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Human Th17 Cells Express High Levels of Enzymatically Active Dipeptidylpeptidase IV (CD26)

Bertram Bengsch,*†‡§,1 Bianca Seigel,*†‡§,1 Tobias Flecken,*†§ Julia Wolanski,* Hubert E. Blum,* and Robert Thimme*

Dipeptidylpeptidase IV (CD26) is a multifunctional ectoenzyme involved in T cell activation that has been implicated in autoimmune pathophysiology. Because IL-17–producing CD4+ T cells (Th17 cells) are important mediators of autoimmune disease, we analyzed the expression of CD26 and its enzymatic function on human Th17 cells. Analysis of CD26 expression on different CD4+ T helper subsets showed that CD26 expression is highest on CD4+ T cells producing type 17 cytokines (e.g., IL-22, IL-17, GM-CSF, or TNF) compared with Th1, Th2, and regulatory T cells. Phenotypic analysis revealed that CD26++CD4+ T cells express the type 17 differentiation molecules CD161, CCR6, IL-23R, and retinoic acid-related orphan receptor-γt. Furthermore, sorted CD26++CD4+ T cells contain >90–98% of Th17 cells, indicating that CD26++ T cells harbor the Th17 lineage. A comparison with CD161 and CCR6 indicated that analysis of CD26 coexpression may improve the phenotypic characterization of Th17 cells. Of note, CD26++ Th17 cells are enriched in the inflamed tissue of patients with hepatitis and inflammatory bowel disease. Functional analysis in migration assays revealed that CD26 expressed on Th17 cells is enzymatically active. Indeed, CD26 negatively regulates the chemotactic CD4+ T cell response to the inflammatory chemokines CXCL9–12 that can be restored by pharmacological blockade of the enzymatic center of CD26. In summary, these results strongly suggest that CD26 may contribute to the orchestration of the immune response by Th17 cells in human inflammatory diseases. They also suggest that the phenotypic analysis of Th17 cells may be facilitated by determination of CD26 expression.  The Journal of Immunology, 2012, 188: 000–000.

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nterleukin-17–producing CD4+ T cells (Th17 cells) play an important role in antimicrobial immunity at epithelial and mucosal barriers, but they are also critically involved in the induction and maintenance of several murine and human inflammatory diseases. Recently, human Th17 cells have been linked to the pathogenesis of rheumatoid arthritis, multiple sclerosis, and inflammatory diseases of the gastroenterologic tract, such as autoimmune hepatitis, primary biliary cirrhosis, and particularly inflammatory bowel disease (1). Th17 cells are able to secrete several potentially proinflammatory cytokines, including IL-17, IL-22, GM-CSF, and TNF, that modulate the tissue response during inflammation (2). Additionally, it has become evident that Th17 cells in inflamed tissues are involved in complex interactions with stromal cells and other immune cells, such as neutrophils, myeloid cells, B cells, and other T cell subsets (3–5). However, the mechanisms responsible for the central role of Th17 cells in inflammatory diseases are currently only poorly understood and warrant further investigation. One major limitation for the analysis of human Th17 cells is the lack of specific phenotypic markers (6). Previous studies have shown that human Th17 cells express the chemokine receptor CCR6, the IL-23 receptor, and the lectin-like receptor CD161 (7), a phenotype that is controlled by the master transcription factor retinoic acid-related orphan receptor (ROR)γt (8).

In this study, we set out to characterize the role of the dipeptidylpeptidase IV (CD26) for Th17 cells. CD26 expression has previously been reported in multiple inflammatory conditions and linked to inflammatory Th1 cells in the pre-Th17 era (9). CD26 upregulation correlates with disease activity in human autoimmune diseases such as rheumatoid arthritis (10) and multiple sclerosis (11–13), diseases that have recently been linked to the presence of pathogenic Th17 cells. However, whether CD26 expression is linked to a specific subset of inflammatory T cells, such as Th17 cells, has not been previously addressed.

CD26 has costimulatory T cell functions, for example through the direct interaction with ADA or caveolin (9, 14), as well as regulatory functions. Indeed, as a dipeptidylpeptidase, CD26 has the ability to N-terminally cleave dipeptides from certain oligopeptide substrates that include incretin hormones, neuropeptides, and several chemokines, and it has been shown that CD26-mediated enzymatic chemokine modification may regulate T cell migration (14). For example, CD26 can cleave and inactivate the chemokines CXCL9–11 and CXCL12 (15–17) that are up-regulated in inflammation and may recruit inflammatory T cells bearing the receptors CXCR3 and CXCR4.

In this study, we found that human Th17 cells are characterized by a high expression of CD26 in the peripheral blood as well as in the inflamed tissue. In contrast, Th1, Th2, and especially regulatory T cells show lower expression levels of CD26. Importantly, CD26++ Th17 cells are enriched in the inflamed tissue in patients with inflammatory bowel disease and viral and autoimmune hepatitis. Migration assays revealed that CD26 expressed by Th17 cells may
enzymatically regulate T cell chemotaxis in response to the inflammatory chemokines CXCL9, CXCL10, CXCL11, and CXCL12. In summary, these results identify CD26 as an enzymatically active molecule expressed by Th17 cells and suggest that it has a biologically active enzymatic role that may contribute to the regulation of inflammatory T cell chemotaxis.

Materials and Methods

Lymphocyte isolation

Human peripheral blood and tissue samples were acquired after obtaining written informed consent from the patients and approval by the Ethics Committee of the Albert-Ludwigs-Universität, Freiburg. Samples were obtained from 23 healthy donors as well as 31 patients with autoimmune or viral hepatitis or inflammatory bowel disease undergoing diagnostic liver biopsies or colonoscopies at the Department of Medicine II (Gastroenterology and Hepatology), University Medical Center Freiburg, Freiburg, Germany. Macropiscopically normal lung tissue samples were obtained from seven patients undergoing lobectomy due to lung cancer at the Department of Thoracic Surgery, University Medical Center Freiburg, Freiburg, Germany. All investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Lymphocytes were isolated and analyzed from patient blood and peripheral tissue essentially as previously described (18).

Flow cytometry

The following reagents were used for polychromatic stainings: anti-CD3-Pacific Blue, anti–CD4-allophycocyanin-H7, anti–CD8-AmCy5, anti–CD8-PE-Cy7, anti–CD8-allophycocyanin-H7, anti–CD26-PE, anti–CD38-PE, anti–CCR6-PerCP-Cy5.5 (clone 11A9), anti–CCR7-PE-Cy7, anti–CXCR3-FITC, anti–CXCR4-PE (BD Biosciences), anti–CD26-FITC, anti–CD45RA-PerCP-Cy5.5, anti–IL-17A-PerCP-Cy5.5, anti–IL-17A-PE, anti–IL-22-PerCP-e70, anti–GM-CSF-Alexa 647, anti–FOXP3-FITC, anti–CD25-allophycocyanin, anti–CD127-Phycoerythrin 50 (eBioscience), anti–CD161-PE, anti–CD161-allophycocyanin (Miltenyi Biotech), anti–CXCR6-PE, anti–IL-22-allophycocyanin, anti–IL-23R-PE (R&D Systems), and anti–CD3-PerCP (BioLegend). Viaprobe (7-aminoactinomycin D; BD Biosciences) was used for dead cell exclusion. For cell labeling, cells were incubated with 40 μM Pacific Blue succinimidyl ester (PBSE; Invitrogen) and washed extensively. Ab staining was performed as previously described (18). Stimulation with PMA and ionomycin for 5 h and intracellular cytokine staining were essentially performed as described (19). Samples were acquired on a FACS Canto II flow cytometer (BD Biosciences) and analyzed with FlowJo v9.2 software (Tree Star). In cell sorting experiments, T cell subsets were sorted following Ab staining using a MoFlo XDP cell sorter (Beckmann Coulter) at the Center of Chronic Inflammatory T cell chemotaxis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software). A Kruskal–Wallis test was performed in Fig. 2. One-way ANOVA and a Tukey multiple comparison test was performed in Figs. 4 and 6. A Wilcoxon test was performed in Figs. 5 and 7. Specificity of IL-17-producing T cell detection in Fig. 1 was calculated using a contingency table for tests based on CD161 expression (CD161+), high levels of CD26 expression (CD26++), or a combination of both (CD26+CD161+) by dividing the number of true negatives by false positives plus true negatives.

Results

High CD26 expression of IL-17-producing CD4+ T cells

We first determined whether human Th17 cells express CD26. In these experiments, T cells were analyzed ex vivo from the peripheral blood of 25 individuals, stimulated with PMA/ionomycin, and costained for IL-17 production and CD26 expression. In agreement with previous studies, we observed that IL-17–producing T cells represented typically <2% of peripheral blood CD4+ T cells (20). Strikingly, as shown in Fig. 1, most (median, 91%) of IL-17–producing CD4+ T cells isolated from the peripheral blood expressed CD26. Because CD4+ T cells derived from PBMCs may have phenotypic and functional differences compared with tissue-infiltrating T cells, we also analyzed liver-derived lymphocytes and lung-derived lymphocytes for IL-17 production. Of note, as displayed in Fig. 1, IL-17 was similarly produced by CD26+CD4+ T cells in the peripheral tissues analyzed. These results suggest that human Th17 cells are characterized by high CD26 expression in the peripheral blood and tissue. However, clearly, not all CD26+CD4+ T cells produced IL-17, raising the possibility that other T helper subsets might also be characterized by high CD26 expression. To address this issue, we sought to determine CD26 expression levels for the major T helper subsets (Th1, Th2, Th17, and regulatory T cells).

Th17, Th1, Th2, and regulatory T cells differ in CD26 expression

To analyze CD26 expression levels on major T helper subsets, PBMCs from 19 healthy donors were stimulated with PMA/ionomycin and cytokines or surface markers expressed by Th1, Th2, regulatory T, and Th17 cells were analyzed (Fig. 2). In the next step, CD26 expression levels were determined on Th cell subsets. Strikingly, as shown in Fig. 2 and Supplemental Fig. 1, the highest CD26 levels were found on CD4+ T cells producing typical type 17 cytokines (e.g., IL-22, IL-17, GM-CSF, TNF). Notably, IFN-γ–producing CD4+ T cells, indicating Th1 cells, showed less CD26 expression. However, although IFN-γ production was generally linked to intermediate CD26 expression levels, IFN-γ production was also found to a lower extent by CD4+ T cells with high CD26 expression (including IL-17, IFN-γ double producing T cells) or low CD26 expression, suggesting some T cell plasticity (Supple-
mental Fig. 1). Th2 cells identified by the production of IL-4, IL-5, IL-13, or the expression of chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) had intermediate CD26 expression levels. Interestingly, the lowest CD26 levels were identified for IL-10–producing CD4+ T cells and CD25hiCD127loFOXP3+ regulatory T cells. Taken together, CD26 expression levels significantly differ between Th17 and Th1, Th2, or regulatory T cells (Fig. 2B). In summary, these findings indeed suggest that different levels of CD26 expression are linked to specific T helper subsets and support the hypothesis that high CD26 expression is linked to CD4+ T cells with type 17 differentiation, but not to Th1, Th2, or regulatory T cells.

The CD26++CD4+ T cell subset coexpresses type 17 differentiation markers

The finding that Th17 cells are linked to the highest CD26 expression levels compared with other CD4+ T helper subsets suggested that a specific CD4+ T cell subset with highest CD26 expression (CD26++) exists that harbors the Th17 lineage. However, because the previous analyses were performed following stimulation with PMA/ionomycin and because this might have resulted in an upregulation of CD26 by cells with initially lower CD26 expression levels, we next asked whether Th17 cells can be identified by CD26 expression levels also ex vivo, prior to stimulation. Thus, we analyzed CD26 expression levels ex vivo and identified three discriminable CD4+ T cell subsets: a population with high CD26 expression (CD26++), a population with intermediate expression (CD26+), and a population that lacks CD26 expression (CD26−) (Fig. 3A). We next asked whether the CD26++ subset was linked to the expression of predescribed Th17-associated molecules such as CD161, the chemokine receptors CCR6 and CXCR6, IL-23R, and the transcription factor RORγt.

Strikingly, as displayed in Fig. 3B by representative FACS plots and overlay histograms, CD26++ cells clearly coexpress CD161 in contrast to CD26+ and CD26− cells (Fig. 3B). Expression of CCR6 and CXCR6 was also observed within the CD26++ subset.

We also determined the expression levels of IL-23R and RORγt after cell sorting of specific CD26 subsets. Of note, mRNA levels of IL-23R and RORγt were significantly increased in sorted CD26++ cells compared with the CD26+ and CD26− subsets, respectively (Fig. 3C). Taken together, these results clearly show that the CD26++CD4+ T cell subset is characterized by the coexpression of type 17 differentiation molecules.

To exclude the possibility that CD26++CD4+ T cells had been activated in vivo prior to isolation, we phenotypically analyzed CD26++ T cells for the expression of the memory and differentiation markers CD127, CCR7, and CD45RA, as well as the activation marker CD38. As shown in Fig. 3D, CD26++ cells clearly coexpress CD127 in contrast to CD26+ and CD26− cells (Fig. 3B). Expression of CCR6 and CXCR6 was also observed within the CD26++ subset.

In summary, these results indicate that already prior to stimulation for IL-17 production, CD26++ cells express typical markers of type 17 differentiation and have not been recently preactivated in vivo, suggesting that CD26++ T cells harbor the Th17 lineage.
Selective production of IL-17 by CD26++CD4+ T cells

Next, we set out to formally validate that CD26++ T cells isolated ex vivo indeed harbor the Th17 lineage. To exclude the possibility of an up- or downregulation of CD26 during the stimulation for IL-17 production, we first FACS-sorted CD4+ T cells depending on the level of CD26 expression into three populations: CD26++, CD26+, and CD26− CD4+ T cells (purity, >98%; Fig. 4A). Subsequently, the cells were rested overnight and stimulated in the presence of PBSE-labeled irradiated feeder cells. As shown in Fig. 4, most Th17 cells (>90%) were found in the sorted CD26++ T cell subset. Th17 cells can be induced in vitro in the presence of TGF-β, IL-6, IL-1β, IL-21, and IL-23 (21). Thus, we next asked whether type 17 skewing conditions may induce Th17 cells from different CD26-expressing T cell subsets. Sorted T cells were cultured under Th17 conditions in the presence of irradiated feeder cells for 14 d. Of note, most (98%) Th17 cells identified within the cultures were derived from CD26++ T cells (Fig. 4). However, type 17 skewing culture conditions also resulted in an upregulation of CD26 expression and, although to a lower extent, of IL-17 production in cultures derived from CD26+ and CD26− CD4+ T cells (Fig. 4). Interestingly, IL-17–producing CD4+ T cells derived from the CD26+ and CD26− subset upregulated CD26 in culture, acquiring the CD26++ phenotype (Fig. 4A). These results suggest that Th17 cells can be differentiated efficiently from CD26++ T cells; however, few CD26+/CD26− T cells may upregulate CD26 expression and IL-17 production under the appropriate conditions. Taken together, these experiments show that the overwhelming majority of Th17 cells are located in the CD26++CD4+ T cell subset ex vivo and in vitro, further suggesting that the CD26++ T cell subset contains the Th17 T cell lineage.

Comparison of CD26 with CD161 and CCR6 expression of IL-17–producing CD4+ and CD8+ T cells

The C-type lectin receptor CD161 and the CC chemokine receptor CCR6 are considered the best phenotypic cell surface markers for human IL-17–producing T cells to date, and Th17 cells have been described by multiple groups to be selectively located within the CD161+ subset and linked to CCR6 expression (6, 22–25). We thus compared the coexpression of CD26 and CD161 or CCR6 on CD4+ T cells and, specifically, Th17 cells. As shown by representative FACS stainings in Figs. 3B and 5A, costaining of CD26 and CD161 reveals a population of CD4+ T cells with high CD26 and CD161 coexpression. Notably, most IL-17–producing CD4+ T cells are located within the CD26++CD4+ T cell subset ex vivo and in vitro, further suggesting that the CD26++ T cell subset contains the Th17 T cell lineage.
with CD161 (Fig. 5E) results in the highest specificity of Th17 cell detection, indicating that a combination of CD26 and CD161 may facilitate the future analysis of Th17 cells. CCR6 was also found to have a high specificity of Th17 cell detection (Fig. 5E). However, in contrast to CD26 and CD161, we observed a strong downregulation of CCR6 expression on CD4+ T cells after stimulation with PMA/ionomycin (Supplemental Fig. 2), which may reduce the utility of CCR6 as a marker for Th17 cells. Because CD161 and CCR6 are also considered the best phenotypic markers for IL-17-producing CD8+ T cells (Tc17), we analyzed whether CD26 expression was similarly linked to IL-17 production and compared it to CD161 and CCR6. As shown in Fig. 5C, we observed a clear link between high CD26 expression and IL-17 production for CD8+ T cells. Furthermore, the subset with high CD26 and CD161 or CCR6 expression contained the Tc17 cells (Fig. 5D). Similarly to Th17 cells, when Tc17 cells did not coexpress both CD26 and CD161, they rather expressed CD26 compared with CD161 or CCR6 (Fig. 5F). Taken together, these results suggest that CD26 expression may be a common signature of type 17 differentiated cells and that a combination of CD26 and CD161 or CCR6 will improve the phenotypic analysis of Th17 and Tc17 cells.

Enrichment of CD26++ Th17 cells in human inflammatory diseases

Th17 cells are implicated in the pathogenesis of several inflammatory autoimmune diseases, including inflammatory bowel disease, autoimmune hepatitis, primary biliary cirrhosis, and viral hepatitis (26, 27). To address whether IL-17-producing T cells in human diseases are also characterized by high levels of CD26 expression, we analyzed tissue specimen from diagnostic biopsies obtained from patients with active inflammatory bowel disease (Crohn’s disease and ulcerative colitis), autoimmune hepatitis, primary biliary cirrhosis, and viral hepatitis. As expected, Th17 cells were enriched in inflamed tissue samples compared with the peripheral blood. Patients with active inflammatory bowel disease were found to have the highest frequency of tissue-infiltrating Th17 cells (Fig. 6A). Clearly, as shown in Fig. 6B, we found a high expression of CD26 on IL-17-producing CD4+ T cells in all tissue samples from inflammatory lesions analyzed. The high frequency of Th17 cells in inflamed intestinal lesions correlated strongly with an increase of IL-17++CD26++CD4+ T cells (Fig. 6C, 6D). Of note, in the inflamed tissue, a much larger fraction of CD26++ T cells produced IL-17.

FIGURE 4. Th17 cells are contained within and derived from sorted CD26++CD4+ T cells. (A) CD4+ T cells were sorted depending on the level of CD26 expression (left columns). Sorted CD26++ T cells were either stimulated for IL-17 production on day 0 after the sort or analyzed following culture under type 17-skewing conditions for 14 d. A representative experiment is shown. (B) Summary of IL-17-producing T cells from sorted CD26 subsets. Th17 cells are significantly enriched within ex vivo sorted CD26++CD4+ T cells (mean, 91%; ***p < 0.001; n = 8) and are induced under type 17-skewing conditions in vitro primarily from sorted CD26++CD4+ T cells (mean, 98%; **p < 0.01; n = 5).
compared with the peripheral blood (Fig. 6C), suggesting that CD26++ T cells may increase their functionality at inflammatory sites. Because the specificity of Th17 cell detection can be further increased by a combination of CD26 with CD161 in healthy donors (Fig. 5E), we analyzed whether the CD26++CD161+CD4+ phenotype correlated with IL-17 production in inflammatory diseases as well. As shown in Fig. 6E, the enrichment of IL-17–producing T cells at the site of disease activity compared with the peripheral blood in Crohn’s disease and ulcerative colitis correlated with the enrichment of CD26++CD161+CD4+ Th17 cells, further indicating that a combination of CD26 and CD161 might be instrumental to identify Th17 cells in inflamed tissue lesions.

Enzymatic regulation of inflammatory chemokine-dependent T cell chemotaxis by CD26-expressing CD4+ T cells

The high expression of CD26 on Th17 cells and the enrichment in inflammatory lesions raised the question whether CD26 may be actively involved in inflammatory processes. CD26 is an ectoenzyme that has several immunologically relevant substrates, such as inflammatory chemokines (e.g., the CXCR3 ligands CXCL9, CXCL10, and CXCL11 and the CXCR4 ligand CXCL12). Several pharmacological inhibitors exist that block the enzymatic activity of CD26. Thus, in a next series of experiments, we asked whether CD26 expressed by Th17 cells is enzymatically active and, if so, whether this activity might be involved in the orchestration of T cell migration by chemokine cleavage. Specifically, we analyzed whether the migration of CD4+ T cells in response to the chemokines CXCL9–12 was influenced by CD26++CD4+ T cells depending on CD26 enzymatic activity. For these experiments, CD26++CD4+ T cells were enriched by bead selection and incubated with the chemokines CXCL9, CXCL10, CXCL11, and CXCL12 in the presence or absence of PI32/98, a specific inhibitor of the enzymatic center of CD26. Subsequently, the chemotactic response of CD4+ T cells was measured in transwell experiments (schematically illustrated in Fig. 7A). Of note, as shown in Fig. 7, the chemotactic migration of CD4+ T cells that express the chemokine receptors CXCR3 and CXCR4 (Fig. 7B) was strongly reduced when CD26++ T cells were added to the CXCL9-, CXCL10-, CXCL11-, and CXCL12-containing wells (Fig. 7C, 7D). Importantly, the addition of the CD26 inhibitor improved the chemotactic response to the chemokines analyzed (p < 0.05 for CXCL12, a trend was observed for CXCL9–11) (Fig. 7C, 7D). These results suggest that CXCL9–12 are cleaved enzymatically by CD26-expressing CD4+CD4+ T cells. They also suggest that enzymatic blockade of CD26 prevents CXCL9–12 cleavage and improves T cell chemotaxis.

Discussion

The first important finding of our study is that human Th17 cells are characterized by a high expression of CD26. Phenotypic analysis of human CD4+ T cells showed that three CD26-expressing CD4+ T cell subsets could be identified; however, IL-17 production is clearly linked to the CD26++CD4+ T cell subset. These CD26++ T cells also coexpress the molecules CD161, IL-23R, CCR6, and...
healthy donors and patients, typically observed in inflamed tissue lesions, where 25–50% of CD26++ T cells produced IL-17 (Figs. 4, 6). In contrast, a strong increase was observed in inflamed tissue of two representative patients. (C) Comparison between the peripheral blood and the inflamed tissue in two representative stainings from patients with ulcerative colitis and Crohn’s disease. (D) The corresponding frequency of IL-17–producing CD4+ T cells and IL-17+/CD26++ T cells in the peripheral blood and inflamed tissue and (E) frequency of CD26+CD161+CD4+ T cells in the peripheral blood and inflamed tissue of two representative patients is shown.

FIGURE 6. CD26 is highly expressed by Th17 cells in human inflammatory lesions. (A) Expression of CD26 was analyzed on IL-17–producing CD4+ T cells isolated from tissue biopsies of patients with hepatitis B virus infection (HBV), hepatitis C virus infection (HCV), autoimmune hepatitis and primary biliary cirrhosis (AIH/PBC), and active inflammatory bowel disease (IBD), including Crohn’s disease and ulcerative colitis. ***p < 0.001. (B) The frequency of IL-17–producing T cells in tissue biopsies was determined and was significantly enriched in IBD patients compared with hepatitis patients. Mean values with SEM are shown. (C) Comparison between the peripheral blood and the inflamed tissue in two representative stainings from patients with ulcerative colitis and Crohn’s disease. (D) The frequency of CD26+ T cells and IL-17+CD26++ T cells in the peripheral blood and inflamed tissue and (E) frequency of CD26+CD161+CD4+ T cells in the peripheral blood and inflamed tissue of two representative patients is shown.

RORγt, which have been previously linked to type 17 differentiation. Of note, these molecules are not linked to T cells with intermediate or absent CD26 expression, indicating that high CD26 expression may be used to identify Th17 cells. Indeed, sorting experiments showed that Th17 cells are almost exclusively located in and can be differentiated from the CD26++ T cell subset. Interestingly, by analyzing Th17 cells, we found that the current "gold standard" phenotypic markers CD161 and CCR6 were expressed to a lesser extent by Th17 cells (Fig. 5F), further emphasizing that analysis of CD26 may be helpful in the future phenotypic characterization of Th17 cells.

However, clearly, as with CD161+ T cells, not all CD26++ T cells produce IL-17 despite the presence of typical type 17 differentiation molecules. We observed that in the peripheral blood of both healthy donors and patients, typically <5% of CD26++ T cells produced IL-17 (Figs. 4, 6). In contrast, a strong increase was observed in inflamed tissue lesions, where 25–50% of CD26++ T cells produced IL-17 upon stimulation. By supplementing inflammatory signals driving Th17 differentiation in vitro, we could observe a strong increase in the frequency of IL-17–producing CD26++ T cells (Fig. 4). These findings raise the possibility that in the peripheral blood, not all CD26++ T cells may have received sufficient stimulatory signals and Th17 differentiation may be incomplete. This might explain why in inflamed tissue lesions with a proinflammatory cytokine environment, a much larger fraction of CD26++ T cells produces IL-17. However, CD26++ T cells do not only produce IL-17, but they may also produce other effector cytokines such as IL-22, GM-CSF, TNF, or IFN-γ (Fig. 2, Supplemental Fig. 1). These findings are in agreement with previous reports showing that Th17 cells may produce these cytokines (28).

This is important to note since the assumption that IL-17 is the critical mediator of Th17 cell function has been challenged by multiple reports in murine model systems of autoimmunity. Indeed, neutralization of IL-17 was inefficient in avoiding Th17-cell mediated disease (29), and several studies suggest that other cytokines produced by Th17 cells, such as GM-CSF, may be more critically involved in inflammation compared with IL-17 (30, 31). Thus, although it is unclear exactly how the individual cytokines produced by CD26++ T cells contribute to the inflammation associated with Th17 cells, it is most likely that the polyfunctional type 17 cytokine profile of CD26++ T cells we observed may actively contribute to Th17-associated pathology.

Another important finding of our study is that Th17 cells have the highest CD26 expression of all Th cell subsets analyzed (Fig. 2, Supplemental Fig. 1). Indeed, Th1 cells identified by IFN-γ production and Th2 cells characterized by the production of IL-4, IL-5 and IL-13 and CRTH2 expression showed CD26 expression levels compatible with the CD26 intermediate subset. In contrast, regulatory T cells identified by the CD25+CD127− FOXP3+ phenotype or IL-10 production even showed a lower CD26 expression. These findings suggest that CD26 expression may be differentially regulated between different human T helper subsets.

However, there clearly is not a tight link between the Th1 phenotype and intermediate CD26 expression since we also observed CD26++ T cells that produced IFN-γ. This finding is not surprising in the light of the polyfunctionality and high degree of plasticity of Th17 cells (28, 32). For example, in a sophisticated fate-mapping study of Th17 cells, Hirota et al. (33) could recently demonstrate that IFN-γ-producing CD4+ T cells in experimental autoimmune encephalitis were once Th17 cells. Th17 cells may convert to Th1 cells under inflammatory conditions (e.g., IL-12, IL-23) and may further contribute to inflammation also in humans, for example in arthritis (32, 34). These “plastic” Th17 cells have been described to maintain some features of Th17 cells, such as CD161 expression. Thus, it is tempting to speculate that the
fraction of IFN-γ–producing CD26++CD4+ T cells (Supplemental Fig. 1) may represent IFN-γ–producing CD4+ T cells that are derived from Th17 cells, but, as previously described for CD161, maintain high levels of CD26 expression.

Another important finding of our study is that CD26 is highly expressed by Th17 cells in inflammatory human diseases, such as Crohn’s disease, ulcerative colitis, primary biliary cirrhosis, autoimmune hepatitis, and viral hepatitis. Our finding that CD26++ IL-17–producing T cells are highly enriched in the inflamed tissues compared with the peripheral blood (Fig. 6C, 6D) is in agreement with previous studies implicating a role of Th17 cells in the pathogenesis of these inflammatory diseases (26, 35), particularly inflammatory bowel disease (36). Interestingly, high levels of CD26 have previously been associated with autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (10, 11). These studies were performed before a role for Th17 cells in autoimmunity and specifically in these diseases was established (20). Hence, although CD26 and IL-17 production were not analyzed simultaneously in these studies, based on our results, it seems likely that both independent observations, the elevated levels of CD26 and IL-17 by inflammatory T cells in multiple sclerosis and rheumatoid arthritis, can be linked to one T cell subset. These findings point toward a biologically important role of CD26 in autoimmunity.

Indeed, by analyzing the enzymatic function of CD26, we could show that CD26 expressed by Th17 cells is biologically active. Specifically, we found that CD26 controls the migration of CD4+ T cells in response to the proinflammatory chemokines CXCL9–12. Importantly, when these chemokines were coincubated with CD26++ CD4+ T cells, a reduced chemotactic migration was observed in transwell migration experiments (Fig. 7). In contrast, blockade of CD26 enzymatic function by a pharmacologic inhibitor (PI32/98) strongly increased the chemotactic response (Fig. 7). These findings suggest that blocking the enzymatic center of CD26 prevents the cleavage of inflammatory chemokines, in full agreement with previous studies that showed that these chemokines are N-terminally truncated by CD26 (14–16). Thus, based on these results we conclude that CD26 expressed by Th17 cells is enzymatically active and can regulate T cell migration by chemokine cleavage. Chemokines are considered to be important orchestrators of T cell migration in inflammation, but only limited insights have been gained into their regulation (37). The finding that type 17-differentiated T cells highly express an important regulating enzyme of inflammatory chemokines hints toward a yet unrecognized role for Th17 cells that may be directly involved in the orchestration of the migration of immune cells to inflamed tissues. Most likely, the cell types regulated by CD26 are inflammatory cells expressing the receptor CXCR3 (including Th1 cells) since all known ligands of CXCR3 (CXCL9–11) are cleaved by CD26. Similarly, cells expressing the CXCL12 receptor CXCR4 may also be regulated by CD26. Despite the general notion of a proinflammatory role of Th17 cells, it is possible that the chemokine cleavage mediated by CD26 represents a negative feedback mechanism. Interestingly, a central role for Th17 cells in regulating inflammatory immune cells via chemokines is further suggested by studies showing that Th17 cells control inflammatory cell subsets by IL-17–dependent mechanisms. For example, IL-17 may regulate the chemokine-dependent mobilization of neutrophils (4). Additionally, Th17 cells may initiate their amplification in a positive feedback loop by IL-17 secretion, inducing the production
of CCL20 (that binds to CCR6) by tissue-resident and recruited cells, resulting in the attraction of additional CCR6+ Th17 cells (38). Thus, Th17 cells may use different mechanisms including CD26 expression and IL-17 production to orchestrate the inflammatory T cell response by regulating inflammatory chemokines.

Recently, Casrouge et al. (39) demonstrated evidence for high CD26 enzymatic activity in chronic hepatitis C virus infection. Specifically, high levels of CD26-cleaved CXCL10 were identified in patients that responded poorly to antiviral therapy. Because the cleaved form of CXCL10 functions as a receptor antagonist, the authors suggested that CD26-mediated cleavage of CXCL10 might contribute to viral persistence. In that study, the source of CD26 was not investigated. In this study, we identified high levels of CD26 expressed by Th17 cells in the intrahepatic T cell compartment in patients with chronic hepatitis C virus infection (Fig. 5A), suggesting that Th17 cells may contribute to chemokine antagonism in hepatitis C virus infection. However, because we also observed a similar Th17 phenotype in other hepatic viral and autoimmune diseases (Fig. 5A), and soluble CD26 may be released by hepatocytes as well (40), further studies will have to clarify whether Th17 cells are indeed a major source of CD26 enzymatic activity in chronic hepatitis C virus infection.

In summary, our study shows that human type 17-differentiated Th17 cells are phenotypically characterized by high CD26 expression in both health and inflammatory disease. It also provides new insights into the immunobiology of human Th17 cells and hints in the existence of different memory T-cell subsets in the peripheral blood and liver. J. Virol. 81: 945–953.


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CD26

CCR6

ex vivo

5h PMA/Ionomycin

CD26

CCR6
Figure S1. **CD26 expression on different CD4+ T helper cell subsets.**

CD26 expression was analyzed on cytokine-producing CD4+ T cells after stimulation with PMA/Ionomycin. Representative FACS plots from one individual (19 tested) gated on CD3+CD4+ T cells are shown. The FACS plots are sorted depending on the rank of CD26 expression (see Fig. 2). Type 17 associated cytokine (IL-17-, IL-22-, GM-CSF and TNF)-producing CD4+ T cells had higher CD26 expression compared cells with a TH1, TH2 or Treg cytokine signature or phenotype.

Figure S2. **Modulation of CCR6 expression during stimulation.**

CCR6 and CD26 expression on CD3+CD4+ T cells was determined ex vivo (left) and following PMA/Ionomycin stimulation for 5 hours (right). A strong downregulation of CCR6 expression was observed. One representative staining out of 5 experiments is shown.