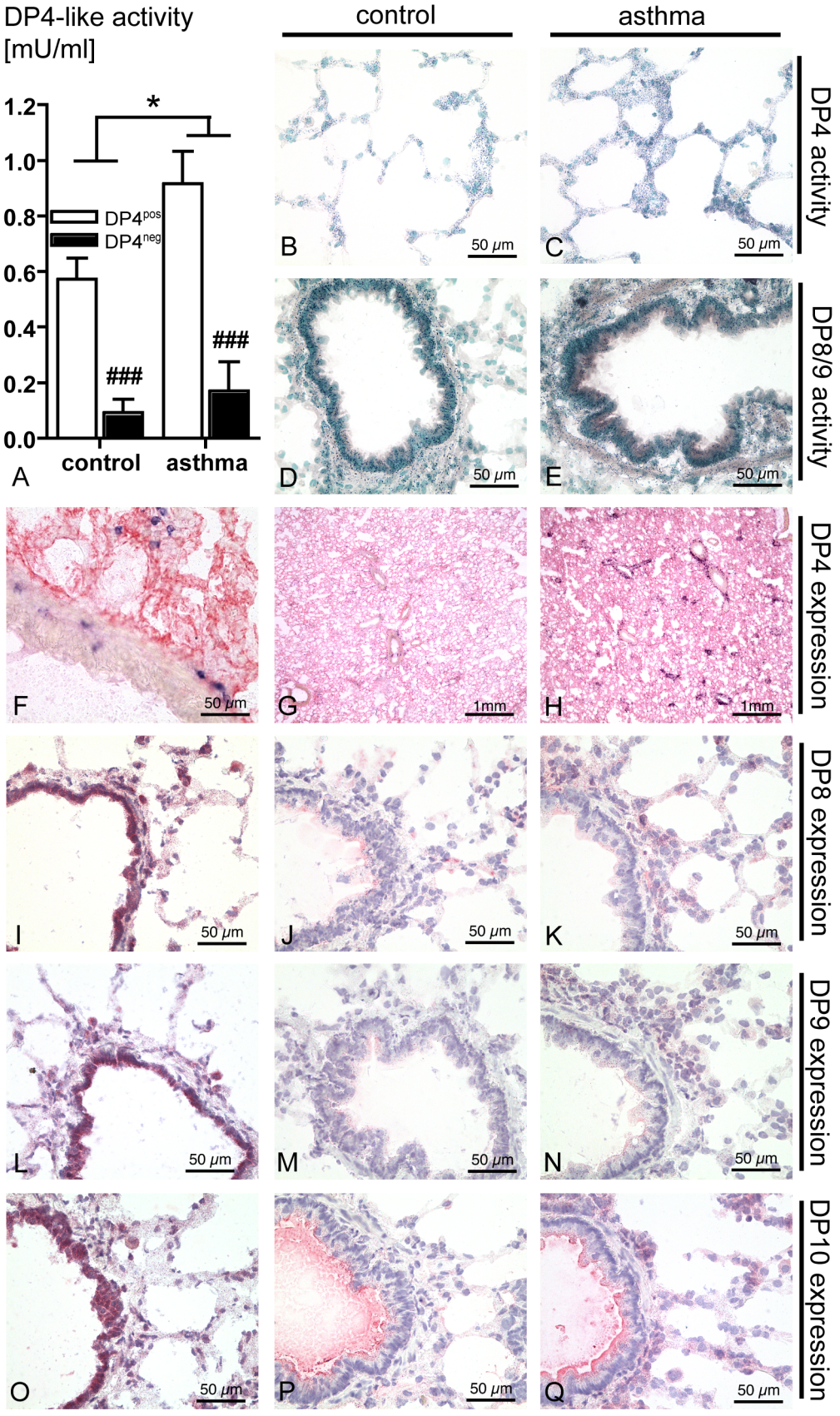

Figure 2 Comparison of the DP4-like peptidases in the lungs. (**A**) DP4-like activity of the bronchoalveolar lavage (BAL) fluid measured by the enzymatic activity assay in vitro

(*p<0.05; ###p<0.0001). Histochemical activity assay of a DP4-positive control lung (**B**), of a DP4-positive asthma lung (**C**), of a DP4-negative control lung (**D**), and of a DP4-negative asthma lung (**E**). (**F,G**) Staining with a DP4 antibody (red) and a T-cell antibody (blue) of a DP4-positive control lung at different magnifications. (**H**) The same staining of a DP4-positive asthma lung. Staining with a DP8 antibody of a control lung without BAL (**I**) and after BAL (**J**), and of an asthma lung after BAL (**K**). Staining with a DP9 antibody of a control lung without BAL (**L**) and after BAL (**M**), and of an asthma lung after BAL (**N**). Staining with a DP10 antibody of a control lung without BAL (**O**) and after BAL (**P**), and of an asthma lung after BAL (**Q**).

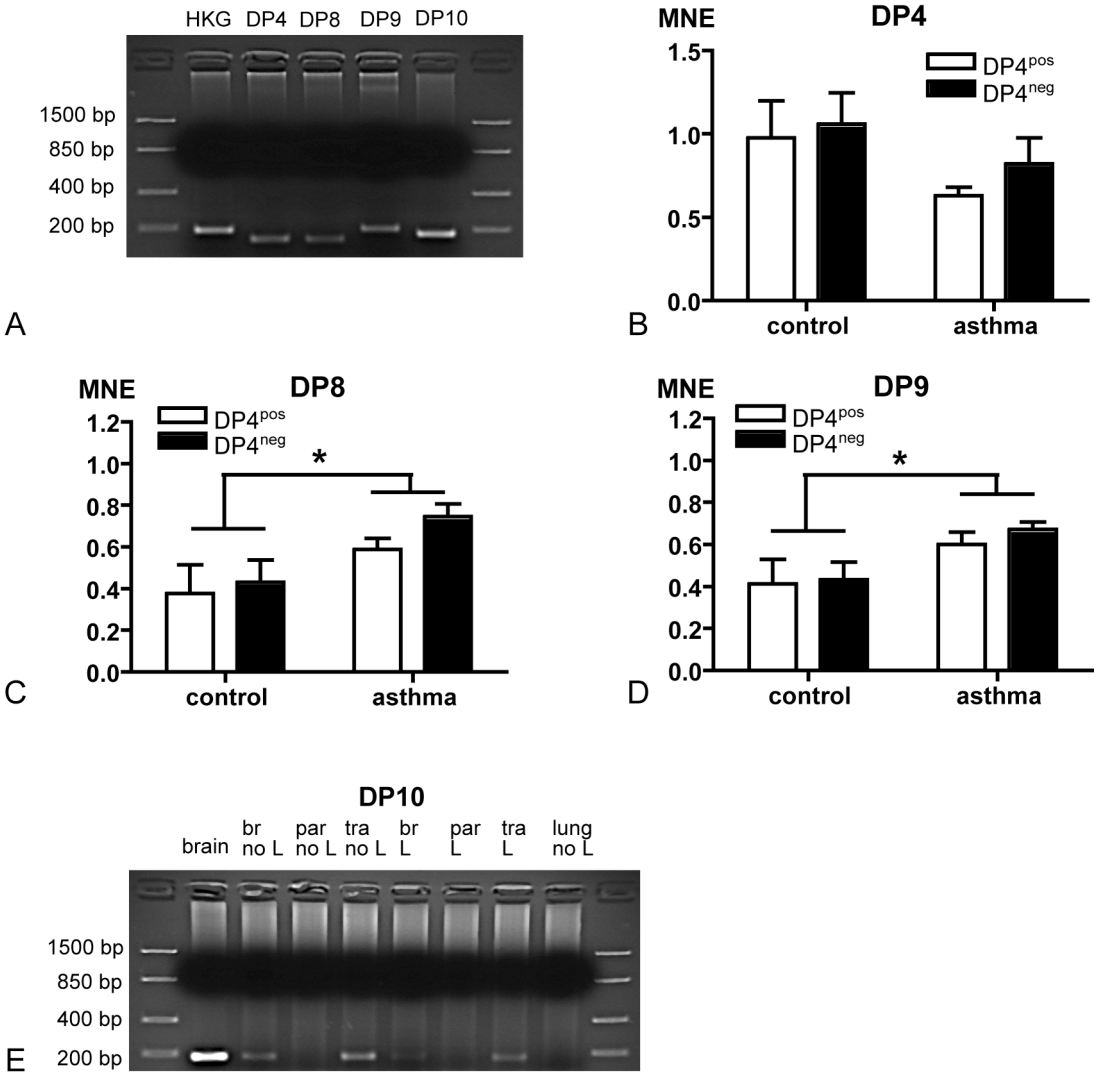

Figure 3 PCR analyses of DP4, DP8, DP9, and DP10. (**A**) Overview of the PCR products resulting from different primer pairs. HKG, housekeeping gene. (**B**) Mean normalized expression (MNE) of DP4 in DP4-positive ($DP4^{pos}$) and DP4-negative ($DP4^{neg}$) lungs of control groups and groups after asthma induction. (**C**) MNE of DP8 in the same groups (*p<0.05). (**D**) MNE of DP9 in the same groups (*p<0.05). (**E**) Detection of a DP10 PCR product only in cDNA from brain, trachea, and bronchi of control lungs and lungs after asthma induction. br, bronchi; par, lung parenchyma; tra, trachea; L, sample after BAL; no L, sample without BAL.

3 Airway-specific Recruitment of T Cells Is Reduced in a CD26-deficient F344 Rat Substrain

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Short title: Fewer T-cell numbers in airways of CD26-deficient rats

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Abstract

Asthma is a chronic inflammatory disease affecting the airways. Increased levels of T cells are found in the lungs after the induction of an allergic-like inflammation in rats, and flow cytometry studies have shown that these levels are reduced in CD26-deficient rats. However, the precise anatomical sites where these newly recruited T cells primarily appear are unknown. Therefore, we quantified the distribution of T cells in lung parenchyma as well as in large, medium, and small airways using immunohistochemical stainings combined with morphometric analyses. The number of T cells increased after the induction of an allergic-like inflammation. However, the differences between CD26-deficient and wild-type rats were not attributable to different cell numbers in the lung parenchyma, but the medium and large sized bronchi revealed significantly fewer T cells in CD26-deficient rats. These sites of T-cell recruitment were further screened using immunohistochemistry and qrtPCR with regard to two hypotheses: 1. involvement of the nervous system, or 2. expression of chemokines with properties of a "T-cell repellent factor" or a T-cell attractor. No topographical association was found between nerves and T cells, but a differential expression of MCP-1 and SDF-1 was revealed in bronchi and parenchyma. Thus, the site-specific recruitment of T cells appears to be a process mediated by chemokines rather than nerve-T-cell interactions. In conclusion, this is the first report showing a differential site-specific recruitment of T cells to the bronchi in a CD26-deficient rat substrain during an asthma-like inflammation.

Keywords: CD26/Dipeptidyl peptidase 4 - T cells - asthma - bronchi - F344 rat substrains

3.1 Introduction

Asthma is a chronic inflammatory disease of the airways, which is mainly characterized by bronchial hyper-responsiveness, airway obstruction, and airway inflammation with an influx of different cell types. The inflammation causes wheezing, coughing, chest tightness, and breathlessness and is accompanied by an elevated production of cytokines and chemokines, as well as edema and mucus hyper-secretion (for review see [1]). An IgE-mediated response very similar to the response in human bronchial asthma can be provoked in different animal models using inhaled allergen challenge. Mice, rats, guinea pigs, and other species, as well as different substrains of species have been established as animal models. Each model has its own advantages, but no model is identical to the conditions found in human disease [2]. Rats as well as other rodents exhibit a monopodial bronchial branching pattern, which is fundamentally different from that of humans, but the more human-like structure of the submucosal and bronchial smooth muscle layers and the existence of bronchial arteries in addition to capillaries and veins in the lamina propria of the bronchi make the rat a good model species for bronchial asthma [3].

In a Fischer 344 (F344) rat model for asthma, we have already documented a dose-dependent recruitment of T cells into the lung [4]. T lymphocytes are thought to play a pivotal role in the pathogenesis of asthma and many disease parameters can be modulated by T-cell recruitment and activation, cell adhesion, and chemokine metabolism. All of these disease-modulating processes are potentially further influenced by CD26 expression and its associated enzymatic activity [5]. CD26 is a multifunctional glycoprotein, which is also called dipeptidyl peptidase 4 (DP4) (DPP4, DPPIV, EC 3.4.14.5). It exists in a membrane bound form as well as in a soluble form, and is capable of regulating various physiological processes by N-terminal cleavage of dipeptides from peptides with L-proline/-alanine at the penultimate position [6], including but not limited to chemokines such as stromal cell derived factor-1 (SDF-1, CXCL12). The lung has been described as the organ with the second highest dipeptidyl peptidase activity of CD26 [7], while the bronchi do not express CD26 [8].

However, at the sites of the bronchi a structural homologue of CD26, DP10, is expressed [8]. The gene of DP10 represents a susceptibility locus for asthma in humans [9] and its protein is associated with Kv4-mediated A-type potassium channels [10] that play a pivotal role controlling cell excitability, especially in the nervous system. In addition to

DP10, the low-affinity pan-neurotrophin receptor $p75^{NTR}$ is expressed in airways [11]. The $p75^{NTR}$ is also expressed on many different cell types in the nervous system and can mediate a variety of different cellular functions, including neuronal survival, cell death, and neurite growth. Recent data also indicate an involvement of neurotrophins in asthma via their binding to $p75^{NTR}$ [12].

An active-site mutation of CD26 in a Fischer rat substrain has caused the retention and degradation of CD26 in the endoplasmic reticulum of these rats [13]. The induction of an allergic-like inflammation in this substrain has shown differences in the recruitment of T cells compared to a CD26-expressing wild-type substrain [14]. These differences were based on flow cytometry analyses of the bronchoalveolar lavage of F344 rat substrains, but morphometric analyses of the distribution of T cells in different compartments of the lungs are still lacking.

The present study investigates the distribution of T cells in lungs of CD26-deficient and wild-type F344 rat substrains under naïve conditions and after the induction of an allergic-like inflammation, with regard to the two hypotheses of either nervous system involvement or a CD26-dependent and chemokine-mediated compartment-specific differential recruitment.

3.2 Materials and Methods

Animals

Male wild-type F344/Ztm rats ($CD26^{pos}$) and male CD26-deficient mutant rats (F344/Crl(Wiga)SvH-Dpp4m) lacking DP4 activity as well as DP4/CD26 expression ($CD26^{neg}$) were used [15]. All animals were housed at the Central Animal Facility (Ztm) of the Hannover Medical School, maintained in a separate minimal barrier-sustained facility, and microbiologically monitored according to Federation of European Laboratory Animal Science Associations (FELASA) recommendations [16]. All research and animal care procedures had been approved by the review board of the Landesamt fuer Verbraucherschutz und Lebensmittelsicherheit (LAVES; Oldenburg, Germany) and were performed according to international guidelines on the use of laboratory animals.

Sensitization and allergen challenge

Two repeated experiments were performed, first with five animals per group, later with four animals per group. At the age of 6 months, CD26-positive and CD26-negative rats in the asthma group were sensitized 14 and 7 days before challenge, as previously described [4]. In brief, in each case sensitization was performed with 1 mg of ovalbumin (OVA) (Sigma, Deisenhofen, Germany) and 200 mg of $Al(OH)_3$ (Sigma) in 1 ml 0.9% (sterile, pyrogen-free) NaCl injected subcutaneously in a hind limb. As a second adjuvant, concentrated preparations of 6×10^9 heat-killed Bordetella pertussis bacilli (kindly provided by Chiron Behring, Marburg, Germany) in 0.4 ml 0.9% NaCl were given intraperitoneally at the same time. Animals were challenged with 7.5% of aerosolized OVA using a Pari LC Star nebulizer (Pari, Starnberg, Germany). Three CD26-positive and three CD26-deficient rats were neither sensitized nor challenged, serving as a control group.

Dissection of animals

The animals were dissected under isoflurane anaesthesia 22 ± 0.5 hr after challenge, as previously described [4]. Briefly, the animals were sacrificed by aortic exsanguination. For bronchoalveolar lavage (BAL) isolation, a cannula was inserted into the trachea in situ and

the lungs were lavaged four times with portions of 5 ml 0.9% NaCl solution. The recovery of fluid was over 90% in all animals. For further analysis, the lungs were excised. Whole left lungs were instilled with 3 ml of Tissue-Tek O. C. T. compound (Miles Inc., Elkhart, IN, USA) mixed 1:4 with PBS and placed on aluminium foil on dry ice. For PCR analyses, the large bronchi and the parenchyma of the right lungs were excised and frozen in liquid nitrogen.

Immunohistochemistry

Analysis of the compartmentalization of T cells, T-cell subpopulations, as well as components of the nervous system in the lung was performed using monoclonal (mAbs) and polyclonal (pAbs) antibodies together with different staining methods.

Two consecutive APAAP stainings [17] were performed on 10 µm acetone-fixed cryostat sections of the whole left lungs with Fast Blue (Sigma) or Fast Red (Sigma) as the detection system for labelled cells as described before [8] with 30 min incubation for T cells (mouse mAb R73; AbD Serotec, Duesseldorf, Germany), CD4 (mouse mAb W3/25; AbD Serotec), CD25 (mouse mAb OX39; AbD Serotec), and over-night incubation for CD26 (mouse mAb 5E8; Hycult Biotechnology b.v., Uden, Netherlands). Sections were counterstained with hemalaun (1:5 in PBS; Merck, Darmstadt, Germany) for 20 sec and covered with Mowiol (Hoechst AG, Frankfurt/Main, Germany).

In addition, immunofluorescence histochemistry was perfomed with a primary pAb raised in rabbits for $p75^{NTR}$ (Millipore, Schwalbach, Germany) together with a primary mouse mAb R73 (AbD Serotec). Rabbit or mouse IgGs in appropriate dilutions were used instead of the primary antibodies as isotype controls. Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Suffolk, United Kingdom), and Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen, Karlsruhe, Germany) were used as secondary antibodies. Counterstaining with Hoechst dye (Invitrogen) was performed in all specimens and the sections were covered with Mowiol (Hoechst AG).

Quantitative histology of lung tissues

For compartmentalization of T cells, the optical dissector method [18] was chosen during the first series of experiments to determine the absolute cell number in the lungs.

Therefore, three acetone-fixed cryostat sections (thickness 40 µm, interval 800 µm) of each rat were evaluated with regard to CD26-expressing and CD26-non-expressing T cells in the different compartments of the lung (parenchyma, bronchi, vessels, alveolar space, perivascular space, and BALT). Absolute T-cell numbers in the lungs were calculated using a Nikon Eclipse 80i microscope (Nikon GmbH, Duesseldorf, Germany) and the Stereo Investigator software (MicroBrightField Inc., Williston, VT, USA). Test fields were generated across the whole section with a constant interval between the test fields in the x- and y-axis.

In the following series of experiments, the cell density of T cells was determined. Three serial cryostat sections (thickness 10 µm, interval 800 µm) were evaluated of at least 4 rats per group as described before [19]. The cells in the lungs were evaluated using a Nikon Eclipse 80i microscope (Nikon) together with the Stereo Investigator software (MicroBrightField). Test fields were generated as described above, T cells were counted, and cell density was defined as:

$$\text{cells per mm}^2 = \frac{\text{number} \times \text{grid size / counting frame}}{\text{total area}} \times 1000000$$

The cells around the bronchi of the lungs were furthermore quantified using a semi-quantitative score (1: <15 cells; 2: 15-30 cells; 3: >30 cells). Small bronchi were defined as bronchi with a diameter <150 µm, medium bronchi were between 150 and 300 µm, and large bronchi were defined as having a diameter >300 µm. At least 5 bronchi of each size were quantified on all sections, and the mean was calculated for each animal.

Representative micrographs were taken with a MicroFire digital microscope camera (Optronics, Goleta, CA, USA).

Quantitative real-time PCR

Tissue samples from shock-frozen bronchi and parenchyma from the lobes of the right lungs were homogenized by means of the homogenizer Precellys with 1.4 mm ceramic beads (5000 rpm, 30 sec; Peqlab, Erlangen, Germany). RNA was prepared using the NucleoSpin RNA II kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's instructions. cDNA preparation and quantitative real-time (qrt) PCR were performed as previously described [20] with RPL13a as a housekeeping gene (HKG).

Briefly, qrtPCR was performed in a Rotor-Gene 3000 (Corbett Research, Hilden, Germany) using the following primers for RPL13a 5' CCC TCC ACC CTA TGA CAA GA 3' and 5' TTC CGG TAA TGG ATC TTT GC 3', for MCP-1 5' CCA GAA ACC AGC CAA CTC TC 3' and 5' CCG ACT CAT TGG GAT CAT CT 3', and for SDF-1 5' GCT CTG CAT CAG TGA CG GTA 3' and 5' TAA TTT CGG GTC AAT GCA CA 3' as well as the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany). For verification, products' melting curves were generated and single amplicons were confirmed by agarose gel electrophoresis. Relative amounts were determined with the Rotorgene software version 4.6 in comparative quantitation mode.

Statistical analysis

Differences among groups were analysed using two-way analysis of variance (ANOVA) with treatment (control versus asthma) and genetic background ($CD26^{pos}$ versus $CD26^{neg}$) being the factors, followed by Fisher's tests for protected last significant differences (PLSD) for post hoc comparisons, if appropriate. Statistically significant effects between the asthma group and the control group are indicated by rhombs (#$p<0.05$; ##$p<0.01$; ###$p<0.001$), and for comparison of $CD26^{neg}$ and $CD26^{pos}$ groups by asterisks (*$p<0.05$). All data are displayed as mean ± SEM.

3.3 Results

Substrain-specific differences in allergen-induced T-cell peribronchial density, but no significant differences in the lung parenchyma

The localization of T cells from CD26-positive and CD26-deficient naïve F344 rats and rats after the induction of an allergic-like inflammation via an OVA-challenge was determined in six different compartments of the lungs (parenchyma, bronchi, vessels, alveolar space, perivascular space, and BALT). The first series of experiments (data not shown) using the optical dissector method showed that the vast majority of T cells were found in the lung parenchyma (up to 90%), and that about 90% of the T cells in wild-type rats were CD26-positive. The absolute T-cell numbers increased significantly in the lung parenchyma of both groups after OVA-challenge, but there was no significant difference between wild-type and CD26-deficient rats. A more precise examination of the bronchi in these experiments showed a significantly different T-cell number around medium and large bronchi of OVA-challenged wild-type and CD26-deficient animals.

Therefore, the experiments and the immunohistochemical stainings were repeated and the T-cell number per mm^2 lung was determined in the parenchyma (Fig. 1a). T cells were stained in blue in wild-type (Fig. 1b) and CD26-deficient (Fig. 1c) naïve rats. Only a small number of these cells was found in the lung parenchyma. However, the T-cell density significantly increased in wild-type (Fig. 1d) and CD26-deficient (Fig. 1e) rats after OVA-challenge. No significant difference was found between wild-type and CD26-deficient rats. CD26 was highly expressed in the lung parenchyma of wild-type rats (Fig. 1b and d), whereas its expression was lacking in CD26-deficient rats (Figures 1c and e).

The T-cell distribution around the bronchi showed differences in the T-cell numbers around the large and medium airways of naïve and OVA-challenged wild-type and CD26-deficient rats (Fig. 2a). The number of T cells in airways of all sizes significantly increased in both substrains after OVA-challenge. No significant difference in the T-cell distribution was observed either in the airways of naïve rats from both substrains, or in the small airways of both substrains after OVA-challenge. However, a significant difference in the T-cell increase around the large and medium airways of wild-type (Fig. 2b) compared to CD26-deficient (Fig. 2c) F344 rats was observed in the OVA-challenged groups.

Characterization of cell types in the bronchial environment

The regions of the differential T-cell distribution were characterized in more detail. First, $CD4^+CD25^+$ cells were stained to determine T-cell subsets around the bronchi. Comparison of the four different groups (Fig. 3a,b,d,e) revealed the highest number of these cells around the airways of OVA-challenged wild-type rats. The higher magnifications illustrate CD4-positive, CD25-positive, and $CD4^+CD25^+$ cells (Fig. 3c and f).

Because nerves and neurotrophins play a critical role during asthma, the expression of the neurotrophin receptor $p75^{NTR}$ was further investigated as a marker for components of the nervous system. The $p75^{NTR}$-positive cells were found in the BALT of lungs after OVA-challenge in follicular-like areas that were mostly free of T cells (Fig. 4a-c). Such $p75^{NTR}$-positive structures were also found around the bronchi (Fig. 4d-f), but no differences between the two substrains were detectable.

Identification of chemoattractants in the lungs

Quantitative real-time PCR analyses revealed differential expression patterns of the chemokines MCP-1 and SDF-1 in the large airways and the lung parenchyma. The expression of MCP-1 was upregulated in the large airways (Fig. 5a) and in the lung parenchyma (Fig. 5b) of both OVA-challenged groups. While the expression of SDF-1 was upregulated in the large airways of unchallenged and OVA-challenged wild-type rats compared with CD26-deficient rats (Fig. 5c), it was equally expressed in the lung parenchyma in all groups (Fig. 5d).

3.4 Discussion

This study demonstrates a compartment-specific and differential increase of T cells in an F344 rat model for an asthma-like inflammation depending on CD26-expression.

Immunostaining of T cells in the lungs revealed that their number increases after the induction of this asthma-like inflammation in CD26-positive and CD26-deficient rats. This is consistent with previous flow cytometric analyses on cell suspensions of whole lobes of the right lungs and BAL fluid [4, 14]. A reduced increase of T cells in the BAL was reported in CD26-deficient rats after OVA-challenge [14]. However, until now, the source of these recruited T cells in the lungs was unknown. Therefore, one aim of the present study was to clarify whether these differentially increased T cells in F344 substrains are equally distributed throughout the whole lungs or whether they accumulate in a certain anatomical compartment of the lungs. After OVA-challenge, staining of whole left lungs revealed an equal distribution of T cells in the lung parenchyma of both substrains, but a significantly different number of T cells between the two substrains around the large and medium airways in the lungs. Surprisingly, this compartment of the lung almost completely lacks CD26 expression as well as DP4 activity [8]. Thus, despite using CD26-deficient and CD26-competent F344 rat substrains, the findings illustrate a difference in an anatomical compartment that largely lacks expression of CD26. This lead to the hypothesis that the reason for this phenomenon might be 1) a different environment of the T cells in this area, 2) the expression or lack of CD26 on the T cells recruited to this site, or 3) other differences, for example in the chemokine pattern at these sites.

Since in humans, the pathology of asthma mostly affects bronchi, any model of this disease should show differences in this compartment. This is the case in the present model. However, the potential mechanisms for the substrain-specific recruitment of T cells to the bronchi remain to be revealed.

We considered either nervous system mediated effects or direct effects mediated via CD26. With regard to the first hypothesis, at least a direct interaction of nervous system-derived factors and T-cell areas around the bronchi appears to be unlikely, since staining for $p75^{NTR}$-positive structures and T-cell areas revealed no overlap. Therefore, the expression of the multifunctional CD26 on the surface of the T cells alone or in combination with a different local chemokine pattern around the bronchi due to their local activity might lead

to this differential increase. Activated T cells that are also differentially distributed in the airways, express more CD26 on their surface, which even potentiates ongoing processes.

First of all, CD26 is a potential mediator of adhesion to extracellular matrix proteins such as collagen and fibronectin [21, 22]. This might retain more CD26-positive T cells in the lungs directly around the bronchi. Interaction of CD26 and fibronectin during adhesion and metastasis of rat breast cancer cells has been shown before [23], but its role under normal conditions has been examined to a lesser extent [24].

The dipeptidyl peptidase activity of CD26 or DP4-like functional homologues might also be a reason for a differential peribronchial increase of T cells, these sites having the first allergen-contact during inhalation of OVA. But – a priori – in the case of blunted degradation in the CD26-deficient animals, a more prominent recruitment to the sites of inflammation would be expected due to a longer half life of those chemokines, being substrates of DP4, e.g. eotaxin/CCL11 [25]. Eotaxin attracts eosinophils via its receptor CCR3 and a strong eosinophilia in the lung should attract many T cells. More eosinophils are expected in CD26-deficient rat substrains in an asthma model, but in our model of an acute asthmatic response, this was not the case [14].

Another potential reason for the differential increase of T cells is their chemotactic potential, which might be modulated by the expression of CD26 on their surface. The monocyte chemoattractant protein 1 (MCP-1) is an example of a T-cell attractant, and an OVA-induced pulmonary T-cell accumulation is abolished in the absence of MCP-1-mediated signals in a mouse model [26]. MCP-1 has been shown to be increased in the BAL fluid of allergic asthmatic patients [27], and a potential association between the gene regulatory region of MCP-1 and asthma susceptibility has been suggested [28]. Expression of the MCP-1 receptor was shown on T cells highly expressing CD26, and only these cells responded to MCP-1 in chemotaxis assays [29]. In our study, qrtPCR revealed a significantly higher expression of MCP-1 in the large airways of wild-type and CD26-deficient OVA-challenged rats compared to unchallenged rats. This might lead to an accumulation around the airways in these rats and the twofold higher expression in the wild-type rats might attract more T cells in this substrain. The differences in the lung parenchyma do not lead to a higher density of T cells in the CD26-deficient rats, which might be due to the lower expression level in the parenchyma compared with the airways.

Another chemoattractant for T cells is SDF-1, if it is expressed at low concentrations. At high concentrations, SDF-1 has been described as a T-cell chemorepellent [30]. It might lead to the direct opposite of chemotaxis, which is called chemofugetaxis or fugetaxis [31], and which might also be a possible explanation for the differential distribution of T cells in the two substrains. SDF-1 is a substrate of CD26 [7] and N-terminal cleavage by CD26 causes its inactivation [32]. Within the airways of asthmatic patients, the immunoreactivity of SDF-1 is significantly increased and its expression is upregulated in asthmatic tissues [33]. If the concentration of SDF-1 is very high in the airways of rats after asthma-induction, and if SDF-1 is not inactivated by CD26 in the CD26-deficient rats, it might repel more T cells in this substrain than it does in wild-type rats. The thymic stroma for example expresses very high levels of SDF-1, which might play a central role in thymocyte emigration [34]. The thymus has been studied recently in wild-type and CD26-deficient F344 rats and differential thymic emigration patterns were revealed in these substrains [35]. SDF-1 is also described as a substrate for the CD26/DP4-functional homologue dipeptidyl peptidase 8 (DP8) [36], which is expressed in the airways of both substrains [8]. A very high concentration of SDF-1 in the airways after OVA-challenge and a lack of inactivation in CD26-deficient rats might prevent the entry of more T cells in this substrain. PCR analyses revealed significant differences between wild-type and CD26-deficient rats under both conditions, which might be due to the degradation of SDF-1 by CD26 and its following upregulation. The expression of SDF-1 in the airways probably does not reach a very high level, which leads to the presumption that it works as a T-cell attractor and not as a T-cell repellent in this model. In this case, it might attract more T cells to the airways of wild-type rats.

In conclusion, we suggest that the differential peribronchial T-cell increase is mediated by the microenvironment at these sites. This needs further examination, in particular, the quantification of local CD26 substrate levels that might mediate attracting or repellent functions, as well as the differentiation between their N-terminal truncated and full-length forms.

3.5 Acknowledgements

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3.7 Figures

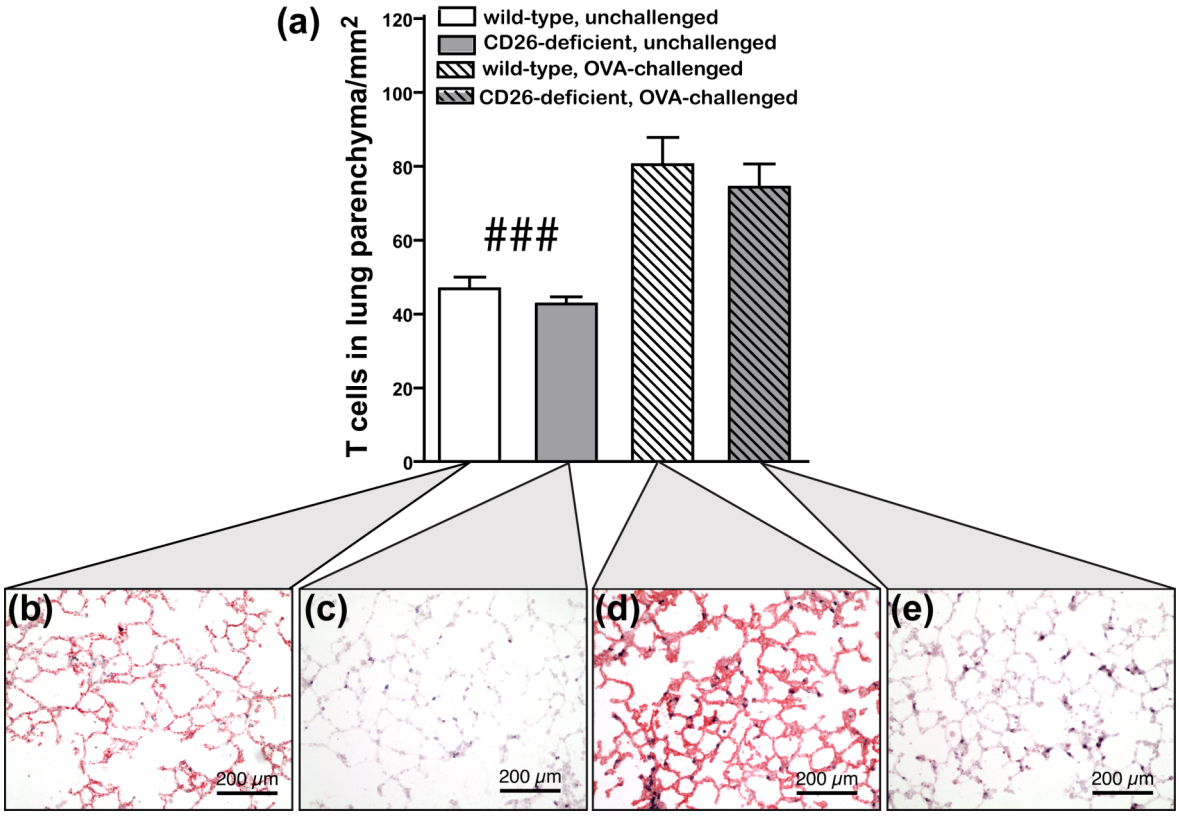

Fig. 1: Histological detection of T cells (blue) and CD26 (red) in naïve lungs and lungs after OVA-challenge. Overview of the T-cell density in the parenchyma (a). Representative micrographs of the lung parenchyma of naïve wild-type rats (b), naïve CD26-deficient rats (c), OVA-challenged wild-type rats (d), and OVA-challenged CD26-deficient rats (e). (###$p<0.001$)

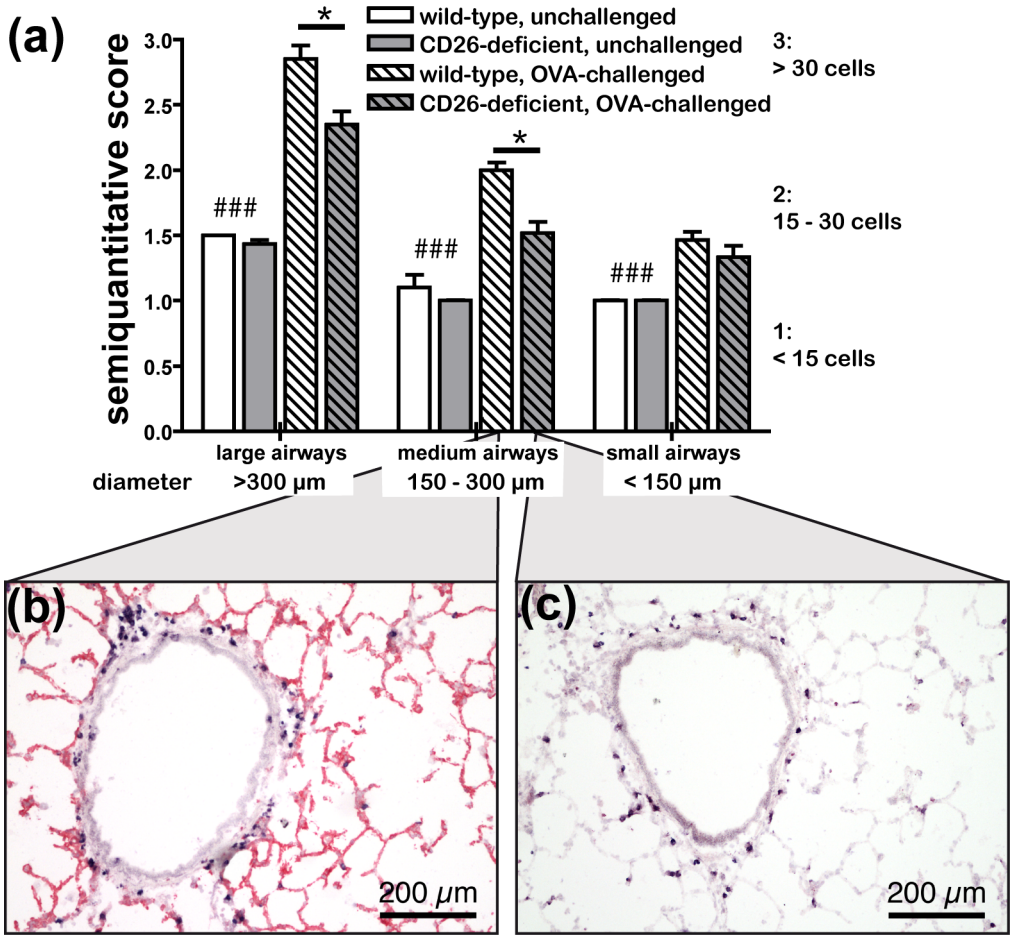

Fig. 2: Histological detection of T cells (blue) and CD26 (red) around the airways of naïve lungs and lungs after OVA-challenge. Overview of the T cell-numbers around the airways (a). Representative micrographs of the medium airways of wild-type OVA-challenged rats (b) and CD26-deficient OVA-challenged rats (c). (###$p<0.001$, *$p<0.05$)

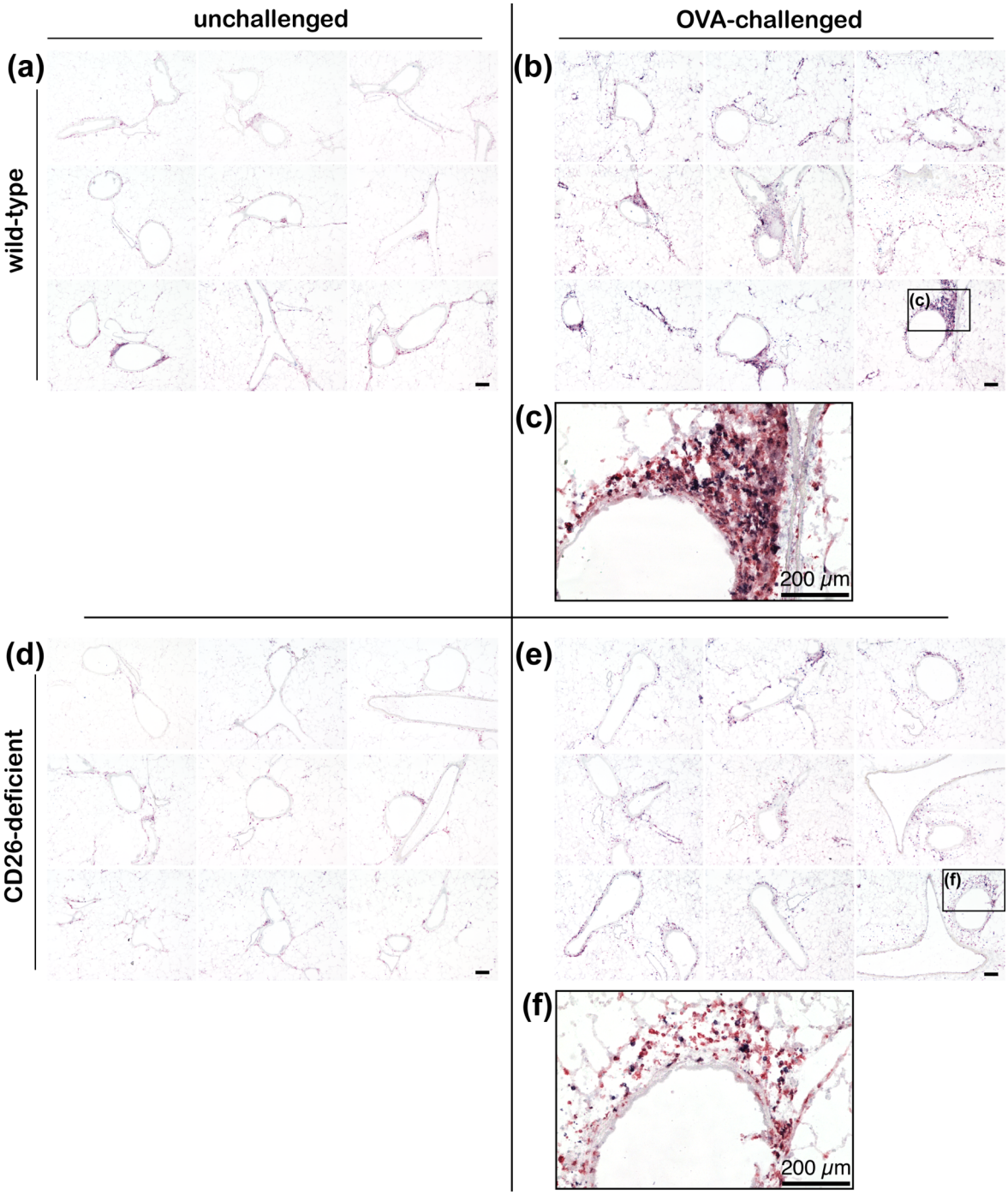

Fig. 3: Histological detection of T-cell subpopulations (CD4 in red, CD25 in blue) in the lungs. Representative micrographs of 3 airways from 3 wild-type animals from the unchallenged (a) and the OVA-challenged group (b), as well as a higher magnification of one airway of an OVA-challenged wild-type animal (c). Representative micrographs of 3 airways from 3 CD26-deficient animals from the unchallenged (d) and the OVA-challenged group (e), and a higher magnification of one airway of an OVA-challenged CD26-deficient animal (f). (all bars = 200 µm)

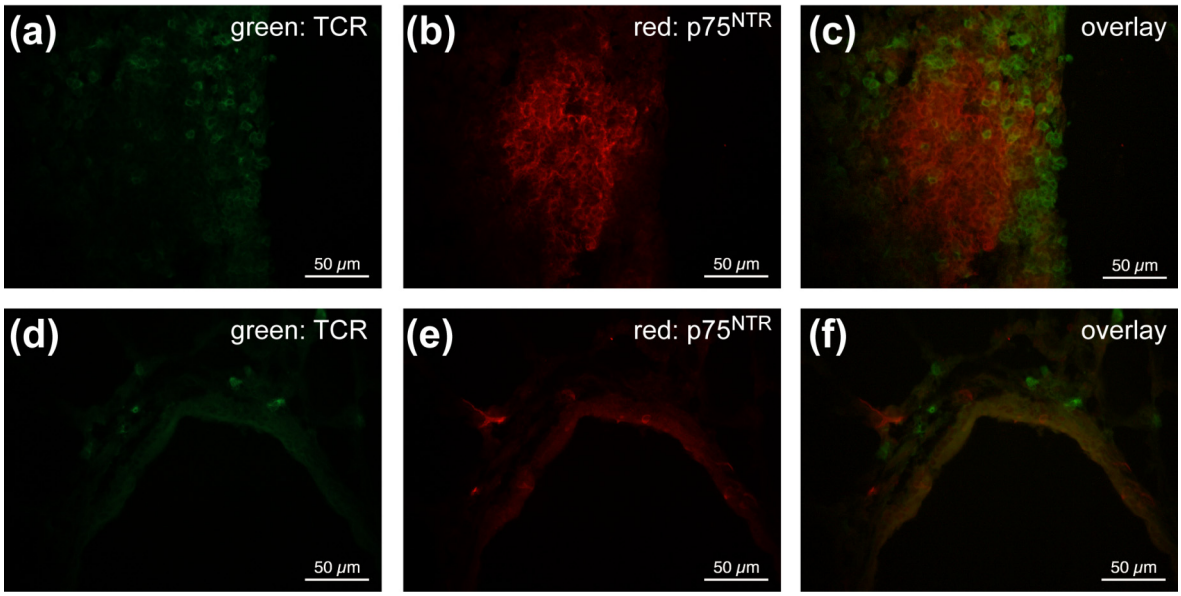

Fig. 4: Immunofluorescent stainings of T cells (green) and $p75^{NTR}$ (red). T-cell receptor (TCR)-positive cells of the bronchus-associated lymphoid tissue (BALT) (a) and $p75^{NTR}$-positive cells (b) were stained and an overlay (c) was produced. Micrographs of an airway with TCR-positive cells (d), $p75^{NTR}$-positive structures (e), and an overlay of both (f).

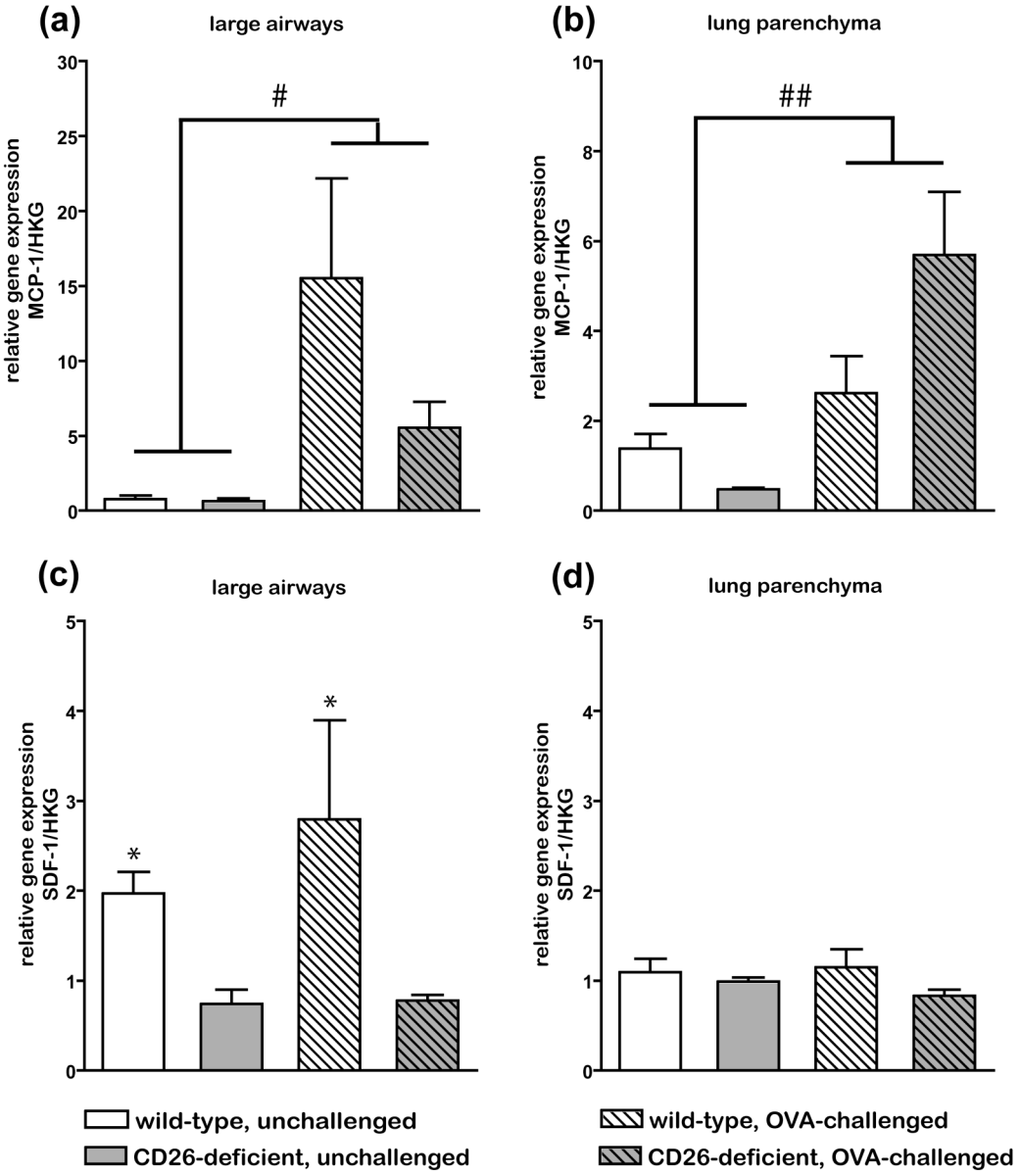

Fig. 5: Quantitative real-time PCR data. Relative gene expression of MCP-1 in the large airways (a) and in the lung parenchyma (b), and of SDF-1 in the large airways (c) and in the lung parenchyma (d). (HKG = housekeeping gene RPL13a; #$p<0.05$, ##$p<0.01$, *$p<0.05$)

4 Transferred T cells Preferentially Adhere in the BALT of CD26-deficient Recipient Lungs during Asthma

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Short title: Role of CD26 in a Coisogenic T-cell transfer

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Abstract

The multifunctional glycoprotein CD26/dipeptidyl peptidase 4 (DP4) has a dipeptidyl peptidase activity, plays a role during T-cell activation, and interacts with several proteins, including extracellular matrix (ECM)-proteins. The latter have been studied mainly in the context of experimental metastasis. The potential role of CD26 for T-cell adhesion could be of major interest. Here, a coisogenic transfer of CFSE-labelled T cells was performed after isolation from CD26-expressing or CD26-deficient F344 rat donors and subsequent cross-transfer to recipients of the other substrain. Their recovery in the lungs was quantified using flow cytometry, a histochemical activity assay, as well as immunohistochemistry and morphometry. Under naïve conditions there were neither differences in the numbers of recovered T cells nor in their preferential anatomical sites of adhesion. The induction of an asthma-like inflammation, however, led to a site-preferential adhesion of T cells in the bronchus-associated lymphatic tissue (BALT). In this compartment of the lungs, surprisingly, significantly more T cells were found in CD26-deficient recipient lungs, regardless of the origin of the transferred T cells. These findings demonstrate a negative regulatory role of the BALT-specific expression of CD26 in T-cell adhesion during an asthma-like inflammation. Considering the pattern of cellular re-distribution it is not very likely that CD26 expressed on T cells and/or endothelial cells represents a significant factor in T-cell adhesion in vivo. Instead, the present findings suggest that the lack of the CD26 peptidase function in BALT might cause an overflow of a T-cell chemoattractant, which yet remains to be identified.

Keywords: asthma; BALT; CD26/dipeptidyl peptidase 4; F344 rat substrains; T cells

Abbreviations: APAAP, alkaline phosphatase anti-alkaline phosphatase; BALT, bronchus-associated lymphatic tissue; CFSE, carboxyfluorescein diacetate succinimidyl ester; DA, Dark Agouti; DP4, dipeptidyl peptidase 4; ECM, extracellular matrix; F344, Fischer 344; HEV, high endothelial venule; Ig, immunoglobulin; mAb, monoclonal antibody; NBT, Nitro Blue Tetrazolium; OVA, ovalbumin; pAb, polyclonal antibody; pNA, paranitroaniline; SDF-1, stromal cell derived factor-1; wt, wild-type

4.1 Introduction

The CD26 antigen is a transmembrane cell surface glycoprotein with dipeptidyl peptidase activity. It is expressed on a variety of different cell types, for example on T cells, B cells, and natural killer cells, and also on endothelial and epithelial cells. CD26 (dipeptidyl pepidase 4, DP4, DPPIV, EC 3.4.14.5) has a widespread distribution in tissues, with the highest expression in the kidney and the second highest in the lung (Mentlein, 1999). Its expression is upregulated on activated T and B cells (Gorrell et al., 1991), as well as on lung tissue after the induction of an allergic-like inflammation (Schade et al., 2008). The dipeptidyl peptidase activity of CD26 leads to modulated receptor specificities of hormones, growth factors, neuropeptides, and chemokines through N-terminal cleavage of dipeptides from peptides with proline or alanine at the penultimate position (De Meester et al., 1999). Cleavage by CD26 alters, for example, the activity of the chemokine stromal cell derived factor-1 (SDF-1, CXCL12) (Sun et al., 2008), and therefore possibly reduces its T-cell chemoattracting potential. CD26 is also involved in T-cell biology as a costimulatory molecule (Tanaka et al., 1993). In addition to these functions, a potential role of CD26 in cell adhesion has been discussed. Binding affinities for fibronectin and collagen have been reported for the cell surface protein CD26 (Bauvois, 1988; Piazza et al., 1989), and these two proteins contribute to the structural integrity of the extracellular matrix (ECM).

CD26 is highly expressed in the lungs, which can be classified in different compartments, such as bronchi and bronchus-associated lymphoid tissue (BALT), perivascular space, bronchoalveolar transition, and lung parenchyma with alveoli and septa. Formation of BALT is age-dependent and varies in different disease-models (Pabst et al., 2008). High endothelial venules (HEVs) are characteristic for BALT and represent the sites of entry in lymphatic tissue. Furthermore, a high density of cells such as lymphocytes is found in the BALT. Morphological studies have shown that CD26 expression is predominantly found in lung parenchyma (Schade et al., 2008). Interaction of lung endothelial CD26 and fibronectin during adhesion and metastasis of rat breast cancer cells was reported in vitro and in vivo (Cheng et al., 1998). Several groups have investigated the association between CD26 expression, tumour-cell adhesion, and metastasis using different carcinoma cell lines, various rat substrains, and by blocking the function of CD26 in models of metastasis (Cheng et al., 1999; Shingu et al., 2003). Fischer 344 (F344) rats used in these studies

exhibit an intermediate DP4-like enzymatic activity compared to other rat strains such as Lewis (lowest activity) or Dark Agouti (highest activity) (Karl et al., 2003). A spontaneous mutation in the CD26 gene in substrains of F344 rats caused a retention of CD26 in the endoplasmic reticulum, which led to its rapid degradation in this substrain (Tsuji et al., 1992). This mutation resulted in an F344 rat substrain devoid of DP4 enzymatic activity and expression, allowing studies on the adhesion properties of CD26 under different conditions in vivo.

The role of CD26 during cell adhesion under normal conditions or in disease models has rarely been examined. So far, only in vitro experiments have been performed investigating its function during adhesion of T cells to endothelial cells (Mattern et al., 1998). For a better understanding of the role of CD26 on T cells and endothelial cells in adhesion processes in vivo, a coisogenic crosswise T-cell transfer was performed. T cells from two substrains were isolated and transferred to the same or the other substrain under normal conditions and in an asthma model. The recovery and spatial distribution of transferred T cells in the lungs were characterized using immunohistochemical stainings and flow cytometry and the expression of a potential T-cell chemoattractant at the sites of differential T-cell distribution was revealed.

4.2 Materials and Methods

Animals

Male wild-type F344 rats ($CD26^{pos}$) from Charles River (F344/Crl(Por)) and male CD26-deficient mutant rats (F344/Crl(DuCrj)SvH-Dpp4m) lacking DP4 activity as well as DP4/CD26 expression ($CD26^{neg}$) were used (Karl et al., 2003). All animals were housed at the Central Animal Facility of the Hannover Medical School, maintained in a separate minimal barrier-sustained facility, and microbiologically monitored according to Federation of European Laboratory Animal Science Associations recommendations (Rehbinder et al., 1996). All research and animal care procedures had been approved by the review board of the Landesamt fuer Verbraucherschutz und Lebensmittelsicherheit (Oldenburg, Germany) and were performed according to international guidelines on the use of laboratory animals.

Sensitisation and allergen challenge

For the induction of an asthma-like inflammation, donor and recipient wild-type and CD26-deficient F344 rats at the age of 8 weeks, were sensitised on day 0 and day 7, as previously described (Skripuletz et al., 2007). In brief, sensitisation was performed with 1 mg of ovalbumin (OVA; Sigma, Deisenhofen, Germany) and 200 mg of $Al(OH)_3$ (Sigma) in 1 ml 0.9% (sterile, pyrogen-free) NaCl injected subcutaneously into a hind limb. As a second adjuvant, concentrated preparations of 6×10^9 heat-killed Bordetella pertussis bacilli (kindly provided by Chiron Behring, Marburg, Germany) in 0.4 ml 0.9% NaCl were given intraperitoneally at the same time. The recipient animals were challenged with 5% of aerosolized OVA using a Pari LC Star nebulizer (Pari, Starnberg, Germany) 1 week after the second sensitisation.

Animals of the control group were neither sensitised nor challenged.

Experimental setup

Initial experiments on the origin of transferred T cells, the sorting method, the time of incubation, and the injected dose resulted in the following experimental setup, which was then used during the crosswise T-cell transfer.

T cells were isolated from mesenteric lymph nodes via a negative selection method based on magnetic-activated cell sorting. Thirty minutes incubation resulted in an average recovery rate of the transferred T cells in the lungs of naïve animals.

The first set of experiments was performed in control animals (Fig. 1). T cells from naïve rats of both substrains were isolated, labelled with CFSE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen, Karlsruhe, Germany), and transferred into naïve animals. The lungs were excised 30 min after the injection of 15×10^6 T cells into the tail vein in 300 µl PBS. Each group consisted of 7 animals.

In the second set of experiments, an asthma-like inflammation was induced (Fig. 2). Therefore, all animals were sensitised twice at intervals of 1 week as described before (Skripuletz et al., 2007). T cells of the donor animals were isolated 6 days after the second sensitisation. After CFSE-labelling of these cells, $15\text{-}25\times10^6$ cells were injected into the tail veins of the recipient animals 12 hr before the challenge with OVA (Sigma). The recipient animals were dissected 22 hr after challenge. Each group consisted of 4 animals.

Isolation and transfer of T cells

Naïve donor and recipient animals were used in the first set of experiments.

In the second set of experiments, all donor and recipient animals were sensitised. The sensitised donor animals were dissected under isoflurane anaesthesia 6 days after the second sensitisation, as previously described (Skripuletz et al., 2007). Briefly, the animals were sacrificed by aortic exsanguination, and EDTA-plasma samples were collected. The mesenteric lymph nodes were removed and cell suspensions prepared using a nylon mesh. The cells were counted in a Neubauer counting chamber and prepared for magnetic-activated cell sorting. The T cells were incubated with unconjugated antibodies (ED9, OX12, RP1, and 10/78; all mouse monoclonal antibodies (mAb) from AbD Serotec, Duesseldorf, Germany) and magnetic beads (goat anti-mouse IgG MicroBeads; Miltenyi Biotec, Bergisch Gladbach, Germany). Negative selection using LD columns (Miltenyi Biotec) resulted in a T-cell purity >93%. Isolated T cells were labelled with CFSE (1 µM for 10 min at 37°C; Invitrogen) and injected into the tail vein. Additional 100 µl of PBS were injected after the injection of T cells to flush the needle.

Dissection of recipient animals

The animals were dissected under isoflurane anaesthesia 30 min after transfer in the control group or 22 ± 0.5 hr after OVA-challenge, as previously described (Skripuletz et al., 2007). Briefly, the animals were sacrificed by aortic exsanguination, and EDTA-plasma samples were collected. A cannula was inserted into the trachea and the lungs were excised from the thorax. Whole left lungs were instilled with 3 ml of Tissue-Tek O. C. T. compound (Miles Inc., Elkhart, IN) mixed 1:4 with PBS and placed on aluminium foil on dry ice. Cell suspensions of the lobes of the right lung were prepared using a nylon mesh for flow cytometry analyses.

Flow cytometry

Flow cytometry was performed with a FACSCanto flow cytometer (BD Biosciences, Heidelberg, Germany) using the FACSDiva software (BD Biosciences). Mononuclear cells were defined according to their size and granularity using the forward and side scatter. The cell numbers were calculated as a percentage of mononuclear cells. Cells were stained with an anti-T-cell mAb R73-FITC (AbD Serotec) to check the purity of the cell suspension.

At least 5000 CFSE-positive T cells in the lung suspensions were counted in a flow cytometer. CFSE-labelled cells in the lung suspensions were measured alone and together with propidium iodide as a marker for dead cells.

Immunohistochemistry

Frozen lung sections (10 µm) were mounted on Poly-L-lysine coated glass slides, and fixed in acetone for 10 min before use. CFSE-positive cells were detected using the DE1 antibody (mouse mAb, 1:100 over-night; Roche Applied Science, Mannheim, Germany) in the APAAP staining procedure with Fast Blue or Fast Red (both from Sigma) as the detection system as described previously (Cordell et al., 1984). Sections were also stained with an antibody against CD26 (mouse mAb 5E8, 1:500 over-night; Hycult Biotechnology b.v., Uden, Netherlands). All sections were counterstained with hemalaun (1:5 in PBS; Merck, Darmstadt, Germany) for 20 sec and covered with Mowiol (Hoechst AG, Frankfurt/Main, Germany). Additional APAAP stainings were performed with a rabbit polyclonal antibody against SDF-1α (1:100 for 30 min; Abcam, Cambridge, United

Kingdom), together with a mouse anti-rabbit antibody (1:50; Dako, Hamburg, Germany) in the APAAP procedure as described before (Schade et al., 2008).

Quantitative histology of lung tissues

Three serial cryostat sections (thickness 10 µm, interval 800 µm) were evaluated from at least 4 rats per group as described before (Schmiedl et al., 2008). The cells in the lungs were evaluated using a Nikon Eclipse 80i microscope (Nikon GmbH, Duesseldorf, Germany) together with the Stereo Investigator software (MicroBrightField, Inc., Williston, VT). Test fields were generated across the whole section with a constant interval between the test fields in the x- and y-axis. DE1-positive cells were counted and cell density was defined as:

$$\text{cells per mm}^2 = \frac{\text{number} \times \text{grid size / counting frame}}{\text{total area}} \times 1000000$$

The BALT, which has a very high density of lymphocytes, was evaluated in more detail. Its total area was measured on the sections and all DE1-positive cells in this area were counted. The number of DE1-positive cells per mm^2 BALT was calculated.

Histochemical activity assay

Acetone-fixed lung sections were treated as described previously (Schade et al., 2008) with the substrate H-Gly-L-Pro-1-hydroxy-4-naphthylamide hydrochloride (kindly provided by Probiodrug, Halle/Saale, Germany) and Nitro Blue Tetrazolium (NBT; Sigma-Aldrich, Steinheim, Germany). Sections of each lung were incubated for 30 min or 20 hr at 37°C in the substrate solution. After fixation in paraformaldehyde, Nuclear Fast Red (Vector Laboratories, Burlingame, CA, USA) was used as a counterstain according to the manufacturer's guidelines. Cleaving of the substrate leads to a precipitation product, which is visible as a blue staining at the sites of enzymatic activity.

Light microscopy investigations were carried out on a Nikon Eclipse 80i microscope and representative pictures were taken with a MicroFire digital microscope camera (Optronics, Goleta, CA).

Enzymatic activity assay in vitro

DP4-like enzymatic activity of the EDTA-plasma samples from the different donor and recipient rats, as well as plasma from a highly DP4-expressing Dark Agouti rat as a positive control, was determined as described before (Frerker et al., 2007). The samples were incubated with the substrate H-glycyl-prolyl-4-nitroaniline hydrochloride (H-Gly-Pro-pNA*HCl; Bachem, Bubendorf, Switzerland) and the release of paranitroaniline (pNA) was measured by an increase in absorbance at 405 nm over time using the PowerWave XS photometer (Bio-Tek Instruments, Bad Friedrichshall, Germany). The assay was composed of 20 µl of the samples in 40 mM HEPES buffer (pH 7.6) and 0.4 mM H-Gly-Pro-pNA*HCl. Prior to the enzymatic reaction, the samples were incubated with the HEPES buffer for 15 min at 37°C. The reaction was initiated by adding the substrate and the release of pNA was measured up to 10 min. One unit is defined as the amount of enzyme necessary to hydrolyze 1 µM of substrate per minute.

Statistical analysis

Differences among groups were analysed using two-way ANOVA with genetic background of donor and genetic background of recipient ($CD26^{pos}$ versus $CD26^{neg}$) being the factors, followed by the Fisher protected least-significance test for post hoc comparisons, if appropriate. Statistically significant effects between $CD26^{pos}$ recipients and $CD26^{neg}$ recipients are indicated by asterisks (*$p<0.05$; ***$p<0.001$). All data are displayed as mean ± SEM.

4.3 Results

The influence of CD26 during adhesion of T cells in the lung was studied using a coisogenic crosswise T-cell transfer in two different experimental setups. Cells transferred from CD26-positive and -negative donors into CD26-positive and -negative recipients were evaluated in the lungs using immunohistochemical stainings and flow cytometry under naïve conditions (Fig. 1) and after the induction of an asthma-like inflammation (Fig. 2).

In our initial kinetic experiments with CD26-positive donors and recipients the highest number of transferred T cells in the lungs was detected after 5 and 30 min, and only few cells were visible after 6, 12, and 24 hr (data not shown). Therefore, naïve CFSE-labelled T cells were transferred into naïve animals and the lungs were removed 30 min after the transfer in the first experimental setup. CFSE-positive T cells were counted by flow cytometry (Fig 3A). In the second experimental setup, the lungs were removed 22 hr after OVA-challenge and CFSE-positive T cells were counted by flow cytometry (Fig. 3B). Additional propidium iodide stainings in these flow cytometric analyses revealed a very high viability of the CFSE-positive cells in all groups (data not shown). Flow cytometric analyses of lung homogenates did not reveal any significant differences in either experimental setup.

Then, the cell density of anti-CFSE stained CFSE-positive T cells on corresponding sections of whole left lungs was determined (Fig. 4). A genotype-specific effect was found in the histo-morphometric analyses of the naïve group (Fig. 4A). More CFSE-positive T cells were found in the lung parenchyma of CD26-deficient recipients (Fig. 4B) compared to CD26-positive recipients (Fig. 4C). No significant differences were found in the parenchyma of lungs from the second experimental setup (Fig. 4D-F).

The use of immunohistochemical antibody stainings and activity assays revealed the expression of CD26 (Fig. 5A) and its activity (Fig. 5B) derived from transferred CD26-positive T cells in the CD26-deficient recipient lungs in the same area on sequential sections. No staining was detectable in the lungs after transfer of CD26-deficient T cells to CD26-deficient animals. DP4-like activity in EDTA-plasma samples was additionally measured in some random samples to verify the functional CD26-expression in the wild-type F344 rats and CD26-deficiency in the mutant substrain (Fig. 5C). The DP4-like

activity of wild-type F344 rats is lower than the DP4-like activity of DA rats. Compared with the activities of other DP4-like peptidases such as DP8 and DP9, the activity in F344-mutant rats is much lower but not absent. No statistical analyses were performed with these random samples.

The overall T-cell numbers counted in the recipient lungs by flow cytometric and histomorphometric analyses did not reveal any genotype-specific differences after OVA-challenge. However, these lungs revealed a compartment-specific accumulation of T cells. In this experimental setup, the highest numbers of T cells were detected in the BALT compared to parenchyma, vessels, and bronchi. This compartment always existed in the lungs after OVA-challenge and at this site, even a genotype-specific effect in the T-cell increase could be found (Fig. 6A). A higher number of transferred T cells was detected in CD26-deficient recipients (Fig. 6B, C) compared to CD26-positive recipients (Fig. 6D, E), and this was independent of the donor's genotype. BALT was barely existent in the recipients of the naïve groups, and therefore, no genotype-specific differences could be found in the BALT of these groups.

To further investigate differences in the BALT of the different substrains after OVA-challenge, staining for the CD26 substrate SDF-1α was performed. It revealed a strong staining of the bronchi in both substrains, while the parenchyma was slightly stained (Fig. 7A,D). The staining of SDF-1α seemed to be more intense in the bronchi of CD26-deficient rats (Fig. 7B) and was detected on a higher number of cells in the BALT of these recipients (Fig. 7C) compared to the bronchi (Fig. 7E) and BALT (Fig 7F) of CD26-positive recipients.

4.4 Discussion

The high enzymatic activity of CD26 in the lungs and its expression on a large population of T cells make these components an ideal system to analyse adhesion features of CD26 in a coisogenic crosswise T-cell transfer under different conditions.

Under naïve conditions, the lungs of the recipient animals were removed 30 min after the transfer. After an intravenous injection of lymphocytes, many of these cells were found in the lungs only a few minutes later, consistent with findings of other groups (Smith and Ford, 1983; Petersen et al., 2006). The T cells might arrive in the lung after a few minutes because it is the first capillary bed, and might spread throughout the animal in the following minutes and hours. Therefore, this finding would represent an unspecific and brief attachment of the detected cells in the lungs. Flow cytometric analyses revealed that the number of detectable T cells did not differ between the substrains, whereas morphometric analyses showed a significant genotype effect. The different compartments of the lung examined with these methods might explain their differential outcome. Namely, in flow cytometric analyses, cell suspensions of the whole lung were examined, but histological analyses were focused on the parenchyma of the lungs. Therefore, this genotype-specific effect might represent a different T-cell number in the vessels, for example. T cells transferred to CD26-positive animals might arrive in the lungs more quickly than T cells transferred to CD26-deficient animals, which is then expressed in different transmigratory kinetics.

A comparison of the different staining methods revealed more CFSE-positive transferred T cells in the lungs than CD26-expressing cells and cells with DP4 activity. As shown before (Schade et al., 2008), DP4-like activity that is detectable in the histochemical activity assay after 30 min incubation is attributable only to DP4 and not to its homologues. Possibly only highly CD26-positive cells can be identified with the anti-CD26 antibody in immunohistochemistry and with the CD26 substrate in the activity assay. Another explanation might be the shedding of CD26 during the procedure. Flow cytometric analyses after the magnetic separation of T cells revealed an expression of CD26 on about 90% of the T cells from wild-type rats. The expression of CD26 was not checked after the CFSE-labelling, but this labelling alone should not alter the expression of cell surface molecules, because it does not change migratory patterns and lymphocyte proliferative responses either (Weston and Parish, 1990).

The asthma-like inflammation was induced with a single OVA-challenge, as described before (Skripuletz et al., 2007), and the protocol used for the transfer of cells was adapted from protocols from adoptive transfer experiments in mice (Hansen et al., 1999). Only a few transferred CFSE-positive cells were detectable in the parenchyma of the lung 22 hr after OVA-challenge. No significant differences were seen in the recipients. Screening of different lung compartments revealed a significantly increased T-cell number in the BALT of CD26-deficient rats compared with the BALT of wild-type rats. This finding was only detectable due to the exact examination of all lung compartments in the morphometric analyses, as flow cytometric analyses failed to detect these low cell numbers. An explanation of the traceability of transferred cells in the BALT might be the very high density of lymphocytes at this site compared to the cell density in the other compartments. These lymphocytes enter the BALT by HEVs, which may have a different pattern of surface molecules than other lung endothelial cells, and the entrance might be enhanced in CD26-deficient rats. Other groups have shown a lymphocyte migration into BALT in a different experimental setup before (Sato et al., 2000). An accumulation of transferred T cells was not found in the BALT of naïve animals, due to the frequent absence and small size of BALT under these conditions (Pabst et al., 2008). In general, a transfer of T cells isolated from lymph nodes does not fulfil ideal conditions in a migration study, because cells from the lymph nodes contain a considerable amount of anchored lymphocytes that would not migrate physiologically (Walter et al., 1995). However, compared to studies from other groups, who determined the physiological migration of thoracic duct lymphocytes (Sato et al., 2000), our results show a similar distribution of T cells in the lungs, for example with their appearance in the BALT, supporting the efficiency of our model.

We hypothesized that a higher number of T cells would be found in the lungs if CD26 were involved, because of its adhesion properties. However, the results failed to support this hypothesis, as more T cells were found in the BALT of CD26-deficient lungs. Results from in vitro studies of other groups also failed to show an involvement of CD26 on T cells in adhesion to endothelial cells (Mattern et al., 1998). Even if the mutant CD26-deficient rats in our study did not represent a protein-knock-out model for CD26, as stated by other groups (Cheng et al., 1999), this would not explain the different distribution of cells found in this case. Therefore, further studies have to be performed to explain the

potential role of CD26 in adhesion and to explain the functions of CD26 expression or its loss in the BALT during asthma-like inflammations.

Our group has suggested a role of CD26 in asthma-like inflammations before, as previous studies showed a less pronounced inflammation in CD26-deficient rats (Kruschinski et al., 2005). Fewer T-cell numbers were found in the BAL and around the medium and large bronchi (unpublished data). Because of the vast number of cells in the BALT of OVA-challenged animals, we did not count T cells in this compartment of the lung. Differences in the T-cell density in the BALT might be due to a differential expression or activity of a T-cell chemoattractant that is a substrate of CD26 at this site.

Therefore, we screened for one of the candidates and found preliminary evidence of a higher expression of SDF-1α in the BALT of CD26-deficient rats. SDF-1 is a substrate of CD26 (Mentlein, 1999) and N-terminal cleavage by CD26 causes its inactivation (Sun et al., 2008). The immunoreactivity of SDF-1 is significantly increased within the airways of asthmatic patients and its expression is upregulated in asthmatic tissues (Hoshino et al., 2003). SDF-1 is not inactivated in CD26-deficient lungs and more SDF-1-positive spots were found in the BALT of these lungs. These observations represent potential reasons for a differential T-cell recovery after asthma induction and the fact that the overall asthma reaction is less pronounced in CD26-deficient rats, leads to the hypothesis that CD26 plays a negative regulatory role in the BALT-specific adhesion of these cells during asthma.

4.5 Acknowledgements

This study was supported by a grant of the German Research Foundation (SFB 587, project B11). We thank Florian Schwichtenhoevel, Susanne Kuhlmann, Susanne Fassbender, Andrea Herden, and Sabine Fiedler for technical assistance, and Sheila Fryk for the correction of the English.

4.6 References

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4.7 Figures

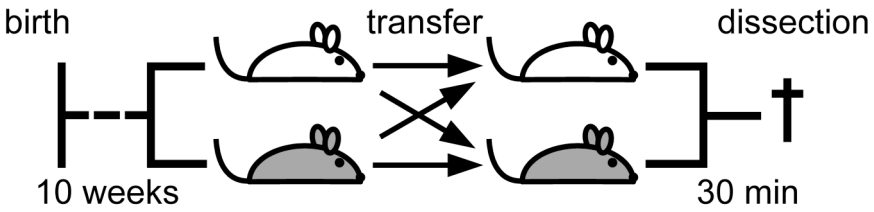

Fig. 1. Schematic overview of the first experimental setup.

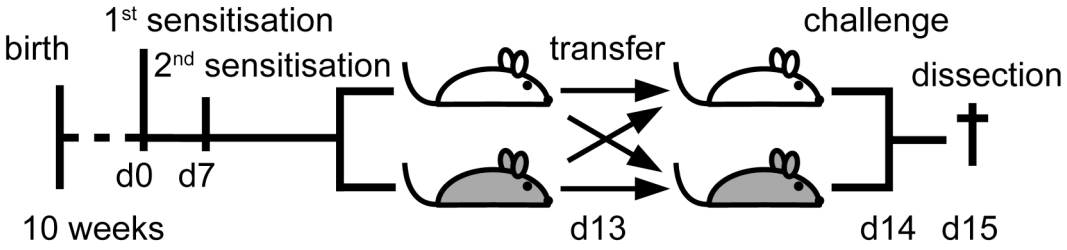

Fig. 2. Schematic overview of the second experimental setup.

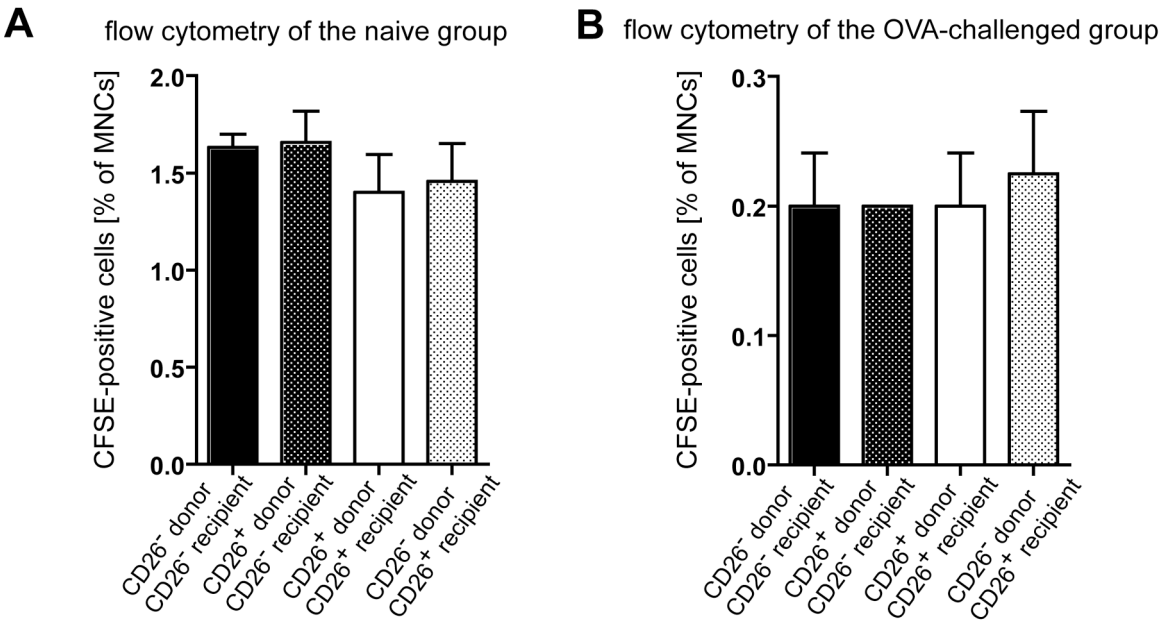

Fig. 3. Flow cytometrical detection of transferred T cells under different conditions in the lung. (A) Flow cytometrical detection of CFSE-positive cells in lung suspensions of the naïve group. (B) Flow cytometrical detection of CFSE-positive cells in lung suspensions of the OVA-challenged group. MNCs, mononuclear cells.

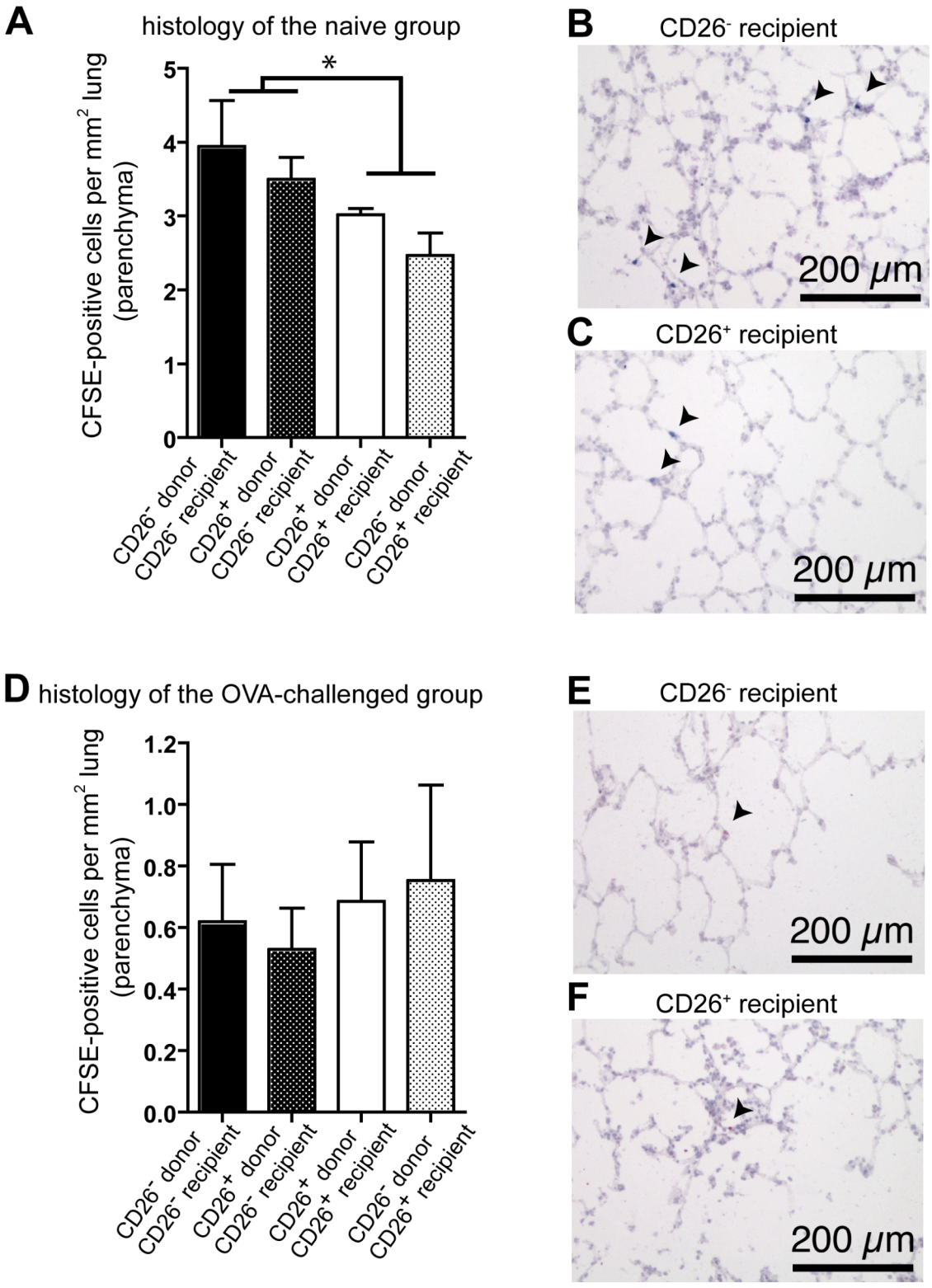

Fig. 4. Histological detection of transferred T cells under different conditions in the lung. (A) Histological detection of CFSE-positive cells per mm^2 lung in the parenchyma of the naïve group. (B, arrowheads) CFSE-positive cells in a representative micrograph of a $CD26^-$ recipient, and (C, arrowheads) of a $CD26^+$ recipient. (D) Histological detection of

CFSE-positive cells per mm^2 lung in the parenchyma in the OVA-challenged group. (E, arrowhead) CFSE-positive cell in the lung parenchyma of a $CD26^-$ recipient, and (F, arrowhead) of a $CD26^+$ recipient. (*$p<0.05$)

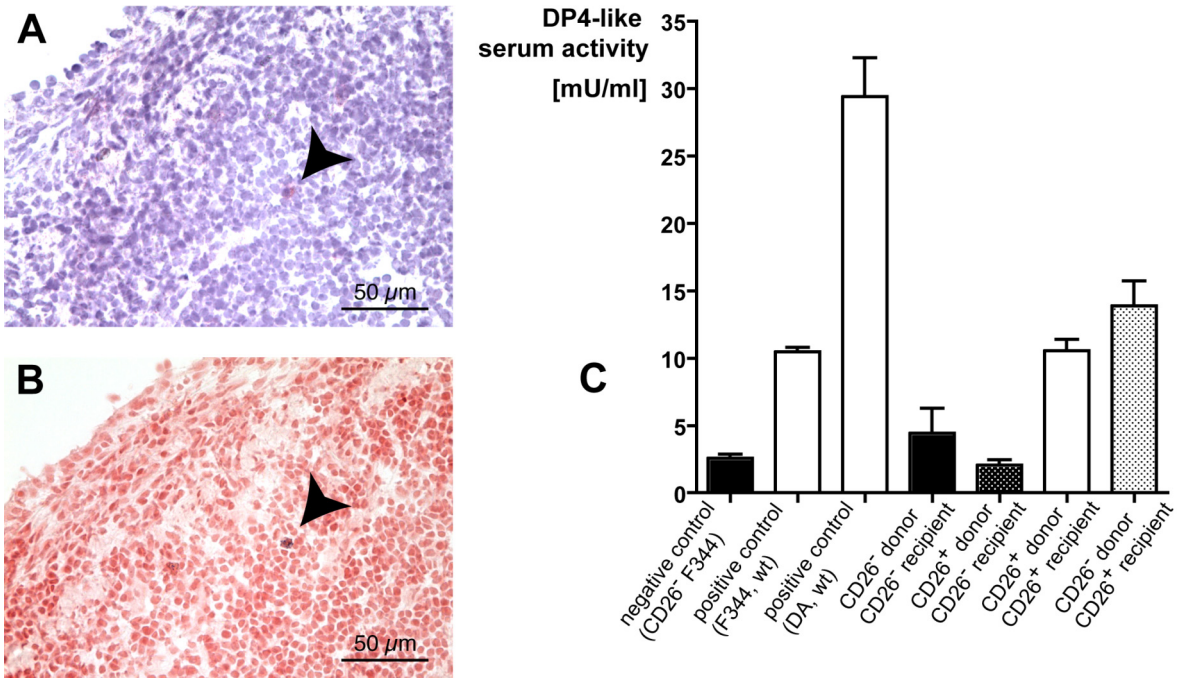

Fig. 5. Detection of CD26 expression and DP4-like activity in $CD26^-$ lungs and DP4-like activity in EDTA-plasma samples of different recipients. (A, arrowhead) CD26-expressing cell in the BALT of a CD26-deficient lung and (B, arrowhead) detection of DP4-like activity in the same area on the following serial section. (C) DP4-like activities in EDTA-plasma samples of all groups including untreated positive and negative controls of different rat strains. wt, wild-type; DA, Dark Agouti.

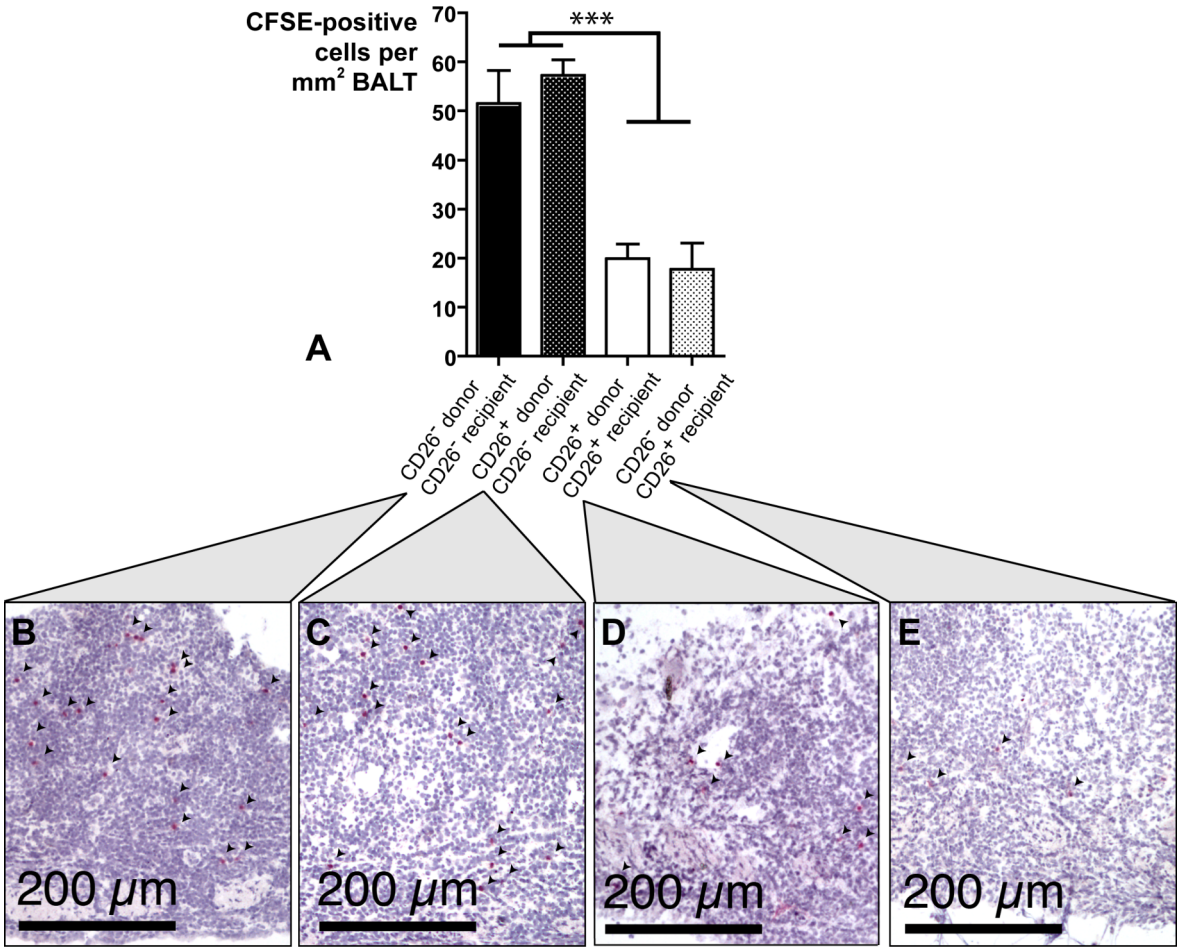

Fig. 6. Histological detection of transferred T cells in the BALT of OVA-treated lungs. (A) Overview of CFSE-positive cells in the BALT. Representative micrographs showing the distribution of (B, arrowheads) $CD26^-/CFSE^+$ cells in the BALT of $CD26^-$ lungs, (C, arrowheads) $CD26^+/CFSE^+$ cells in the BALT of $CD26^-$ lungs, (D, arrowheads) $CD26^+/CFSE^+$ cells in the BALT of $CD26^+$ lungs, and (E, arrowheads) $CD26^-/CFSE^+$ cells in the BALT of $CD26^+$ lungs. (***$p<0.001$)

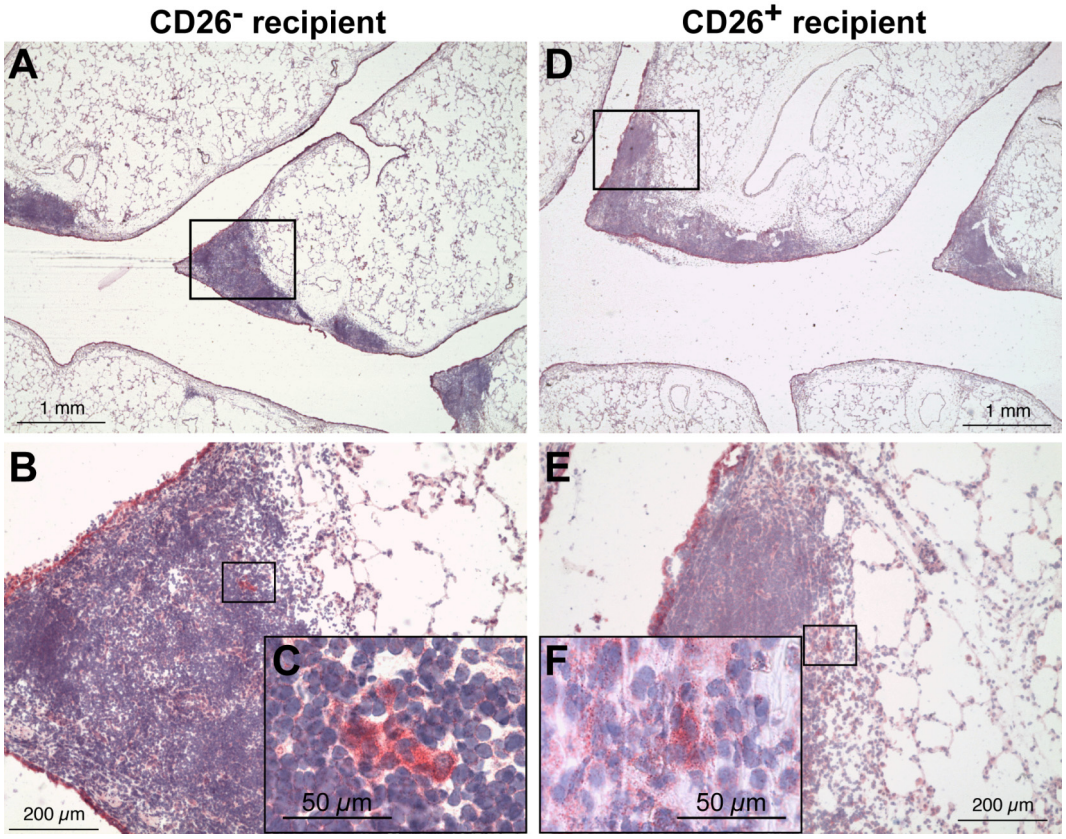

Fig. 7. Histological detection of SDF-1α in the lungs. Representative micrographs showing (A) the distribution of SDF-1α in the lungs of $CD26^-$ rats, (B) magnification of the BALT, and (C) magnification of SDF-1α-positive cells in the BALT of $CD26^-$ lungs. Representative micrographs showing (D) the distribution of SDF-1α in the lungs of $CD26^+$ rats, (E) magnification of the BALT, and (F) magnification of SDF-1α-positive cells in the BALT of $CD26^+$ lungs.

5 Contribution

The article “Regulation of expression and function of dipeptidyl peptidase 4 (DP4), DP8/9, and DP10 in allergic responses of the lung in rats” by J. Schade, M. Stephan, A. Schmiedl, L. Wagner, A. J. Niestroj, H.-U. Demuth, N. Frerker, C. Klemann, K. A. Raber, R. Pabst, and S. von Hörsten published in the Journal of Histochemistry and Cytochemistry, Vol. 56, No. 2, pp. 147-155 (2008) was written by myself. I carried out most of the methods and evaluations described in this article, except for the RNA isolation and following reverse transcription, and the dissection of the animals.

The article “Airway-specific recruitment of T cells is reduced in a CD26-deficient F344 rat substrain” by J. Schade, A. Schmiedl, A. Kehlen, T. Z. Veres, M. Stephan, R. Pabst, and S. von Hörsten submitted in Clinical and Experimental Immunology is under revision at the moment and was written by myself. I carried out most of the methods and evaluations described in this article, except for the quantitative real-time PCR.

The article “Transferred T cells preferentially adhere in the BALT of CD26-deficient recipient lungs during asthma” by J. Schade, A. Schmiedl, M. Stephan, R. Pabst, and S. von Hörsten submitted in Immunobiology was written by myself and I carried out all of the methods and evaluations described in this article.

6 Discussion

6.1 Use of animal models

Modelling diseases in animals is frequently used in biomedical research to study the pathogenesis of diseases and to evaluate potential treatments. Validity and predictability of such models is critical for the value of preclinical research. A detailed characterization of disease processes in these models represents, therefore, an important step. A priori, already the decision of the species used for the induction of a particular disease has far-reaching consequences.

Due to the advanced transgenic technology, mice are commonly used as mammalian models, but also other rodents such as rats and guinea pigs, and higher mammals such as dogs, cats, sheep, pigs, and monkeys are used. Each species has its own advantages and disadvantages and no model is fully identical to the conditions found in human diseases.

In contrast to several other diseases, asthma does not occur in animals, therefore, only asthma-like inflammations can be induced in animals by sensitising and challenging them according to given protocols. A common protocol used for rat asthma models is a twofold sensitisation with ovalbumin (OVA), aluminium hydroxide ($Al(OH)_3$), and *Bordetella pertussis* on day 0 and day 7, followed by an inhalation challenge with aerosolized OVA on day 14 [59]. This provocation leads to an acute airway response and a late-phase reaction in rats, but not in mice [60]. Furthermore, the morphology of the airways shows some differences. Rats, as well as other rodents, exhibit a monopodial bronchial branching pattern, which is fundamentally different compared to human's dichotomal branching pattern. Mice and rats have rather thin layers of submucosa and bronchial muscles, but compared to mice, the structure of the submucosal and bronchial smooth muscle layers is more pronounced in rats. Mice lack bronchial arteries, but rats, just like humans, have bronchial arteries, capillaries, and veins in the subepithelial connective tissue within the bronchial wall, which make the rat a good model species for bronchial asthma [61].

The multiplicity of different genetic techniques to silence or knockout specific genes in animals even simplifies the possibilities to investigate the impact of these genes and their products in different diseases. In different rat strains, the expression and activity of DP4 is varying and due to a spontaneous mutation even absent in some substrains.

Fischer 344 (F344) rats for example exhibit an intermediate DP4-like enzymatic activity compared to other rat strains such as Lewis (lowest activity) or Dark Agouti (highest activity) [62]. A spontaneous mutation in the DP4 gene in two substrains of F344 rats (F344/Crl(Wiga)SvH-Dpp4m and F344/Crl(DuCrj)SvH-Dpp4m) causes the retention of DP4 in the endoplasmic reticulum (ER), which leads to its rapid degradation in these substrains [63]. Thus, these animals do not express functional DP4 on their cell surfaces, their DP4-like activity is reduced to a minimum, and they are considered as DP4-deficient [64]. The availability of different rat substrains with varying levels of DP4 facilitates their use to examine the impact of DP4 and its loss under normal conditions and in models of different diseases *in vitro* and *in vivo*.

6.2 Expression of different dipeptidyl peptidases in the lungs

DP4 is a multifunctional protein, and the lung is the organ with the second-highest DP4-like activity [15]. The detection of DP4-homologous peptidases in the last decade raised the question if they account for functions of DP4 and if they are involved in diseases according to these functions [65]. Therefore, not only the expression pattern and activity of DP4 were studied in the lungs in the first part of this thesis, but also the expression patterns of DP4-homologues were examined. First of all, the expression patterns of DP4, DP8, and DP9 in the lungs were characterized by means of their peptidase activity. A histochemical activity assay was established and performed with a substrate that is specific for DP4, DP8, and DP9 [66], which facilitated the detection of these three peptidases in lungs of wild-type rats. In lungs of DP4-deficient rats, only a staining for DP8 and DP9 was expected. In addition to the genetic deficiency model used in this study, also a pharmacological inhibition of DP4 was performed in this assay. Genetic deficiency and pharmacological inhibition both revealed the same results, namely different activity patterns for DP4 and DP8/9. While this assay produced a strong staining for DP4 activity in the lung parenchyma of the wild-type rats, the activity of DP8/9 was weak and preferentially found in the bronchi. To distinguish between DP8 and DP9 expression in the lungs and to further verify the expression patterns of the three functional homologues DP4, DP8, and DP9, as well as the structural homologous DP10, immunohistochemistry with specific antibodies was performed. These stainings supported the results of the histochemical activity assay and revealed a similar staining for DP8, DP9, and DP10 in the bronchi, whereas DP4 was

strongly expressed in the parenchyma of lungs from wild-type rats but was lacking in DP4-deficient rats. The expression pattern of DP4 in wild-type rat lungs is in accordance with the pattern in human lungs, where DP4 expression was not detectable in the bronchial epithelium [67, 68].

Additional quantitative real-time (qrt) PCR analyses revealed the expression of DP4, DP8, and DP9 in lung samples of all rat substrains. DP4 mRNA expression in DP4-deficient rats was not surprising, as a functionally inactive form of DP4 is expressed in these animals due to a spontaneous mutation. The primer pairs used for this analysis lay outside the mutated region and therefore, amplified DNA of the mutated DP4 was expressed on a normal level, as described before [69]. In addition, the functional homologues of DP4, DP8 and DP9, did not show any compensatory effects due to the lack of DP4 expression in mutant rats, consistent with studies in DP4 knockout mice [70].

PCR analyses of DP10 expression revealed a strong expression of DP10 in the brain and a weaker expression in the trachea [58, 71], while expression in lung samples was not detectable [72]. PCR analyses of isolated bronchi revealed a very weak expression of DP10 in the bronchi, consistent with findings from the histological analyses.

6.3 Expression of dipeptidyl peptidases in asthmatic rat lungs

Various physiological processes can be regulated by the N-terminal cleavage of substrates by DP4. For example cleavage of chemokines often modifies their activity status as well as their receptor specificity. The additional functions of DP4 as a costimulatory molecule in T-cell activation [4] and in cell adhesion by binding of ECM molecules [5] might also contribute to a role of DP4 in diseases. All properties of DP4 might be involved in the pathogenesis of asthma, as it is partly mediated by chemokines, activated T cells are one of the key players in this disease, and the adhesion of T cells in the lungs might be mediated via collagen binding. DP4 homologous peptidases might also participate in the course of asthma due to their site-specific expression in the lungs. Additionally, genetic studies have already identified the gene of the human DP10 as a locus for asthma susceptibility [58]. Therefore, a potential regulation of DP4 and its homologues in the pathogenesis of allergic asthma was studied in the next part of this thesis.

A histochemical activity assay was performed that revealed a slight upregulation of DP4-like activity on lung slices after asthma-induction in wild-type rats. This upregulation was also visible on lung slices stained with an antibody against DP4. These findings were further verified in the BAL of these lungs by an *in vitro* activity assay. The documented upregulation of DP4-like activity in the BAL fluid is contrary to observations from others [68]. They did not observe differences between allergic asthmatics and healthy non-smokers. As DP4-like activities in the BAL fluid and the lung might at least partially depend on activated CD4-positive T-cells [73], an unaltered T-cell number in allergic asthmatics would explain their results [74]. Previous studies from our group have shown an elevated number of activated DP4-positive T-cells after asthma induction in a rat model [59], which is consistent with findings in adult patients with allergic asthma [75]. These studies together with the important role of T cells in the pathogenesis of asthma [57] support the presented upregulation of DP4-like activity.

PCR analyses of lung samples revealed an upregulation of DP8 and DP9, but no regulation of DP4 was detectable 22 hours after asthma-induction. The significant upregulation of DP4 activity in the BAL might be due to a rapid response of DP4 to the stimulus and therefore, its mRNA expression might already be on the downslope 22 hours after challenge. Elevated DP8 levels have been documented in activated lymphocytes [43, 65], and the contribution of DP8/9 to the overall DP4-like activity and their necessity for T-cell proliferation have been demonstrated using selective inhibitors [26, 46]. Therefore, the upregulation of DP8 and DP9 furthermore suggests its involvement in diseases where T-cell activation occurs.

In contrast to the regulation of the functional homologues of DP4, regulation of the DP4-structural homologue DP10 was not detectable in the lungs. However, genetic studies in humans revealed an association of the gene of DP10 with asthma prevalence [58] and DP10 is described to associate with Kv4-mediated A-type potassium channels [51]. These facts together with the fact that DP10 is exclusively expressed in the bronchi of the lungs, might point to an involvement of the bronchus-associated nervous system in the pathogenesis of asthma.

As asthma is a disease of the airways and DP8 and DP9 are primarily expressed and also upregulated in the bronchi, these results suggest that these peptidases specifically respond to the inflammatory stimulus. Therefore, not only DP4 might be involved in the

pathogenesis of asthma, but also its homologues DP8, DP9, and DP10 might contribute to this disease. Further studies are necessary to elucidate the role of these peptidases in allergic diseases. But as different patents from the University of Sydney were granted in the meantime that protect DP8 and DP9 in all forms, including their gene sequence and antibodies against them (WO 01/19866, WO 02/34900), studies regarding these peptidases had to be stopped.

6.4 Distribution of T cells in the lungs of naïve and asthmatic rats

Studies with wild-type F344 rats and DP4-deficient rat substrains in our novel model of an asthma-like inflammation in F344 rats revealed that DP4 is directly associated with the recruitment of T cells to the lungs and with IgE production. Less T cells were found in the bronchoalveolar lavage (BAL) of DP4-deficient rats, as well as lower OVA-specific IgE-levels in serum, which represents a lower asthmatic-like reaction in these substrains [76]. Also a dose-dependency in the recruitment of T cells and eosinophils in F344 wild-type rats was demonstrated together with allergen-specific early airway responsiveness and antigen-specific IgE-levels [59]. As the previous part of this thesis revealed a site-specific expression of different dipeptidyl peptidases and their regulation during asthma, the next part of this thesis focussed on the distribution of T cells that are one of the key players in this disease. Previous studies regarding the recruitment of T cells to the lungs were based on flow cytometric analyses of the BAL and of cell suspensions from whole lobes of the right lungs. Consequently, following studies were based on immunohistochemical stainings and morphometrical analyses. Immunostaining of T cells in the lungs revealed that their number increased after the induction of an asthma-like inflammation in wild-type and DP4-deficient rats. This was consistent with previous flow cytometric analyses [59, 76]. In the lung parenchyma, these T cells were equally distributed in both substrains. Examination of the large, medium, and small airways revealed significant differences between the two substrains, with more T cells in the large and medium bronchi of wild-type rats. A similar distribution was documented for activated DP4/CD4-positive T cells. Surprisingly, these results illustrated a difference between DP4-expressing and DP4-deficient rats in a compartment that almost completely lacks DP4 expression as well as DP4 activity [77]. Therefore, this effect might be due to a different microenvironment surrounding the T cells in this area, which interacts with DP4.

6.5 Differences in the peribronchial microenvironment

As a potential interaction of DP10 with the bronchus-associated nervous system was suggested before [77], colocalization studies of T cells together with nervous system-derived factors like $p75^{NTR}$ were performed by immunohistochemistry. No overlap of these $p75^{NTR}$-positive structures and T-cell areas was revealed. In the meantime, other studies have documented that dendritic cell-nerve clusters are sites of T cell proliferation in allergic airways [78], and verified our findings showing no direct interaction between nerves and T cells at these sites. Therefore, we investigated whether the expression or lack of DP4 and its resulting functions, potentially together with a differential chemokine pattern at these sites, might be responsible for the presented outcome.

Screenings for two potential T-cell chemoattractors were performed in qrtPCR studies with material from parenchyma and large bronchi of wild-type and DP4-deficient naïve and OVA-challenged rats, namely MCP-1 and SDF-1. MCP-1 is an attractor of T cells that is not cleaved by DP4 [79]. The absence of MCP-1-mediated signals results in abolished pulmonary T-accumulation in a mouse model [80]. It is increased in the BAL fluid of allergic asthmatics [81] and its gene regulatory region is potentially associated with asthma susceptibility [82]. T cells highly expressing DP4 carry receptors for MCP-1 and respond in a chemotaxis assay [83]. As qrtPCR analyses in our study revealed a significantly higher expression of MCP-1 in the large airways of wild-type and DP4-deficient OVA-challenged rats compared to unchallenged rats, MCP-1 might be responsible for the accumulation of T cells at these sites. The twofold higher expression of MCP-1 in the wild-type rats might attract even more T cells to the airways, which results in a substrain-specific difference in this compartment. The overall increased number of T cells in the lung parenchyma after OVA-challenge corresponds to an increased expression of MCP-1 in the lung parenchyma. However, no substrain-specific differences were found.

In contrast to MCP-1, qrtPCR analyses of SDF-1 did not reveal any treatment-specific differences, but a significant substrain-specific difference in the large airways. SDF-1 is a substrate of DP4 and N-terminal cleavage by DP4 causes its inactivation [84]. Its higher level of expression in the large airways of wild-type rats might be caused by the degradation of SDF-1 by DP4 and its following upregulation. The further increase of SDF-1 after OVA-challenge in the large airways of wild-type rats might result in a stronger expression of this DP4-substrate and therefore attract more DP4-positive T cells to these

sites. The upregulation of DP4 on the surface of activated T cells [17] might even potentiate these processes. These findings therefore suggest that the microenvironment around the airways mediates the differential peribronchial T-cell increase via DP4.

As DP4 is a potential mediator of adhesion to ECM proteins, this function of DP4 might also explain a higher number of T cells around the airways of wild-type rats. Interaction of lung endothelial DP4 and fibronectin in adhesion and metastasis of rat breast cancer cells has been shown before [36], but its role under normal conditions has not been examined in depth.

6.6 Adhesion properties of DP4 in a crosswise transfer of T cells

Because DP4 is highly expressed in the lungs and on a large population of T cells, a cross-transfer of T cells was performed and their recovery was evaluated in the lungs under naïve and asthmatic conditions in the last part of this thesis. The use of wild-type and DP4-deficient F344 rats as donors and recipients in a crosswise transfer even facilitates the differentiation between adhesion properties of lung DP4 and DP4 on T cells in this study. The recovery of the transferred T cells in the recipient lungs under naïve conditions and after the induction of an asthma-like inflammation was evaluated using flow cytometry and immunohistochemical stainings.

Initial experiments on the origin of transferred T cells, the sorting method, the injected dose, and the time of incubation were performed. The mesenterial lymph nodes were chosen instead of the spleen, because relatively more T cells were found in the lymph nodes. Negative selection via a method based on magnetic-activated cell sorting was chosen since positive selection might activate the selected T cells and fluorescence-activated cell sorting resulted in a vast number of dead cells. Different injected doses were tested and 15×10^6 T cells seemed to be sufficient for transfer. Thirty minutes incubation resulted in an average recovery rate of the transferred T cells in the lungs of naïve animals. Therefore, the lungs of the recipient animals were removed 30 min after the transfer under naïve conditions in the first experimental setup. According to the protocol used in our rat asthma model, the lungs were removed 22 hr after the induction of an asthma-like inflammation in the second experimental setup. Subsequent flow cytometric analyses of lobes from the right lungs did not reveal any significant differences in all groups.

Corresponding morphometric analyses resulted in a significant genotype-specific effect under naïve conditions with less CFSE-positive cells in wild-type rats. The different lung compartments examined with these methods might explain their differential outcome. Namely, in flow cytometric analyses, cell suspensions of the whole right lungs were examined, but histological analyses were focused on the parenchyma of the lungs. This effect might represent a different T-cell number in the vessels, for example. Intravenous injection of lymphocytes leads to a quick accumulation of these cells in the lungs [85, 86], potentially because it is the first capillary bed. T cells transferred to wild-type rats might arrive in the lungs more quickly and might already be on their way out of the lungs after 30 min compared to T cells transferred to DP4-deficient rats. Only very few cells were detectable after the induction of an asthma-like inflammation in the second experimental setup.

While flow cytometric analyses were performed with cell suspensions from lobes of the right lungs, histological morphometric analyses facilitated the examination of different lung compartments, such as parenchyma, bronchi, and bronchus-associated lymphatic tissue (BALT). The exact examination of the BALT revealed a significantly increased T-cell number in CD26-deficient rats compared with wild-type rats. A potential explanation of the traceability of transferred cells in this compartment might be the very high density of lymphocytes at this site compared to the cell density in the other compartments. Such a BALT-specific accumulation has been shown before after the transfer of physiologically migrating thoracic duct lymphocytes [87]. Our results are similar, although lymphocytes from lymph nodes were used that potentially contain a considerable amount of anchored lymphocytes, and would not migrate physiologically [88]. Lymphocytes enter the BALT by high endothelial venules (HEVs) that may express different surface molecules than other lung endothelial cells that have been used in studies with cancer cells [36]. BALT is frequently absent or small-sized in naïve rats [89], therefore no differences were found in these animals.

Furthermore, DP4-positive cells were additionally detected using an antibody against DP4 and in a histochemical activity assay. Both staining methods revealed DP4-positive cells in DP4-negative lungs, although fewer DP4-positive cells than CFSE-positive cells were found. This might be explained by a shedding of DP4 during the procedure. Flow cytometric analyses after the magnetic separation of T cells revealed an expression of DP4

on about 90% of the T cells from wild-type rats. The expression of DP4 was not checked after the CFSE-labelling, but this labelling alone should not alter the expression of cell surface molecules, because it does not change migratory patterns and lymphocyte proliferative responses either [90]. Because propidium iodide stainings in the flow cytometry did not reveal many dead cells, possibly only highly DP4-positive cells can be identified with the antibody and in the activity assay.

An additional screening of the BALT was performed with an immunohistochemical staining for one of the DP4-substrates that represents a T-cell chemoattractor. Staining for SDF-1 provided preliminary evidence for a higher expression in the BALT of DP4-deficient rats. SDF-1 was reported to be upregulated in qrtPCR analyses of the large airways of wild-type rats in the previous part of this thesis. No differences were found in the parenchyma, and BALT was not included in these analyses. The findings in the large airways were explained by the inactivation of SDF-1 by DP4 and its following upregulation. More T cells were found peribronchially in the wild-type rats in that study, but there were no differences in the numbers of the transferred cells around the bronchi in the coisogenic transfer model, potentially due to the overall low cell numbers. The BALT-specific accumulation of the transferred cells was documented in DP4-deficient rats, independent of the genotype of the transferred cells. SDF-1 is not inactivated by DP4 in these DP4-deficient rats and more SDF-1-positive spots were found in the BALT of their lungs. Therefore, the expression of SDF-1 in its activated or inactivated form might represent one of the reasons for a differential recruitment of the T cells to the BALT. In addition, the fact that the overall allergic inflammation is less pronounced in DP4-deficient rats [76] leads to the hypothesis that DP4 plays a negative regulatory role in the BALT-specific adhesion of these cells during asthma.

As several groups have investigated the association between DP4-expression, tumour-cell adhesion, and metastasis in different experimental setups [37, 38] and revealed a role of DP4 in adhesion processes, we hypothesised that a higher number of T cells would be found in the lungs if DP4 is involved. We expected a clear-cut outcome in the cross-transfer studies with either more transferred T cells in DP4-positive lungs or more DP4-positive T cells in the recipient lungs. However, the results of these experiments failed to support our hypothesis, as more T cells were found in the BALT of DP4-deficient lungs. Results from other groups also failed to verify an involvement of DP4 on T cells in

adhesion to endothelial cells [39]. Although other studies stated that the mutant DP4-deficient rats used in our study do not represent a protein-knock-out model for DP4 [37], this does not explain our results. The role of DP4 in adhesion processes, especially under normal conditions or conditions different from cancer, still remains unclear. The fibroblast activation protein (FAP) is a functional homologue of DP4 and forms heterodimers together with DP4 [24]. It is only expressed on reactive stromal fibroblasts of epithelial cancers and healing wounds [42]. Therefore, it might as well be involved in cell adhesion, possibly as a heterodimer with DP4, which would explain a decreased metastasis in cancer-models if DP4 is non-existent.

6.7 Conclusion

In conclusion, this thesis gives an insight in the expression and potential functions of DP4 in normal and asthmatic rat lungs, and for the first time analysed the role of DP4 in the recruitment and adhesion of T cells to the lungs.

The F344 rat model used in this thesis has been shown to represent many features of human bronchial asthma and DP4 was shown to be involved in the asthmatic reaction in this model. This thesis clearly shows the necessity of molecular biological, biochemical, and morphological, as well as morphometrical methods to determine the distribution, enzymatic activity, and expression levels of DP4 and its homologues in the lungs of wild-type and DP4-deficient rats.

A differential and site-specific DP4-like expression and function in the lungs, together with differences in the peribronchial T-cell accumulation after the induction of an asthma-like inflammation, strongly suggest a pathophysiologically significant role of DP4 in asthma. This role might be mediated by chemokines that represent substrates of DP4. Further studies have to be performed to quantify local chemokine-levels, to reveal N-terminal truncated and full-lengths forms of DP4-subtrates in the lungs, and to determine the impact of DP4 homologous peptidases in all processes that were thought to be mediated by DP4. The exact examination of the lung compartments in this thesis revealed totally different outcomes in the compartments, with special regard to the expression of chemokines and peptidases. Therefore, one has to bear in mind that organs cannot always be regarded as a whole, but that all compartments have to be examined in detail due to their potential

different functions. The same is necessary for the DP4-like peptidases that might have similar functions, but do not have to.

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8 Curriculum Vitae

Personal details

Name	Jutta Schade
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Date of birth	19th September 1980
Place of birth	Papenburg, Germany

Education and qualifications

since 2005	Scientific assistant and doctoral student in a project of the SFB 587 (“Immune reactions of the lung in infection and allergy“) at the Institute of Functional and Applied Anatomy, Hannover Medical School, Germany
2004 – 2005	Diploma thesis at the Helmholtz Centre for Infection Research (former German Research Center for Biotechnology), Braunschweig, Germany Title: „Creation of a recombinant adenovirus with an anti-TLR2 intrabody“
2000 – 2005	Study of biology, Carl von Ossietzky University, Oldenburg, Germany
2000	Abitur (high school diploma), Staatliches Gymnasium, Papenburg, Germany
1987 – 2000	Primary and Grammar School

Research experience

March 2003	Laboratory work in the Lung Pharmacology, Research Center Borstel, Germany
July 2000	Laboratory work in the Erken Laboratory, University of Uppsala, Sweden

Conferences/workshops

May 2008	American Thoracic Society, International Conference 2008, Toronto, Canada: **Schade J**, Schmiedl A, Stephan M, Pabst R, von Hörsten S: CD26 as a mediator in the differential increase of T cells during an allergic-like inflammation in rat lungs.
April 2008	3rd Conference on Dipeptidyl Peptidases and Related Proteins, Antwerp, Belgium: **Schade J**, Schmiedl A, Stephan M, Pabst R, von Hörsten S: DPP4 expression contributes to the influx of T cells to the bronchi of rat lungs during an allergic-like inflammation.
March 2008	4th Spring School on Immunology, German Society of Immunology, Ettal, Germany: **Schade J**: Dipeptidyl peptidase 4 and related peptidases in rat lungs.
November 2006	Autumn Meeting of the Section of Cell Biology, *Deutsche Gesellschaft für Pneumologie und Beatmungsmedizin e.V.*, Mainz, Germany: **Schade J**, Stephan M, Wagner L, Pabst R, Schmiedl A, von Hörsten S: Hochregulation der Prolin-spezifischen Peptidasen DP4, DP8 und DP9 im Asthma bronchiale.

September 2006

23rd Annual Workshop of the *Anatomische Gesellschaft*, Wuerzburg, Germany:

Schade J, Stephan M, Wagner L, Niestroj AJ, Frerker N, Pabst R, Schmiedl A, von Hörsten S:

Die Bedeutung prolinspezifischer Peptidasen beim Asthma bronchiale - Histochemischer Nachweis von DP4- und DP4-ähnlicher Enzymaktivität auf Lungenschnitten.

9 Publication list

Original publications

Frerker N, Wagner L, Wolf R, Heiser U, Hoffmann T, Rahfeld JU, **Schade J**, Karl T, Naim HY, Alfalah M, Demuth HU, von Horsten S (2007) Neuropeptide Y (NPY) cleaving enzymes: structural and functional homologues of dipeptidyl peptidase 4. Peptides 28(2):257-268

Skripuletz T, Schmiedl A, **Schade J**, Bedoui S, Glaab T, Pabst R, von Horsten S, Stephan M (2007) Dose-dependent recruitment of CD25+ and CD26+ T cells in a novel F344 rat model of asthma. Am J Physiol Lung Cell Mol Physiol 292(6):L1564-1571

Schade J, Stephan M, Schmiedl A, Wagner L, Niestroj AJ, Demuth HU, Frerker N, Klemann C, Raber KA, Pabst R, von Horsten S (2008) Regulation of expression and function of dipeptidyl peptidase 4 (DP4), DP8/9, and DP10 in allergic responses of the lung in rats. J Histochem Cytochem 56(2):147-155

Forssmann U, Stoetzer C, Stephan M, Kruschinski C, Skripuletz T, **Schade J**, Schmiedl A, Pabst R, Wagner L, Hoffmann T, Kehlen A, Escher SE, Forssmann WG, Elsner J, von Horsten S (2008) Inhibition of CD26/dipeptidyl peptidase IV enhances CCL11/eotaxin-mediated recruitment of eosinophils in vivo. J Immunol 181(2):1120-1127

Klemann C, **Schade J**, Pabst R, Leitner S, Stiller J, von Horsten S, Stephan M (2009) CD26/dipeptidyl peptidase 4-deficiency alters thymic emigration patterns and leukcocyte subsets in F344-rats age-dependently. Clin Exp Immunol 155(2):357-365

Frerker N, Raber K, Bode F, Skripuletz T, Nave H, Klemann C, Pabst R, Stephan M, **Schade J**, Brabant G, Wedekind D, Jacobs R, Jörns A, Forssmann U, Straub RH, Johannes S, Hoffmann T, Wagner L, Demuth HU, von Horsten S (2009) Phenotyping of congenic dipeptidyl peptidase 4 (DP4) deficient Dark Agouti (DA) rats suggests involvement of DP4 in neuro-, endocrine, and immune functions. Clin Chem Lab Med 47(3):275-287

Schade J, Schmiedl A, Kehlen A, Veres TZ, Stephan M, Pabst R, von Horsten S (2009, Clin Exp Immunol, under revision) Airway-specific recruitment of T cells is reduced in a CD26-deficient F344 rat substrain.

Schade J, Schmiedl A, Stephan M, Pabst R, von Horsten S (2009, Immunobiol, submitted) Transferred T cells preferentially adhere in the BALT of CD26-deficient recipient lungs during asthma.

Abstract publications

Schade J, Stephan M, Wagner L, Niestroj AJ, Frerker N, Pabst R, Schmiedl A, von Hörsten S: Die Bedeutung prolinspezifischer Peptidasen beim Asthma bronchiale - Histochemischer Nachweis von DP4- und DP4-ähnlicher Enzymaktivität auf Lungenschnitten. DOI: 10.3337/anatges.2006.0003

Schade J, Stephan M, Wagner L, Niestroj AJ, Pabst R, Schmiedl A, von Hörsten S: Hochregulation der Prolin-spezifischen Peptidasen DP4, DP8 und DP9 im Asthma bronchiale. Pneumologie. 61, 2007; DOI: 10.1055/s-2007-967222

Schade J, Schmiedl A, Stephan M, Pabst R, von Hörsten S: DPP4 expression contributes to the influx of T cells to the bronchi of rat lungs during an allergic-like inflammation. Clin Chem Lab Med. 46(4):A33, April 2008

Schade J, Schmiedl A, Stephan M, Pabst R, von Hörsten S: CD26 as a mediator in the differential increase of T cells during an allergic-like inflammation in rat lungs. Am J Respir Crit Care Med. 177(Abstracts Issue):A364, April 2008

10 Erklärung zur Dissertation

Hierdurch erkläre ich, dass die Dissertation “Expression and potential functions of dipeptidyl peptidase 4 (CD26) in the asthmatic rat lung” selbstständig verfasst und alle benutzten Hilfsmittel sowie evtl. zur Hilfeleistung herangezogene Institutionen vollständig angegeben wurden.

Die Dissertation wurde nicht schon als Diplom- oder ähnliche Prüfungsarbeit verwendet.

Hannover, den 6. April 2009

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(Unterschrift)

Jutta Schade

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