3 Materials and methods

3.1 Materials

3.1.1 Animals

Homozygous CD26$^{-/-}$ mice on C56BL/6 genetic background obtained from Dr. Marguet (Marguet et al., 2000), 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.

3.1.2 Cells and bacteria

Jurkat cell line (human leukemia T cell line)	ATCC-Nr. TIB-152
BW cell line (mouse thymoma T cell line)	Dr. A. von Boin (von Bonin et al., 1997)
CHO cell line (Chinese Hamster Ovary cell line)	ATCC Nr. CCL-6
CHO/CD26 cells (CHO transfectants of rat CD26 cDNA)	Dr. H. Fan (Fan et al., 1997)
E. coli HB 101	Stratagene (Amsterdam, The Netherlands)
E. coli SCS 110 (DNA adenine methylase and cytosine methylase deficiency)	Stratagene (Amsterdam, The Netherlands)

3.1.3 Plasmids

pF-162	Plasmid pRc/CMV carrying the rat CD26 gene with mutation of stop codon into *Sal* I endonuclease digestion sequence Dr. H. Fan

The following plasmids were from Clontech Laboratories (California, USA):

pEGFP-N1	Reporter vector encodes a red-shifted variant of wild-type GFP
pTRE2	Response vector of inducible expression system
PTK-Hyg	Selection vector encodes a hygromycin resistant gene
pTet-on	Regulator vector encodes the reverse tet-responsive transcriptional activator

3.1.4 Antibodies

Antibodies for cell surface staining

FITC-conjugated anti-mouse CD3 mAb	Biosource (Nivelles, Belgium)
FITC-conjugated anti-mouse CD4, CD8 and CD45RB mAb	Pharmingen (Heidelberg, Germany)
R-PE-conjugated anti-mouse CD4, CD8 and NK1.1 mAb	Pharmingen (Heidelberg, Germany)
FITC-conjugated anti-mouse CD19, CD26 and CD45 mAb	Southern Biotechnology (Birmingham, USA)
R-PE-conjugated anti-mouse CD19, CD25 and CD71 mAb	Dianova (Hamburg, Germany)
Purified anti-rat CD26 mAb Purified anti-rat CD26 pAb	Research group of Prof. Reutter
R-PE-conjugated anti-mouse IgG FITC-conjugated anti-rabbit IgG	Sigma (Missouri, USA)

Antibodies for intracellular cytokine staining

FITC-conjugated anti-mouse IFN-γ mAb	Pharmingen (Heidelberg, Germany)
R-PE-conjugated anti-mouse IL-4 mAb	R&D (Minneapoil, USA)

Antibodies for measurement of immunoglobulin production

Purified anti-mouse Ig pAb Purified anti-mouse IgE mAb	Pharmingen (Heidelberg, Germany)
Biotin-conjugated anti-mouse IgG1, IgG2a and IgE mAb Mouse IgM, IgG1, IgG2a and IgE standards	Pharmingen (Heidelberg, Germany)
Biotin-conjugated anti-mouse IgG (γ-chain specific) Biotin-conjugated anti-mouse IgM (μ-chain specific) Mouse IgG reagent Grade	Sigma (Missouri, USA)

Others

Anti-mouse CD4 Microbeads	Miltenyi Biotec (Bergisch Gladbach, Germany)

3.1.5 Reagents

General salts and buffer regents	Sigma (Missouri, USA) Carl Roth (Karlsruhe, Germany) Merck Eurolab (Hannover, Germany)

Mediums and regents for cell culture

RPMI 1640 medium	Biochrom (Berlin, Germany)
Alpha medium	Biochrom (Berlin, Germany)
Fetal calf serum	Kraeber (Wedel, Germany)
L-Glutamine	BioWest (Essen, Germany)
Penicillin/streptomycin	Biochrom (Berlin, Germany)
Hygromycin	Calbiochem (Damstadt, Germany)
G418 sulfate	PAA laboratories GmbH (Linz, Austria)
Yeast extract	Roth (Karlsruhe, Germany).
Peptone	Roth (Karlsruhe, Germany).
Agar	Roth (Karlsruhe, Germany).
kanamycin	Sigma (Missouri, USA)
Ampicillin	Sigma (Missouri, USA)

Mitogens

LPS (lipopolysaccharide)	Calbiochem (Damstadt, Germany)
PWM (pokeweed mitogen)	Sigma (Missouri, USA)
ConA (concanavalin A)	Sigma (Missouri, USA)
PHA (Phytohemagglutinin)	Sigma (Missouri, USA)

Molecular weight standards

Prestained protein molecular weight standard (for gel electrophoresis)	Sigma (Missouri, USA)
Protein molecular weight standard (for gel filtration chromatography)	Bio-rad (Bichmond, USA)
GeneRuler™ 1 Kb DNA Ladder	MBI Fermentas (ST.Leon-Rot, Germany)

Restriction endonucleases (RE)

All of restriction endonucleases and buffers were purchased from MBI Fermentas (ST.Leon-Rot, Germany).

Sources of other special regents and materials were indicated in the text of Methods.

3.2 Methods

3.2.1 Purification and three dimensional structure elucidation of homogeneous dimeric CD26/DPPIV with enzyme acitivity

3.2.1.1 Expression and solubilization of rat DPPIV/CD26

CHO/CD26 cells were cultured in Alpha medium supplemented with ribonucleoside (G, 2.5 mg. A, C, T, 5mg in 500 ml), 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin, 100 IU/ml penicillin and 600 mg/l geneticin. Cells were harvested from 30 dishes (10 cm in diameter) and solubilized in 15 ml solubilization buffer by vortexing at 4°C overnight. Cell debris was pelleted by centrifugation at 18,000 rpm at 4°C for 30 min. The clear supernatant was used directly for immunochromatography.

Solubilization buffer: 10 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, pH 7.8
1:1000 Protease-inhibitor cocktail (Sigma, P 2714)
1% Trion

3.2.1.2 Isolation of rat CD26/DPPIV with enzyme activity by immunoaffinity chromatography

3.2.1.2.1 Preparation of immunoaffinity column

330 mg Protein-A-Sepharose Cl 4B (Amersham Pharmacia, Uppsala, Sweden) was swelled for 30 min in PBS. Then sepharose was centrifuged for a short time (4000-6000 rpm). After discarding the supernatant, sepharose was incubated at 4°C in the antibody solution (0.5 ml 13.4 mAb in 10 ml steril PBS) overnight. Subsequently, the sepharose was centrifuged and washed twice with PBS, one time with 0.2 M triethanolamine (pH 8.2). 10 ml coupling reagent was added to sepharose and incubated at room temperature for 45 min with shaking. Then the sepharose was centrifuged and the supernatant was discarded. The reaction was stop using 10 ml 50 mM ethanolamin (pH 8.8). The sepharose was whirled up for a short time and incubated at room temperature for 10-20 min. Finally, the sepharose coupled with antibody was washed for three times with PBS. This affinitycolumn was kept at 4°C in PBS containing 0.02% NaN_3.

Coupling reagent: 0.2 M Triethanolamine
50 mM Diethylpimelimidate (DMP)
Adjust pH to 8.2 with NaOH.

3.2.1.2.2 Isolation of rat CD26/DPPIV with enzyme activity using affinity column

NaN_3 was washed out of the affinity column with PBS. The supernatants of solubilization of CHO/CD26 cells were loaded onto the column at a flow rate of 0.5 ml/min at 4°C overnight. After extensive washing with (a) ca. 40 ml RIPA for 2 h (b) ca. 40 ml Prewash for 2 h (c) ca. 20 ml PBS for 30 min, bound protein was eluted using elution buffer. Fractions were collected and promptly neutralized with 1/30 volume 0.5 M NaH_2PO_4, and stored at 2-4°C.

RIPA: 50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% Triton-x-100, 1% Na-Deoxycholate, 0.1% SDS.

Prewash: 10 mM Tris-HCl pH 7.2, 1 M NaCl, 0.1% Triton-x-100.
Elution buffer: 50 mM Diethylamine pH 10.8.

3.2.1.3 Isolation of homogeneous dimeric CD26/DPPIV through size-exclusion fast protein liquid chromatography (SE-FPLC)

The immunopurified DPPIV/CD26 isolated from affinitycolumn was concentrated to 250-300 µl by using an Ultrafree-4 centrifugation unit with a Biomax-30 membrane (UFV4BTK25, Millipore, Schwalbach, Germany). The concentrate was loaded onto a Superdex 200 column (Pharmacia) which had been equilibrated with PBS. Proteins were eluted out at a flow rate of 0.25 ml/min with equilibrium buffer. The molecular weight of the obtained protein was determined based on the elution profile of standard proteins.

3.2.1.4 Determination of CD26/DPPIV enzyme activity

5 µl sample and 2 µl substrate (Gly-Pro-4-Nitroanilide, Bachem, Bubendorf, Switzerland) were incubated at 37°C in 18 µl incubation buffer for 30 min. Then the reaction was stopped by adding 100 µl stop solution. The absorbance at 405 nm were measured with microplate reader as described by Nagatsu (Nagatsu et al., 1976).

Substrate solution: 10 mg/ml L-Gly-L-Pro-p-Nitroanilidtosylate in distilled H_2O.
Incubation buffer: 100 mM Tris-HCl, pH 8.0.
Stop solution: 1 M Na-Acetate, pH 4.5.

3.2.1.5 Quantitative analysis of protein by BCA protein assay reagent kit (Pierce, Rockford, USA)

20 µl sample, BSA standard (Pierce, Rockford, USA), or distilled H_2O as blank was added into each well of 96-well microplate in triplicate, respectively. 200 µl BCA working reagent (Regent A:B = 50:1) was added to each well. The plate was incubated at 37 °C for 30 min with shaking, and was read at 570 nm with microplate reader. The protein concentration was calculated according to standard curve.

3.2.1.6 Determination of protein purity by SDS-PAGE and non-denaturing PAGE

24 µl sample mixed with 6 µl of loading buffer were heated at 100°C for 5 min, then loaded on the 7.5% SDS-gel and separated by electrophoresis. The gel was stained with silver staining and dried for storage.

To run non-denaturing PAGE, the gel, loading and running buffer did not contain SDS. Samples were not heated. Electrophoresis was performed under ice-cold condition. Other steps were the same as mentioned above.

Silver staining: After electrophoresis, the gel slab was soaked in fixation solution for 20 min and rinsed three times with washing solution (5 min for each). Then the gel was incubated in sodium thiosulfate solution for 1 min. Solution was discarded and the gel was quickly rinsed with distilled water. Subsequently, silver nitrate solution was added to the gel for 12 min. Following washing with distilled water, the gel was developed in developing solution. When a sufficient degree of staining was obtained, staining was quenched by discarding the developing solution, washing with water and replacement with fixation buffer without formaldehyde. 10 min later, the gel was washed with washing buffer and dried for storage.

5 x Loading buffer:	15% SDS, 50% Glycerol, 0.015% Bromophenol blue, 25% 2-Mercapto-propandiol, 300 mM Tris-HCl, pH 6.8.
10 x Running buffer:	25 mM of Tris-HCl, 190 mM of Glycine, 3.5 mM of SDS, pH 8.8.
Fixation solution:	50% Methanol, 12% Acetic acid, 0.05% Formaldehyde in distilled water.
Washing solution:	Ethanol : H_2O = 1:1
Sodium thiosulfate solution:	20 mg $Na_2S_2O_3 \cdot 5H_2O$ in 100 ml distilled water.
Silver nitrate solution:	0.4 g $AgNO_3$, 150 µl 37% Formaldehyde, in 200 ml distilled water.

Developing solution: 12 g Na_2CO_3, 1 mg $Na_2S_2O_3 \cdot 5H_2O$, 100 µl 37% Formaldehyde, in 200 ml distilled water.

Staining and developing solutions were freshly prepared. Formaldehyde, silver nitrate and sodium thiosulfate were added to solutions immediately prior to use.

3.2.1.7 Sample preparation for cryo-transmission electron microscopy (Ludwig et al., 2003)

Droplets (5 µl) of the protein solution were placed on hydrophilized (glow discharge treatment for 30 s at 8 W in a BALTEC MED 020 device from Baltec, Liechtenstein) carbon-coated copper grids and blotted to create an ultrathin layer of the solution. A droplet of 2% (m/v) phosphotungstic acid (PTA) was subsequently applied for 30 s and blotted again. The grids were eventually propelled into liquid ethane at its freezing point (89 K) using a custom made plunging device. Liquid nitrogen was used as storage medium. The specimen preparation procedure was essentially the same as described earlier for the 3D-structure determination of influenza HA (Bottcher et al., 1999). The embedding matrix had a somewhat higher contrast than that of the conventional vitreous-ice preparation. Despite the relatively high acceleration voltage of the microscope (160 kV) and the chosen "close-to-focus" imaging conditions, a better signal-to-noise ratio is obtained and the beam sensitivity is reduced (De Carlo et al., 2002). Comparison of the X-ray crystallographic structure of HA with our three-dimensional EM-reconstruction revealed no significant structural difference within the limits of the achieved resolution of 10 Å.

3.2.1.8 Cryo-Electron microscopy (Ludwig et al., 2003)

Vitrified samples were transferred into a Tecnai F20 FEG using a Gatan cryoholder and –stage (Model 626). Samples were constantly cooled by LN2 during imaging to maintain a sample temperature of T = 93 K. Imaging was performed at 160 kV accelerating voltage at a defocus value of 600 nm, which corresponds to a first zero of the contrast transfer function at 13 Å (C_s = 2.0 mm). Micrographies were recorded following the low-dose protocol of the microscope at a primary magnification of 65 473x.

3.2.1.9 Image processing (Ludwig et al., 2003)

Micrographs were screened using a laseroptical diffractometer. Images were selected for further processing, which were free of aberrations, charging, drift, misalignments etc. The selected micrographs were then digitised using the Heidelberg "Primescan" drum scanner (Heidelberger Druckmaschinen AG, Heidelberg, Germany) at a resolution of 4 µ (6350 dpi), which corresponds to a pixel resolution of 0.61 Å on the negative.

All subsequent steps of the image processing procedure were performed with IMAGIC 5 software (Image Science GmbH, Berlin, Germany). 11,121 single molecules were interactively selected and used for further processing. The three-dimensional reconstruction was calculated using the "angular reconstitution" (Van Heel, 1987) as described elsewhere (Böttcher et al., 1999). Fourier shell correlation (FSC) (Van Heel and Harauz, 1986) of two different 3D reconstructions, each of which included half of the final class averages, was done to assess the resolution at a 0.5 cutoff. For the final reconstruction the resolution was determined to be ~14 Å.

For docking experiments of the crystal structure of POP, the Situs Software Package Version 2.0 (http://situs.scripps.edu/index.html) was used to fit the high-resolution X-ray structure into the EM density maps. The docking procedure was performed following the protocol described by Wriggers and Birmanns (2001) (Wriggers and Birmanns, 2001).

3.2.2 The role of CD26/DPPIV in immunoregulation

3.2.2.1 Examination of CD26/DPPIV expression in mouse organs with western blot

Organs (liver, kidney, heart, lung spleen and muscle) from 6-8-week-old mice were cleaned with washing buffer and homogenized for 1 min with an Ultra-Turrax homogenizer in 5 ml washing buffer. The homogenates were centrifuged at 20,000 rpm 4°C for 30 min. The pellets were resuspended and homogenized for 30 sec in 2 ml solubilization buffer (3.2.1.1). The homogenates were centrifuged at 35,000 rpm 4°C for 30 min. The protein concentration and DPPIV enzyme activity of supernatants were

quantified as described in 3.2.1.5 and 3.2.1.4. Then 25 µg protein per well was separated by SDS-PAGE (3.2.1). The CD26/DPPIV protein was detected by western blot.

After electrophoresis separation, the proteins were transferred onto a nitrocellulose membrane at 150 V/250 mA in ice-cold transfer buffer for 60 min. Then the membrane was stained with 0.1% Ponceau-red for 1 min, washed with 1% acetic acid for several times. The molecular weight marker and sample lanes were marked out with pencil. The nitrocellulose membrane was decolourized in PBS.

Subsequently, the membrane was incubated at 4°C in 5% milk solution in 0.1% Tween/PBS overnight to block unspecific binding sites. After washed for 3 x 5 min with 0.1% Tween/PBS, the nitrocellulose membrane was hybridized with rabbit anti rat CD26 serum (1:2000) in 1% BSA/PBS at 4°C overnight. The membrane was washed for 3 times with 0.1% Tween-20/PBS and hybridized with the second antibody (horseradish peroxidase conjugated goat-anti-rabbit IgG-antibody in 1% BSA/PBS, 1:5000) at room temperature for 2 h. Before detection, the membrane was washed for 5 times with 0.1% Tween-20/PBS. For detection, 1 ml substrate solution was added to the membrane. Then the membrane was covered with a piece of kodak BioMax MR film and the film was exposed for different time.

Washing buffer:	1 mM $NaHCO_3$, 0.5 mM $CaCl_2$, pH 7.0.
Transfer buffer:	25 mM of Tris-HCl, 114 mM of Glycine, 10% of Ethanol.
Substrate solution:	10 µl 6.8 mM Plovinuric acid in DMSO, 1 ml 1.25 mM Luminol in 0.1 M Tris-HCl (pH 8.5), 3 µl 3% H_2O_2.

3.2.2.2 Cell culture

Cells were cultured at 37°C in suitable medium in a humidified atmosphere with 5% CO_2.

MSLs	RPMI 1640 medium
Jurkat cells	RPMI 1640 medium

CHO cells	Alpha medium supplemented with ribonucleoside (G, 2.5 mg, A, C, T, 5mg in 500ml)

Besides special additives indicated above, all kinds of mediums were supplemented with 2 mM glutamine, 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin and 100 IU/ml penicillin. Stable transfectants were cultured in correspondent selection medium supplemented with G418 (600 µg/ml). For double stable transfectants of Jurkat cells, medium was supplemented with G418 (600 µg/ml) and hygromycin (200 µg/ml).

3.2.2.3 Cell counting

Cells were counted with a Coulter Z series equipment (Coulter electronics, Miami, USA).

3.2.2.4 Preparation of lymphocytes from C57BL/6 mouse spleens

Mice were killed by cervical dislocation. The spleens were removed, suspended and washed for three times with sterile PBS; then pressed through stainless steel mesh (100 mesh) into a culture plate using a sterile syringe plunger. The homogenates of spleen cells were centrifuged at 200 rpm for 3 min. The suspension was transferred to a new tube and the cell clumps in the bottom were discarded. The cell suspension was centrifuged at 1400 rpm for 6 min. Cell pellets were washed twice with PBS. Then 1.5 x 10^8 cells were resuspended in 1 ml Tris-HCl-buffered NH_4Cl solution for lysing erythrocytes. After kept at room temperature for 10 min, the cell suspension was further diluted with 10-fold volume of medium, mixed and centrifuged. Finally cell pellets were resuspended in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin.

Spleen lymphocytes were further purified by depletion of monocytes on plastic plates at 37°C for 4 h.

Cell viability was assessed with trypan blue exclusion.

Tris-HCl-buffered NH_4Cl solution: 9 volumes of 0.83% (w/v in distilled H_2O) NH_4Cl, 1 volume of Tris-HCl (2.06%, w/v in distilled H_2O, pH 7.65), adjust pH to 7.2.

3.2.2.5 Separation of peripheral blood lymphocytes by density centrifugation over Ficoll separation solution

After anesthesia, peripheral blood samples were collected from the vena cava posterior and anticoagulated with heparin (Biochrom Berlin, Germany). Samples were first diluted with the same amount of PBS. The diluted blood samples were then layered carefully onto the same volume of Ficoll separation solution (Biochrom Berlin, Germany) and centrifuged at 1800 rpm for 30 min without brake. Lymphocyte layer at the interface was carefully transferred into a new tube. The lymphocytes were washed twice with 10-fold volume of PBS at 1400 rpm for 10 min. Finally, the lymphocytes were resuspendended in 1% BSA/PBS for further analysis of cell subpopulations.

3.2.2.6 Analysis of lymphocyte subpopulations by flow cytometry

The following mAbs were used in multiparameter flow cytometric analysis: FITC-conjugated anti-CD3, FITC-conjugated anti-CD19, anti-CD26, FITC-conjugated anti-CD4, PE-conjugated anti-CD8, PE-conjugated anti-NK1.1 mAb. 1×10^6 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 for three times with PBS and resuspended in 0.3 ml PBS. Fluorescence analysis was performed on FACScan cytometer (Becton Dickinson, Sanjose, CA, USA). Lymphocytes were gated on the basis of both forward and side scatter and a mean of 10,000 cells were analyzed. All samples were measured within 2 h. CELLQuest software was used to analyze the percentage of different lymphocyte subpopulation.

3.2.2.7 Separation of $CD4^+$ cells by magnetic cell sorting (MACS)

Lymphocyte suspension was prepared from mouse spleens as described in 3.2.2.4. Cells were washed twice and centrifuged, the supernatant was removed completely and cell pellets were resuspended in 0.5% BSA/PBS (45 µl per 10^7 cells). MACS CD4 MicroBeads

were added to cell suspension (5 µl per 10^7cells). Cell suspension was mixed well and incubated at 6-12°C for 15 min. Then cells were washed with 20-fold volume of 0.5% BSA/PBS and centrifuged at 1400 rpm for 10 min. The supernatant was removed completely, and cell pellets were resuspended (500 µl buffer per 10^8 cells) for magnetic separation. The cell suspension was applied onto a LS^+ separation column prewashed with 3 ml 0.5% BSA/PBS and placed in the magnetic field of MidiMACS separator. The cell suspension was allowed through the column and the column was rinsed with 3 × 3 ml buffer. The eluate was collected as non-CD4 cells. The column was removed from the magnetic field of separator and placed on a new collecting tube. $CD4^+$ cells were flushed out in 5 ml 0.5% BSA/PBS using plunger supplied with the column.

3.2.2.8 Stimulation of mouse spleen lymphocytes *in vitro*

Mouse spleen lymphocytes (MSLs) were cultured in RPM1 1640 medium. 180 µl cell suspension (2 x 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 blank at 37°C in 5% CO_2 atmosphere. Proliferation was detected by Alamar Blue Assay. For determination of cytokine production, culture supernatants were harvested at the different time-points, centrifuged at 3000 rpm for 5 min to remove the cells, and the cell free supernatants were stored at –20°C for further analysis.

3.2.2.9 Determination of proliferation and activation of mouse spleen lymphocytes

MSLs were stimulated *in vitro* as described as 3.2.2.8. 20 µl per well of Alamar blue reagent (Biosource, Nivelles, Belgium) was added in the final 6 h of the culture period. At different times, the extinction was measured using a micro ELISA autoreader at 570 and 600 nm, respectively. The proliferation rate was calculated according to the Biosource protocol.

3.2.2.10 Assay of lymphocyte subsets secreting different cytokines

4 x 10^6 MSLs were cultured in 2 ml RPM1 1640 medium with or without PWM (0.5 µg/ml) for 48 h. Monensin was added to cell culture during the final 6 h to block cytokine secretion. At 48 h, the cells were harvested and washed twice with cold PBS by spinning at 1400 rpm for 7 min. Then the cells were stained with anti-mouse FITC- or PE-conjugated anti-CD4 mAb as described 3.2.2.6. When surface staining was ready, cells were resuspended in 1ml of cold 4% paraformaldehyde fixative and incubated at room temperature for 10 min. Following fixation, cells were washed twice in 2 ml permeabilization buffer. After centrifugation, cell pellets were gently resuspended in 100 µl permeabilization buffer with 10 µl PE-conjugated anti-mouse IL-4 or 0.5 µl FITC-conjugated anti-mouse IFN-γ mAb at room temperature for 45 min in the dark. Cells were washed twice using 2 ml permeabilization buffer and resuspended in 300 µl PBS for FACS analysis.

Paraformaldehyde fixative:	Dissolve 4.0 g of Paraformaldehyde in 100 ml of PBS (10 mM Phosphate buffered saline, pH 7.4) by heating the solution at 56°C for about 1 h.
Permeabilization buffer:	Disolve 0.1% (w/v) Saponin, 0.05% (w/v) NaN_3 in PBS.

3.2.2.11 Immunization *in vivo*

3.2.2.11.1 Immunization with PWM

Mice were immunized with an intraperitoneal (i.p.) injection of 40 µg PWM dissolved in 200 µl PBS on day 0 and 13, respectively. Blood samples were collected from the animals' tails for antibody measurement and from the posterior vena cava for cytokines test. Blood samples were allowed to clot for 4 h at room temperature. Sera were transferred into new tubes after centrifugation and stored at –20°C for further analysis.

3.2.2.11.2 Immunization with OVA (ovalbumin)

On day 0, mice were sensitized with intraperitoneal (i.p.) injection of 20 µg of OVA (Sigma) dissolved in 0.3 ml endotoxin-free PBS and 0.2 ml Alu-Gel-S Suspension (Serva electrophoresis, Heidelberg, Germany). The animals received the second injection of this adjuvant-OVA mixture on day 5. On day 12, mice were challenged by exposure to an aerosol of 1% OVA in PBS for 40 min. Inhalation was carried out in a plastic chamber (27 x 20 x 10 cm). The chamber has an attachment to allow entry of aerosol from an ultrasonic nebulizer (1-5 µm particles by manufacture's specifications, Molel. no. SW-966V 230V, Shining World Health Care Co, Taipei). The other end of the box has two small holes for the maintenance of continuous airflow. Blood samples were collected from the animals' tails for antibody measurement and by cardiac puncture for cytokines test. Blood samples were allowed to clot at room temperature for 4 h. Sera were transferred in new tubes after centrifugation and stored at –20°C for further analysis.

3.2.2.12 Bronchoalveolar lavage (BAL)

BAL was performed at 36 h after aerosol challenge. Mice were killed by cervical dislocation. A median sternotomy was performed. The trachea was dissected free and canulated. The lungs were lavaged twice with 1ml-aliquot of PBS. The samples were then pooled and centrifuged. The cell-free BAL fluid was stored at –20°C until analysis. The cell pellet was resuspended in PBS. Cell numbers were counted in a hemocytometer. Smears of BAL fluid cells were stained with Giemsa & May-Grünwald solution and cell differential was enumerated based on morphology and staining profile.

3.2.2.13 Lung histology

After BAL, lungs were inflated with 1 ml tissue freezing medium instilled through a tracheostomy tube. Tissues were frozen immediately in liquid nitrogen and stored at –20°C until sectioning. Sections of 10µm thickness were mounted on slides and stained with hematoxylin & eosin.

3.2.2.14 RNA isolation and reverse transcription-PCR

Total RNA was isolated from lung tissue using Qiagen RNeasy Midi kit (Qiagen) according to the manufacturer´s instruction. cDNA was synthesized using RevertAid minus First strand cDNA synthesis kit (Fermentas). PCR was performed using Quantitative PCR detection kit – mouse Th1/Th2 set 2 (Biosource) with Taq DNA polymerase (Fermentas). One fifth of PCR product was analyzed on 2% agarose gel by electrophoresis.

3.2.2.15 Measurement of immunoglobulin production

Microtiter plates (96-well) were coated with 100 µl/well of 2 µg/ml (in PBS) anti-mouse Ig polyclonal antibodies to detect IgM, IgG, IgG1 and IgG2a, or with the same volume of 2 µg/ml (in PBS) anti-mouse IgE mAb to detect IgE (pH 7.5). After overnight incubation at 4°C, unbound antibodies were removed by washing with 0.9% (wt/vol) NaCl containing 0.1% (wt/vol) Tween 20^{TM}. Then the plates were incubated in 1% BSA/PBS at room temperature for 1 h to block the unspecific binding. 100 µl appropriately diluted samples or standards were added to the microtiter plate and incubated at room temperature for 2 h. Plates were washed again for three times. Biotin-labeled anti-IgM, anti-IgG (Sigma), anti-IgG1, anti-IgG2a, or anti-IgE mAbs (PharMingen) were added and incubated at room temperature for 1 h. After intensive washing, plates were then incubated for further 1 h at the presence of alkaline phosphatase-labeled streptavidin (PharMingen). The plates were washed for six times, followed by addition of disodium 4-nitrophenyl phosphatehexahydrate (Sigma) substrate solution (1 mg/ml in substrate buffer) 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.

Substrate buffer: 97.0 ml/l Diethanolamine, 0.1 g/l $MgCl_2 \cdot 6H_2O$, 0.02% NaN_3 in distilled H_2O, pH 9.8.

3.2.2.16 Measurement of cytokine production

Cytokine production (IL-2, 4, 5, 6, 10, 13, IFN-γ) in supernatants of cultured MSLs and sera was determined by ELISA kit (R&D Systems, Minneapolis, USA) referring to the procedure provided by manufacturer. Serum samples and samples with high concentration of cytokines were appropriately prediluted with Calibrator Diluent.

3.2.2.17 Statistical analysis

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

3.2.3 Establishment of inducible expression system of rat CD26 in Jurkat cells

3.2.3.1 Culture of *E. coli* HB 101 and SCS 110

E.coli was cultured on LB-agar or LB liquid media. For positive clones' selection, medium was supplemented with antibiotics (50 µg/ml of kanamycin for pEGFP-N1, or 50 µg/ml of Ampcillin for pTRE2 and pTet-on, at final concentration).

LB liquid medium:	10 g NaCl, 5 g Yeast extraction, 10 g Peptone in 1 l deionized H_2O
LB-agar	15 g Agar in 1 l LB liquid medium

3.2.3.2 Preparation of competent *E. coli* cells

1 ml *E.coli* HB 101 or SCS 110 cultures were incubated in a sterile 500 ml flask with 100 ml fresh medium and cultured at 37°C, 225 rpm to an optimum OD_{600} values between 0.4-0.7. Subsequently, *E. coli* cells were incubated on ice for 10 min, then centrifuged at 5000 rpm for 10 min. Pellets were resuspended in 20 ml pre-chilled 0.1 M/l $CaCl_2$ and incubated on ice for 30 min. After centrifugation, the supernatants were removed. 1 ml pre-chilled 0.1 M/l $CaCl_2$ was added to cell pellets, mixed gently and incubated on ice for 1 h. The prepared competent cells were aliquoted, and stored with 15% glycerol at -80°C until use.

3.2.3.3 Transformation of competent cells

1-5 ng plasmid DNA or half amount of a ligation preparation (5-10 µl volume) was added to 100 µl of competent cells and mixed gently with pipette. After incubation on ice for 20 min, at 42°C for 2 min, and at room temperature for 5 min, 1 ml LB medium (without antibiotic) was added. Cells were cultured with shaking at 37°C for 1 h, then incubated on LB-agar plate over night. The transformed clones were selected by appreciate antibiotic.

3.2.3.4 Preparation of plasmid by QIAGEN plasmid purification kit (QIAGEN, Hilden, Germany)

Referring to the procedure in QIAGEN plasmid purification handbook.

3.2.3.5 Preparation and separation of insert rat CD26 DNA from plasmid pF-162

60 µl plasmid DNA (2.4 µg/µl), 10 µl *Sal* I, 20 µl buffer O^+ and 110 µl sterile distilled H_2O were mixed and incubated at 37°C overnight. The digested plasmid DNA mixed with 1/6 dye solution was loaded on 1% preparation agarose gel for electrophoretic separation. After staining of the gel in ethidium bromide solution for 10 min, the band of 3194 bp was cut under the UV light of 360 nm. DNA was extracted from gel by using QIAquick gel extraction kit (QIAGEN). 110 µl DNA extract was further digested with *Hind* III (110 µl DNA extract, 10 µl *Hind* III and 20 µl buffer Y^+) at 37°C overnight. After electrophoresis and gel extraction, the insert DNA of 2336 bp were isolated, resuspended in 90 µl H_2O and stored at -20°C for ligation.

3.2.3.6 Preparation of vector DNA from pEGFP-N1

100 µl plasmid DNA (1.8 µg/µl), 10 µl *Hind* III, 20 µl buffer Y^+ and 70 µl sterile distilled H_2O were mixed and incubated at 37°C overnight. On the next day, 600 µl ethanol and 20 µl 3 M Na-acetate were added to the reaction system. After incubation at -80°C for 1-2 h, plasmid DNAs were precipitated by centrifugation at 13,000 rpm for 10 min. Then plasmid DNA was washed twice with 200 µl 80% ethanol (pre-chilled at -20°C) and dried at room temperature. This pDNA was further digested by *Sal* I (170 µl sterile distilled H_2O, 10 µl

Sal I, 20 µl buffer O^+) at 37 °C overnight. The pDNA digested by *Hind* III and *Sal* I were isolated by electrophoretic separation on preparative gel and extracted using QIAquick gel extraction kit. For dephosphorylation, 90 µl DNA, 10 µl bacterial alkaline phosphatase (BAP) buffer and 1 µl BAP were mixed and incubated at 60°C for 4 h. Then another 1 µl BAP was added. The mixtures were incubated again at 60°C for further 4 h. The dephosphorylated DNA was extracted with phenol-chloroform, and resuspented in 20 µl H_2O following ethanol precipitation.

3.2.3.7 Ligation of insert and vector DNA

5 µl vector DNA, 15 µl insert DNA, 6 µl T4-ligase-buffer, 1 µl T4-ligase, and 3 µl distilled H_2O were mixed and incubated at 14°C over night. 15 µl of ligate mixture was transformed into competent cells of *E.coli* SCS 110 or HB 101 strains.

3.2.3.8 Preparation of insert DNA encoding fusion protein CD26/GFP

Plasmid pGFP-N1/CD26 was first digested with *Xbal* and then *Hind* III. The same digestion procedure as described in 3.2.3.5 was applied. The band of 3109 bp was isolated as insert DNA.

3.2.3.9 Preparation of vector DNA from plasmid pTRE2

Plasmid pTRE2 was first digested with *Xbal* and then *Hind* III. The same digestion procedure as described in 3.2.3.6 was applied.

3.2.3.10 Transfection of Jurkat cells with Tet-on plasmid and selection of positive clones

4 x 10^6 Jurkat cells in 400 µl hypoosmolar electroporation buffer (Eppendorf, Woburn, USA) were pipetted into electroporation cuvette. 5-10 µg linearized Tet-on plasmid (digested with *Sal* I) was added and transferred into cells by electroporation (240V, 40µs 1× puls using a gene pulser, Eppendorf, Hamburg, Germany). The cells were kept at room temperature for 10 min, then cultured in RPMI 1640 medium (w/o phenolred, antibiotics,

supplemented with 2 mM Glutamine, 10 % FCS) for 48 h. Subsequently, medium was replaced with RPMI 1640 selection medium containing 100 µg/ml streptomycin, 100 IU/ml penicillin, 1 mg/ml G418. Fresh medium was changed everyday in the first week and every two days in the second week. Two weeks later, cells were diluted into different concentration in microplate for single positive clone's selection.

3.2.3.11 Transfection of Jurkat cells (tet-on positive clone) with pTRE2/CD26/GFP

Based on the same procedure as described in 3.2.3.10, 5-10 µg linearized pTRE2/CD26/GFP and pTK-Hyg were co-transfected into G418 resistant clones (3.2.3.10). The double-stable transfectants were selected in the presence of hygromycin and G418. Subsequently, the cells were cultured on fibronectin (Sigma) coated plates and CD26 expression in these transfectants was induced by incubation in 100 ng/ml doxycycline (Sigma) overnight. The positive cells were determined by fluorescence microscope and FACScan cytometry was used for CD26/GFP positive cells sorting.