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REGULAR ARTICLE

Jenny Wah · Anne Wellek · Marion Frankenberger · Pia Unterberger · Ulrich Welsch · Robert Bals

Antimicrobial peptides are present in immune and host defense cells of the human respiratory and gastroinstestinal tracts

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Abstract Previous studies have implicated antimicrobial peptides in the host defense of the mammalian intestinal and respiratory tract. The aim of the present study has been to characterize further the expression of these molecules in non-epithelial cells of the human pulmonary and digestive systems by detailed immunohistochemical analysis of the small and large bowel and of the large airways and lung parenchyma. Additionally, cells obtained from bronchoalveolar lavage were analyzed by fluorescent activated cell sorting and immunostaining of cytospin preparations. hBD-1, hBD-2, and LL-37 were detected in lymphocytes and macrophages in the large airways, lung parenchyma, duodenum, and colon. Lymphocytes positive for the peptides revealed a staining pattern and distribution that largely matched that of CD3-positive and CD8-positive T-cells. Macrophages with positive staining for the antimicrobial peptides also stained positively for CD68 and CD74. In view of the morphology of the LL-37-positive and hBD-2-positive mucosal lymphocytes, they are probably also B-cells. Thus, antimicrobial peptides of the defensin and cathelicidin families are present in a variety of non-epithelial cells of mucosal organs. These findings confirm that antimicrobial peptides have multiple functions in the biology of the mucosa of these organs.

Keywords Antimicrobial peptide · Defensin · Innate immunity · Cathelicidin · Lung · Gastrointestinal tract · Host defense · Human

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A. Wellek · R. Bals
Hospital of the University of Marburg, Department of Internal Medicine, Division of Pulmonology,
Philipps University Marburg,
35033 Marburg, Germany

J. Wah · P. Unterberger · U. Welsch
Department of Anatomy, Chair II,
Ludwig Maximilian University,
80336 München, Germany

M. Frankenberger
Clinical Cooperation Group "Inflammatory Lung Diseases",
GSF-Institute for Inhalation Biology
and Asklepios Fachklinik Gauting,
82131 Gauting, Germany

R. Bals (✉)
Department of Internal Medicine, Division of Pneumonology,
Hospital of the University of Marburg,
Baldingerstrasse 1,
35043 Marburg, Germany
e-mail: bals@mailer.uni-marburg.de
Tel.: +49-6421-2864994
Fax: +49-6421-2868987

Introduction

Antimicrobial peptides are effector substances of the innate immune system (Boman 1995; Koczulla and Bals 2003). In addition to their direct antimicrobial function, they act as mediators of inflammation (Yang et al. 1999), as ion channels (Lencer et al. 1997), or as a stimulus of angiogenesis (Koczulla et al. 2003), wound repair, chemotaxis (Agerberth et al. 2000), or cell proliferation (Heilborn et al. 2003).

One of the best characterized families of antimicrobial peptides in vertebrates is that of the defensins, which are small cationic cystine-rich peptides of broad antimicrobial activity (Zasloff 2002). α-Defensins have been isolated primarily from myeloid-derived cells, such as neutrophils and macrophages, in which they reside in cytoplasmic granules and contribute to non-oxidative microbial killing (Lehrer et al. 1991). Four human β–defensins have been identified, and their biology has been analyzed. The genes of further putative defensins have been identified by screening databases of genomic DNA (Schutte et al. 2002). Expression of human β-defensins was initially described in epithelial cells of the skin (Harder et al. 1997) and of the gastrointestinal (Takahashi et al. 2001; O'Neil et al. 1999) and the respiratory (Bals et al. 1998a; McCray and Bentley 1997; Goldman et al. 1997; Williams et al. 1990; Garcia et al. 2001a,b) tracts. Recently, the genes of hBD-1 and hBD-2 have been found to be expressed in monocytes and macrophages. hBD-2 peptide has been detected in alveolar macrophages (Duits et al. 2002).

Another family of peptide antibiotics comprises the cathelicidins, which are characterized by a highly conserved signal sequence and pro-region ("cathelin") but show substantial heterogeneity in the C-terminal domain that encodes the mature peptide (Bals and Wilson 2003; Lehrer and Ganz 2002). The only human cathelicidin, LL-37/hCAP-18, has been cloned from cDNA isolated from human bone marrow (Larrick et al. 1995; Agerberth et al. 1995). LL-37/hCAP-18 has been isolated from myeloid cells and subsequently been detected in epithelial cells of the skin and the gastrointestinal, urinary, and respiratory tracts (Frohm et al. 1997; Bals et al. 1998b; Gudmundsson et al. 1996). Secreted forms of these peptides have been detected in wound and airway-surface fluid and have been associated with increased inflammatory activity. Based on the available data, antimicrobial peptides of the β-defensin and cathelicidin families thus have direct antimicrobial function and in addition act as inflammatory or angiogenic mediators.

The aim of the present study has been to test whether host defense and immune cells of the respiratory and gastrointestinal tracts express hBD-1, hBD-2, or LL-37/hCAP-18. We have used immunohistochemistry and the analysis of bronchoalveolar lavage (BAL) cells by fluorescent activated cell sorting (FACS) and have detected all three peptides in neutrophils, lymphocyte subsets, and macrophages.

Materials and methods

Tissue

Tissue samples were obtained from surgical procedures at the Hospital of the University of Munich. They were fixed in formaldehyde (5%) for at least 24 h and subsequently embedded in paraffin via serial dilutions of ethanol and xylol. Sections were cut at a thickness of 4 µm. Tissue was taken from patients undergoing surgical therapy for lung cancer. Only macroscopically and microscopically healthy material was used.

Immunohistochemistry

Polyclonal antisera against hBD-1, hBD-2, and LL-37 were produced in rabbits as described by Bals et al. (1990, 1998a,b). We applied a two-step indirect immunohistochemical method. Sections were deparaffinized in xylol (20 min), hydrated in serial dilutions of ethanol, and left in phosphate-buffered saline (PBS, pH 7.4) for 5 min. To eliminate the activity of endogenous peroxidase, the sections were treated with 3% H_2O_2 for 5 min. This procedure resulted in complete inhibition of all non-specific peroxidase activity (data not shown). After three washes in PBS, the sections were incubated with a 1:5 dilution of goat serum in PBS for 20 min to reduce unspecific background staining, incubated with various dilutions of the primary antibodies in PBS (1:50 and 1:500) for 18 h at 4°C, washed three times in PBS, incubated with the biotinylated secondary goat anti-rabbit antibody (1:1,000 in PBS) for 30 min, washed in PBS, and incubated with peroxidase-conjugated streptavidin for 20 min (1:150 dilution with PBS). After several washes, drops of 6 mg 3,3-diaminobenzidine-tetrahydrochloride in 10 ml distilled water with 2 ml 3% H_2O_2 were applied as chromogen. The sections were dehydrated in serial dilutions of ethanol, treated with xylol, and mounted. Negative controls lacked the primary antibody or were incubated with the corresponding pre-immune serum. For colocalization of positive signal with cell-type-specific stains, we applied antibodies to CD2, CD3, CD4, CD8, CD20, CD68, or CD74 on adjacent serial sections. The antibodies were obtained from BioGenex (San Ramon, Calif., USA; CD20), DAKO (DakoCytomation, Hamburg, Germany; CD8, CD68), or Zymed (South San Francisco, Calif., USA; CD3, CD4, CD74). The staining intensity of cell types was evaluated by using a semiquantitative scoring system: no staining (–), low staining (+), intermediate staining (++), and strong staining (+++). The results were evaluated by three independent investigators and averaged.

BAL cells

Patients and lavage BAL was performed for the intracellular detection of antimicrobial peptides. After informed consent, lavage was performed during fiber-optic bronchoscopy by instilling 160 ml 0.9 % saline solution in 20-ml aliquots into the lingula or middle lobe and withdrawing the fluid immediately. Total cell counts were determined, and cytocentrifuge smears were prepared for cytologic analysis. Differential cell counts of 400 cells were made by Giemsa-May-Grünwald staining (Diff-Quick, Dade Behring, Switzerland). All patients underwent the lavage procedure for diagnostic purposes.

Staining of intra-cellular antimicrobial peptides by FACS The detection of intra-cellular defensins was performed with 1×10^6 BAL cells per sample. Samples were obtained from patients with fibrosis (n=4), sarcoidosis (n=4), and infectious lung infiltrates (n=4). The polyclonal antibodies as described were used together with the corresponding pre-immune serum as a control. In brief, cells were pelleted and fixed in 200 µl Cytofix/Cytoperm (Becton Dickinson, Heidelberg, Germany) for 20 min on ice. After two washing steps with PBS/2% fetal calf serum (FCS), cells were resuspended in 90 µl Permwash (Becton Dickinson), and 10 µl defensin-specific antibody was added and incubated for 20 min on ice. After one washing step with PBS/2% FCS, cell pellets were resuspended in 90 µl Permwash and 8.5 µl goat anti-rabbit-IgG fluorescein isothiocyanate (FITC)-conjugated (MEDAC, Hamburg, Germany) and incubated for 20 min on ice. Cells were then immediately analyzed in a Coulter EPICS XL flow cytometer (Coulter, Krefeld, Germany). The discrimination of different cell population in the lavage fluid was performed by gating on forward versus side scatter. This method has been previously used to discriminate BAL cell types (Maus et al. 1997).

Staining of cytospin preparations Cells prepared as cytospins as described above were fixed for 15 min in 4% formaldehyde/5% acetic acid and washed with PBS. Immunohistochemical detection was performed as described above.

Results

Antimicrobial peptides are expressed in BAL cells

The aim of the present study was to test whether human antimicrobial peptides are expressed in host defense or immune cells of the pulmonary or gastrointestinal tract. Initially, we stained cytospin preparation of BAL cells. We found the expression of hBD-1, hBD-2, and LL-37/hCAP-18 in neutrophils, macrophages, and lymphocytes in samples from ten control patients with normal total numbers and differential counts (Fig. 1).

We next aimed to confirm these results by means of intra-cellular staining and FACS analysis of cells from BAL. The discrimination of the various cell populations in the lavage fluid was performed by gating on forward versus side scatter. Figure 2 shows the absolute fluorescence intensity in alveolar macrophages given in delta mean channels for the three peptides. Using pre-immune serum as a control, we detected positive signals for all peptides in lymphocytes and macrophages. hBD-1 was most abundant in alveolar macrophages from patients with infiltrates, followed by sarcoidosis and fibrosis. The most intense signal for hBD-2 was also detected in macrophages of patients with infectious infiltrates, followed by sarcoidosis and fibrosis. LL-37 was detected with a similar pattern in macrophages of all patients. Almost the same pattern of expression of peptides hBD-1, hBD-2, and LL-37 could be detected in the lymphocyte population of the BAL fluid, but all three peptides were weaker in expression compared with the alveolar macrophages (data not shown).

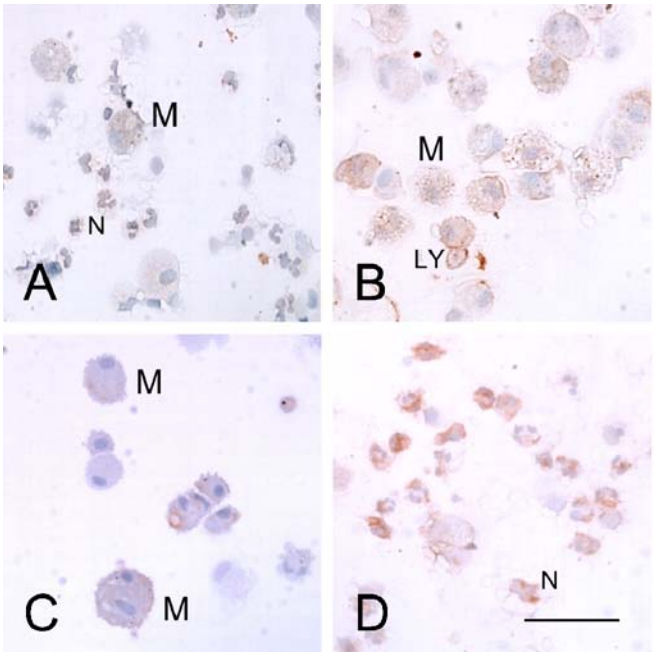

Fig. 1 Staining of bronchoalveolar lavage (BAL) cells prepared as cytospins with antibodies to hBD-1 (**a**), hBD-2 (**b**), or LL-37 (**c**, **d**). **d** Cells from a lavage rich in neutrophils. Cells were identified by their characteristic morphological appearances (*M* macrophage, *N* neutrophil, *LY* lymphocyte). *Bar* 20 μm

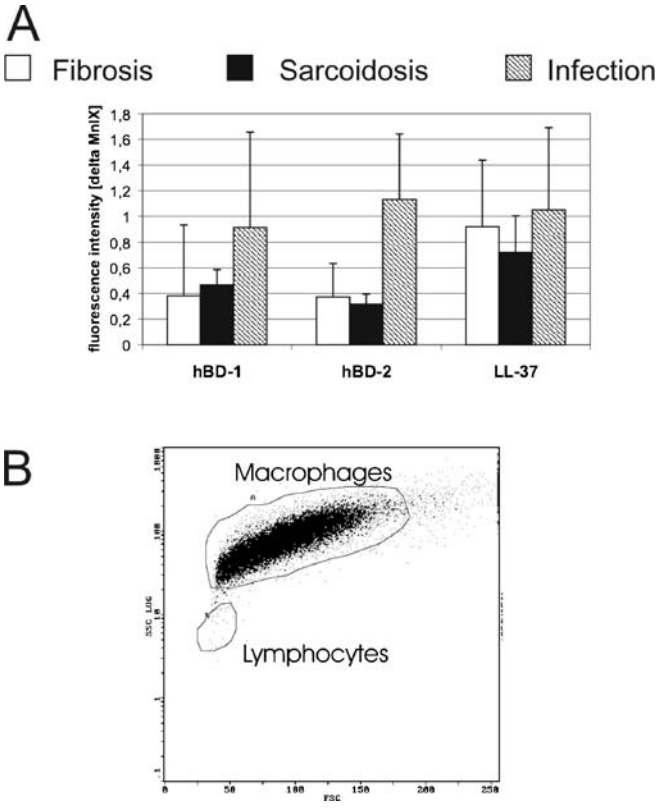

Fig. 2 Detection of intra-cellular antimicrobial peptides by FACS. **a** Delta mean channels for the three peptides are displayed. **b** Discrimination of the different cell populations (macrophages, lymphocytes) in the lavage fluid was performed by gating on forward versus side scatter

Tissue distribution of antimicrobial peptides in non-epithelial cells

Based on the expression data from the lavage cells, we next performed an immunohistochemical analysis of lung sections to characterize the tissue distribution of peptide expression.

Airways, lung parenchyma hBD-1, hBD-2, and LL-37 are known to be expressed in surface epithelia of the respiratory tract. These results were confirmed in the present study (data not shown). In addition to epithelial cells, several non-epithelial cells were intensely labeled. To categorize the positive cell types, we stained adjacent serial sections with a variety of antibodies directed against cell-type-specific markers (see above).

Numerous lymphocytes in the subepithelial connective tissue and between the glandular acini of the large airways gave a positive immunoreaction for the three peptides

(Fig. 3b,d). Some of the positive lymphocytes were also found in the epithelium. In the distal airways and alveolar region, alveolar macrophages stained with variable intensities with the hBD-1 and LL-37 antibodies (Fig. 3a,e). The hBD-2 antibody gave a strong granular staining pattern in large macrophages; smaller macrophages exhibited fainter cytoplasmic staining (Fig. 3c). This antibody also stained lymphocytes in the lamina propria, among which oval-shaped cells were particularly common in the region of the bronchial glands. Moreover, intra-alveolar lymphocytes and pneumocytes type II stained positively for the three peptides (Fig. 3a,c,e). Numerous intra-vascular neutrophils stained

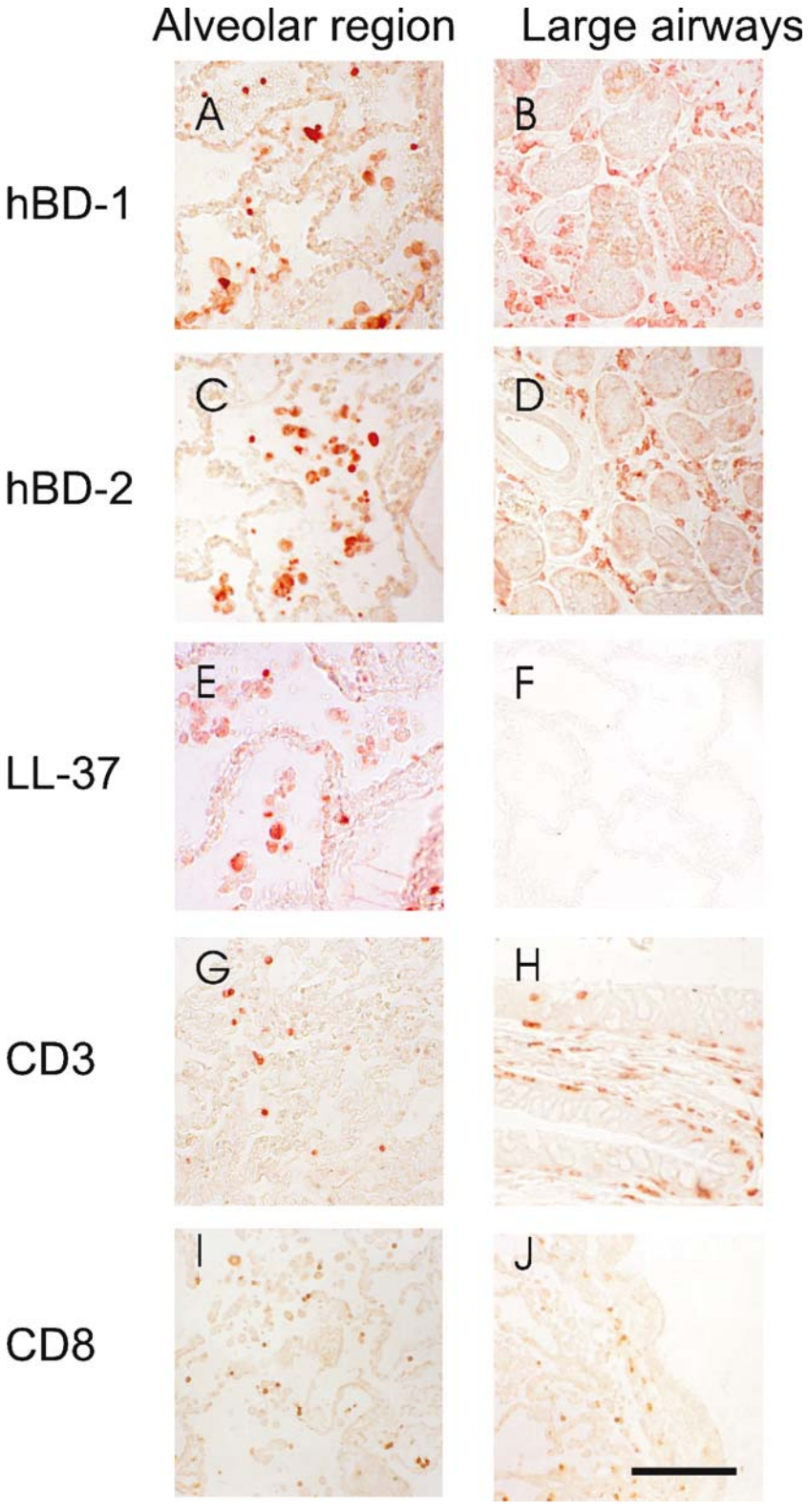

Fig. 3 Staining of sections of large airways and lung parenchyma with antibodies to hBD-1 (**a**, **b**), hBD-2 (**c**, **d**), or LL-37 (**e**). The antibodies stain different non-epithelial cells that were identified by using cell-type-specific antibodies to CD3 (**g**, **h**) and CD8 (**i**, **j**). **f** Negative control with pre-immune serum as first antibody (corresponding to LL-37). The parenchymal structures in the column *large airways* are glands. *Bar* 50 μm

strongly with LL-37. The results are summarized in Table 1. Cells positive for hBD-1 or hBD-2 were also stained for CD8 or CD68 (hBD-1) or for CD8, CD68, or CD74 (hBD-2), respectively. Cells positive for LL-37 also stained for CD8, CD68, or CD74. On the basis of morphological criteria alone (nucleus, shape), some of the LL-37-positive and hBD-2-positive cells were probably plasma cells, especially in the region of the bronchial glands.

Intestinal tract We next asked whether these results could be extended from the respiratory to the gastrointestinal tract. Epithelial cells of the duodenum and the colon stained variably but positively for the presence of the peptides (data not shown). In the lamina propria of the duodenum, numerous lymphocytes and variable numbers of macrophages exhibited positive immunostaining for hBD-1 and hBD-2 (Fig. 4a,c). Several endocrine epithelial cells gave a positive immunoreaction for hBD-2 and LL-37 (Fig. 4c,e). These cells could be ascribed to endocrine cells and individually also occurred in the glands of Brunner. In the colon, numerous small lymphocytic cells of homogeneous size and macrophages in the lamina propria stained positively for hBD-1, hBD-2, and LL-37 (Fig. 4b,d,f). The cells of lymphatic aggregates, which morphologically represented T-cell areas, stained positively for the three peptides studied. The antibody to LL-37 stained single endocrine epithelial cells strongly (data not shown). The tissue distribution of the peptides in non-epithelial cells of the gastrointestinal tract is summarized in Table 1. The hBD-1 staining pattern correlated with CD3-, CD4-, CD8-, or CD68-positive cells in the small intestine and with CD8- or CD74-positive cells in the colon. hBD-2-positive cells stained also for CD8, CD68, or CD74 in the duodenum and for CD3 or CD68 in the colon. LL-37-positive cells stained also for CD8.

Table 1 Summary of the immunohistological analysis of sections of the respiratory and gastrointestinal tracts. The staining intensity of cell types was evaluated by using a semiquantitative scoring system: – no staining, + low staining, ++ intermediate staining, +++ strong staining

Tissue	hBD-1	hBD-2	LL-37
Respiratory tract			
Lymphocytes in lamina propria (large airways)	+	+	+
Pneumocytes II	+	–	+
Alveolar macrophages	+	++	+
Lymphocytes (alveolar region)	++	++	+
Intra-vascular neutrophils	–	–	++
Gastro-intestinal tract			
Endocrine cells (duodenum)	–	++	+++
Macrophages in lamina propria (duodenum)	+	++	–
Lymphocytes in lamina propria (duodenum)	+++	++	–
Lymphatic follicles in lamina propria (parafollicular areas; duodenum)	++	+	–
Endocrine cells (colon)	–	+++	+++
Macrophages in lamina propria (colon)	+	+	–
Lymphocytes in lamina propria (colon)	++	++	+/++
Lymphatic follicles (parafollicular areas; colon)	++	++	–

Taking only morphological criteria, a number of the LL-37-positive and hBD-2-positive cells probably also represented plasma cells.

Discussion

The main finding of our study is the detection of antimicrobial peptides of the defensin and cathelicidin families in host defense and immune cells throughout the mucosa of the respiratory and the gastrointestinal tract. The expression of hBD-1, hBD-2, and LL-37 in epithelial cells of different organs has been confirmed in the present study. Recently, the genes for hBD-1 and hBD-1 have been found to be expressed in monocytes and macrophages; the presence of the peptide hBD-2 has also been documented in alveolar macrophages (Duits et al. 2002). LL-37 was initially isolated from myeloid cells and has recently been found to be expressed in intra-vascular lymphocytes (predominantly B and γδ T-cells), NK cell, and monocytes/macrophages and in various cell lines of these cells (Agerberth et al. 2000). Our observations reveal that mainly macrophages and cytotoxic T-lymphocytes contain these antimicrobial peptides. The morphology of a subset of the immunostained lymphocytes resembles the morphology of plasma cells.

The immunohistochemical detection of peptides is a technically challenging procedure. The diffusibility of the small peptides together with their positive charge could easily result in artifacts and pseudolocalization. Consequently, the obtained results have to be interpreted with caution, and additional methods other than immunohistochemisty have to be used. The results of the present study are in concordance with earlier studies showing the expression of the genes for these peptides in the cells of interest (Bals et al. 1998a,b; Duits et al. 2002; Agerberth et al. 2000).

The histological distribution patterns show that the defense and immune cells with positive staining for antimicrobial peptides are largely confined to the mucosal membrane of the organ systems studied. This underlines the importance of the mucosa for the integrity of the organism. In the intestinal tract, the peptide-containing lymphocytes occur both in the lamina propria and in the basal zone of the epithelium, whereas the macrophages are restricted to the lamina propria. Here, they are mainly found at the tips of the villi (small intestine) or in a subepithelial zone (colon). Obviously, the intra-epithelial lymphocytes together with the epithelium form the "front line" for the protection of the intestinal mucosa. In the bronchial mucosa, the distribution pattern of the lymphocytes resembles that of the intestinal tract. In the area of the alveoli, however, intra-alveolar macrophages are the predominant "professional" defense cells expressing antimicrobial peptides.

Antimicrobial peptides are effector substances of the innate immune system. Several studies have highlighted the host defense function of antimicrobial peptides in vivo. Investigations of a human bronchial xenograft model have revealed decreased antimicrobial activity of airway-surface fluid after the inhibition of hBD-1 synthesis by antisense oligonucleotides (Goldman et al. 1997). Mice deficient in

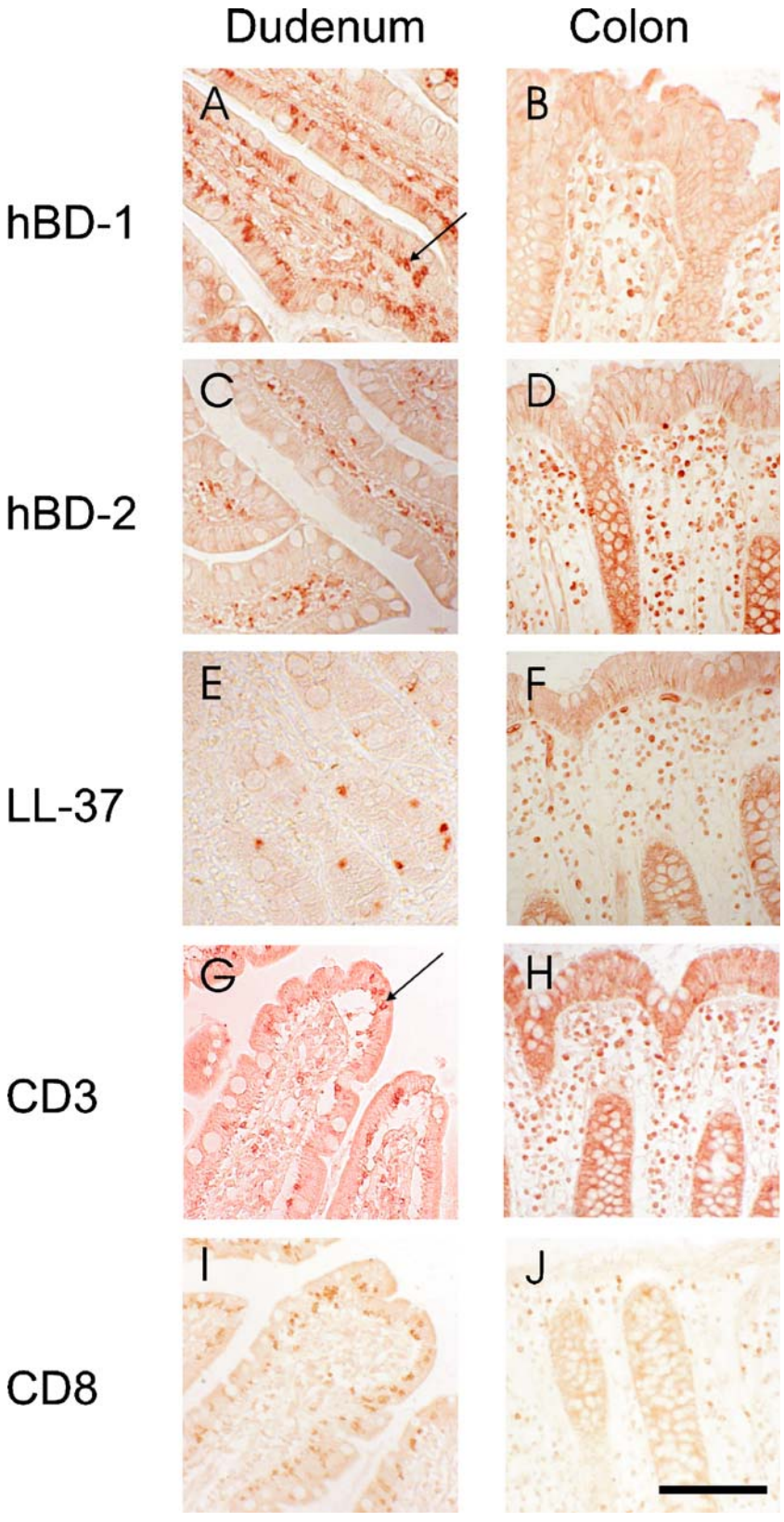

Fig. 4 Staining of sections of the small (duodenum) and large intestine (colon) with antibodies to hBD-1 (**a**, **b**), hBD-2 (**c**, **d**), or LL-37 (**e**, **f**). The antibodies stained different non-epithelial cells that were identified by using cell-type-specific antibodies to CD3 (**g**, **h**) and CD8 (**i**, **j**) (*arrows* intra-epithelial lymphocytes as indicated by positive CD3 staining). *Bar* 50 μm

an antimicrobial peptide, viz. mouse β-defensin-1 (mBD-1), exhibit delayed clearance of *Haemophilus influenzae* from lung (Moser et al. 2002). Mice with deleted CRAMP, the murine homolog of LL-37, show more prominent infection after cutaneous inoculation of bacteria (Nizet et al. 2001). Moreover, overexpression of LL-37 produced by viral gene transfer results in the augmentation of the innate host defense in a bronchial xenograft model of cystic fibrosis and in

murine models of pneumonia and septic shock (Bals et al. 1999a,b). In addition to their role as endogenous antibiotics, evidence is accumulating that defensins and cathelicidins bind to endogenous receptors and induce various cellular processes. hBD-1 and hBD-2 bind to a chemokine receptor known as CCR-6 (Yang et al. 1999). This receptor is found on immature dendritic and memory T cells ($CD4^+/CD45RO^+$), and consequently, these findings are interpreted as a link between innate and adaptive immune mechanisms mediated by defensins. hBD-3 and hBD-4 chemoattract monocytes by mechanisms that have not yet been clarified (Garcia et al. 2001a,b). Additionally LL-37 binds to formyl peptide receptor-like 1 (FPRL1), a promiscuous receptor expressed on a variety of cells including neutrophils, monocytes, and lymphocytes (Yang et al. 2000). By activation of this G-protein-coupled receptor, LL-37 attracts neutrophils, monocytes, and CD4 T cells, activates mast cells (Niyonsaba et al. 2001), and induces angiogenesis (Koczulla et al. 2003). These data indicate that antimicrobial peptides are multifunctional molecules. The expression of these peptides in "professional" host defense and immune cells is possibly relevant for two scenarios. First, cells that express antimicrobial peptides are equipped with endogenous antibiotics and contribute to the direct microbicidal activities as an effector mechanism of host defense. Second, based on the role of antimicrobial peptides as mediators, the secretion of antimicrobial peptides probably results in the activation of other cells, such as endothelial, epithelial, and inflammatory cells.

The role that antimicrobial peptides play in the respiratory tract is still speculative. Based on the functions described above, they are probably involved in the protection of the lung from micro-organisms by a direct antimicrobial effect. Furthermore, they act as mediators of inflammation and related processes. Thus, antimicrobial peptides are probably important for the homeostasis of the respiratory tract and in the development of diseases.

In the present study, we describe the expression of hBD-1, hBD-2, and LL-37 in some of the principal inflammatory cell types of the lung. Future studies should address the question of whether peptides released from these cells contribute to host defense or to inflammatory reactions.

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