Deficiency of CD26 results in a change of cytokine and immunoglobulin secretion after stimulation by pokeweed mitogen

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To investigate the role of CD26 in the immune system, CD26 gene knockout mice with C57BL/6 background were used to study the immune response after stimulation with PWM. CD26$^{-/-}$ mice display an apparently normal phenotype. However, in their spleen lymphocyte population the percentage of CD4$^+$ T cells is lower, and that of NK cells is higher, than that in CD26$^{+/+}$ mice. In their peripheral blood, CD26$^{-/-}$ mice present a conspicuously decreased proportion of CD4$^+$ NKT lymphocytes. *In vitro,* the PWM-stimulated IL-4 production was decreased by 60–80% in the supernatants of spleen lymphocytes of CD26$^{-/-}$ mice compared to that of CD26$^{+/+}$ mice, whereas levels of IL-10 and IFN-γ were increased. No significant differences were found in the production of IL-2, IL-5, IL-6 and IL-13 between knockout and wild-type mice. After immunization of mice with PWM *in vivo*, serum levels of total IgG, IgG1, IgG2a and IgE were markedly lower in CD26$^{-/-}$ mice than those in CD26$^{+/+}$ mice, while no difference was found in IgM production. Further analysis of cytokine levels *in vivo* revealed a reduced IL-4, IL-2 and delayed IFN-γ production in sera of CD26$^{-/-}$ mice upon immunization with PWM. These results indicate that CD26 contributes to the regulation of development, maturation and migration of CD4$^+$ T, NK and NKT cells, cytokine secretion, T cell-dependent antibody production and immunoglobulin isotype switching of B cells.

1 Introduction

CD26, also known as dipeptidyl peptidase IV (DPPIV) (EC 3.4.14.5), is a multifunctional type II transmembrane glycoprotein [1]. It has been shown to play a crucial role in T cell activation and immune system functions [2–5]. This protein is expressed constitutively on epithelial cells of kidney, intestine and bile duct, and on several types of endothelial cells and fibroblasts, as well as leukocyte subsets such as T, B and natural killer (NK) lymphocytes and macrophages [6]. Like other ectopeptidases involved in immunologically relevant functions, such as CD10 (neutral endopeptidase, EC 3.4.24.11) and CD13 (aminopeptidase N, EC 3.4.11.2), the expression of CD26/DPPIV is strictly ontogenetically controlled. The expression level of CD26 on T cells is tightly regulated, and its density is markedly enhanced after T cell activa-

tion [7]. The number of CD26^{bright} cells and/or CD26 antigen density is higher during the active phase of autoimmune diseases, such as rheumatoid arthritis [8], multiple sclerosis, Graves' disease, Hashimoto's thyroiditis and sarcoidosis [5], and decreased during immunosuppression, as in AIDS [9, 10], Down's syndrome [11] and common variable hypogammaglobulinemia [5].

This enzyme is capable of cleaving N-terminal dipeptides from polypeptides with either proline or alanine resides in the penultimate position. Several cytokines, hematopoietic growth factors, neuropeptides and hormones share the X-Pro or X-Ala motif at their N terminus. As with N-terminal cyclization, the presence of a proline near the N terminus serves as structural protection against nonspecific proteolytic degradation. Most CD26/DPPIV-mediated truncation of natural substrates has drastic effects on the biological activity or function. Some substrates of this enzyme are closely related to the immune function, such as substance P, neuropeptide Y, endomorphin-2 [12], GLP-1 (glucagon-like peptide), GIP (glucose-dependent insulinotropic polypeptide) [13], RANTES (regulated on activation normal T cell

expressed and secreted) [14, 15], eotaxin [16], MDC (monocyte-derived chemokine) [17] and SDF-1α and SDF-1β (stromal derived factors) [18].

Synthetic inhibitors of the enzymatic activity of CD26 have been shown to suppress certain immune reactions *in vitro* and *in vivo* [19]. Inhibition of the enzymatic activity of CD26 by the use of specific inhibitors suppresses T cell proliferation *in vitro* and decreases antibody production in mice immunized with bovine serum albumin *in vivo* [20]. Whether the origin of the depressive effect on immunoglobulin secretion is T cell mediated or can be attributed solely to B cells is not yet clear.

It is now established that the T cell surface molecule CD26 is involved in T cell activation by a costimulatory pathway [21, 22]. On human T cells, CD26 exhibits a costimulatory function and plays an important role in the immune response via its ability to bind adenosine deaminase (ADA) [23] and mediate signaling by direct interaction with the cytoplasmic domain of CD45. CD26 promotes aggregation of lipid rafts and facilitates colocalization of CD45 to the T cell receptor signaling molecules p56Lck, ZAP-70 and TCRζ, thereby enhancing protein tyrosine phosphorylation of various signaling molecules and subsequent IL-2 production [24]. However, unlike other costimulatory molecules on the T cell, such as CD28, no receptor has been identified for CD26.

CD26 is a highly glycosylated membrane protein. The *N*-glycan structure contributes to the stability of this molecule [25] and is involved in the interaction with the extracellular matrix proteins, collagen and fibronectin [26–28]. The central domain of the extracellular part contains 10 of the 12 cysteine residues that are essential for formation of the disulfide bridges that constitute part of the functional structure. The collagen and ADA-binding domains are located within the cysteine-rich domain [29]. The recently solved crystal structure of CD26 will lead to a better understanding concerning the multifunctional properties of this protein [30].

Although a lot of evidence for the importance of CD26 in immunoactivation have been reported, the molecular mechanism of this activation has not yet been elucidated. In addition to *in vivo* targeting of CD26 with antibodies and/or specific inhibitors, knockout experiments and overexpression of CD26 should provide additional insights into the underlying molecular mechanisms. To investigate the role of CD26 in the immune system, CD26 gene knockout mice with C57BL/6 background [13] were used to study the activation and differentiation of lymphocytes after the application of different stimulators. In this study we investigated the influence of CD26 deficiency on cytokine and immunoglobulin secretion in response to pokeweed mitogen (PWM).

2 Results

2.1 The percentage of CD4+ cells is decreased, while that of NK cells is increased in spleen lymphocyte population of CD26−/− mice

To investigate whether CD26 expression influences the development of lymphocytes, the percentages of subsets of mouse spleen lymphocytes (MSL) were analyzed by flow cytometry. As shown in Fig. 1A, the percentage of CD3+ cells in CD26−/− MSL was 17% lower as compared to the values in CD26+/+ MSL (30.54±3.99% *vs.* 36.71±3.17%, *p*<0.01), while the percentage of CD19+ cells was not significantly different between knockout and wild-type mice (57.38±3.65% *vs.* 55.04±2.06%). In the CD3+ subpopulation of MSL, no difference was detected in the percentage of CD8+ cells between these two kinds of mice (11.58±2.09% *vs.* 13.24±2.03%). However, the percentage of CD4+ cells in MSL of CD26−/− mice was decreased by 23% as compared to that in
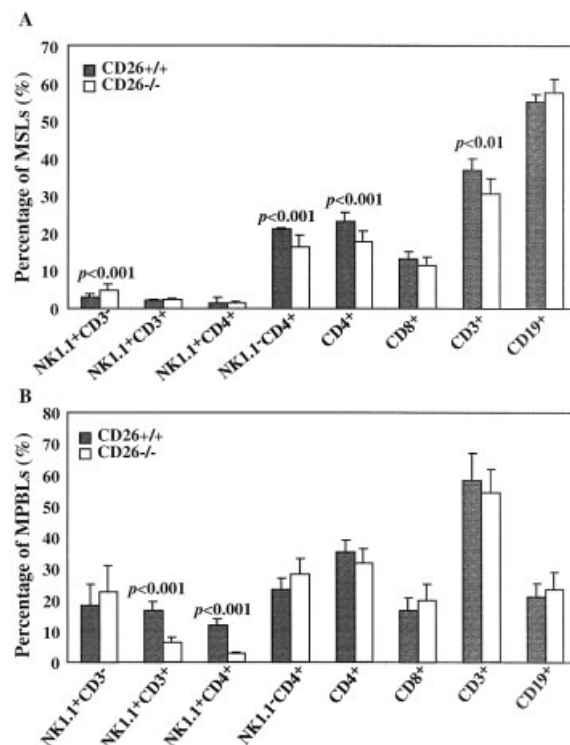

Fig. 1. Percentage of MSL (A) and MPBL (B) subpopulations. Lymphocytes were separated and analyzed by flow cytometry. The values represent the mean ± SD of 7 mice (A) or 20 mice (B).

MSL of CD26$^{+/+}$ mice (17.89±2.75% *vs.* 23.06±2.33%, $p<0.001$). This indicates that reduction of CD3$^+$ lymphocytes is caused by a decrease of the CD4$^+$CD3$^+$ population. In contrast, the proportion of spleen NK1.1$^+$CD3$^-$ (NK) cells was elevated by 67% in CD26$^{-/-}$ mice (4.93±1.47% *vs.* 2.95±0.78%; $p<0.001$), whereas the percentage of NK1.1$^+$CD3$^+$ (NKT) cells was similar in MSL of both knockout and wild-type mice.

2.2 The percentage of CD4$^+$ NKT cells is lower in the PBL population of CD26$^{-/-}$ mice

According to the data shown in Fig. 1B, in the mouse PBL population (MPBL), no difference was found in the percentages of CD3$^+$, CD4$^+$ or NK cells between the two groups of mice. However, the percentage of NKT cells in CD26$^{-/-}$ mice was decreased to 40% of that in CD26$^{+/+}$ mice (6.53±1.48% *vs.* 16.49±3.00 %, $p<0.001$). Subpopulations of NKT lymphocytes were further analyzed. We found that the decreased NKT lymphocytes were NK1.1$^+$CD4$^+$ cells, which represented only 22% of those in CD26$^{+/+}$ mice (2.69±0.44% *vs.* 12.06±1.92%; $p<0.001$).

2.3 CD26 deficiency results in a decreased proliferation of MSL after stimulation by PWM or Con A

To examine the influence of CD26 deficiency on the immune response, MSL from CD26$^{-/-}$ and CD26$^{+/+}$ mice were stimulated with the mitogens PHA, PWM, Con A and LPS. Subsequently, the proliferation of MSL was measured by the Alamar Blue assay. No significant differences in proliferation rates were observed after stimulation with PHA or LPS, but the proliferation rates of CD26$^{-/-}$ MSL were decreased by about 20% ($p<0.05$) after stimulation by PWM or Con A (Fig. 2).

2.4 MSL from CD26$^{-/-}$ mice secrete markedly less IL-4, but more IFN-γ and IL-10 after stimulation by PWM

To determine whether the expression of CD26 influences T cell differentiation and cytokine secretion, we analyzed the cytokine production of MSL stimulated with PWM *in vitro*. The levels of different cytokines in the supernatants were measured by ELISA at different times after stimulation. Fig. 3 shows that stimulation with PWM resulted in an augmented cytokine release into the supernatant. Differences in the IL-4 secretion of CD26$^{+/+}$ and CD26$^{-/-}$ MSL were particularly observable at 72 h and 96 h after stimulation. At these time points, supernatants from CD26$^{+/+}$ MSL contained 2.5- and 3.4-fold more IL-4,
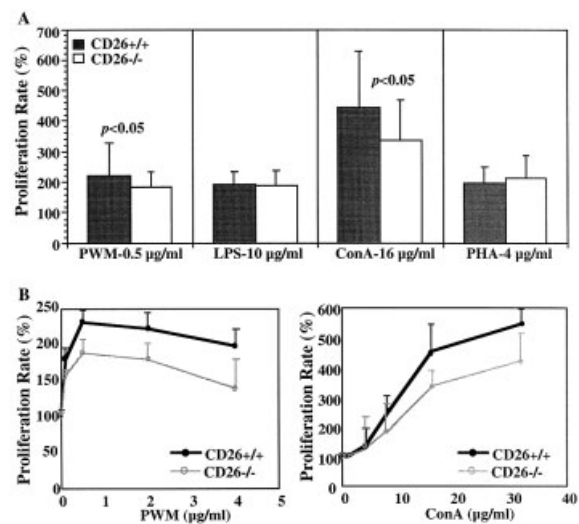

Fig. 2. (A) Influence of CD26 deficiency on the proliferation of MSL stimulated by different mitogens. The values represent the mean ± SD of seven mice. (B) Proliferation of MSL stimulated by PWM or Con A with different concentrations. Data are shown as triplicates of one of three representative experiments. Lymphocytes were stimulated as described in Sect. 4.4. The proliferation rate was determined by the Alamar Blue assay.

respectively, than those from CD26$^{-/-}$ MSL ($p<0.001$) (Fig. 3A). In contrast, CD26$^{-/-}$ MSL produced 1.6-fold more IFN-γ and 1.4-fold more IL-10 than CD26$^{+/+}$ MSL at 72 h after stimulation ($p<0.05$) (Fig. 3B, C), although the magnitude of the differences was much smaller than that of IL-4 production. No significant differences were found in the production of IL-2, IL-5, IL-6 and IL-13 (Fig. 3D–G).

2.5 CD26$^{-/-}$ mice produce markedly less IgG after immunization by PWM

The reduced IL-4 production by CD26$^{-/-}$ MSL prompted us to examine whether differentiation and immunoglobulin production of B lymphocyte were dependent on the expression of CD26. Mice were immunized with PWM at days 0 and 13, and the concentrations of different immunoglobulin classes in sera were measured by ELISA at different times after immunization. Fig. 4A and B show that the levels of IgM and IgG in serum reached a peak at days 6 and 19 after immunization. We found that both kinds of mice showed similar amounts of IgM production at each of the indicated times (Fig. 4A); however, CD26$^{-/-}$ mice showed significantly lower IgG levels, especially after primary immunization. At day 6, the increment in serum IgG levels of CD26$^{+/+}$ mice was 1,400 μg/ml (from 1,700 to 3,100 μg/ml), while in the sera of CD26$^{-/-}$ mice this increase was only 450 μg/ml (from 1,250 to
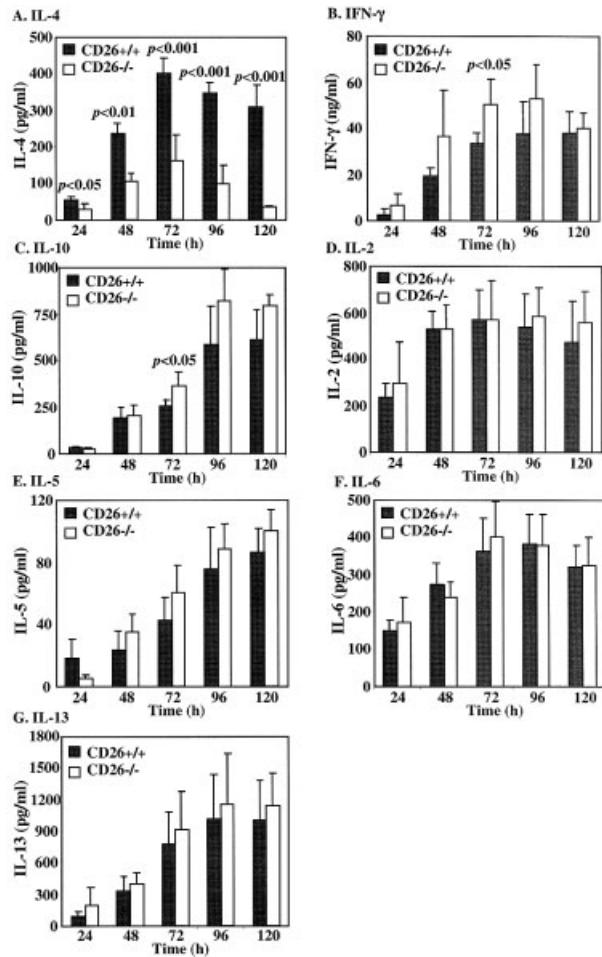
Fig. 3. Levels of IL-4, IFN-γ, IL-10, IL-2, IL-5, IL-6 and IL-13 in the supernatants of MSL after stimulation by PWM (0.5 μg/ml). Lymphocytes were stimulated as described in Sect. 4. Cytokine concentrations were measured by ELISA. The values represent the mean ± SD of a minimum of seven mice at each time point.

Fig. 4. Levels of IgM and IgG in the sera of mice after immunization with PWM. Mice were immunized twice on days 0 and 13 (40 μg/mouse, i.p.), respectively. Blood was taken at the indicated times. IgM and IgG concentrations in sera were analyzed by ELISA. The values represent the mean ± SD of a minimum of five mice at each time point.

1,700 μg/ml) (Fig. 4B). The IgG increase in CD26$^{-/-}$ mice was only one third of that in CD26$^{+/+}$ mice. Thus, deficiency of CD26 resulted in a decreased IgG production after primary immunization, but it did not result in decreased IgM production.

2.6 CD26 deficiency impaired B cell immunoglobulin isotype switching to IgG1, IgG2a and IgE

To further examine whether CD26 deficiency influences immunoglobulin isotype switching of B cells, the production of IgG1, IgG2a and IgE was measured at different time points after immunization of mice with PWM. As shown in Fig. 5, the concentrations of all these anti-
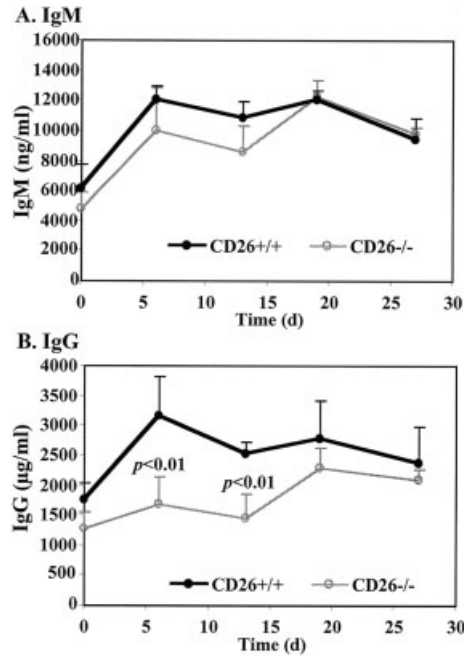
bodies were significantly lower in CD26$^{-/-}$ mice. Until day 13 after primary immunization, IgG1 production in CD26$^{-/-}$ mice increased barely and IgG2a production elevated slightly, in comparison with CD26$^{+/+}$ mice (Fig. 5A, B). The production of IgE was increased and reached a peak at day 19 after immunization. The maximal level of serum IgE in CD26$^{-/-}$ mice was lower than in CD26$^{+/+}$ mice (Fig. 5C).

2.7 Reduced IL-4 and IL-2, and delayed IFN-γ secretion in sera of CD26$^{-/-}$ mice immunized by PWM

The lower IgG production as well as the impaired immunoglobulin isotype switching in CD26$^{-/-}$ mice led us to determine the levels of the cytokines IL-2, IL-4, IL-5 and IFN-γ in sera after immunization by PWM. As shown in Fig. 6, injection of PWM induced a rapid elevation of these cytokines in sera. IL-2, IL-4, IL-5 and IFN-γ concentrations peaked at 2, 4, 4 and 12 h after immunization, respectively. All these cytokines dropped down to basic level 36 h after immunization (data not shown). In sera of CD26$^{-/-}$ mice the maximal value of IL-4 was 38% ($p < 0.01$) (Fig. 6C), and that of IL-2 about 50% ($p < 0.05$) of that in CD26$^{+/+}$ mice (Fig. 6A). The maximum of
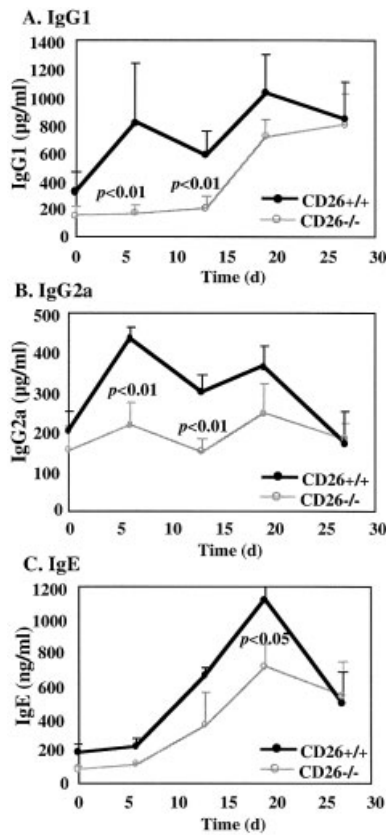
Fig. 5. Levels of IgG1, IgG2a and IgE in the sera of mice after immunization with PWM. Mice were immunized twice on days 0 and 13 (40 μg/mouse, i.p.), respectively. Blood was taken at the indicated times. IgG1, IgG2a and IgE concentrations in sera were analyzed by ELISA. The values represent the mean ± SD of a minimum of five mice at each time point.

IFN-γ production was not affected; however, a delayed IFN-γ elevation was observed in CD26$^{-/-}$ mice. At 2 h after immunization, CD26$^{-/-}$ mice secreted only half the amount of IFN-γ as compared to CD26$^{+/+}$ ($p<0.05$) (Fig. 6B). No significant difference was observed in IL-5 production (Fig. 6D).

3 Discussion

Although CD26$^{-/-}$ mice display an apparently normal phenotype, a subpopulation analysis of MSL reveals that they present a lower percentage of CD3$^+$ T lymphocytes together with a higher percentage of NK cells in their MSL population (Fig. 1A). Further analysis showed that the diminishing T lymphocytes in MSL are mainly CD4$^+$ cells (helper/memory population) (Fig. 1A). In the CD4$^+$ lymphocyte population, no significant difference was found in the ratio of memory *vs.* naive lymphocytes
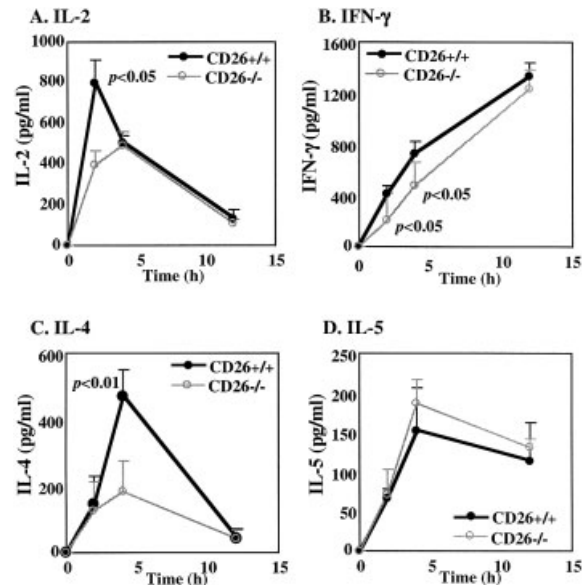

Fig. 6. Levels of IL-2, IFN-γ, IL-4 and IL-5 in the sera of mice after stimulation by PWM. Mice were immunized on day 0 (40 μg/mouse, i.p.). Blood was taken at the indicated times. Cytokine concentrations were measured by ELISA. The values represent the mean ± SD of six mice at each time point.

between CD26$^{-/-}$ and CD26$^{+/+}$ mice (data not shown). On the other hand, in MPBL of CD26$^{-/-}$ mice, the percentage of T cells as well as their subpopulations, CD4$^+$ and CD8$^+$ cells, was not significantly changed, in contrast with the marked decrease in the proportion of NKT cells. This decrease is due to the reduction of the CD4$^+$ NKT cells (Fig. 1B). These results suggest an involvement of CD26 expression in development, maturation and migration of CD4$^+$, NK and NKT cells. Ruiz et al. [31] have suggested that CD26 is ontogenetically controlled during T cell maturation. However, there was no evidence about the influence of CD26 expression on the development and maturation of NK and NKT cells. We showed for the first time that the deficiency of CD26 expression results in an increase of NK cells in MSL and a decrease of NKT cells in MPBL.

CD4$^+$ cells are T helper cells, which, upon activation, produce a number of different cytokines and play a role in the activation and/or proliferation of B cells, cytotoxic T lymphocytes and macrophages. NK cells are important effector cells involved in innate immunity against tumors and a variety of pathogens. They also participate in the induction and regulation of subsequent immune responses via release of various cytokines and chemokines, such as IFN-γ and TNF-α [32]. NKT cells are a distinct population of mature lymphocytes that co-express NK receptor and T cell antigen receptors. Upon *in vivo* stimulation, NKT cells produce large amounts of both

IL-4 and IFN-γ [33]. Thus, the changes of percentages of CD4$^+$, NK and NKT cells observed in the present work may be expected to influence the immune response of CD26$^{-/-}$ mice. Therefore, this prompted us to examine the response of CD26$^{-/-}$ mice to produce cytokines and immunoglobulins after stimulation *in vitro* and *in vivo*.

The activation and proliferation of MSL were determined after stimulation with different mitogens *in vitro*. A reduction in the proliferation rates was detected in CD26$^{-/-}$ MSL stimulated by Con A or PWM (Fig. 2). To further investigate the influence of CD26 expression on lymphocyte activation and differentiation, the production of different cytokines of MSL was analyzed after stimulation *in vitro*. Compared to Con A, PWM was found to be a better stimulator of IL-2 and IL-4 secretion. The IL-2 and IL-4 levels showed distinct maxima at 72 h after stimulation by PWM. These maxima were much greater than those following stimulation by Con A (data not shown). Thus, PWM was used as the stimulator in the following experiments. The deficiency of CD26 affected mostly IL-4 secretion (Fig. 3) after PWM stimulation. We further tried to identify IL-4 and IFN-γ producing subsets in MSL. FACS analysis showed that IL-4 was produced by CD4$^+$ lymphocytes, while the IFN-γ-producing cells consisted of not only CD4$^+$ but also CD4$^-$ populations. The lower level of IL-4 in the supernatants of CD26$^{-/-}$ MSL is probably due to the lower percentage of CD4$^+$ cells or to the impaired capacity of CD4$^+$ cells to produce IL-4. In addition, considering the capacity of NK cells to produce IFN-γ, an increased percentage of NK cells in CD26$^{-/-}$ MSL also should have contributed to the inhibition of IL-4 and enhancement of IFN-γ.

The cytokine levels in sera of both strains of mice were also measured after immunization by PWM *in vivo*. Similar to the results from the *in vitro* test, deficiency of CD26 affected mainly IL-4 secretion (Fig. 6A). In contrast to that seen in the *in vitro* assay, deficiency of CD26 not only influenced the secretion of the Th2 cytokine IL-4, but also the production of Th1 cytokines, IL-2 and IFN-γ. Its deficiency results in a reduced IL-2 and delayed IFN-γ secretion (Fig. 6C, D). CD26 may exert its influence on the immune system through two mechanisms. On the one hand, this protein exhibits a costimulatory function on T cell activation via its ability to mediate signaling through the direct interaction with the cytoplasmic domain of CD45 [24], which could influence the differentiation of T lymphocytes and hence their cytokine production. On the other hand, some substrates of DPPIV, such as RANTES, eotaxin, MDC, SDF-1α and SDF-1β, have a direct effect on the immune response. Deficiency of this enzyme could also reduce the Th1 response, and hence reduce the Th1 cytokine (IL-2 and IFN-γ) levels in sera. Furthermore, CD26$^{-/-}$ mice exhibited significantly

lower percentage of CD4$^+$NKT cells in their PBL. NKT cells are one of the earlier players involved in cytokine production and immune response initiation. Thus, *in vivo*, decreased NKT cells in peripheral blood of knockout mice may produce much less IFN-γ and IL-4 upon stimulation. Our results support the notion that CD26 expression is involved in the regulation of cytokine secretion.

In the present study, a conspicuous decrease in the serum concentrations of IgG (also subclasses IgG1 and IgG2a) and IgE was found in CD26$^{-/-}$ mice immunized with PWM (Figs. 4, 5). PWM has been reported to stimulate antibody production and to induce immunoglobulin class switching from IgM to IgG. The PWM-induced immunoglobulin production is highly T cell dependent [34]. The B cell response to T cell-dependent antigen requires cognate interaction between T and B cells as well as interactions between T cell-derived lymphokines and B cells [35]. Recently, a number of studies have demonstrated that Th2 cells are not unique in their ability to induce T cell-dependent B cell responses, but both Th1 and Th2 cells and cytokines are capable of supporting B cell clonal expansion and antibody synthesis [36, 37]. IL-2 stimulates proliferation of both B and T lymphocytes [38]. IL-4 induces the activation and differentiation of B cells, and thus it reinforces the antibody response and plasma cell isotype switching to produce IgG1 and IgE, while IgG2a isotype switching is promoted by IFN-γ [39]. Therefore, the reduced levels of these cytokines in MPBL of CD26$^{-/-}$ mice correspond to the disturbed humoral response of these animals. Our results indicate that the reduced IL-4 and IL-2 production, combined with delayed IFN-γ secretion in sera of CD26$^{-/-}$ mice, contribute to decreased immunoglobulin production. The impaired class switching to IgG1 and IgE in CD26$^{-/-}$ mice is related to the decreased serum IL-4 concentration, whereas the delayed IFN-γ secretion is responsible for the disturbed switching to IgG2a.

In conclusion, our results show that CD26 is involved in development, maturation and migration of CD4$^+$ T, NK and NKT cells, in cytokine secretion, and T cell-dependent antibody production and isotype switching of B cells.

4 Materials and methods

4.1 Animals and stimulator

Adult, 8–12-week-old homozygous CD26$^{-/-}$ mice on C56BL/6 genetic background [13], and wild-type C57BL/6 mice were kept under specific pathogen-free conditions. Most experiments were performed on males, although no sex-related differences were found. Mitogens PWM, Con A, PHA

(all purchased from Sigma, MO), and LPS (Calbiochem, Schwalbach, Germany) were used as stimulators *in vivo* and *in vitro*.

4.2 Separation of MSL and MPBL

MSL were isolated from mouse spleen by lysing erythrocytes in isotonic ammonium chloride solution as described previously [40], and further purified by depletion of monocytes on plastic plates for 4 h at 37°C. Peripheral blood samples were anticoagulated with heparin (Biochrom Berlin, Germany). PBL were isolated from whole blood by density centrifugation over Ficoll separation solution (Biochrom). Cells were counted with Coulter Z series equipment (Coulter Electronics, Miami, FL).

4.3 Analysis of lymphocyte subpopulations by flow cytometry

The following mAb were used in multiparameter flow cytometric analysis: FITC-conjugated anti-CD3 (Biosource, Nivelles, Belgium), FITC-conjugated anti-CD19 (Southern Biotechnology Associates, Birmingham, AL), FITC-conjugated anti-CD4, PE-conjugated anti-CD8, PE-conjugated anti-NK1.1 mAb (all purchased from PharMingen, Heidelberg, Germany). Lymphocytes were incubated with antibodies in 1% (wt/vol) BSA/PBS at 4–8°C for 0.5 h in the dark. Cells were washed three times with PBS and analyzed on a FACScan cytometer (Becton Dickinson, San Jose, CA). CellQuest software was used to analyze the percentage of different lymphocyte subpopulations.

4.4 Stimulation *in vitro*

MSL were cultured in RPM1 1640 medium supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (Biochrom). Cell suspension (180 μl; 2×10^6 cells/ml) were added to each well of 96-well tissue culture plates, and incubated with 20 μl stimulators (diluted in PBS) or 20 μl PBS as control at 37°C in 5% CO_2 atmosphere. Alamar blue reagent (Biosource, Nivelles, Belgium) was added during the final 6 h of the culture period, and proliferation was measured at 72 h by the Alamar blue assay. The extinction was measured using a micro ELISA autoreader at 570 and 600 nm. The proliferation rate was calculated according to the Biosource protocol. For determination of cytokine production, culture supernatants were harvested at the indicated time points, centrifuged at 3,000 rpm for 5 min to remove the cells, and the supernatants were stored at –20°C for further analysis.

4.5 Immunization protocol *in vivo*

Mice were immunized with an intraperitoneal (i.p.) injection of 40 μg PWM dissolved in 200 μl PBS on days 0 and 13. Blood samples were collected from the animals' tails for

antibody measurement and by cardiac puncture for cytokines test. Blood samples were allowed to clot for 4 h at room temperature. Sera were placed in new tubes after centrifugation and stored at –20°C for further analysis.

4.6 Measurement of cytokine production

Cytokine production in supernatants of MSL and sera was determined by ELISA kit (R&D Systems, Minneapolis, MN) according to the procedure provided by manufacturer.

4.7 Measurement of immunoglobulin production

Microtiter plates (96-well) were coated with 100 μl/well of 2 μg/ml anti-mouse Ig polyclonal antibodies (BD PharMingen) to detect IgM, IgG, IgG1 and IgG2a, or with the same volume of 2 μg/ml anti-mouse IgE mAb to detect IgE in PBS (pH 7.5). After overnight incubation at 4°C, plates were washed with 0.9% (wt/vol) NaCl containing 0.1% (wt/vol) Tween 20^{TM} and blocked for 1 h at 37°C in 1% (wt/vol) BSA/PBS; 100 μl appropriately diluted samples or standards (BD PharMingen) was added to the microtiter plate and incubated for 2 h at room temperature. Plates were washed again three times. Biotin-labeled anti-IgM, anti-IgG (Sigma), anti-IgG1 anti-IgG2a, or anti-IgE mAb (BD PharMingen) were added and incubated for 1 h at room temperature. Plates were then incubated for a further 1 h in the presence of alkaline phosphatase-labeled streptavidin (BD PharMingen). The plates were washed three times, followed by addition of p-nitrophenyl orthophosphate, disodium 6 H_2O (Sigma) substrate solution and incubation at room temperature for 15 min. The reaction was stopped with 1 M sodium carbonate and the plates were analyzed with an ELISA reader at 405 nm. The concentration of immunoglobulins was calculated by comparison with standard samples.

4.8 Statistical analysis

Data were expressed as mean ± SD and compared using Student's *t*-test.

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