Involvement of CD26 in differentiation and functions of Th1- and 1 Th17-subpopulations of T lymphocytes

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Abstract

CD26, acting as a co-stimulator of T cell activation, plays an important role in the immune system. However, the role of CD26 in the differentiation of T cell subsets, especially of new paradigms of T cells, such as Th17 and Tregs, is not fully clarified. In the present study, the role of CD26 in T cell differentiation was investigated in vitro. CD26 expression was analysed in the different subsets of human peripheral blood T lymphocytes after antigen stimulation. Here, the percentage of CD4+ cells significantly increased and most of these cells were co-expressed with CD26, suggesting a close correlation of CD26 expression with the proliferation of CD4+ cells. Subsequently, after antigen stimulation, CD26 high-expressing cells (CD26high) were separated from CD26 low-expressing cells (CD26low) by magnetic cell sorting. We found that the percentages of cells secreting Th1-typical cytokines (IL-2, IFN-γ), Th17-typical cytokines (IL-6, IL-17, IL-22) or expressing Th17-typical biomarkers (IL-23R, CD161, CD196) in the CD26high group were markedly higher than in the CD26low group. In addition, a co-expression of CD26 with IL-2, IFN-γ, IL-17, IL-22, and IL-23R in lymphocytes was demonstrated by fluorescence microscopy. These results provide direct evidence that the high expression of CD26 is accompanied by the differentiation of T lymphocytes into Th1 and Th17, indicating that CD26 plays a crucial role in regulating the immune response.

Introduction

CD26/DPPIV (Dipeptidyl peptidase) is a multifunctional integral type II transmembrane glycoprotein with a broad cell-surface distribution [1]. As serine proteases, DPPIV cleaves the dipeptides after proline or alanine at the penultimate position of the N-terminus of several bioactive peptides, and thereby modulates their activities in diverse biological processes [2]. Besides its enzyme activity, CD26 was also shown as a co-stimulator involved in T-cell activation and differentiation by its interaction with other cellular molecules, such as adenosine deaminase (ADA), receptor-type protein tyrosine phosphatase (CD45), CARMA1 and caveolin-1 [3, 4]. The expression of CD26 in T lymphocytes is differentially regulated during T cell development. As an activation marker of T cells, CD26 is mainly expressed on $CD4^+$ T cells, and it is thought to be a marker of T helper type 1 cells [5, 6]. Although both Th1 and Th2 cells express CD26, Th1 cells express three- to six-fold more CD26 protein than Th2 cells [7]. Other studies have indicated that CD26 expression induced the cytokine production of Th1 cells, including IL-2, IFN-γ, IL-10, and IL-12 [8]. *In vivo* , CD26-deficiency decreased the production of IL-2 and IL-4, delayed the production of IFN-γ in sera of mice after pokeweed mitogen (PWM)-stimulation, and increased secretion of IL-4, IL-5, and IL-13 in bronchoalveolar lavage (BAL) after ovalbumin-induced airway inflammation [9, 10]. In recent years, a new major effector population of $CD4^+$ T cells has been defined and designated as Th17 cells [11]. One of the Th17 signature cytokines is IL-17 which is a pro-inflammation factor. Besides of IL-17, Th17 cells can produce

other pro-inflammatory cytokines, including IL-22, IL-26, and IFN-γ [12]. Recent studies have shown that Th17 cells express IL-23R, lectin-like receptor CD161 and chemokine receptor CCR6 (CD196) [13, 14]. It has been reported that human Th17 cells express also a high level of DPPIV/CD26 [15]. However, the role of CD26 in the differentiation of Th17 cells has not been clearly investigated. Besides Th17 cells, regulatory T cells (Tregs) are another subpopulation of T helper cell. Tregs modulate the immune activities through their immunosuppressive effect on other self-reactive T cells thereby contributing to the maintenance of immunologic self-tolerance [16]. Previous studies found that the majority of human Tregs strongly and constitutively express CD25 ($CD25^{high}$), and a fork-head transcription factor (Foxp3) is required for the development and function of $CD4^+CD25^+$ regulatory T cells and regards as one of the specific markers of Tregs [16]. Recently we have demonstrated a delayed allogeneic skin graft rejection in CD26-deficient mice. During graft rejection, the concentration of IL-17 in serum and the percentage of cells secreting IL-17 in mouse peripheral blood lymphocytes (MPBLs) were both significantly lower while the percentage of regulatory T cells (Tregs) was significantly higher in MPBLs of $CD26^{-/-}$ mice than in those of $CD26^{+/+}$ mice [17]. To further investigate the role of CD26 in the differentiation of Th17 subpopulations of T lymphocytes, in this work, the correlation of CD26 expression with the differentiation of subsets of lymphocytes after antigen stimulation was investigated *in vitro* . We demonstrated that CD26 is closely involved in regulating the differentiation and functions of Th1 and Th17 subpopulations of T lymphocytes.

Materials and methods

Separation of human peripheral blood lymphocytes

Healthy human blood collection was performed according to the German Ethics laws, and approval (EA4/106/13) was obtained from the Ethics Committee of Charité Universitätsmedizin Berlin. Lymphocytes from peripheral blood were isolated using Ficoll density gradient centrifugation (GE Healthcare, Sweden). The isolation process was performed according to the manufacturer´s instructions.

Activation of human lymphocytes by antigen stimulation

Previous studies have indicated that certain monoclonal antibodies (mAbs) against human CD3 can activate T cells and induce the T cell proliferation. According to Schwinzer´s protocol [17], human peripheral blood lymphocytes (HPBLs) were stimulated by immobilized anti-human CD3 mAb (OKT3, IgG2a) (Thermo Fisher Scientific, USA). Briefly, each of 100 µL PBS with 2 µg/mL anti-CD3 mAb (stimulated group) or without antibodies (PBS, as negative control) were immobilized in a well of 96-well plates overnight. After removal of PBS buffer, 200 µL lymphocyte culture including 2×10^5 fresh isolated lymphocytes in RPMI-1640 growth medium (supplemented with 10% FBS, 100 µg/mL streptomycin and 100 UI/mL penicillin) were cultured directly in each well of the 96-well plate with or without immobilized antibody at 37°C in a humidified atmosphere with 5% CO_2 for 72 h.

Measurement of lymphocytes proliferation

The proliferation of lymphocytes after stimulation was measured by flow cytometry after cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) Assay Kit (Thermo Fisher Scientific, USA) according to the instructions of the manufacturer.

Separation of $CD26^+$ cells by magnetic cell sorting (MACS)

MACS MicroBeads (Miltenyi Biotec, Germany) were used for the separation of cells expressing CD26. Lymphocytes were collected at day three after stimulation. At first, the mouse anti-human CD26 mAb (anti-CD26 mAb_{350} prepared in our own laboratory) was used to label the lymphocytes for 1 h at 4°C. Following two washing steps, magnetic MicroBeads labeled with anti-mouse IgG were added to the cells and incubated further for 15 min at 4°C. After a washing step, cells were loaded into the column, which was pre-placed in the magnetic field of a suitable MACS Separator (Miltenyi Biotec, Germany). The unlabeled cells were collected after flow-through with two times wash processes. The labeled $CD26^+$ cells were bound to the column. After removing the column from the separator and placed in a suitable collection tube, the labeled $CD26^+$ cells were separated from the column and flushed out by help of a plunger. Finally, two groups of

cells, CD26 high-expressing ($CD26^{high}$) group and CD26 low-expressing ($CD26^{low}$) group, were obtained and then analyzed by flow cytometry.

Analysis of co-expression of CD26 with each of the cytokines or markers of different subpopulations of lymphocytes by flow cytometry

All the cell labelling with immune fluorescence-conjugated antibodies was performed in 1% (w/v) BSA/PBS at 4°C for 1 h in the dark. For the determination of co-expression of CD26 with cell surface markers of lymphocyte subpopulations, lymphocytes were incubated with both FITC-conjugated anti-human CD26 and PE-conjugated corresponding antibody simultaneously. For determination of the co-expression of CD26 with intracellular cytokines, after incubation with FITC-conjugated-anti-human CD26, the cells were washed and fixed with 4% formaldehyde for 5 min, washed again and subsequently permeabilized with 0.1% Triton X-100 in PBS for 10 min. After further wash steps after permeabilization, the cells were then incubated with PE-conjugated corresponding antibody. Fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated antibodies (direct against CD26, CD4, CD8, CD69, CD25, CD71, IL-2, IFN-γ, IL-4, IL-13, IL-6, IL-17, IL-22, IL-23R), as well as allophycocyanin (APC)-conjugated anti-FoxP3 antibodies were obtained from ImmunoTools (Friesoythe; Germany). Allophycocyanin (APC)-conjugated anti-CD161 and Per-conjugated anti-CD196 antibodies were provided by MACS Miltenyi Biotec (Bergisch Gladbach, Germany). After the immunofluorescent cells were resuspended in FACS buffer and measured by flow cytometry, the WinMDI 2.9 software was used to analyze the percentages of different lymphocyte subpopulations or cytokine secreting cells.

Fluorescence immunomicroscopy

The immunofluorescence staining of cell surface or intracellular proteins was performed as above. Thereafter, cells were resuspended in 20 μL PBS after twice washing steps with PBS and covered on a slide with a thin layer. After air-drying, cell layers were added with mounting solution (Termo Fische Scientefic, USA) and covered by coverslips for fluorescence microscopy. Images were made at a magnification of 600×.

Statistical analysis

All data represent the mean value ± SD from a minimum of five independent experiments with at least five healthy donor HPBLs samples, and each experiment was repeated more than three times. The statistical differences of values were calculated using ANOVA-analysis. Differences between groups were considered significant at $p < 0.05$;$p < 0.01$; $p < 0.005$ and$p < 0.001$; p- values were calculated with a Chi-square test.

Results

Part of the lymphocytes were activated and proliferated, and the expression of CD26 was upregulated after antigen stimulation

Three days after stimulation by immobilized anti-CD3 mAb, the activation of HPBLs was determined by the measurement of expression of different lymphocyte activation markers (CD69, CD25, CD71, and CD26). In comparison to non-activated control cells, the percentage of $CD26^+$ HPBLs was significantly increased after stimulation by 85% (33±8% *vs.* 61±14% of total HPBLs,$p < 0.001$) **(Figure 1A)** , while the percentages of $CD69^+$ and $CD71^+$ cells were 6-fold and 5-fold compared to control cells (54.29± 20.87% *vs.*9.07±7.28%, $p < 0.01$; 30.6±14% *vs.* 5.8±2.46%,$p < 0.05$), respectively, and the percentage of $CD25^+$ HPBLs was 68% higher than the value in the control group (17.65±6.58% *vs.* 10.49±9.41%) **(Figure 1B)** . These results indicate that a substantial part of the HPBLs was activated after stimulation with immobilized anti-CD3 mAb.

To determine the proliferated new generations of lymphocytes after stimulation, the CFSE assay was used. As shown in **Figure 1C** and**D** , at day three after stimulation, the stimulated group (hollow black histogram) showed five additional peaks that represent five increased generations of HPBLs whereas the PBS control group (shaded red histogram) showed only one peak remaining in the original position, indicating that no new generation was generated. These results provide evidence that the lymphocytes proliferated and increased

by up to five new generations after stimulation compared to the lymphocytes of the PBS control group that had not proliferated within three days.

Increased percentages of $CD4^+$-, $CD4^+CD26^+$- and $CD8^+CD26^+$-HPBLS after stimulation

In order to clarify the role of CD26 in lymphocyte differentiation, the percentages of $CD4^+$ T lymphocytes (T helper cells) and $CD8^+$ T lymphocytes (T cytotoxic cells), as well as the percentage of cells that were co-expressing each of these two subpopulation markers with CD26 were analyzed after stimulation. As shown in **Figure 2** , after stimulation, the percentage of $CD4^+$ cells was increased from 32.57±8.91% to 54.72±12.85% of total HPBLs while the percentage of $CD8^+$ cells did not increase significantly. This result suggests a strong proliferation of the T helper subpopulation ($CD4^+$) of T lymphocytes after stimulation. Further analysis revealed that after stimulation the percentage of cells that were co-expressing CD4 and CD26 ($CD4^+CD26^+$) in total HBPLs was 2.8-fold of that in the control group (39.98% *vs.* 14.43%). In the stimulated $CD4^+$ subpopulation, about 73% of the $CD4^+$ cells were co-expressed with CD26, while in the control $CD4^+$ subpopulation only 40% of the $CD4^+$ cells were co-expressed with CD26 (**Figure 2A, B**). As previously known, CD26 is a co-stimulator of T cell activation; the increased T helper cells ($CD4^+$) after stimulation were mostly co-expressed with CD26 observed in the present work, indicating that the activation and proliferation of $CD4^+$ cells are closely related to CD26 expression.

While the percentage of $CD8^+$ cells did not increase significantly after stimulation, we found that the percentage of $CD8^+CD26^+$ cells in the stimulated group was about 2.1 times that of the control group (14.28±3.35%*vs.* 6.72±4.21%). In the stimulated group approx. 40% of $CD8^+$ cells were co-expressing CD26, compared with 21% of the CD8 cells in the control group **(Figure 2C, D)** . The increased $CD8^+CD26^+$ cells suggest that CD26 is also related to the activation of $CD8^+$cells. Interestingly, the percentage of total $CD8^+$cells was not increased significantly. Since cell survival analysis showed that almost no dead lymphocytes were observed after stimulation (data not shown), it suggests that T cytotoxic $CD8^+$cells hardly proliferated, or their proliferation rate was much slower than that of $CD4^+$ cells.

Higher percentages of $CD4^+$, $CD4^+CD26^+$ and $CD8^+CD26^+$ cells in $CD26^{high}$ group

For further analysis of the correlation of CD26 to T cell differentiation, after stimulation, $CD26^+$cells were separated using MACS MicroBeads conjugated with anti-mouse IgG after binding of $CD26^+$ -lymphocytes with anti-human CD26 mAb **(Figure 3)** . After separation, two groups of cells were obtained: CD26 low-expressing group ($CD26^{low}$) and CD26 high-expressing group ($CD26^{high}$). The expression profiles of $CD4^+$ and $CD8^+$ and their co-expression with CD26 on surfaces of cells in the $CD26^{low}$ and the $CD26^{high}$ group were analyzed. As shown in **Figure 4** , the percentage of $CD4^+$ cells in $CD26^{high}$ group was 2.2-fold of that in $CD26^{low}$ group (62.70±14%*vs.* 28.28±9%, p <0.005), while the percentage of $CD8^+$ cells was lower in $CD26^{high}$group compared to the $CD26^{low}$ group (32.24%±5%*vs.* 45.11±9%, p <0.05). Further analysis showed that the percentage of $CD4^+CD26^+$cells in the $CD26^{high}$ group was 6-fold of that in the $CD26^{low}$ group (44.27±15% *vs.* 7.13±7%,p <0.01) **(Figure 4A, B)** , while the percentage of $CD8^+CD26^+$ cells in the $CD26^{high}$ group was about 3.5-fold of that in the $CD26^{low}$ group (12.93±6% *vs* . 3.72±0.9%,p <0.05) **(Figure 4C, D)** . These results showed that the expression of CD26 occurred mostly in T helper cells ($CD4^+$) and only a small part of T cytotoxic cells ($CD8^+$) expressed CD26 after stimulation, indicating activation of most T helper cells ($CD4^+$) but only a few T cytotoxic cells ($CD8^+$). In consideration of the greatly increased percentages of $CD4^+$ cells and $CD4^+CD26^+$ cells after stimulation, it is undoubtedly that CD26 is closely involved in the proliferation of T helper cells ($CD4^+$).

Higher secretion of Th1- and Th17-typical cytokines or expression of Th17 molecular markers in cells of $CD26^{high}$ group

To investigate the association of CD26 expression with CD4 cell differentiation, the percentages of T helper subpopulations were determined by flow cytometry after cells were labeled with fluorescence-conjugated antibodies against corresponding cytokines or cell surface markers. The results showed that the percentages of cells secreting Th1-typical cytokine IL-2 and IFN-γ in the $CD26^{high}$ group were significantly higher than those in the $CD26^{low}$ group (**Figure 5A)** . The percentage of cells secreting IL-2 in the $CD26^{high}$

group was approximately three times that of the $CD26^{low}$ group (25.93±5.39% *vs.* 8.89±5.85%), and the percentage of cells secreting IFN-γ in the $CD26^{high}$ group was about seven times that of the $CD26^{low}$ group (30.17±11.14%*vs.* 4.45±2.63%). Similarly, in **Figure 5B** , the percentages of cells secreting Th17-typical cytokines (IL-6, IL-17, IL-22) or expressing biomarkers (IL-23R, CD196 and CD161) were evidently higher in the $CD26^{high}$ group than in the $CD26^{low}$ group. The percentages of cells secreting IL-6 or lL-17 in the $CD26^{high}$ group were about 7-fold of that in the $CD26^{low}$ group (28.11% *vs.* 4.12%, 31.28% *vs.* 4.32%). The percentage of cells secreting IL-22 in the $CD26^{high}$ group was 5.4-fold compared to the $CD26^{low}$ group (31.05% *vs.* 5.74%). The percentage of cells expressing IL-23R was even higher in the $CD26^{high}$ group, 7-fold of that in the $CD26^{low}$ group (35.93% *vs.* 4.98%). In addition, the percentages of cells expressing Th17 surface biomarkers CD196 and CD161 in the $CD26^{high}$ group were 2.8-fold and 3-fold of that in the $CD26^{low}$ group (34.73%*vs.* 12.35%, 42.52% *vs.* 13.59%), respectively. Histogram analysis showed that the expression levels of Th1 and Th17 typical cytokines (IL-2, IFN-γ, IL-6, IL-17, IL-22) or a Th17 typical surface marker (IL-23R) in the cells of the $CD26^{high}$ group were much higher in relation to the cells of $CD26^{low}$group **(Figure 5C)** . These results suggest that the expression of CD26 is involved in the regulation of the differentiation and functions of Th1 and Th17 subpopulations of T lymphocytes.

On the other hand, the percentages of cells secreting Th2-typical cytokines either IL-4 or IL-13 showed exceptionally low and no significant differences between the $CD26^{high}$ group and the $CD26^{low}$ group **(Figure 5A)** . Similarly, the histogram analysis showed that there were no significant differences in the expression levels of Th2 cytokines (IL-4 and IL-13) in cells between the $CD26^{high}$ group and the $CD26^{low}$group **(Figure 5C)** . In addition, the percentages of cells expressing molecular markers of regulatory T cells ($CD25^{+}Foxp3^{+}$ or $CD4^{+}Foxp3^{+}$) in the $CD26^{high}$ group did not have significant differences to that in the $CD26^{low}$ group **(Figure 5D)** . These results suggest that the CD26 expression is not correlated to the differentiation and functions of Th2 and Tregs subpopulations of T lymphocytes after antigen stimulation.

Co-expression of CD26 with Th1- or Th17-typical cytokines in cells of $CD26^{high}$ group

The association of CD26 expression to the differentiation of Th1 or Th17 subset was further analyzed by determination of the co-expression of CD26 with each of Th1 typical cytokines (IL-2 or IFN-γ), Th17 typical cytokines (IL-6, IL-17, IL-22), or Th17 specific surface marker (IL-23R). In comparison to CD26 ^{low} group, the percentages of cells that were co-expressing CD26 with each of these cytokines were obviously higher in the $CD26^{high}$ group**(Figure 6A)** . The percentages of cells that were co-expressing CD26 with IL-2 ($CD26^{+}$ $IL\text{-}2^{+}$) or IFN-γ ($CD26^{+}IFN\text{-}\gamma^{+}$) in the $CD26^{high}$group were 3.5- and 3-fold of that in the $CD26^{low}$group (20.31% *vs.* 5.83% and 15.66% *vs.* 5.18%), respectively. Notably, the percentages of cells that were co-expressing CD26 with IL-17 ($CD26^{+}$ $IL\text{-}17^{+}$), IL-6 ($CD26^{+}$ $IL\text{-}6^{+}$) or IL-22 ($CD26^{+}$ $IL\text{-}22^{+}$) in the $CD26^{high}$ group were nearly 6-fold, 5-fold and 6.5-fold of that in the $CD26^{low}$ group (20.14%*vs.* 3.43%, 14.81% *vs.* 3% and 18.64% *vs.*2.86%), respectively. Also, the percentage of cells that were co-expressing CD26 with Th17 marker IL-23R ($CD26^{+}$ $IL\text{-}23R^{+}$) in $CD26^{high}$ group was 6-fold compared to the $CD26^{low}$ group (23.14% *vs.* 3.7%)**(Figure 6A)** .

Fluorescence microscopy detected that the CD26 protein was predominantly located on the cell plasma membrane, while IL-2, IFN-γ, IL-17, and IL-22 were present in the cytosol, and IL-23R was also mainly located on the cell surface. After merging the photos, CD26 was found to be co-expressed with IL-2, IFN-γ, IL-17, IL-22, or IL-23R **(Figure 6B)** in the same lymphocytes. Since IL-2 and IFN-γ are typical Th1 cytokines, the co-expression of Th1-cytokines with CD26 suggests a correlation of CD26 to the differentiation and function of Th1 cells. Similarly, IL-17 and IL-22 are typical Th17 cytokines, and IL-23R is a typical Th17 cell surface marker. Therefore, the co-expression of Th17-cytokines or -markers with CD26 suggests a correlation of CD26 to the differentiation and function of Th17 cells.

Discussion

CD26 was determined as one of co-stimulator for T cell activation [18], and the co-stimulatory effect of CD26 for T cell activation could be mediated by the interaction of CD26 with the ecto-adenosine deaminase (ADA), tyrosine phosphatase CD45, CARMA1 or caveolin-1 [4, 19]. In the present work, antigens of lymphocytes

were stimulated by using an immobilized anti-CD3 mAb (OKT3, IgG2a) to further investigate the role of CD26 in T cell differentiation [20]. Three days after stimulation, the activation of lymphocytes was determined by the enhanced expression of lymphocyte activation markers CD69, CD71, CD25 or CD26 (Figure 1A and B). CD69 is one of the earliest cell surface antigens expressed by T cells following activation. It acts as a co-stimulatory molecular and surface marker for T cell activation and proliferation [21]. CD71 (the transferrin receptor) and CD25 (the IL-2 receptor) are other two molecular surface markers of T cell activation and proliferation [22]. The significant increase in the expression of CD69, CD71, and CD25 indicates that most of the lymphocytes are activated after stimulation. In addition, CD26 expression was also significantly upregulated after stimulation**(Figure 1A)** suggesting an association of CD26 to the activation of T lymphocytes, which is consistent with previous studies [23]. After stimulation, the co-expression level of CD26 with $CD4^+$ or $CD8^+$ was increased markedly**(Figure 2)** , which indicates that the expression of CD26 is related not only to the activation of $CD4^+$ cells, but also to a certain extent, the activation of CD8 cells. A previous study reported that a unique pattern of CD26-high expression was identified on influenza-specific $CD8^+$ T cells but not on $CD8^+$ T cells specific for cytomegalovirus, Epstein Barr virus or HIV, which suggested that high CD26-expression may be a characteristic of long-term memory cells [24]. A later study indicated that $CD26^+CD8^+$ cells belong to the early effector memory T cell subsets. The CD26 mediated co-stimulation of $CD8^+$ cells provokes effector function via granzyme B, tumor necrosis factor-α, IFN-γ and Fas ligand [25]. The role of CD26 in the differentiation and function of $CD8^+$ cells needs further investigation.

Thereafter the proliferation of lymphocytes was analyzed after stimulation. It was found that in comparison to lymphocytes without stimulation (PBS control), which did not proliferate, the antigen stimulated lymphocytes proliferated up to five generations**(Figure 1C, D)** . Further analysis showed that after stimulation, the percentage of $CD4^+$ cells in total HBLs was increased significantly while the percentages of $CD8^+$did not change (**Figure 2)** . The up-regulated percentage of $CD4^+$ cells suggests that the immobilized anti-CD3 mAb triggered mainly the proliferation of $CD4^+$ lymphocytes [26]. It was found that the percentage of $CD4^+$cells in the $CD26^{high}$ group was significantly higher than in the $CD26^{low}$ group, and most of the CD4 cells were co-expressed with CD26 **(Figure 4A, B)** . Previously, Ohnuma et al. have reported that CD26 was thought to be mostly expressed by memory T helper cells, and its expression was preferential on $CD4^+$ cells and associated with T cell activation as a costimulatory molecule [4, 5]. Blockade of CD26-mediated T cell co-stimulation with soluble caveolin-1 induced anergy in $CD4^+$ cells [27]. Besides studies on the involvement of CD26 in the activation and proliferation of $CD4^+$ T cells *in vitro* , *in vivo*investigation using CD26 knockout mice presented a decreased percentage of $CD4^+$ cells [10]. $CD4^+$ cells are T helper cells and they can secret different cytokines upon T cell activation and these cytokines play a crucial role in the activation and/or proliferation of other effector cells, such as B cells, cytotoxic T cells and macrophages. The higher percentage of $CD4^+$cells in $CD26^{high}$ group and CD26 high expression in activated $CD4^+$ cells observed in the present work further confirms that CD26 expression is involved not only in the activation, but also in the proliferation and in further bioprocesses and functions of $CD4^+$ cells.

After activation, $CD4^+$ cells proliferate and differentiate into different subpopulations. Th1 and Th2 are the two main and earliest defined subpopulations of T helper cells [28]. Th1 cells can potentially produce large amounts of IFN-γ and IL-2 cytokines while Th2 effector cells are characterized by the production of IL-4 and IL-13 [29]. In present work, after cell sorting of CD26-expressing cells, the percentages of cells secreting each of Th1-typical cytokines IFN-γ and IL-2 in the $CD26^{high}$ group were significantly higher than in the $CD26^{low}$ group**(Figure 5A, C)** . Moreover, most of cells secreting IFN-γ or IL-2 were co-expressing CD26 **(Figure 6)** . In a previous study, the up-regulation of CD26 expression on $CD4^+$ cell surfaces was identified to be related to the production of Th1 cytokines [4]. It was reported that the solid-phase immobilized anti-CD26 mAb had a comitogenic effect by inducing $CD4^+$ lymphocytes proliferation and enhancing IL-2 production in conjunction with submitogenic doses of anti-CD3 [30]. The inhibitor of DPPIV/CD26 enzyme activity has been suggested to be able to reduce the production of IL-2, IL-6 and IFN-γ of human and mouse T cells under mitogen stimulation [8]. Supporting these findings, the results of the present work showed that the expression of CD26 is associated with the differentiation of Th1 cells. Th1 is an important subset of T

helper cells. The positive relation between the activation of $CD4^+$ cells and CD26 expression **(Figure 4A, B)**benefits the differentiation of $CD4^+$ cells into a Th1 subset.

Interestingly, the percentages of cells secreting Th2-typical cytokines IL-4 or IL-13 were not only very low (<5%) in $CD26^{low}$ and $CD26^{high}$ groups, but they also didn´t present any difference between the both kinds of cell groups **(Figure 5A)** . As one of the main subpopulations of T helper cells, Th2 subset is often recognized as an opposite of Th1 cells since Th2 cytokines may suppress the activity and proliferation of Th1 cells during immune responses [31]. Our results indicate that CD26 expression is not related to the differentiation of $CD4^+$ cells into the Th2 subset after antigen stimulation.

Besides Th1 and Th2 subsets, Th17 and Tregs are other two important subsets of T helper subpopulations. Th17 is a more recently identified subset of $CD4^+$ cells [11], which is distinct from classic Th1 and Th2 subsets [32]. These cells originate from naive $CD4^+$ precursor cells mainly in the presence of TGF-β and IL-6, and their differentiation requires IL-23 [32]. As a novel member of $CD4^+$ T subset, it is important to clarify the role of CD26 in the differentiation and function of Th17 cells. After cell sorting, the percentage of cells secreting Th17 typical cytokines (IL-17 and IL-22), or expressing Th17 molecular markers (IL-23R, CD161, CD196) were found to be significantly higher in the $CD26^{high}$ group than in the $CD26^{low}$group **(Figure 5B)** . Moreover, most of cells secreting IL-17 and IL-22 or expressing IL-23R, CD161 and CD196 were co-expressed with CD26**(Figure 6)** . This indicates an involvement of CD26 in the differentiation of $CD4^+$ cells into Th17 subset. A previous study showed that Th17 cells express a high level of CD26, and the phenotypic analysis of Th17 cells could be identified by the CD26 expression [15]. Th17 cells play an important role in preventing the pathogen invasion through secreting pro-inflammatory cytokines. Clinical research found that CD26 was related to some diseases which involved the immune response initiated by Th17 cells through inducing chronic inflammation or autoimmunity, like rheumatoid arthritis and multiple sclerosis [33].

Recently, it has been reported that inhibition of the enzyme activity of CD26 by sitagliptin reduced the proliferation and Th1/Th17 differentiation of human lymphocytes*in vitro* [34], and the deficiency of CD26 or the inhibition of DPPIV enzyme activity induced the reduction of IL-17 expression and increased the allograft acceptance *in vivo* [35, 36]. We have also shown recently that CD26-deficiency resulted in a delayed allogeneic skin graft rejection after allogeneic skin transplantation. The concentrations of serum IgG, including its subclasses IgG1 and IgG2a, were significantly reduced in $CD26^{-/-}$ mice during graft rejection. The secretion levels of the cytokines IFN-γ, IL-2, IL-6, IL-4, and IL-13 were significantly reduced whereas the level of the cytokine IL-10 was increased in the serum of $CD26^{-/-}$ mice compared to $CD26^{+/+}$mice. Additionally, the concentration of IL-17 in serum and the percentage of cells secreting IL-17 in mouse peripheral blood lymphocytes (MPBLs) were both significantly lower while the percentage of regulatory T cells (Tregs) was significantly higher in MPBLs of $CD26^{-/-}$ mice than in those of $CD26^{+/+}$ mice [17]. In line with the results of these *in vivo* experiments, the results of the present *in vitro* study confirm that the expression of CD26 is not only highly correlated to the differentiation of Th1 and Th7, but also plays an important role in the differentiation and function of Th1 and Th7. It is precisely because CD26 plays an indispensable role in the differentiation and function of Th1- and Th17-lymphocytes, which results in a lack of effective Th1 and Th17 cells when CD26 is absent under relevant pathological conditions. The present study provides more insight into the role of CD26 for the function of Th17 cells and related diseases and will support future research in this field.

It is reported that CD26 can be used as a negative selection marker for Tregs [37]. In the present study, the percentages of Tregs were very low in $CD26^{high}$ and $CD26^{low}$ groups, and no significant difference was found between the two groups**(Figure 5D)** , indicating that the expression of CD26 is not necessary for the differentiation of Tregs after immobilized anti-CD3 mAb stimulation.

In conclusion, CD26 is not only an activation marker for T lymphocytes, but its expression is closely related to the subsequent proliferation, differentiation, and functions of T lymphocytes. Considering that the balance between Th1 and Th2 and the balance between Th17 and Tregs play a prominent role in immune responses, our results in this study demonstrated that the high expression of CD26 is beneficial to the differentiation

of T lymphocytes into Th1 and Th17 subpopulations after antigen stimulation, indicating a crucial role of CD26 in regulating the immune response to the inflammation and autoimmune reactions. The correlation of CD26 with the differentiation balance between Th1 and Th2 and between Th17 and Tregs observed in this study provides more insights into the role of CD26 in related diseases. The important role of CD26 in immune regulation suggests that it would become a therapeutic target for related diseases [38] .

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Figure legends

Figure 1. The activation and proliferation of T lymphocyte after stimulation. After 72 h of the stimulation with immobilized anti-CD3 mAb, the activation of lymphocytes was determined by the measurement of the expression of T lymphocyte activation markers (CD69, CD71, CD25, CD26). The proliferation of lymphocytes was analysed by CFSE assay. PBS treated cells were used as controls. (**A**) Percentages of $CD26^+$-HPBLs in control group and stimulated group. (**B**) Percentages of $CD69^+$-, $CD71^+$- and $CD25^+$-HPBLs in control group and stimulated group. Data represented mean value ± SD from a minimum 5 independent experiments with at least 5 healthy donor HPBLs samples, and each experiment was repeated more than 3 times.*P* -values were calculated with a Chi-square test. (**C**) Histogram of the proliferated generations of lymphocytes after stimulation by immobilized anti-CD3 mAb (anti-CD3) or only PBS as control (PBS) for three days. The shaded histogram represents the original generation (0g) of PBS control group at day 3. The hollow histogram indicates the increased 5 generations (1g, 2g, 3g, 4g and 5g) of the stimulated group three days after stimulation. (**D**) The dot plots show the proliferated generations of lymphocytes analyzed by flow cytometry.

Figure 2. Percentages of $CD4^+$ and $CD8^+$ cells, and cells co-expressing each of these surface markers with CD26 after stimulation. (**A**) Percentages of $CD4^+$ and $CD4^+CD26^+$ cells in control group and stimulated group. (**C**) Percentages of $CD8^+$ and $CD8^+CD26^+$ cells in control group and stimulated group. Data represented mean value ± SD from five independent experiments with five healthy donor HPBLs samples, and each experiment was repeated more than three times. *P* -values were calculated with a Chi-square test. The dot plots show one typical experiment for the analysis of percentages of $CD4^+$ and $CD4^+CD26^+$ cells (**B**) and percentages of $CD8^+$ and $CD8^+CD26^+$ cells (**D**) by flow cytometry.

Figure 3. Separation of $CD26^{low}$ and $CD26^{high}$ lymphocytes by magnetic cell sorting (MACS).(**A**) Procedure of separation of $CD26^{low}$ and $CD26^{high}$ lymphocytes by MACS. After stimulation for three days, lymphocytes were labeled with the mouse anti-human CD26 mAb (anti-CD26 mAb_{350} prepared in our own laboratory) for 1 h at 4°C. Following the two washing steps, magnetic MicroBeads labeled with anti-mouse IgG were added to the cells and incubated further for 15 min at 4°C. After a washing step, cells were loaded into the column which was pre-placed in the magnetic field of a suitable MACS Separator (Miltenyi Biotec, Germany). The unlabeled cells were collected after flow-through with two times wash processes. The labeled $CD26^+$ cells were bound to column, and then flushed out after removing the column from the separator by help of a plunger. (**B**) Analysis of CD26 expression in CD26 high-expressing ($CD26^{high}$) group and CD26 low-expressing ($CD26^{low}$) group by flow cytometry. Data represented mean value ± SD from a minimum five independent experiments with at least five healthy donor HPBLs samples. (**C**) The dot plots show one typical experiment for analysis of CD26 expression.

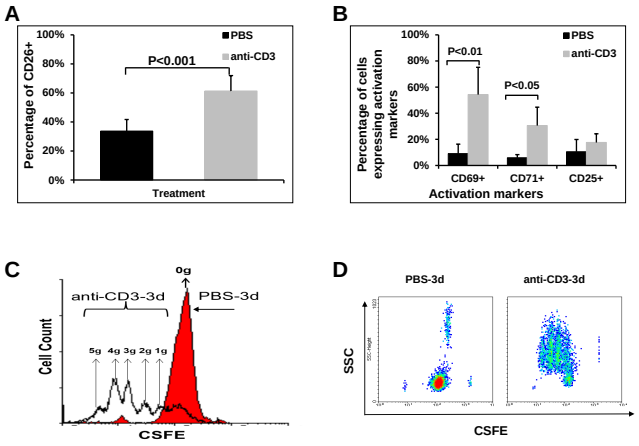
Figure 4. Percentages of $CD4^+$, $CD8^+$, $CD4^+CD26^+$and $CD8^+CD26^+$ cells in $CD26^{low}$ and $CD26^{high}$ groups.(**A**) Percentages of $CD4^+$ and $CD4^+CD26^+$ cells in $CD26^{low}$ and $CD26^{high}$ group. (**C**) Percentages of $CD8^+$ and $CD8^+CD26^+$ cells in $CD26^{low}$ and $CD26^{high}$ group. Data represented mean value ± SD from seven independent experiments with seven healthy donor HPBLs samples, and each experiment was repeated more than three times. Dot plots show the percentages of $CD4^+$, $CD4^+CD26^+$cells (**B**), $CD8^+$, and $CD8^+CD26^+$ cells (**D**) in $CD26^{low}$ and $CD26^{high}$ group.

Figure 5. Percentage of cells secreting different cytokines in the $CD26^{low}$ and the $CD26^{high}$groups. After separation, the cells in the $CD26^{low}$group and the $CD26^{high}$ group were labe-

led with different monoclonal antibodies against-cytokines or surface markers at 4°C for 30 min and then measured by flow cytometry. (**A**) The percentages of cells secreting Th1- and Th2-typical cytokines in $CD26^{low}$ and $CD26^{high}$ group. (**B**) The percentages of cells secreting Th17-typical cytokines or expressing Th17-typical biomarkers in $CD26^{low}$ and $CD26^{high}$ group. (**C**) Overlay histograms demonstrate the relative expression of cells secreting different cytokines in $CD26^{low}$ and $CD26^{high}$groups. The black line indicates the values of $CD26^{low}$group cells while the color lines indicated the values of $CD26^{high}$ group cells. (**D**) The percentages of cells expressing Tregs-typical biomarkers in $CD26^{low}$and $CD26^{high}$ group. Data represented mean value ± SD from a minimum of five independent experiments with at least five healthy donor HPBLs samples, and each experiment was repeated more than three times.

Figure 6. Co-expression of CD26 with each of Th1- or Th17-typical cytokines or surface markers in the cells of $CD26^{low}$ and $CD26^{high}$ groups. Lymphocytes were harvested at 72 h after stimulation and were double-stained with the FITC-conjugated anti-CD26 mAb and PE-conjugated anti-IL-2, anti-IFN-γ, anti-IL-17, anti-IL-6, anti-IL-22, or anti-IL-23R mAb. (**A**) Percentages of cells co-expressing CD26 with each of Th1-typical cytokines (IL-2, or IFN-γ), Th17-typical cytokines (IL-6, IL-17, IL-22) or Th17-typical surface marker (IL-23R) in the $CD26^{low}$ and $CD26^{high}$ groups. Data represented mean value ± SD from a minimum five independent experiments with at least five healthy donor HPBLs samples, and each experiment was repeated more than three times. (**B**) Co-expression of CD26 with Th1- or Th17-typical biomarkers was observed by fluorescence microscopy. Images were made at 600× magnifications. Co-expressing of CD26 with IL-2, IFN-γ, IL-17, IL-22, or IL-23R in some lymphocytes indicated by the merged images.

Figure 1



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