Investigation on the role of CD26 in Th1 and Th17 cell differentiation and allogeneic graft rejection

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Disputation: 26-04-2018

For my family

献给我的家人

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1. Introduction

CD26, also known as dipeptidyl peptidase IV (DPPIV), is an integral type II transmembrane glycoprotein. As a co-stimulator of T cell activation, CD26 plays an important role in the immune system. Due to its enzyme activity, CD26/DPPIV is involved in many biological processes through the degradation of its substrates. Moreover, CD26/DPPIV is associated with many diseases.

1.1 CD26/Dipeptidyl peptidase IV (DPPIV)

1.1.1 Expression of CD26/DPPIV

CD26/DPPIV has a broad cell-surface distribution. It is constitutively expressed and widely distributed in mammalian tissues, mainly on epithelial and endothelial cell surfaces, fibroblasts as well as activated lymphocytes [1]. CD26/DPPIV is involved in several immunologically relevant functions. Its expression is strictly controlled during T cell maturation [2]. In human and mammalian lymphocytes, CD26/DPPIV is mostly expressed on T cell populations and is expressed at low density when the lymphocytes at resting state but strongly up-regulated following T cell activation [3]. In resting peripheral blood cells, the high expression level of CD26 was found on the surfaces of a small subpopulation of T cells ($CD26^{bright}$ cells), which belong to the $CD45RO^{+}$ population of memory T cells [4]. The expression of CD26 is up-regulated during the active phase of some autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, Grave´s disease, Hashimoto´s thyroiditis and sarcoidosis, while the expression of CD26 is decreased during immunosuppression, as in AIDS, Down´s syndrome and common variable hypogammaglobulinemia [5]. Furthermore, some studies revealed that the expression of CD26/DPPIV has a correlation with T helper subsets. Higher expression of CD26/DPPIV was found on activated Th1 and Th0 cells in comparison to Th2 cells [6]. Besides the integral membrane form of CD26/DPPIV, a soluble form of CD26/DPPIV without an intracellular tail and transmembrane regions exists and it is identified on the basis of its enzyme activity, immune-reactivity and ADA binding [7]. The soluble CD26 was found in high concentration in seminal plasma, and in plasma and cerebrospinal fluid,

the concentration was low [8]. Interestingly, the concentration of soluble CD26/DPPIV in serum varies in different diseases [9].

1.1.2 Structure of CD26/DPPIV

CD26/DPPIV is expressed as a homodimer which comprises of two identical subunits of approximately 110 kDa molecular mass. It is made up of 776 and 760 amino acid residues in humans and mice, respectively [10, 11]. The amino acid sequence of CD26/DPPIV is highly conserved among different species. The homology is about 92% between rat and mouse and 85% between rat and human. The intracellular N-terminus domain of CD26/DPPIV is a short cytoplasmic domain containing 6 amino acid residues, the middle is a hydrophobic transmembrane domain of 22 amino acid residues, and both domains serve as signal peptides and membrane anchor [12, 13]. The third part is the extracellular domain which can be divided into three regions with specific characteristics. The N-glycan-rich region of rat CD26/DPPIV adjacent to the membrane domain contains 5 out of 8 N-glycosylation sites. It is responsible for the biological stability and processing of the protein [14]. The central region of rat CD26/DPPIV is the cysteine-rich domain containing 10 of 12 cysteines. The disulfide bridge is built in the cysteine-rich region, and it is responsible for the formation of the functional conformation of CD26/DPPIV [15]. Homodimerization induced by this domain is essential for the enzyme activity of this protein. The C-terminal region of CD26/DPPIV is the catalytic center [16].

The crystal structure of CD26/DPPIV from various mammals was resolved [17, 18]. In humans, crystal structure revealed a homodimer of DPPIV. Each subunit comprises two structural domains: the N-terminal 8-bladed β-propeller domain and the C-terminal α/β-hydrolase domain. The cysteine- and N-glycan-rich- domains are packed within the β-propeller domain. The catalytic triad is at the interface of the two domains [17]. The crystal structure of the free form of CD26/DPPIV reveals two potential channels through which substrates can access the active site. One is in the β-propeller domain; the other one is a side opening formed at the interface of the β-propeller and hydrolase domain [19]. The β-propeller domain is composed of an unusual eightfold repeat of blades. Each blade comprises of four strands of antiparallel β-sheets. The β-propeller domain defines a funnel-shaped, solvent-filled tunnel that extends from the lower face of the β-propeller to

the active site. The lower face of the funnel, distal to the hydrolase domain, has a diameter of approximately 15 Å. The size of the distal side openings reveals why the substrates and inhibitors of DPPIV must possess a limited chain length of about 80 amino acid residues. The complex of DPPIV with its inhibitor revealed that the catalytic site was located in the large cavity formed between the α/β-hydrolase domain and an 8-bladed β-propeller domain [20].

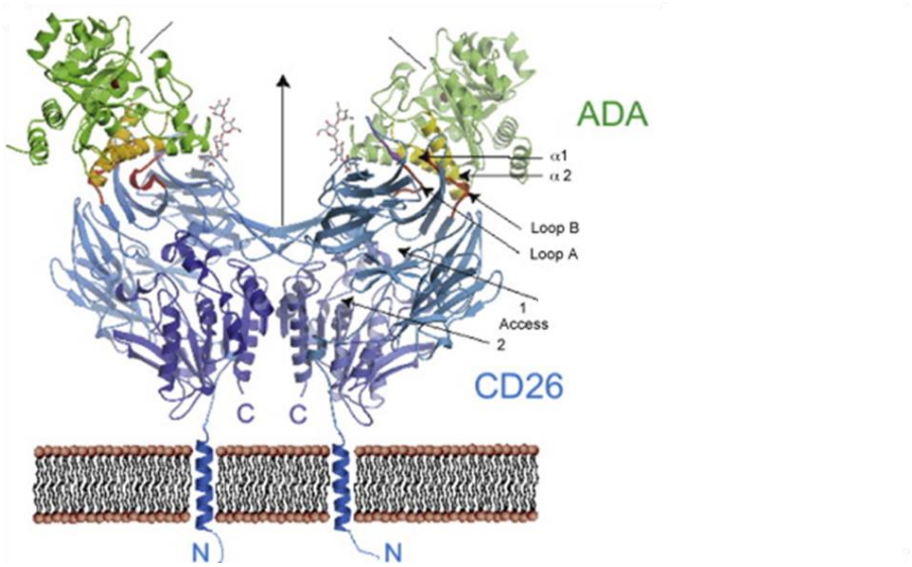


Figure 1.1: Crystal structure of human-DPPIV (hDPPIV) in complex with bovine adenosine deaminase (bADA)

Figure 1 shows the crystal structure of human DPPIV (hDPPIV) in complex with bovine adenosine deaminase (bADA). The membrane and membrane anchor (not seen in the electron density) are drawn schematically. The view is normal to the pseudo-2-fold axis (vertical arrow) that relates the two hDPPIV·bADA in $(hDPPIV{\cdot}bADA)_2$. The domains of hDPPIV are *violet* and *blue* for the α/β hydrolase and β-propeller domains, respectively, bADA is shown in *green*, active site Zn^{2+} is shown as a *red sphere*. The *stick* points the oligosaccharide at conserved DPPIV-Asn^{229}. *Access arrow 1* points to the entrance of the substrate to the active site through the β-propeller, and *arrow 2* points through the side opening of DPPIV [21].

1.1.3 Enzyme activity and substrates of CD26/DPPIV

CD26/DPPIV is a serine protease. It can cleave off dipeptides with proline or alanine at the penultimate position of the N-terminus of various bioactive peptides. The cleavage of DPPIV was shown to modulate the activities of the peptides and influence their functions in a diversity of biological processes [21]. A wide range of polypeptides with the sequence

Xaa-Pro/Ala at their N-terminus are substrates of DPPIV. The substrates with Pro are better hydrolyzed than the corresponding ones with Ala. However, not all polypeptides with this sequence are substrates of DPPIV, as in the case of intact Interleukin-2 and G-CSF [22]. The length of the peptide plays an important role in the determination of DPPIV substrates. Certain peptides with other small amino acids up to 80 residues at the second position are also cleaved by DPPIV at low rates, while peptides longer than 80 residues are unlikely to be potential substrates of DPPIV [23]. The enzyme activity of DPPIV is able to degrade its substrates to small peptides and amino acids, in order to make them suitable for transport and utilization. In addition, DPPIV degrades the substrates to inactivated or activated forms during the important biological process. Physiological substrates of DPPIV include neuropeptides Y and YY, peptide hormones glucagon-like peptides 1, 2 (GLP-1, GLP-2) and glucose-dependent insulinotropic polypeptide (GIP), chemokines (C-X-C chemokine receptor type 4/CXCR4), growth factors (mannose-6-phosphate/insulin-like growth factor II receptor/M6P/IGF-IIR) [5]. The cleavage of DPPIV results in different effects on different substrates. Patients with diabetes mellitus type-2 exhibit an attenuated insulinotropic action of GIP and a reduction in meal-stimulated levels of GLP-1 [24, 25]. The degradation of GIP and GLP by DPPIV inactivates their insulinotropic activity. In addition, DPPIV inactivates eotaxin at its main receptor CCR3, and this N-terminal truncated product is an antagonist to the intact eotaxin. However, the truncation of interferon-γ inducible protein (IP-10) does not abolish its biological activity [26]. Substance P is cleaved by DPPIV into an active state which modulates T cell function [27]. Some substrates of DPPIV are involved in immune functions, such as regulated on activation, normal T cell expressed and secreted (RANTES), Stromal-derived factor-1 (SDF-1), IP-10, macrophage-derived chemokine (MDC) and monocyte chemotactic protein-2 (MCP-2) [28].

Many peptides with proline or alanine in the second position of N-terminus can be considered substrates of DPPIV. Truncation by DPPIV, the substrates may alter their biological functions, or generate different binding abilities to their receptors which will lead to different consequences. For example, eotaxin, a substrate of DPPIV, its chemotactic potency for blood eosinophils and its signaling capacity through CCR3 could be reduced 30 times after truncation by DPPIV [29]. In **Table 1** shows a summary of selected known substrates which are studied more of DPPIV and the consequences of the

degradation, which is modified from De Meester *et al*.[5]; Hildebrandt *et al.* [28]; and C. Klemann *et al*.[30].

Table 1.1: Selection of known peptides as substrates for DPPIV

Substrate	**N-terminus**	**Biological effect**	**Consequences**
GLP-1	His-Ala-Glu	inactivation	Loss of potent insulinotropic and blood glucose-normalizing effect
GLP-2	His-Ala-Asp	inactivation	Loss of potent insulinotropic blood glucose-normalizing effect
GIP	His-Ala-Asp	inactivation	Loss of potent insulinotropic effect
PHM	His-Ala-Asp	inactivation	-
GRF (1-44)	Try-Ala-Asp	inactivation	-
Substance P	Arg-Pro-Lys	inactivation	Modulates T cell function
Peptide YY	Try-Pro-Ile	Alteration receptor specify	Modulation of receptor specificity
Neuropeptide YY	Try-Pro-Ser	Alteration receptor specify	Loss of YI-receptor mediated functions
Fibrinogen	-	Hydrolysis	Inhibition of fibrinogen polymerization
RANTES (CCL5)	Ser-Pro-Tyr	Altered receptor specify	Inhibition of monocyte chemotaxis with simultaneous enhancement of T cell migration
SDF-1α (CXCL12)	Lys-Pro-Val	degradation	Cleavage of SDF-1a by DPPIV leads to reduced chemotactic activity and promotes HIV infection via its receptor CXCR4
Eotaxin (CCL11)	Gly-Pro-Ala	inactivation	Loss of ability to attract eosinophils

MDC (CCL22)	GP-YG-A	Change in receptor preference	Change of its ability to attract monocytes
IP-10 (CXCL10)	Val-Pro-Leu	inactivation	Loss of chemotactic ability for CD4 positive T cells
MCP-1	Gln-Pro-Asp	inactivation	Loss of monocyte chemotactic function
MCP-2	Gln-Pro-Asp	inactivation	Loss of monocyte chemotactic function

Table showing part of known peptides which are substrates of DPPIV. Abbreviations: **GLP**: glucagon-like peptide**;** GIP: **gastric inhibitory peptide;** PHM: **peptide histidine methionine;** GRF: **growth hormone-releasing factor;** RANTES: **regulated on activation normal T cell expressed and secreted;** SDF-1: **stromal cell-derived factor 1;** MDC: **macrophage-derived chemokine;** IP-10: **interferon-inducible protein 10;** MCP: **monocyte chemotactic protein.**

1.1.4 The role of CD26/DPPIV in immune regulation

1.1.4.1 As a co-stimulator in T cell activation

The CD3/T cell receptor (TCR) complex plays a central role in T cell activation and function; however, this alone is not sufficient to induce T cell activation. It requires a second co-stimulatory crosslinking of TCR to induce T cell activation and proliferation. CD26 is one of such co-stimulatory molecules [4]. Some studies have indicated that CD26 can deliver a potent co-stimulatory signal for T-cell activation [2, 31]. It was described for the first time that the basal expression level of CD26 was very low on the surface of resting T cells, but the expression was strongly up-regulated on the activated T cells [32]. The administration of certain anti-CD26 monoclonal antibodies suggested an enhancement of anti-CD3 mAb-driven T cell activation and the inhibition of DPPIV could suppress anti-CD3 mAb-driven signaling pathways [33]. Some evidence indicates that CD26 interacted with protein tyrosine phosphatase CD45 in lipid rafts, thereby enhanced protein tyrosine phosphorylation of various signaling molecules, and then induced T cell activation and proliferation [34]. In addition, it has been reported that the interaction of CD26 with caveolin-1 can lead to the up-regulation of CD86, which can enhance the

interaction of CD86 and CD28 on T cell surfaces, this can subsequently induce the antigen-specific T cell proliferation and activation [35]. Other studies indicate an involvement of CD26 in T cell activation through its interaction with CARMA1, which may lead to the activation of NF-kB signaling pathway and T cell proliferation [36]. Furthermore, it was reported that CD26 can have a costimulatory effect on T-cell activation via its interaction with ADA (see section 1.1.4.3).

1.1.4.2 Involved in T cell differentiation

$CD4^+$ T cells play a critical role in mediating adaptive immunity to a variety of pathogens. After TCR activation, naive $CD4^+$ T cells may differentiate into one of several lineages of T helper cells, including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17) and T regulatory cells (Tregs), which are defined by their cytokine patterns or surface marker production [37]. The initial two major groups of differentiated $CD4^+$ T cells are Th1 and Th2 cells [38]. Th1 cells secrete IFN-γ as their signature cytokine and they are also good IL-2 producers. While Th2 cells fail to secrete IFN-γ, their signature cytokines are IL-4, IL-13 and IL-5 [37]. It has been reported that CD26 is associated with T cell differentiation as well as being a co-stimulator of T cell activation. The expression of CD26 is differentially regulated between different T cell subsets. As a marker of T cell activation, CD26 is mainly expressed on $CD4^+$ T cells and it is thought to be a marker of T helper 1 cells [39]. On lymphocytes of patients allergic to birch pollen, high expression of CD26 was shown to correlate with Th1/Th0 phenotype and mostly on IFN-γ-producing cells [6]. In multiple sclerosis (MS) disease, $CD4^+$ memory T cells with high CD26 expression are enriched for Th1 markers [39]. Although both Th1 and Th2 cells express CD26, Th1 cells express three- to six-fold more CD26 protein than Th2 cells, and the co-stimulatory function of CD26 for T cell activation and proliferation is proportional to the expression level of CD26 on T cell surfaces [40]. Other studies have indicated that CD26 expression may induce the cytokines production of Th1 cells, including IL-2, IFN-γ, IL-10 and IL-12 [41, 42]. In addition, the expression level of CD26 is more related to the production of Th1 cytokines than with Th2 cytokines [40]. *In vivo,* CD26-deficiency may decrease the production of IL-2 and IL-4 in sera and delay the production of IFN-γ of mice after pokeweed mitogen (PWM)-stimulation [43].

In recent years, new major effector populations of $CD4^+$ T cells has been defined and designated as Th17 cells [44]. One of the Th17 signature cytokines is IL-17 which is a pro-inflammation factor. Besides IL-17, Th17 cells are able to produce other several pro-inflammatory cytokines, including IL-22, IL-26 and IFN-γ [45]. Previous studies have shown that Th17 cells express IL-23R, lectin-like receptor CD161 and chemokine receptor CCR6 (CD196) [46-48]. As a pro-inflammatory cell subset, Th17 cells are shown to be involved in various diseases such as in autoimmune disease, tumor immunity as well as organ transplantation [49-51]. It was found that the inhibitor of CD26/DPPIV could inhibit the differentiation of Th1 and Th17 cells [52]. Human Th17 cells express a high level of DPPIV/CD26 [53]. 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 cells. Tregs modulate the immune activities through their immunosuppressive effect on other self-reactive T cells thereby contributing to the maintenance of immunologic self-tolerance [54]. Tregs are capable of recognizing both self and non-self antigens and control various immune responses. The majority of human Tregs strongly and constitutively express CD25 ($CD25^{high}$). Past studies found that forkhead transcription factor (Foxp3) is required for the development and function of $CD4^+CD25^+$ regulatory T cells and is one of the specific markers of Tregs [55]. The activation, expansion and survival processes of Tregs are controlled by various accessory molecules and cytokines, such as IL-2 which has been indicated as a contributor for Tregs survival [56, 57]. The depletion or functional alteration of Tregs may lead to autoimmune disease, allergy, malignancies, as well as transplant rejection [54]. In addition, the balance between Th17 and Tregs is crucial for immune homeostasis. However, the therapeutic application of regulatory T cells is hampered by the lack of suitable extracellular markers, which complicates the identification or isolation of Tregs [58]. There are currently very few investigations about the role of CD26 in the function of Tregs. A previous study found that $CD4^+CD25^{high}$- or $CD4^+FoxP3^{high}$-lymphocytes express lower level of CD26 compared within $CD4^+CD25^{low}$- or $CD4^+FoxP^{-/low}$-lymphocytes [59].

As a co-stimulator of T cell activation, CD26 has been suggested to be involved in many immune diseases. Further research on the role of CD26 in T cell differentiation, especially following the emergence of new paradigms of T cells, such as Th17 and Tregs, will

contribute to the elucidation of the molecular mechanisms of the role of CD26 in related immune diseases.

1.1.4.3 As a binding partner of adenosine deaminase (ADA)

Adenosine deaminase (ADA) is an enzyme involved in purine metabolism. It can catalyze the hydrolytic deamination of adenosine and 2-deoxyadenosine which are toxic to lymphocytes to inosine and 2-deoxyinosine [60]. It was first demonstrated in 1993 that CD26 is identical with ADA binding protein [61]. ADA is presented in many different mammalian tissues as an ectoenzyme and is involved in the development and function of lymphoid tissues. The deficiency of ADA in human may cause impairment of T and B cell function which lead to severe combined immune-deficiency disease (SCID). The interaction of ADA and CD26 may induce a co-stimulatory effect in T cell activation, which leads to increased productions of some proinflammatory cytokines, such as IFN-γ, TNF-α and IL-6 [60]. The crystal structure of CD26 in combination with ADA reveals a highly amphiphilic interface of the binding area between CD26 and ADA, which elucidates that the binding of both proteins does not influence the activities of both enzymes [62].

1.1.4.4 As a regulator of chemokine function

Chemokines are a group of secreted bioactive molecules, consisting of about 100 amino acids. They were identified to be produced from immune cells and contribute to the maturation and trafficking of leukocytes. Chemokines are classified into four main subfamilies (CXC, CC, XC and CX3C chemokines) based on structural, functional and genetic criteria. The major role of chemokines is to guide the migration of cells as a chemoattractant. They regulate multiple cell functions in the immune system. Some chemokines are involved in the recruitment of leukocytes to the infection and inflammation sites in response to the bacterial infection or physical damage by viruses.

A large number of human chemokines have been found to be substrates of CD26/DPPIV, such as CXCL2 (MIP2-alpha, macrophage inflammatory protein 2-alpha), CXCL6 (GCP-2, granulocyte chemotactic protein 2), CXCL9 (HuM1G, monokine induced by IFN-γ),

CXCL10 (inflammatory protein-10), CXCL11 (IFN-γ-inducible T cell chemoattractant), CXCL12 (SDF-1α and 1β), CCL3 (macrophage inflammatory protein-1 isoform LD78), CCL4 (MIP, macrophage inflammatory protein-1), CCL5 (RANTES), CCL11 (eotaxin) and CCL22 (MDC, monocyte derived chemokine) [63]. The cleavage by DPPIV can either activate or inactivate the chemokines or change their receptor specificity thereby leading to different chemotaxis to their target cells and induce different target cells recruitment. SDF-1α has a chemo-attractant activity for lymphocytes and monocytes, and plays an important role in the trafficking, exporting and homing of bone marrow. It was reported that SDF-1α can inhibit viral infection by T-tropic strains of HIV-1. The degradation of SDF-1α by DPPIV leads to the inactivation of SDF-1α, which then loses its binding and signaling activity for receptor CXCR4 [64]. In contrast to SDF-1α which is inactivated by DPPIV cleavage, the chemoattractant activity of CCL5 (RANTES) is modulated following the cleavage by DPPIV. CCL5 promotes the recruitment of monocytes, eosinophils, basophils and NK cells via the interaction to its receptors. CCL5 is a more powerful activator of CCR5, but with less affinity for CCR1 and CCR3. The truncation of CCL5 by DPPIV regulates its receptor selectively, abolishes the signaling between CCL5 with CCR1 and CCR3, but does not affect the signaling through CCR5 [65]. These observations indicate that CD26 potentially modulates the extravasation and migration of immune cells by regulating the activities of their chemokines.

1.1.4.5 As a mediator of cell adhesion

CD26 has been shown to mediate cell adhesion and migration through its interaction with extracellular matrix proteins, such as fibronectin and collagens [66, 67]. Different collagens have different affinities to CD26/DPPIV. For instance, collagen I and III have the strongest binding ability to CD26/DPPIV, while collagen II, IV and V have moderate binding affinities. The binding ability between CD26/DPPIV and collagen VI is the lowest [16]. Other works have also shown that the interaction between CD26 and ADA is important for the regulation of epithelial cells and lymphocytes adhesion [68].

1.2 CD26/DPPIV and diseases

CD26 is a multifunctional protein. In clinical research, many observations have linked CD26 to some clinical diseases, such as diabetes mellitus, HIV infection, asthma, cardiovascular disease, rheumatoid synovium, Middle East respiratory syndrome, coronavirus infection, as well as organ transplantation failures [69-74].

1.2.1 In Diabetes Mellitus Type-2

Diabetes mellitus type-2 is a chronic metabolic disorder. It is manifested from two main pathophysiological defects in insulin action and secretion. The impaired action and secretion of insulin leads to an increased production of hepatic glucose and then disrupt the physiological glucose homeostasis. The main insulin secretion results from the incretin response, mainly the effects of GIP and GLP-1 [75] which contribute to the physiological glucose homeostasis. The use of GIP and GLP-2 are considered to be good therapies for the treatment of type-2 diabetes. However, the limiting factor to their use is that they are rapidly degraded *in vivo* by DPPIV [69, 76]. Therefore, alternative therapeutic approaches are required. Two strategies for the improvement of the pharmacokinetics of GLP-1 and GIP were proposed: GLP-1R agonists which are administered subcutaneously and DPPIV inhibitors which are administered orally. A number of structural modifications of GLP-1 and GIP were used to against DPPIV degradation. Exenatide-3 and -4 are the first GLP-1 receptor agonists approved by the U.S. Food And Drug Administration (FDA) in 2005 for the treatment of type 2 diabetes [77]. Some structural modifications of GIP such as modifications of its N-terminal Tyr^7 and Glu^9 were used to safeguard against DPPIV degradation [78, 79]. Another strategy is to develop DPPIV inhibitors as drugs in the treatment of type 2 diabetes. A series of DPPIV inhibitors, such as NVP-LAF237, P32/9，Omarigliptin, Saxagliptin, Alogliptin, Linagliptin, Gliptins *et al.*, were used to improve the glucose tolerance *in vivo* [80-85]. DPPIV inhibitors are a promising treatment option for type II diabetes.

1.2.2 In HIV infection and AIDS

Acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), is one of the most devastating pandemics ever at the global level. HIV is an enveloped, single-stranded positive-sense RNA virus. The envelop glycoprotein structure of HIV-1 is essential for the viral entry into the target host cells. It consists of two subunits: external (gp120 proteins) and transmembrane (gp41 proteins) [86]. The HIV infection is mediated by the binding of gp120 to CD4 on host cells, forming a heterotrimeric complex. The formation of the complex effects conformational changes of gp120 which induces its binding to the co-receptor CCR5 (CXCR4 for HIV-2). The binding of gp120 with CCR5 leads to the exposure of gp41 fusogenic peptide and then initiates the fusion of the viral membrane with the target cell membrane [87]. The normal ligands of CCR5 are RANTES, MIP-1β and MIP-1α. These ligands are able to suppress HIV-1 infection *in vitro* through their binding with CCR5 [88]. Although CD26/DPPIV does not serve directly as a co-receptor of the HIV infection, many studies suggest an association of CD26/DPPIV with HIV infection and AIDS immune response [70]. Some previous work demonstrated that low CD26-expressing cells are more prone to HIV infection while high CD26-expressing cells are resistant to HIV infection [89]. The low CD26-expressing cells may reduce the binding of CD26 to ADA. This in turn may be responsible for the increased concentration of adenosine in the microenvironment around the cells which may suppress T cell immune response [70]. However, in contrast, other groups have reported that the high expression of CD26 may affect HIV infection as the inhibition of CD26/DPPIV may in turn inhibit HIV1 infection [90]. In addition, the transcription transactivator of HIV1 (TAT) can interact with CD26 expressed on the target cell surfaces, which may induce the apoptosis of target cells [91]. Moreover, CD26/DPPIV may be involved in HIV infection through the cleavage of its substrates. RANTES, whose receptors are CCR1, CCR3 and CCR5, may inhibit HIV infection via its binding to CCR5. This chemokine has been confirmed to be substrates of CD26/DPPIV. The cleavage of RANTES by DPPIV may abolish the signaling provided by the interaction between RANTES and CCR1 or CCR3, resulting in the induction of HIV-specific T cell cytotoxicity. However, the truncation was shown not to affect the signaling provided by the interaction of RANTES and CCR5 [92]. SDF-1α is a ligand for CXCR4 and blocks HIV infection through its binding to CXCR4. The

cleavage of SDF-1α by DPPIV reduces its binding to CXCR4; subsequently, it may promote HIV infection [93].

1.2.3 In inflammatory and autoimmune diseases

Inflammatory disease is characterized by common inflammation which is produced by the immune response of the body to remove the harmful stimuli, such as bacteria and viruses. Inflammation is mediated by various immune cells and molecules. It can be classified into acute and chronic. The acute inflammation normally occurs over a short period of time, ranging from a few minutes to a few hours after the injury of tissues. It is initiated by the resident immune cells in the involved tissues, mainly resident macrophages, dendritic cells, neutrophils, mast cell and Kupffer cells [94]. If the pathogens causing acute inflammation are not resolved within a short time, the inflammation may pass to a longer term-chronic phase. The chronic inflammation may occur over few years. In chronic inflammation, one of the main immune cells is lymphocyte. The proinflammatory cytokines secreted by T lymphocytes such as IFN-γ, IL-6, IL-17, IL-23, tumor necrosis (TNF) and granulocyte-macrophage colony stimulating factor are critical for the inflammatory reaction [95, 96]. However, excess inflammatory cytokines can drive many medical disorders and autoimmune diseases, including allergy, asthma, arthritis, inflammatory bowel disease, multiple sclerosis *et al.* [97]. It was reported that the expression of CD26/DPPIV on monocytes and $CD4^+$ lymphocytes was increased in active chronic Rheumatoid Arthritis [98]. Multiple sclerosis (MS) is a prototypic Th1/Th17 chronic autoimmune disease. The plasma level of CD26 and its DPPIV activity are significantly lower in MS patients [99]. *In vivo*, the autoimmune disease encephalomyelitis can be suppressed by DPPIV inhibition both in preventive and therapeutic fashion [100]. The enzymatic activity of CD26/DPPIV and the numbers of $CD26^+$ T cells are both increased under allergic airway inflammation. The deficiency of CD26/DPPIV presents protective effect in experimental asthma [101]. However, Yan *et al.* found that the deficiency of CD26 enhanced the ovalbumin-induced airway inflammation, suggesting a careful control of the application of DPPIV inhibitor in clinic allergic disease [102]. The application of DPPIV inhibitor in clinical immunosuppressive therapy still needs to be further investigated.

1.3 CD26/DPPIV and skin transplantation

1.3.1 Skin transplantation

Skin is the largest organ of the human body. The primary function of the skin is to protect the body from physical and chemical attack from the environment, water loss, and invasion of pathogens [103]. The loss or damage of the skin is one of the most challenging problems in healthcare. More than 11 million people suffer from burn injuries worldwide annually [104]. Autografting and allografting are the two main strategies for the treatment of skin injury or large wound [105]. Autografting, also known as an autologous transplant, means that the donor skin is taken from a different site on the same individuals´ body. Autografting could avoid the problem of immunogenicity. However, it is much limited when the burn area is very large. The patients may be lacking appropriate donor skin sites, or another new wound needs to be created to provide the donor skin. The allograft skin has to be used for skin transplantation under this situation. Allografting, also known as allogenic graft transplant, means that the donor and recipient are of the same species but they are different individuals. It is one of the most important approaches for treating the large-area burn. However, it is one of the market´s challenges of clinical treatments because of the allogeneic graft rejection. Isografting is an exception in allograft transplant. It means that the donor and recipient are two individuals who are genetically identical (e.g. monozygotic twins). Isografting is anatomically identical to allografting; however, it does not trigger an immune response. The limitation of isografting is the small probability to find genetically identical donors [106]. Another strategy for treatment of skin injury or large wound is xenografting or xenotransplantation, which means that the skin donor is from different species. Although xenotransplantation is a good way to solve the shortage of donor skin, it increases the high risk of immune rejection and easily causes disease and may lead to the death of the recipients [105].

1.3.2 Skin transplantation rejection

After skin transplantation, the grafts should start to develop blood vessels and connect to the skin around it within a short time. If these blood vessels do not begin to form shortly after the surgery, it may be a sign that the graft is rejected by the recipient body. The

recipients´ immune system then destroys the allografts until the grafts fall completely. The allogenic skin graft rejection is a complex immune reaction process. Firstly, because the skin is not a primary vascularized graft, the skin grafts have to establish their own blood vascularization after skin transplantation. Following vascularization, antigen-presenting cells (APCs) of the donor skin migrate out of the graft and are infiltrated into the draining lymph nodes of the recipient. These cells can present donor antigens which may be recognized by the recipient T cells directly (direct antigen-presentation) or through the host APCs (indirect antigen presentation). Following allorecognition, the recipient´s T cells become activated. The activated cytotoxic $CD8^+$ T cells (CTL) may induce the apoptosis of the donor alloantigen-expressing target cells through perforin-granzyme and Fas-Fas ligand pathway, leading to an acute rejection of the grafts. The $CD4^+$ T helper cells aid the activation and function of CTL through the secretion of cytokines [107], which can cause chronic transplant rejection.

Cytokines secreted by different T cell subsets play a crucial role in the immune rejection not only through the effect on the activation of effector T cells but also on the activation and function of innate immune cells, such as macrophages [108]. However, many cytokines have been found to possess diverse and potential contradictory effects on organ allograft rejection. In acute rejection, Th1 cells are infiltrated into grafts predominantly where they can release proinflammatory cytokines and then induce the activation of natural killer cells and macrophages [109]. IL-2 is one of such proinflammatory cytokines. It is released by Th1 cells and plays a role in a tug-of-war between T effector cells and T regulatory cells [110]. IL-2 is one of the important factors for the growth of T effector cells during the allogenic immune response. In addition, IL-2 is also necessary for the expansion of regulatory T cells. Tregs and T helper cells can compete for the consumption of IL-2 depending on activation status and spatial localization of the cells [111]. IFN-γ is another main cytokine of Th1 cells. It has double effects in organ transplantation. It has been reported that lack of IFN-γ greatly reduces the induction of MHC and suppresses the generation of CTL in organ allografts. However, IFN-γ also plays a protective role in the early stage of immune response to vascularized organ allograft [112]. IL-4, a typical cytokine of Th2 cells, was reported to partly mediate the tolerance in allograft rejection through promoting IL-10 and IgG1 production. Other studies have however provided

conflicting results that the inhibition of Th2 cells may prolong allograft survival [113, 114].

Off late, the role of new T cell subsets such as Th17 cells during transplant rejection is getting more attention. Th17 cells can secrete several proinflammatory cytokines with IL-17 being the main one. It was found that the number of Th17 cells and the secretion level of IL-17 were increased after allogenic skin transplantation [51]. On the contrary to Th17 cells, Tregs play a critical role in the maintenance of immune tolerance in transplant rejection. They can negatively regulate the immune response of other effector T cells by secreting immune suppressing cytokines such as IL-10 and TGF-β, or via CTLA-4 signaling pathway [115]. The balance between Th17 cells and Tregs is critical for allograft rejection and immunological tolerance [108]. IL-6 was reported to play an important role in regulating the balance between Th17 cells and Tregs [116]. Although the rejection of allogeneic skin transplants is principal to a T cell-dependent process, the innate immune reaction also contributes to the graft-specific immune rejection. For example, the macrophages can be activated by some proinflammatory cytokines after the allogeneic transplantation, and then migrate into the graft sites to destruct the grafts [117]. NK cells are another kind of innate immune cells that play important roles in allogeneic transplantation [118].

1.3.3 CD26/DPPIV in organ transplantation

Several observations indicate a relevance of CD26 in transplant rejection. To begin with, CD26 acts as an important co-stimulatory and activation marker of Th1 cells which are associated with the early stage of transplant rejection [6, 53]. Secondly, recent studies have suggested that CD26 is a potential positive marker of Th17 cells and a negative marker of Tregs [53, 59]. In addition, CD26 has been shown to be involved in skin transplantation through its degradation of some chemokines which can influence the chemotaxis of immune cells towards grafts after skin transplantation, such as monocyte chemotactic proteins (MCP-1, -2, -3), macrophage colony-stimulating factor (M-CSF or CSF-1), and RANTES [119]. As substrates of DPPIV/CD26, MCPs and RANTES can be truncated by DPPIV followed by the alteration of their chemotactic activities [5]. It was found that MCP with an amino-terminal Lys can be cleaved by CD26/DPPIV and may result in the

inactivation of its chemotaxis; however, MCP with an NH2-terminal pGlu remained unaffected [120]. The truncation of CCL5/RANTES by DPPIV is reported to decrease its binding to CCR1 and CCR3 but increases its binding to CCR5 which contribute to macrophage recruitment in renal grafts [119].

More studies indicate the involvement of CD26/DPPIV in organ transplantation. The CD26 expression was shown to be strongly increased in patients with end-stage kidney disease after kidney transplantation [121]. The loss of CD26 protease activity in recipient mice could improve transplant efficiency in hematopoietic stem transplantation *in vivo* [122]. In clinical research, the inhibition of DPPIV could enhance the engraftment of cord blood transplantation in patients with hematological malignancies [123]. In addition, other studies suggest the involvement of CD26 in graft-versus-host diseases (GVHDs). It was found that the chronic GVHD of the lungs was caused in part by IL-26(+)CD26(+)CD4 T cells [124]. The humanized anti-CD26 monoclonal antibody was shown to contribute to the prevention of the acute graft-versus-host disease [125]. However, the role of CD26 in allogeneic skin transplantation is yet to be fully clarified. Allogeneic skin transplantation is one of the most efficient and encouraging approach to treating large area burn. The elucidation of the mechanism of CD26 in the allogeneic skin graft rejection is therefore necessary and urgent.

2. Aims of this work

As a multifunctional protein, CD26 was shown to be involved in the activation of T lymphocytes. The clarification of the role of CD26 in the proliferation, differentiation and function of T cells, especially in the new paradigms of T cells, such as Th17 and Tregs, will contribute to the elucidation of the molecular mechanisms of the role of CD26 in the immune response. Two parts of the work should be done as follows:

2.1 To investigate the role of CD26 in the activation, proliferation and differentiation of human peripheral blood T lymphocytes through the analysis of the expression levels of different T cell subpopulations in different CD26-expressing groups after antigen stimulation in an *in vitro* system.

2.2 To investigate the role of CD26 in immune rejection of skin allogeneic transplantation in an *in vivo* system using CD26-deficient mice. The elucidation of the role of CD26 in immune rejection is expected to play a crucial role in developing novel strategies to inhibit the graft rejection and improve the therapeutic effect in clinical organ transplantation.

3. Materials and methods

3.1 Materials

3.1.1 Animals

Homozygous CD26$^{-/-}$ mice on the C56BL/6N genetic background were obtained originally from Dr. Marguet [69] and bred in Forschungseinrichtungen für Experimentelle Medizin (FEM) of Charité under specific pathogen-free conditions. The wild-type C56BL/6N and the donor mice BALB/c were obtained from FEM of Charité and kept under specific pathogen-free conditions. Experiments were performed on males and females; there was no sex-related difference [43]. The animals were treated according to the German Law on the Protection of Animals and the permission (G0071/14) was obtained from the State Animal Welfare Committees.

3.1.2 Cells

Human lymphocytes	healthy donors

3.1.3 Primers

Primers for the genotyping of CD26 knockout mice:

CD26$^{+/+}$ (WT)	Forward	5′ TCCATAGCATCGTGGCTGAG 3′
	Reverse	5′ TAAACACCACCCACAACCCG 3′
CD26$^{-/-}$(KO)	Forward	5′ ACTCCATAGCATCGTGGCTG 3′
	Reverse	5′ CGATGTTTCGCTTGGTGGTC 3′

3.1.4 Kits

Kits for the measurement of the cytokines secretion

Human IL-2	R&D Systems (Minnesota, USA)
Human IFN-γ	R&D Systems (Minnesota, USA)
Human IL-4	R&D Systems (Minnesota, USA)
Human IL-6	R&D Systems (Minnesota, USA)
Human IL-13	R&D Systems (Minnesota, USA)
Human IL-10	R&D Systems (Minnesota, USA)
Human IL-5	R&D Systems (Minnesota, USA)
Mouse IFN-γ	Thermo Fisher Scientific (Massachusetts, USA)
Mouse IL-2	Thermo Fisher Scientific (Massachusetts, USA)
Mouse IL-4	Thermo Fisher Scientific (Massachusetts, USA)
Mouse IL-6	Thermo Fisher Scientific (Massachusetts, USA)
Mouse IL-10	R&D (Minneapolis, USA)
Mouse IL-5	R&D (Minneapolis, USA)
Mouse IL-13	R&D (Minneapolis, USA)
Mouse IL-17	Biolegend (London, United Kingdom)

Kit for the measurement of cell apoptosis

Annexin V/PI Apoptosis Detection Kit	Thermo Fisher Scientific (Massachusetts, USA)

Kit for the measurement of lymphocytes proliferation

CellTrace™ CFSE Cell Proliferation Kit	Thermo Fisher Scientific (Massachusetts, USA)

3.1.5 Antibodies

Antibodies for human lymphocytes surface staining

FITC-conjugated anti-CD26	ImmunoTools (Friesoythe, Germany)
PE/FITC-conjugated anti-CD4	ImmunoTools (Friesoythe, Germany)
PE/FITC-conjugated anti-CD8	ImmunoTools (Friesoythe, Germany)
PE-conjugated anti-CD19	ImmunoTools (Friesoythe, Germany)
PE-conjugated anti-CD69	ImmunoTools (Friesoythe, Germany)
PE-conjugated anti-CD25	ImmunoTools (Friesoythe, Germany)
PE-conjugated anti-CD71	ImmunoTools (Friesoythe, Germany)
PE-conjugated anti-CD38	ImmunoTools (Friesoythe, Germany)
APC-conjugated anti-CD161	Miltenyi Biotec (Bergisch Gladbach, Germany)
Per-conjugated anti-CD196	Miltenyi Biotec (Bergisch Gladbach, Germany)
PE-conjugated anti-CD11a	Miltenyi Biotec (Bergisch Gladbach, Germany)
PE-conjugated anti-CD11b	Miltenyi Biotec (Bergisch Gladbach, Germany)
PE-conjugated anti-CD11c	Miltenyi Biotec (Bergisch Gladbach, Germany)
PE-conjugated anti-CD54	Miltenyi Biotec (Bergisch Gladbach, Germany)

Antibodies for human lymphocytes intracellular staining

These antibodies are all from ImmunoTools (Friesoythe, Germany)

PE-conjugated anti-IL-2
PE-conjugated anti-IFN-γ
PE-conjugated anti-IL-4
PE-conjugated anti-IL-13
PE-conjugated anti-IL-6
PE-conjugated anti-IL-17
PE-conjugated anti-IL-22
PE-conjugated anti-IL-23R
PE-conjugated anti-TNF-α
APC-conjugated anti-FoxP3

Antibodies for mice lymphocytes staining

APC-conjugated anti-CD3	Biolegend (London, United Kingdom)
PE-conjugated anti-NK1.1	Biolegend (London, United Kingdom)
FITC-conjugated anti-CD19	Biolegend (London, United Kingdom)
FITC-conjugated anti-CD4	Biolegend (London, United Kingdom)
PE-conjugated anti-CD8	Biolegend (London, United Kingdom)
PE-conjugated anti-CD25	Miltenyi Biotec (Bergisch Gladbach, Germany)
APC-conjugated anti-FoxP3	Miltenyi Biotec (Bergisch Gladbach, Germany)
PE-conjugated anti-IL-17	Biolegend (London, United Kingdom)
Biotin-conjugated anti-mouse IgG, IgG1, and IgG2a mAb	
Purified anti-mouse Ig pAb	ImmunoTools (Friesoythe, Germany)
Mouse IgG, IgG1, IgG2a standards	Pharmingen (Heidelberg, Germany)

Antibodies for the mice immunohistostaining

Rat anti-mouse mAb against CD14	R&D (Minneapolis, USA)
Rat anti-mouse mAb against CD3	Biolegend (London, United Kingdom)
Rat anti-mouse mAb against CD4	Biolegend (London, United Kingdom)
Rat anti-mouse mAb against CD8	ImmunoTools (Friesoythe, Germany)
FITC-conjugated anti-rat IgG	R&D (Minneapolis, USA)

3.1.6 Solutions

Solutions for the electrophoretic analysis of nucleic acids:

TAE-buffer	0.04 M Tris
	5 mM Na-acetate
	2 mM EDTA
	pH 8.0

5x Sample buffer	25 % Glycerine
	50mM EDTA
	0.1% Bromo-phenol blue

Sources of other special reagents and materials have been indicated in the text of Material section.

3.1.7 Reagents

RPMI 1640 medium	Biochrom (Berlin, Germany)
Fetal calf serum	Kraeber (Wedel, Germany)
Alkaline phosphatase-labeled streptavidin	Pharmingen (Heidelberg, Germany)
Penicillin/streptomycin	Biochrom (Berlin, Germany)
Ficoll-Paque PLUS	GE Healthcare (Uppsala, Sweden)
Anti-human IgG Microbeads	Miltenyi Biotec (Bergisch Gladbach, Germany)
Accuprime-*pfx*-Supermix	Thermo Fisher Scientific (Massachusetts, USA)
1 Kb DNA ladder	Thermo Fisher Scientific (Massachusetts, USA)
Other regents	Sigma (Missouri, USA)

3.2 Methods

3.2.1 Separation of human peripheral blood lymphocytes

Healthy human blood collection was performed according to the German Ethics laws, and approval (EA4/106/13) 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. Briefly, blood samples were first diluted with the same volume of balanced salt solution. The diluted blood samples were then layered carefully onto Ficoll-Paque Plus and centrifuged at 1400 rpm for 30 min at

room temperature. Then the upper layer of plasma was aspirated out using a sterile Pasteur pipette and the lymphocyte layer at the interface was carefully transferred into a new tube. The lymphocytes were washed twice with at least three volumes of balanced salt solution and then centrifuged at 1400 rpm for 10 min at room temperature. Finally, they were resuspended in the medium for cell culture.

Balanced salt solution (pH 7.6):

Anhydrous D-glucose	0.01 %
$CaCl_2 \cdot 2H_2O$	5.0×10^{-6} M
$MgCl_2 \cdot 6H_2O$	9.8×10^{-5} M
KCl	5.4×10^{-4} M
TRIS	0.0145 M
NaCl	0.126 M

3.2.2 Cell culture

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

3.2.3 Activation of human lymphocytes by antigen stimulation

Previous studies have indicated that certain monoclonal antibodies (mAbs) against human CD3 can induce T cell proliferation. To achieve this following Schwinzer´s protocol [126], stimulation of human peripheral blood lymphocytes (HPBLs) was performed by incubation of the lymphocytes for three days under stimulation with 2 µg/mL immobilized anti-human CD3 mAb (OKT3, IgG2a) (Thermo Fisher Scientific, USA). Briefly, 100 µL PBS with 2 µg/mL anti-CD3 mAb (stimulated group) or 100 µL PBS as control was immobilized in a 96-well plate overnight. After isolation, 2×10^5 lymphocytes were cultured directly in each well of the 96-well plate with immobilized antibody or PBS. The culture medium of the lymphocytes is RPMI-1640 growth medium supplemented with 10% FBS, 100 µg/mL streptomycin and 100 UI/mL penicillin. The cells are cultured at 37°C in a humidified atmosphere with 5% CO_2.

3.2.4 Measurement of cell survival rate

The survival rate of lymphocytes after stimulation was measured using Annexin V/PI Apoptosis Kit. In normal live cells, phosphatidylserine (PS) is located on the cytoplasmic surface of the cell membrane. However, in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane. Annexin V labeled with FITC can identify apoptotic cells by binding to PS on the outlet leaflet of the cells. Propidium iodide (PI) can stain dead cells by binding to the nucleic acid of the cells. The procedure was performed according to the instructions of the manufacturer (Thermo Fisher Scientific, USA). Briefly, lymphocytes were harvested at day three after stimulation and washed with PBS. After centrifugation, the supernatant was discarded. The lymphocytes at the bottom were incubated with FITC-conjugated Annexin V and PI in 1x Annexin-binding buffer for 15 min at room temperature (in the dark). After the incubation period, the cells were washed once with 1x Annexin-binding buffer and then measured by flow cytometry.

3.2.5 Measurement of lymphocytes proliferation

The proliferation of lymphocytes after stimulation was measured by Carboxyfluorescein succinimidyl ester (CFSE) Assay Kit (Thermo Fisher Scientific, USA). Carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) is a novel cell-tracing fluorescent dye used to examine the proliferative activity of cells by labeling of a parent generation. CFDA-SE diffuses into cells, where the acetate groups on the molecule are cleaved to yield a highly fluorescent derivative (CFSE). CFSE is retained in the cells and can be detected by flow cytometry. The dye dilution in flow cytometry can be used for tracing several cell generations. The procedure was performed according to the instructions of the manufacturer. Briefly, cells were stained with the stock dye solution in protein-free medium for 20 min at room temperature. Then they were washed with complete medium to remove the remaining dye solution. After the washing step, the cells were cultured under normal conditions. Three days after stimulation, the cells were collected, washed once with PBS, and then measured by flow cytometry.

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

MACS MicroBeads (Miltenyi Biotec, Germany) were used for the separation of CD26-expressing cells. Lymphocytes were collected at day three after stimulation, After centrifugation, the supernatant was removed and the lymphocytes at the bottom were collected for further procedure. At first, mouse anti-human CD26 mAb (named 350 which was prepared in our own laboratory) was used to label the cells for 1 h at 4°C. Following the two washing steps, anti-mouse IgG labeled with magnetic MicroBeads was added to the cells and incubated for 15 min at 4°C. After a washing step, cells labeled with magnetic 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. The column was then removed from the separator and placed in a suitable collection tube. The labeled $CD26^+$ cells were flushed out and collected using the plunger supplied with the column. Finally, two groups of cells $CD26^{high\text{-}expressing}$ ($CD26^{high}$) and $CD26^{low\text{-}expressing}$ ($CD26^{low}$) groups were obtained and then analyzed by flow cytometry.

3.2.7 Fluorescence immunomicroscopy

For cell surface staining, CD26-expressing lymphocytes were incubated with FITC-conjugated anti-human CD26 and PE-conjugated anti-human IL-23R at 4°C for 1 h. For the intracellular staining, after incubation with FITC-conjugated-anti-human CD26, the cells were washed and fixed with 4% formaldehyde. After a washing step, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, washed and were then incubated with PE-conjugated anti-human IL-2, anti-IFN-γ, anti-IL-17, or anti-IL-22 at 4°C for 1 h. The cells were then washed twice with PBS and were resuspended in 20 μL PBS and then covered on a slide with a thin layer. After air-drying, mounting solution was added to the slide, and the slide was covered by coverslips and left to air dry. The slides were then assessed by fluorescence microscopy. Images were acquired made at a magnification of 400x.

3.2.8 Flow cytometry analysis

All the incubation steps were performed in the dark. Cells for the surface staining were collected and incubated with fluorescence-conjugated antibodies in 1% (w/v) BSA/PBS at 4°C for 1 h. Cells for the intracellular staining were first fixed with 4% formaldehyde, after a washing step, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. After two washing steps with PBS, cells were incubated with fluorescence-conjugated antibodies in 1% (w/v) BSA/PBS at 4°C for 1 h. Then there were two further washing steps and finally the cells were resuspended in FACS buffer and analyzed by flow cytometry (BD Biosciences). WinMDI 2.9 software was used to analyze the percentages of different lymphocyte subpopulations or cytokine-secreting cells.

3.2.9 Polymerase Chain Reaction (PCR)

PCR reactions for genotyping purposes were carried out with Accuprime-*pfx*-Supermix kits. The procedure was done according to the manufacturer´s instructions. The total volume of a standard PCR reaction sample was 25 µL which was constituted as follows:

Template DNA	1–200 ng
Primer (forward and reverse)	200 nM each
Accuprime-*pfx*-Supermix	22.5 µL

The reaction was carried out in a Touch-Down-Thermo cycler (Gradient) with the following program (with modification of annealing temperatures depending on the primer used):

Denaturation	1 cycle	94°C	5 min
	30–35 cycles		
Denaturation		94°C	30 sec
Annealing		50–60°C	30 sec
Elongation		72°C	1 min pro kb of the amplified DNA
	1 cycle	72°C	5 min

Primers used in the amplification of DNA by PCR are listed in the Material section of the present work. After each PCR, 5–10 μL aliquot of PCR products was analyzed by agarose gel electrophoresis. The electrophoresis runs with 70–80 volts for 45–60 min (Bio-Rad, USA). The gels were then stained for visualization by incubating them for 15 min in an EB (Ethidium Bromide) bath (0.5 μg/mL) in TAE buffer. Ethidium bromide intercalates in the DNA and fluoresces thus makes the DNA fragments visible under UV light (366 nm).

3.2.10 Murine tail-skin transplantation

To prepare the tail skin from donor mice, the donor mice were sacrificed by cervical dislocation and the entire tail was swabbed with 70% ethanol. The tail skin of the donor was incised to an area of 1.0×1.0 cm^2. The recipient mice were anesthetized with isoflurane inhalation, a circumferential band was shaved on the back of the recipient mice. The shaved back of the recipient was cleaned with 70% ethanol and allowed to dry. The back skin of the recipient mice was cut to a 1.0×1.0 cm^2 graft bed. The bed skin was removed from the recipient and the skin graft was placed into the graft bed. The four corners of the grafts were stitched up and the mice were wrapped over with bandages. The mice were placed in a clean cage and heated by a red-lamp until they moved freely.

3.2.11 Scoring for rejection of skin grafts

Seven days after transplantation, bandages were removed; skin grafts were monitored daily and recorded by photographs up to 15 days. The skin rejection was scored by the necrotic areas (wrinkled skin) of the skin grafts. The necrotic areas were roughly estimated by visual inspection and six (0–5) different score levels were defined according to the percentage of the necrotic area of grafts. Fully intact smooth grafts or less than 20% necrotic area of the grafts scored 5, between 20%–40% necrotic area of the grafts scored 4, 40%–60% necrotic area of the grafts scored 3, and 60%–80% necrotic area of the grafts scored 2, 80%–100% (but not removed) scored 1, grafts fully removed had a score of 0.

3.2.12 Preparation of mouse spleen lymphocytes

Mice were sacrificed by swift cervical dislocation. The spleens were removed using the forceps and then washed with sterile PBS in clean tubes. After washing, the spleens were placed on a stainless steel mesh (100 μm mesh), and then pressed and mashed through into a petri dish using a plunger end of the sterile syringe. The homogenates of spleen cells were centrifuged at 300 rpm for 3 min. After the centrifugation, the suspension was transferred to a new tube and the cell clumps in the bottom were discarded. The cell suspension was further centrifuged at 1400 rpm for 6 min. Then the supernatant was removed and cell pellets were washed twice with PBS. After cell counting, 1.5×10^8 cells were resuspended in 1 mL Tris-HCl-buffered NH_4Cl solution to lyse the erythrocytes. After incubation at room temperature for 8 min, the cell suspension was further diluted with a 10-fold volume of medium to stop erythrocytes lysis. The cell suspension was further mixed and centrifuged. Finally, cell pellets were resuspended in RPMI1640 medium containing 10% fetal calf serum, 2 mM glutamine, 100 IU/ mL penicillin and 100 μg/mL of streptomycin.

Spleen lymphocytes were further purified by depleting monocytes on plastic plates at 37°C for 4 h. Cell viability was assessed using trypan blue exclusion.

3.2.13 Separation of mouse peripheral blood lymphocytes

After anesthesia, mice peripheral blood samples were collected with MiniCollect Tubes (Greiner Bio-One, Austria) and erythrocytes were lysed with lysing solution (BD FACS™ Lysing Solution). BD FACS 10x lysing solution was diluted with ddH_2O into 1x lysing solution 1 h before use. 1 mL blood was mixed with 1 mL 1x FACS lysing solution by gently pipetting up and down, and was then kept for 10 min at room temperature until the blood supernatant becomes clear. Ten-fold volume of PBS was added to the blood cells to stop the lysis process. After a centrifugation step at 1200 rpm for 10 min at room temperature, the supernatant was removed and the cell pellets were washed twice with PBS. The separated lymphocytes were used for the further analysis.

Cell viability was assessed with trypan blue staining exclusion.

3.2.14 Bicinchoninic Acid Assay

The mice graft tissues were collected at indicated time points. The graft tissues were homogenized in lysis buffer and then centrifuged (see section 3.2.17). The supernatant of the lysate was then transferred into new tubes. The protein concentration in the lysate was determined using Bicinchoninic Acid Assay (Thermo Fisher Scientific, USA), according to the manufacturer´s instruction. Briefly, 20 µL dilutions (1µg/20 µL to 10 µg/20 µL) of a BSA standard (Pierce, Rockford, USA), or distilled water as blank were transferred into wells of a 96-well microplate. Individual diluted lysate samples amounting to 20 µL to be quantified were also added to individual wells in the 96-well microplate. BCA working reagent (200 µL) (Reagent A and B at a ratio 50:1) was added to each well and the plate was incubated for 30 min at 37^{o}C. All measurements were done in duplicates. The extinction of the samples was then determined at 570 nm on a microplate reader (TECAN, Austria). The protein concentrations were calculated with reference to the standard curve.

3.2.15 Measurement of immunoglobulin production

Blood samples were collected from mice tails and clotted for 1 h at room temperature. After centrifugation, the serum was transferred into new tubes and stored at –80°C for further analysis. To quantify serum immunoglobulins, ELISA was used as described previously [43]. Briefly, anti-mouse Ig polyclonal antibody was used as capture antibody for IgG, IgG1, and IgG2a. Biotin-labelled anti-IgG, anti-IgG1, or anti-IgG2a was used as detecting antibody. Alkaline phosphatase-labeled streptavidin (PharMingen) and disodium 4-nitrophenyl phosphate hexahydrate (Sigma) substrate solution (1 mg/mL in substrate buffer) were used for the chromogenic reaction. After 15 min of incubation, the chromogenic reaction was stopped using 1 M sodium carbonate. The plates were analyzed using an ELISA reader at 405 nm.

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.

3.2.16 Measurement of cytokines secretion by HPBLs and in mice sera

Three days after antigen stimulation, human peripheral blood lymphocytes were collected and then centrifuged. The supernatant was transferred to new tubes for the further measurement. The concentrations of cytokines secreted by lymphocytes were determined through the measurement of the cell suspension using ELISA Kits from R&D system (IL-2, IFN-γ, IL-4, IL-6, IL-10, IL-5, and IL-13). Interleukin concentrations in mice serum were determined by ELISA Kits from eBioscience (IFN-γ, IL-2, IL-4, and IL-6), R&D Systems (IL-10, IL-5 and IL-13) and Biolegend (IL-17). The procedure was done according to the instructions provided by the corresponding manufacturer.

3.2.17 Measurement of cytokines secretion of graft tissues

The mice graft tissues were collected at indicated time points. The procedure for preparing tissue homogenates was performed according to the instructions of Invitrogen Company. Briefly, the graft tissues were homogenized in 1.5 mL lysing buffer per gram and then centrifuged at 18000 rpm at 4^{o}C for 15 min. After centrifugation, the supernatant of the lysate was transferred into new tubes. The protein concentration in the lysate was determined by Bicinchoninic Acid Assay (see section 3.2.14). The levels of cytokines in graft tissues were analyzed through the measurement of the lysate with the same amount of proteins (40μg) by ELISA kits from eBioscience (IFN-γ, IL-2, IL-4, and IL-6) and R&D Systems (IL-10, IL-5 and IL-13).

Lysing buffer: 10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100.

3.2.18 Immunohistofluorescence analysis

After tail-skin transplantation, the skin grafts of the recipient mice were collected at indicated time points and placed onto pre-labeled tissue base molds. The entire tissue blocks were covered with optimal cutting temperature compound (Sakura, USA) and then stored at –80°C until ready for sectioning. The desired thickness of frozen sections was 5–7 μm. The tissue sections were pre-warmed for 1 h at room temperature before use. After a washing step with PBS, the tissue sections were continually performed with

immunohistofluorescence staining. Briefly, tissue sections were first incubated with primary antibodies overnight at 4^{o}C. After two washing steps with PBS, the tissue sections were further incubated with FITC-labeled secondary antibodies for 1 h. Following two washing steps, the sections were incubated with Hoechst 33342 (Thermo Fisher Scientific) for nuclear staining. The tissues from the tails of the donor mice served as control.

3.2.19 Statistical analysis

All data were obtained from three or more independent experiments and the values represent the mean ± SD of at least 5 numbers for each group. 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 $p<0.001$.

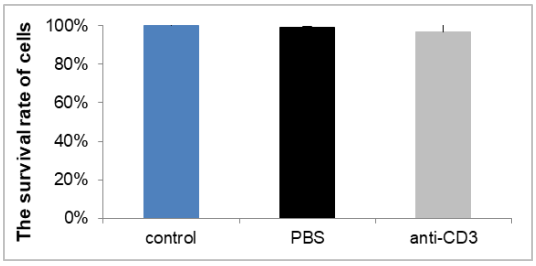
4. Results

4.1 The role of CD26 in T cell proliferation and differentiation

4.1.1 Determination of cell survival rate after stimulation

It was reported that certain immobilized monoclonal antibodies (mAbs) against human CD3 can instead antigens to trigger off the CD3/TCR-dependent pathway and induce T cell activation and proliferation [126]. In order to study the role of CD26 in CD3/TCR-dependent T cell proliferation and differentiation, human peripheral blood lymphocytes (HPBLs) were isolated and stimulated with the immobilized anti-CD3 monoclonal antibody (OKT3, IgG2a) (Thermo Fisher Scientific; USA). Three days after stimulation, the survival rate of HPBLs was tested by flow cytometry after staining with FITC-Annexin V/PI. The survival rate of the cells was more than 95% in both PBS control group and stimulated group (Figure 4.1A). This confirmed the state of the cells and allowed further analysis could be done after stimulation.

A



B

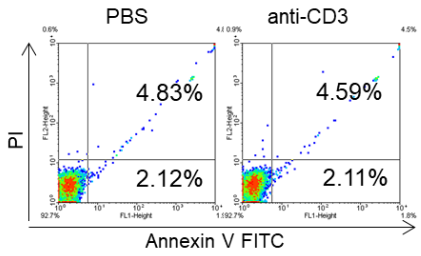


Figure 4.1: Analysis of the survival rate of lymphocytes after stimulation using FITC-Annexin V/PI Assay. The lymphocytes were collected at 72 h after stimulation or PBS treatment. After centrifugation, the supernatant was removed and the lymphocytes at the bottom were stained with FITC-Annexin V/PI-kit according to the instructions of the kit (see section 3.2.4), the unstained cells were used as blanks. (A) The statistical analysis of the cell survival rate. Data are shown as mean value ± SD of five separated experiments. (B) Data shown is a typical representative of five experiments.

4.1.2 Analysis of the lymphocytes activation after stimulation

Three days after immobilized anti-CD3 mAb stimulation, the activation of HPBLs was determined through the measurement of lymphocyte activation markers (CD69, CD25, CD71, and CD26). As shown in Figure 4.2B, the percentage of $CD26^+$ HPBLs was increased by 28% from 33±8% in control group to 61±14% in stimulated group (p<0.001). In comparison to the control group (treated with PBS), the percentages of $CD69^+$ and $CD71^+$ cells in the stimulated group were 6-fold and 5-fold of that in control group (54.29± 20.87% *vs.* 9.07±7.28%, p<0.01; 30.6±14% *vs.* 5.8±2.46%, p<0.05), respectively (Figure 4.2A). The percentage of $CD25^+$ HPBLs was 68% higher in the stimulated group than the value in control group (17.65±6.58% *vs.* 10.49±9.41%). The results indicate that part of the HPBLs were activated after immobilized anti-CD3 mAb stimulation.

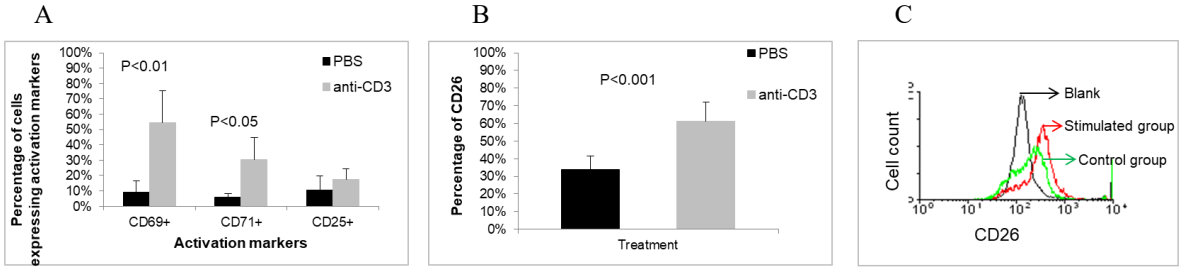


Figure 4.2: Percentage of cells expressing each of the activation markers on surfaces of HPBLs after stimulation. After 72 h of the stimulation, lymphocytes were collected and stained with FITC- or PE-conjugated antibody against human-CD69, CD25, CD71, or CD26, and then measured using flow cytometry. (A) Percentage of $CD69^+$-, $CD71^+$- or $CD25^+$-HPBLs in control group and stimulated group (n>5). (B) Percentage of $CD26^+$ cells in control group and stimulated group (n>5). (C) Histogram of CD26 expression in stimulated group (red line) and control group (PBS treatment) (green line). The cells of PBS group without staining used as blank (black line).

4.1.3 Proliferation of lymphocytes after stimulation

In order to determine the proliferation of lymphocytes after stimulation, the carboxyfluorescein succinimidyl ester (CFSE) assay was used to measure the generations of the lymphocytes three days after stimulation. As shown in Figure 4.3B, at day three after stimulation, the stimulated group (hollow black histogram) had five additional peaks which represent five increased generations. However, the PBS control group (shaded red histogram) had only one original peak indicating that no new generation was produced.

These results indicate that lymphocytes of PBS control group did not proliferate within the three days; however, the lymphocytes proliferated and increased up to five generations after stimulation.

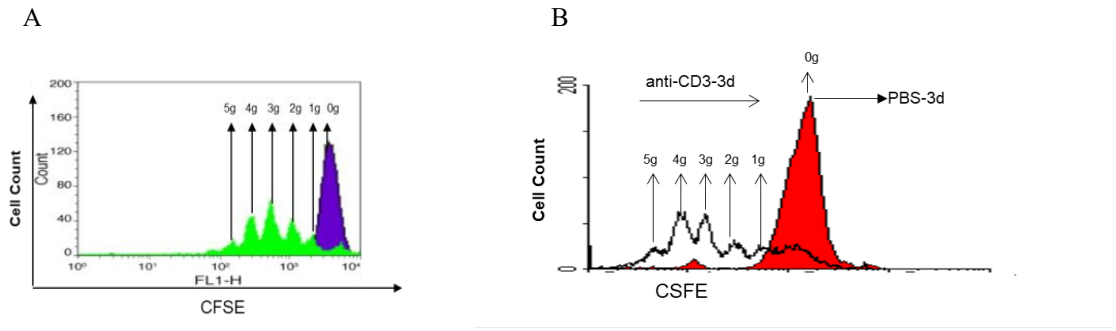


Figure 4.3: Measurement of lymphocyte proliferation using CSFE assay

Lymphocytes were stained with CSFE solution according to manufacturer´s instructions (see section 3.2.5) and then cultured in the plate which was immobilized with anti-CD3 mAb or treated with PBS. Three days after stimulation, lymphocytes were collected and measured by flow cytometry. PBS treated cells were used as controls. (A) The principle example of the assay. The purple peak represents the parent generation (0g), the green peaks represent successive generations (1g is the first generation, 2g is the second generation, 3g is the third generation, 4g is the fourth generation, and 5g is the fifth generation). (B) Histogram of the generations of lymphocytes. The shaded red histogram represents the PBS control group at day 3. The hollow black histogram indicates the generations of the stimulated group after 3 days stimulation.

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

In order to clarify the association of CD26 with lymphocyte differentiation, the percentages of $CD4^+$ T lymphocytes (T helper cells), $CD8^+$ T lymphocytes (T cytotoxic cells) and $CD19^+$ B lymphocytes, as well as the percentage of cells co-expressing each of these subpopulation markers with CD26 after stimulation were investigated. As the results shown in Figure 4.4A, the percentage of $CD4^+$ cells in HPBLs was increased by 12% after stimulation (44.72±8.91% *vs.* 32.57±12.85%), while the percentage of $CD8^+$ or $CD19^+$ cells was not increased significantly after stimulation. These results suggest a strong proliferation of the $CD4^+$ subpopulation after stimulation. Further analysis found that the percentage of cells co-expressing CD4 and CD26 in total HBPLs was 2.2-fold after stimulation of that in the control group (32.38% *vs.* 14.43%). This suggests that in the stimulated group, 73% of the $CD4^+$ cells were co-expressed with CD26 ($CD4^+CD26^+$) (red

line in the left panel of Figure 4.4A), while in the control group, only 25% of the $CD4^+$ cells were co-expressed with CD26 (red line in the left panel of Figure 4.4A). This implies that in $CD4^+$ cells the percentage of cells co-expressing CD4 and CD26 was increased 2-fold after stimulation. As previously known, CD26 is a co-stimulator of T cell activation, the increased T helper cells ($CD4^+$) after stimulation is mostly co-expressed with CD26 observed in the present work indicating that the activation and proliferation of $CD4^+$ cells are related to CD26 expression.

While the percentage of $CD8^+$ cells was only increased from 31.10% to 34.45% after stimulation. Further analysis found that the percentage of $CD8^+CD26^+$ cells in the stimulated group was about 2.1-fold of that in control group (14.28±3.35% *vs.* 6.72±4.21%). This indicates that forty percent of $CD8^+$ cells were co-expressed with CD26 ($CD8^+CD26^+$) in the stimulated group in comparison to 21% in the control group (red line in the middle panel of Figure 4.4A). The increased $CD8^+CD26^+$ cells after stimulation indicate that CD26 is also related to the activation of $CD8^+$ cells. Interestingly, the percentage of total $CD8^+$ cells did not change after stimulation. While cell survival analysis indicated that no dead lymphocytes were observed after stimulation, this may imply that T cytotoxic cells were activated after stimulation but probably did not proliferate or the proliferation rate of $CD8^+$ cells was slower than that of $CD4^+$ cells.

Other results show that the percentage of B cells ($CD19^+$) did not significantly increase after stimulation. The percentage of $CD19^+CD26^+$ cells did not differ between the control group and stimulated group. This suggests that B lymphocytes ($CD19^+$) did not proliferate after stimulation.

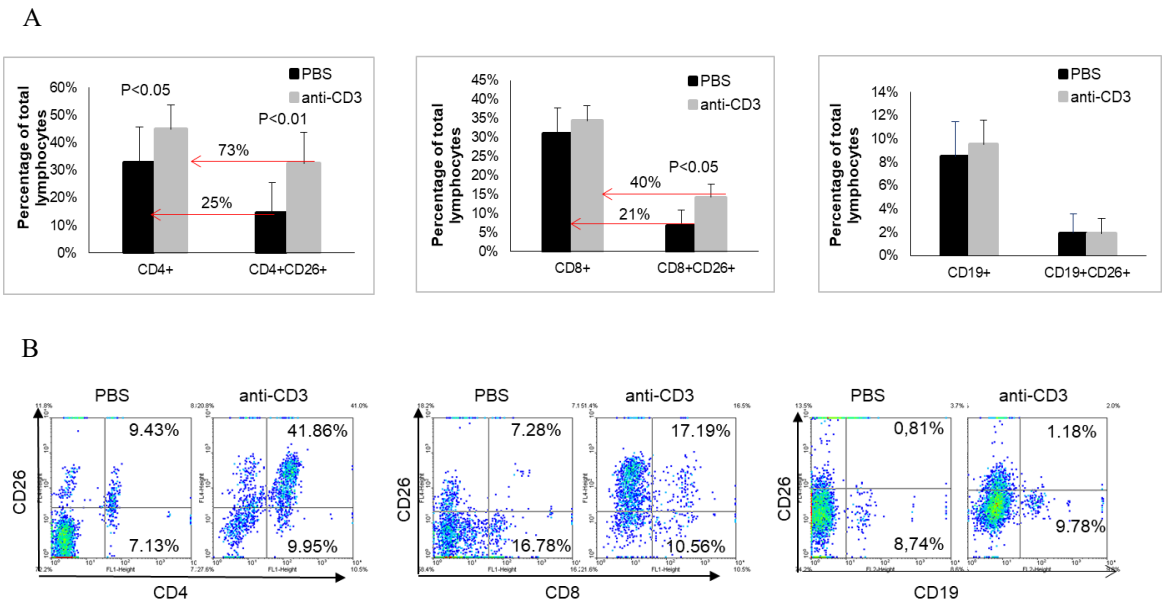


Figure 4.4: Percentages of $CD4^+$, $CD8^+$ and $CD19^+$ cells, and cells co-expressing each of these surface markers with CD26 after stimulation. Data represented mean value ± SD of HPBLs samples from a minimum 5 separated experiments with 5 healthy donor lymphocytes. (A) Percentages of $CD4^+$, $CD4^+CD26^+$, $CD8^+$, $CD8^+CD26^+$, $CD19^+$, and $CD19^+CD26^+$ cells in control group and stimulated group. (B) FACS dot plot of the expression of $CD4^+CD26^+$, $CD8^+CD26^+$ and $CD19^+CD26^+$ cells, respectively.

4.1.5 Secretion of IL-2, IFN-γ, IL-4, IL-6, and IL-13 of HPBLs after stimulation

After stimulation, HPBLs were activated and proliferated significantly. In order to investigate the differentiation of T lymphocytes, the levels of cytokines secreted by the stimulated lymphocytes were analyzed using ELISA kits. As shown in Figure 4.5, on day 3 after stimulation, the levels of cytokines IL-2, IFN-γ, IL-4, IL-6, and IL-13 in the cell culture medium of the stimulated group were all significantly higher in comparison to the control group. The concentration of IL-2 was 25-fold higher in the cell culture medium of the stimulated group than in the control group (2600 pg/mL *vs.* 100 pg/mL). The concentration of IFN-γ was increased to 500 µg/mL in the cell culture medium of the stimulated group in comparison to 100 pg/mL in the control group. In addition, the concentration of IL-6 was 7-fold higher in the stimulated group when compared to the control group (800 pg/mL *vs.* 100 pg/mL). The secretion levels of IL-4 and IL-13 were also increased after stimulation. However, the difference of IL-4 secretion levels in the

stimulated group and control group (42 pg/mL *vs.* 72 pg/mL, $p<0.01$) was not as big as that of IL-13 (80 pg/mL *vs.* 5 pg/mL, $p<0.001$).

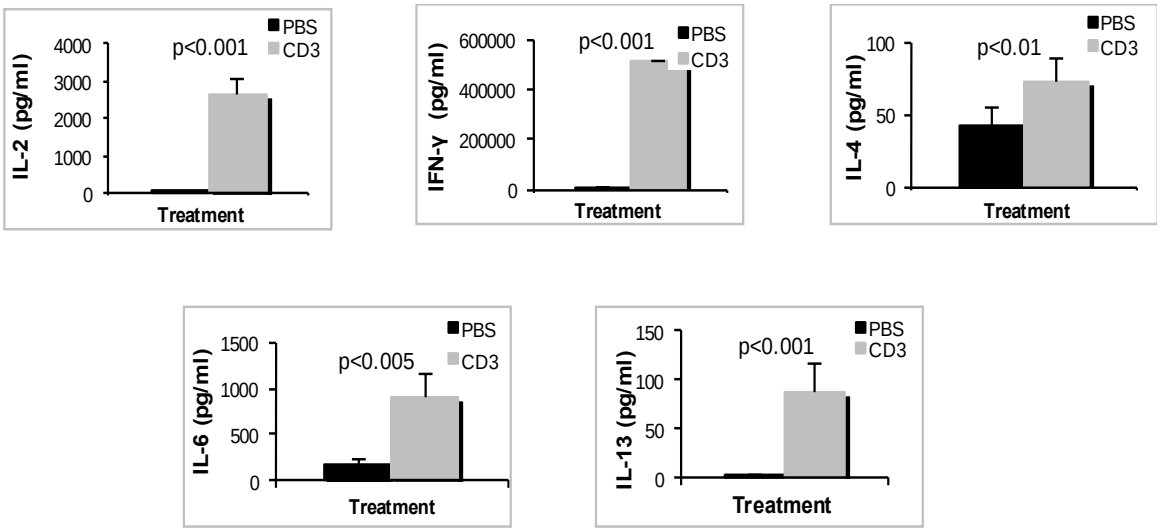


Figure 4.5: The cytokine secretion profiles of HPBLs after stimulation

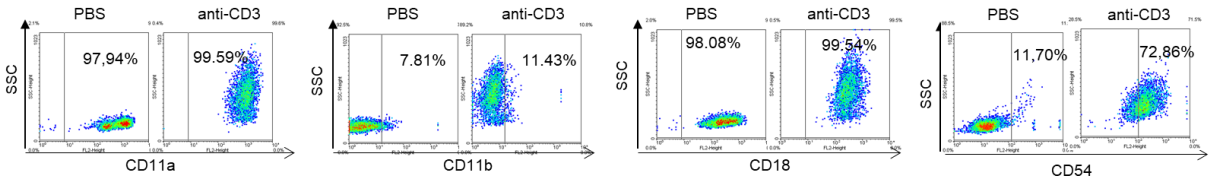
Three days after stimulation, the cell culture suspensions of HPBLs were collected. After centrifugation, the supernatant was transferred into new tubes. Different cytokine levels in the supernatant were measured with ELISA kits. The values represent the mean value ± SD of samples from minimum 7 of healthy donors in each group.

4.1.6 Increased expression of CD11b and CD54 after stimulation

Cell adhesion between T lymphocytes and other cells is an important step in the immune response. The critical adhesion molecules mediating this process include lymphocyte function-associated antigen CD4, CD8, LFA-1, CD11 and the ligand of CD11 (ICAM-1/CD54) (Intercellular Adhesion Molecule 1) [127]. CD11a, a subset of CD11, is the α (alpha) component of various integrins especially ones in which the β (beta) component is CD18, play an important role in the mediation of lymphocytes adhesion. Besides CD11a, CD11b is another subtype of CD11. CD11b was reported to play an important role in the process of lymphocyte adhesion during virus infection. In the present study, the expression levels of adhesion molecules CD11a, CD11b, CD18 and CD54 on the surfaces of HPBLs were analyzed after stimulation. As shown in Figure 4.6B, the percentage of $CD11a^+$ and $CD18^+$ cells between the stimulated group and the control group were comparable. However, the percentage of $CD11b^+$ and $CD54^+$ cells increased after stimulation. The

percentage of $CD11b^+$ cells was 7.71% in control group and it was increased to 9.79% after stimulation, while the percentage of $CD54^+$ cells was 5.7-fold higher in the stimulated group than the control group (84.88% *vs.* 12.61%).

A



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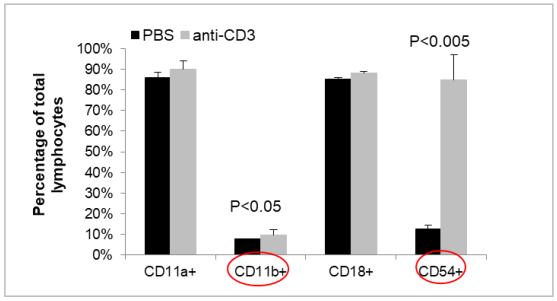

Figure 4.6: Percentage of cells expressing adhesion molecules CD11a, CD11b, CD18, or CD54 on surfaces of HPBLs after stimulation.

(A) Flow cytometry analysis of expression of CD11a, CD11b, CD18 and CD54 molecules on HPBLs after 72 h of stimulation. (B) Diagrammatic representation of the percentage of cells expressing CD11a, CD11b, CD18 or CD54 after stimulation. Data represented mean value ± SD of three independent experiments.

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

To analyze further of the role of CD26 in T cell differentiation after stimulation, $CD26^+$ cells were separated using MACS MicroBeads conjugated with anti-mouse IgG after binding of lymphocytes with anti-human CD26 mAb (350 strain from our lab). After separation, two group cells were obtained: CD26 low-expressing group ($CD26^{low}$) and CD26 high-expressing group ($CD26^{high}$). The percentage of $CD26^+$ cells in the $CD26^{high}$ group was more than 70%, while that in the $CD26^{low}$ group was only 14% (Figure 4.7A). 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 after cell sorting. As shown

in Figure 4.7B, 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 than that in $CD26^{low}$ group (32.24%±5% *vs.* 45.11±9%, $p<0.05$). These results suggest that the activation and proliferation of T helper cells ($CD4^+$) present a positive correlation with CD26 expression. Further analysis showed that the percentage of $CD4^+CD26^+$ cells in the $CD26^{high}$ group was 5-fold higher than that in the $CD26^{low}$ group (44.27±15% *vs.* 7.13±7%, $p<0.01$), further suggesting that the activation and proliferation of $CD4^+$ cells are associated with CD26 expression. The percentage of $CD8^+CD26^+$ cells was about 2.5-fold (12.93±6% *vs.* 3.72±0.9%, $p<0.05$) higher in the $CD26^{high}$ group than the value in the $CD26^{low}$ group (Figure 4.7B). The higher percentage of $CD8^+CD26^+$ cells in the $CD26^{high}$ group may imply that the activation of T cytotoxic cells ($CD8^+$) is also related to CD26 expression. The lower percentage of $CD8^+$ cells in the $CD26^{high}$ group may indicate that the proliferation rate of T cytotoxic cells ($CD8^+$) is lower than that of T helper cells ($CD4^+$) after stimulation.

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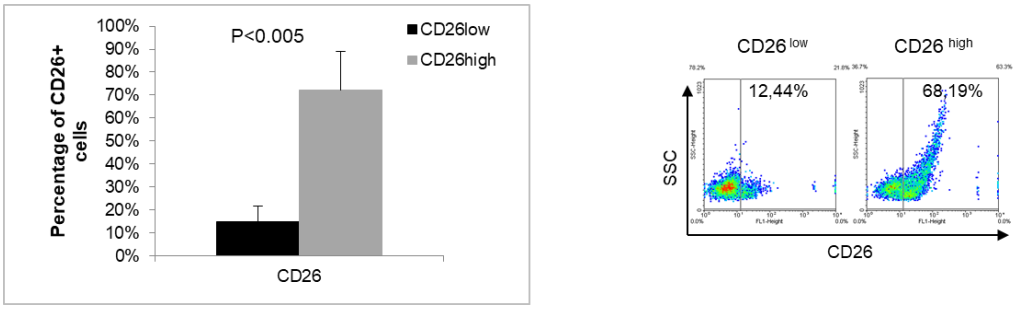

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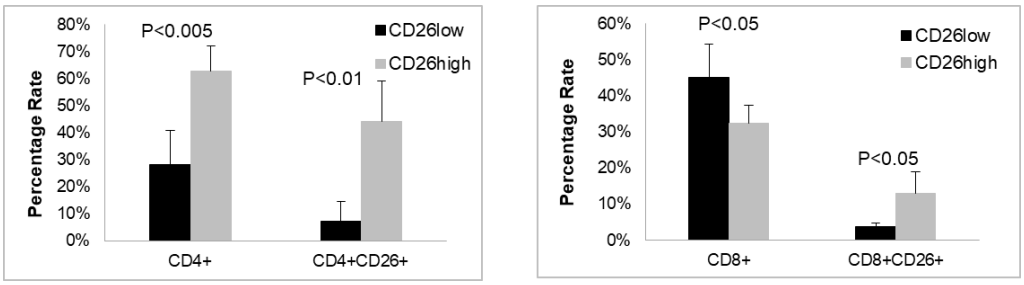

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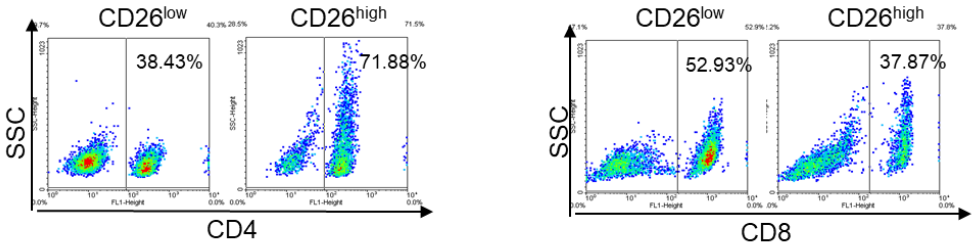

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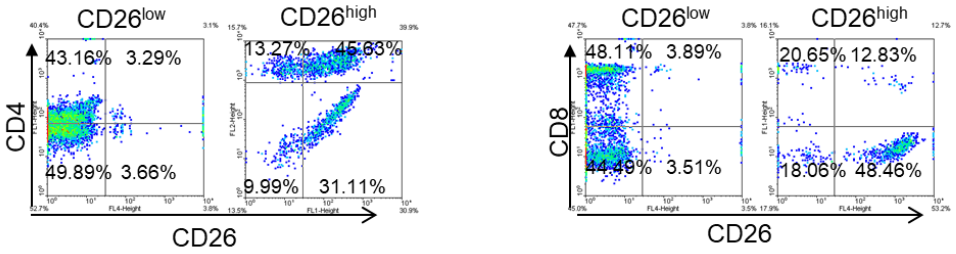

Figure 4.7: Percentages of CD4$^+$, CD8$^+$, CD4$^+$CD26$^+$ and CD8$^+$CD26$^+$ cells in CD26^{low} and CD26^{high} groups. (A) Percentages of CD26$^+$ cells in CD26^{low} and CD26^{high} groups (n>7). (B) Percentages of CD4$^+$ and CD4$^+$CD26$^+$, CD8$^+$ and CD8$^+$CD26$^+$ cells in CD26^{low} and CD26^{high} group (n>7). (C) Dot plots show the percentages of CD4$^+$ and CD8$^+$ cells in CD26^{low} and CD26^{high} group. (D) Dot plots show the percentages of CD4$^+$CD26$^+$ and CD8$^+$ CD26$^+$ cells in CD26^{low} and CD26^{high} group.

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

To further investigate the association of CD26 expression with CD4 cell differentiation, the percentages of T helper subpopulations were determined using fluorescence-conjugated antibodies against corresponding cytokines or cell surface markers and analyzed by flow cytometry. The results showed that the percentages of cells secreting Th1-typical cytokine IL-2 or IFN-γ in the CD26^{high} group were significantly higher than that in the CD26^{low} group (Figure 4.8A). The percentage of cells secreting IL-2 in the CD26^{high} group was about 3-fold of that in 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 7-fold of that in the CD26^{low} group (30.17±11.14% *vs.* 4.45±2.63%). Similarly, the percentages of cells secreting Th17-typical cytokines (IL-6, IL-17, or IL-22) or expressing biomarkers (IL-23R, CD196 or 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 both 6-fold higher in comparison to 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 of that in 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 percentage of cells expressing Th17 surface biomarkers CD196 or CD161 was 1.8-fold or 2-fold higher in the $CD26^{high}$ group than in the $CD26^{low}$ group (34.73% *vs.* 12.35%, 42.52% *vs.* 13.59%, respectively). Histogram analysis showed that the expressing level of cells secreting Th1 or Th17 typical cytokines (IL-2, IFN-γ, IL-6, IL-17, or IL-22) or expressing Th17 typical surface marker (IL-23R) in the $CD26^{high}$ group was much higher than that in the $CD26^{low}$ group (Figure 4.8B). These results suggest that the expression of CD26 is closely related to the differentiation and functions of Th1 and Th17 subpopulations of T lymphocytes.

On the other hand, the percentage of cells secreting Th2-typical cytokines either IL-4 or IL-13 showed no significant differences in the two groups after cell sorting. They were notably very low in the two groups. The percentage of cells secreting IL-14 was 4.78% in the $CD26^{low}$ group and 4.14% in the $CD26^{high}$ group. The percentage of cells secreting IL-13 was 3.67% in the $CD26^{low}$ group and 4.29% in the $CD26^{high}$ group, respectively. Similarly, the histogram analysis showed that no much difference in the expression levels of cells secreting Th2 cytokines (IL-4 and IL-13) in the $CD26^{high}$ group and the $CD26^{low}$ group (Figure 4.8B). These results suggest that CD26 expression is not related to the differentiation of Th2 subpopulation of T lymphocytes after stimulation.

Additional results showed that the percentage of cells expressing molecular markers of regulatory T cells ($CD25^{+}Foxp3^{+}$ or $CD4^{+}Foxp3^{+}$) in the $CD26^{high}$ group did not have significant differences within the $CD26^{low}$ group. This indicated lack of correlation between the differentiation of Tregs and CD26 expression.

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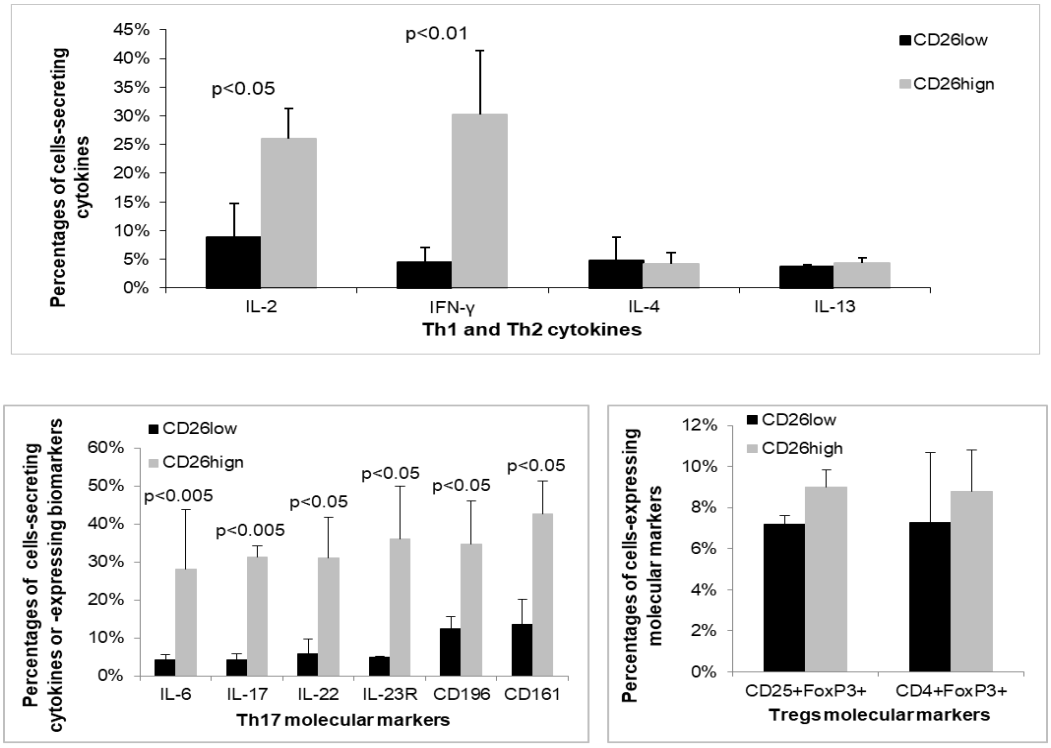

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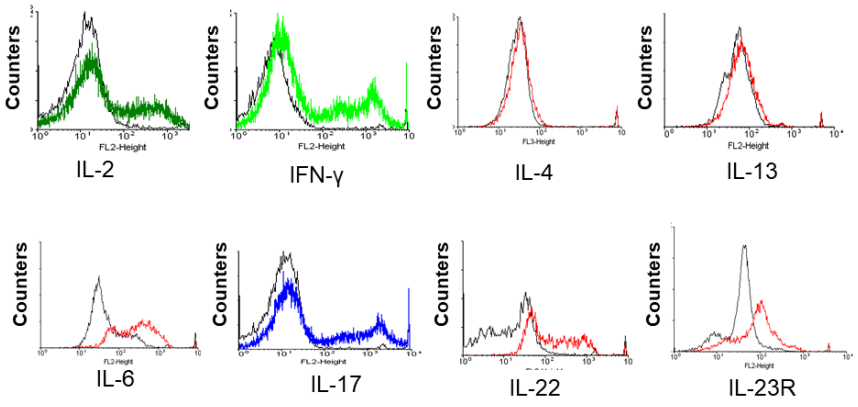

Figure 4.8: Percentage of cells secreting different cytokines in the CD26^{low} and the CD26^{high} groups after separation of CD26-expressing cells. After separation, the cells in the CD26^{low} group and the CD26^{high} group were labeled with different monoclonal antibodies against-cytokines or surface markers at 4^{o}C for 30 min and then measured by flow cytometry. (A) Statistical analysis of the percentages of cells secreting different cytokines in CD26^{low} and CD26^{high} group (n>5). (B) 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.

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

In order to further determine the association of CD26 with Th1 or Th17 subset differentiation, the percentage of cells co-expressing each of Th1 typical cytokines (IL-2 or IFN-γ) or Th17 typical cytokines (IL-6, IL-17, IL-22, or IL-23R) with CD26 in two groups was analyzed. Results show that the percentage of cells co-expressing each of these cytokines with CD26 was obviously higher in the $CD26^{high}$ group (Figure 4.9). The percentage of cells co-expressing CD26 with IL-2 or IFN-γ in the $CD26^{low}$ group was only 28% or 33% of the value in the $CD26^{high}$ group (5.83% *vs.* 20.31% of IL-2, 5.18% *vs*. 15.66% of IFN-γ), respectively. Notably, the percentage of cells co-expressing CD26 with IL-17 ($CD26^{+}IL\text{-}17^{+}$) was significantly higher in the $CD26^{high}$ group (20.14%) in comparison to that in the $CD26^{low}$ group (3.43%). Moreover, the percentage of $CD26^{+}IL\text{-}6^{+}$ cells in $CD26^{high}$ was 5-fold of that in the $CD26^{low}$ group (14.81% *vs.* 3%). The percentage of $CD26^{+}IL\text{-}23R^{+}$ cells in $CD26^{high}$ was 6-fold of that in the $CD26^{low}$ group (23.14% *vs.* 3.7%). In addition, the percentage of cells co-expressing CD26 with IL-22 ($CD26^{+}IL\text{-}22^{+}$) was 40% more in the $CD26^{high}$ group than in the $CD26^{low}$ group (18.64% *vs.* 12.86%).

Fluorescence microscopy detected CD26 protein predominantly located on the cell plasma membrane. While IL-2, IFN-γ, IL-17, or IL-22 was detected in the cytosol, IL-23R was also detected on the cell plasma membrane. After merging the photos, CD26 was found to be co-expressed with IL-2, IFN-γ, IL-17, IL-22, or IL-23R (Figure 4.10). Since IL-2 and IFN-γ are typical Th1 cytokines, the co-expression of Th1-cytokines with CD26 suggests an association 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 an association of CD26 to the differentiation and function of Th17 cells.

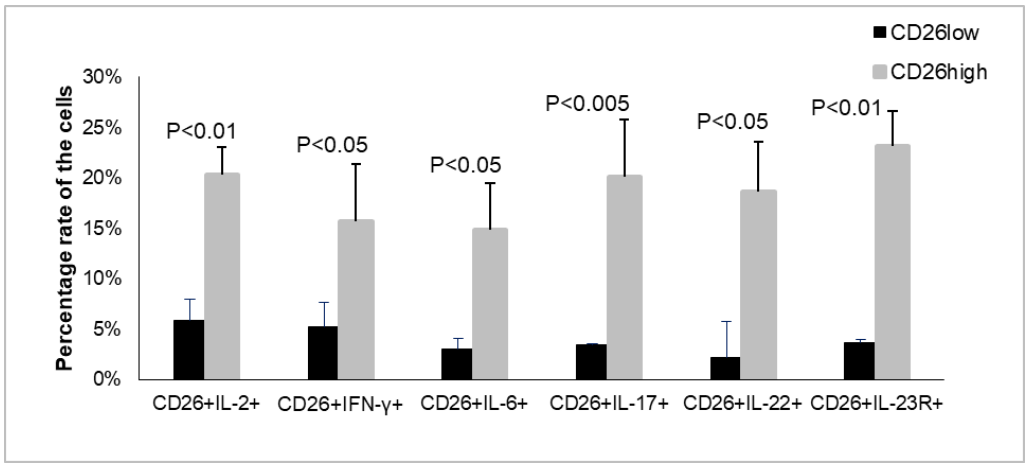

Figure 4.9: Percentages of cells co-expressing CD26 with each of Th1-typical cytokines (IL-2, or IFN-γ) or Th17-typical cytokines (IL-6, IL-17, or IL-22) or surface marker (IL-23R) in the $CD26^{low}$ and $CD26^{high}$ groups.

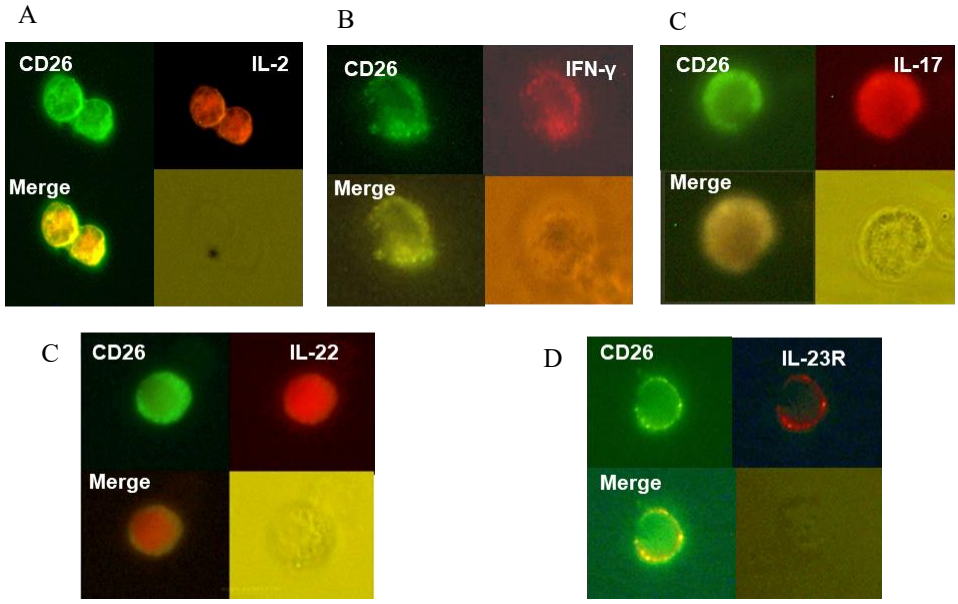

Figure 4.10: Co-expression of CD26 with each of Th1-typical cytokines (IL-2 or IFN-γ), or Th17-typical cytokines (IL-17 or IL-22) or surface marker (IL-23R) in lymphocytes.

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-22, or anti-IL-23R mAb. After a washing step, cells were fixed on culture slides and observed by fluorescence microscopy. Images were made at 600x magnifications. Co-expressing of CD26 with IL-2, IFN-γ, IL-17, IL-22, or IL-23R indicated by the merged images.

4.1.10 Higher percentages of CD11b$^+$ and CD54$^+$ cells in CD26^{high} group

An involvement of CD26 in cell adhesion and migration has been reported through its interaction with extracellular matrix proteins. In this work, it was observed that the percentage of CD11b$^+$ or CD54$^+$ lymphocytes was up-regulated after stimulation (Figure 4.6B). In order to investigate whether the expression of adhesion molecule CD11b or CD54 was associated with CD26 expression, the percentage of CD11b$^+$ cells or CD54$^+$ cells was analyzed in CD26^{high}- and CD26^{low}-group by flow cytometry after cell separation. As shown in Figure 4.11B, the percentage of CD11b$^+$ cells in the CD26^{high} group was 80% higher as compared to the value in the CD26^{low} group (27.56±9.78% *vs.* 15.23±4.6%, $p<0.01$). Similarly, the percentage of CD54$^+$ cells in the CD26^{high} group was 40% higher in comparison to that in the CD26^{low} group (96.30±10.11% *vs.* 69.10±10.24%, $p<0.005$). The data suggest an association of CD26 expression with the expression of adhesion molecules CD11b and CD54.

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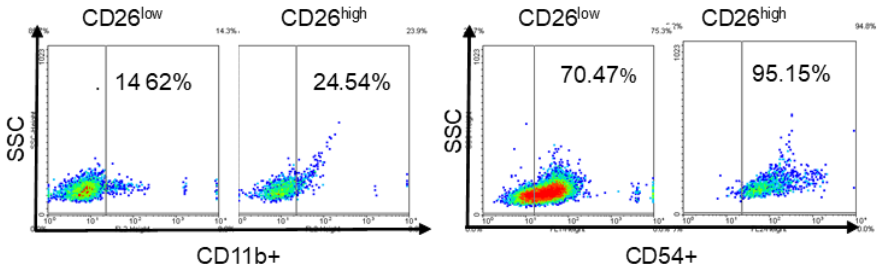

B

	CD26^{low}	CD26^{high}
CD11b+ ($p<0.01$)	15.23±4.6%	27.56±9.78%
CD54+ ($p<0.005$)	69.10±10.24%	96.30±10.11%

Figure 4.11: Percentage of cells expressing adhesion molecules CD11b or CD54 in CD26^{low} and CD26^{high} group.

(A) FACS dot plot of the percentages of CD11b$^+$ cells and CD54$^+$ cells in CD26^{low} and CD26^{high} groups by flow cytometry with one representative experiment. (B) Statistical analysis of the percentages of CD11b$^+$ and CD54$^+$ cells in CD26^{low} and CD26^{high} groups. Data represented as mean value ± SD of a minimum of 4 independent experiments with 4 different healthy donors.

4.2 The involvement of CD26 in allogeneic skin graft rejection

4.2.1 Identification of homozygous of CD26 knockout mice

According to Marguet *et al.*, the CD26 knockout mice were generated by replacing a 2.3-kb *Pst*I-*Sph*I fragment in the 13.5-kb *Sal*I-*Eco*RI CD26 genomic fragment derived from the B10.A-derived l clone 10 with a neo gene (Figure 4.12A) [69]. For genotyping of the mice, different primers were designed for polymerase chain reaction (PCR) amplification. Primers (F1 and R1), designed for genotyping of wildtype mice ($CD26^{+/+}$), were used to amplify 749 bp DNA fragments across *Pst*I restriction site and Exon 1 of CD26 gene. Primers (F2 and R2), designed for genotyping of CD26 knockout mice ($CD26^{-/-}$), were used to amplify 1325 bp DNA fragments across *Pst*I restriction site and *neo* gene (Figure 4.12A). The DNA sample of each mouse was used for PCR with primers of $CD26^{-/-}$ mice or $CD26^{+/+}$ mice. After PCR and electrophoresis, the resulting DNA fragment with only 759 bp is from homozygous of $CD26^{+/+}$ mouse, with only 1325 bp is from homozygous of $CD26^{-/-}$ mouse, with both fragments of 759 bp and 1325 bp is from the heterozygous mouse. As shown in Figure 4.12B, the number 2, 3, 4, 5, 7 were homozygous $CD26^{-/-}$ mice, the number 6 was $CD26^{+/+}$ mouse, number 1 and 8 were heterozygous mice. After genotyping, the homozygous $CD26^{-/-}$ mice were used for the further experiment. The data is one representative result. All homozygous $CD26^{-/-}$ mice used in our experiments were identified by genotyping.

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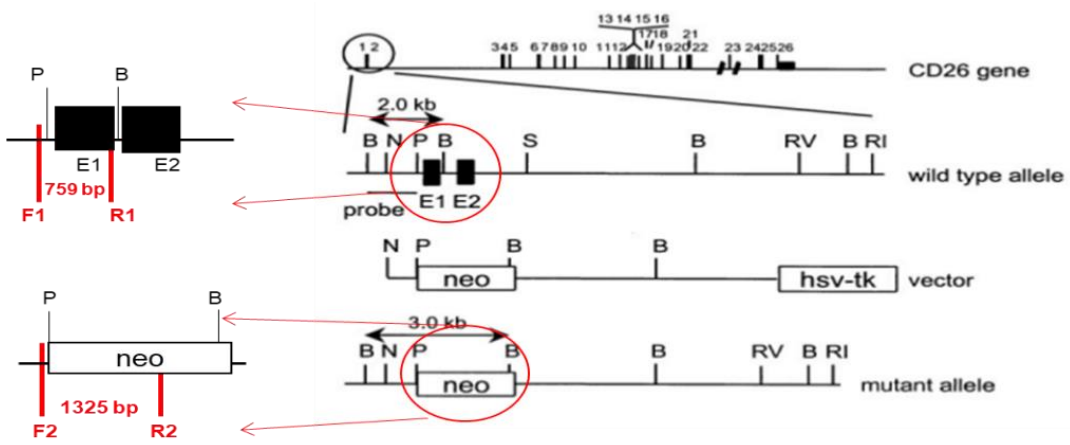

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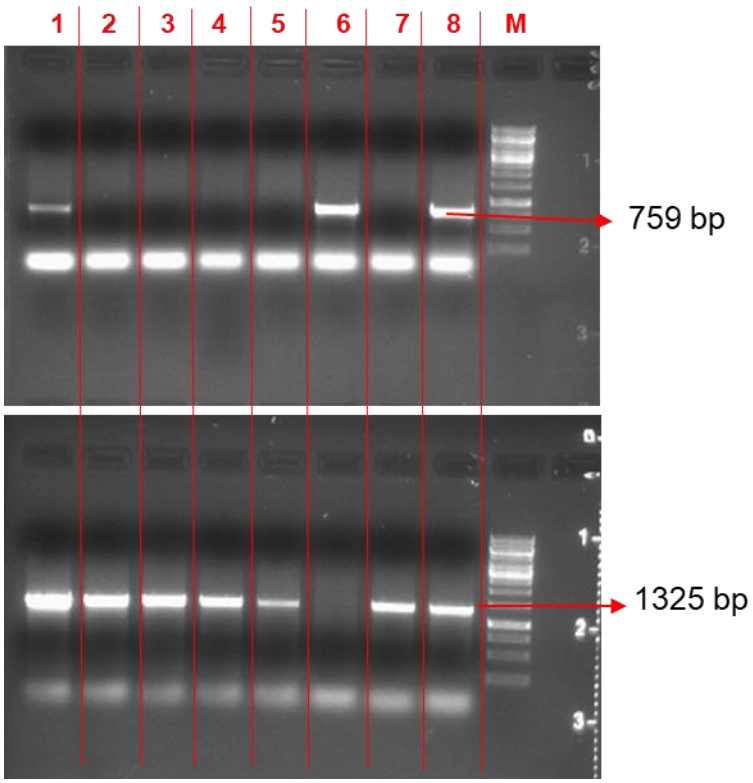

Figure 4.12: Identification of the homozygous of CD26 knockout mice after genotyping with PCR by electrophoresis. (A) Targeting strategy. E1: exon 1; E2: exon 2; neo: neo gene. Restriction sites: B, *Bgl*II; N, *Nco*I; P, *Pst*I; S, *Sph*I; RI, *Eco*RI; RV, *Eco*RV [69]. F1: Forward primer for wildtype mice ($CD26^{+/+}$), R1: Reverse primer for $CD26^{+/+}$ mice, F2: Forward primer for knockout mice ($CD26^{-/-}$), R2: Reverse primer for $CD26^{-/-}$ mice. The DNA fragment amplified with primers F1 and R1 was 759 bp and DNA fragment amplified with primers F2 and R2 was 1325 bp. (B) One representative result of the genotyping after PCR and electrophoresis.

4.2.2 Murine tail-skin transplantation

The procedure of the mice tail-skin transplantation was performed according to the introduction from https://www.jove.com/video/634/murine-skin-transplantation. As shown in Figure 4.13, the donor mice were 8-weeks male BALB/c. The recipient mice were 8–12 weeks homozygous $CD26^{-/-}$ mice on the C57BL/6N genetic background and wild-type C57BL/6N mice. The grafts were prepared from the tail skins of BALB/c donor mice with the same size and incised to the graft beds of the recipient mice. The graft beds were

prepared on the back of the recipient mice. Seven days after transplantation, the bandages were removed and the rejection of grafts was observed.

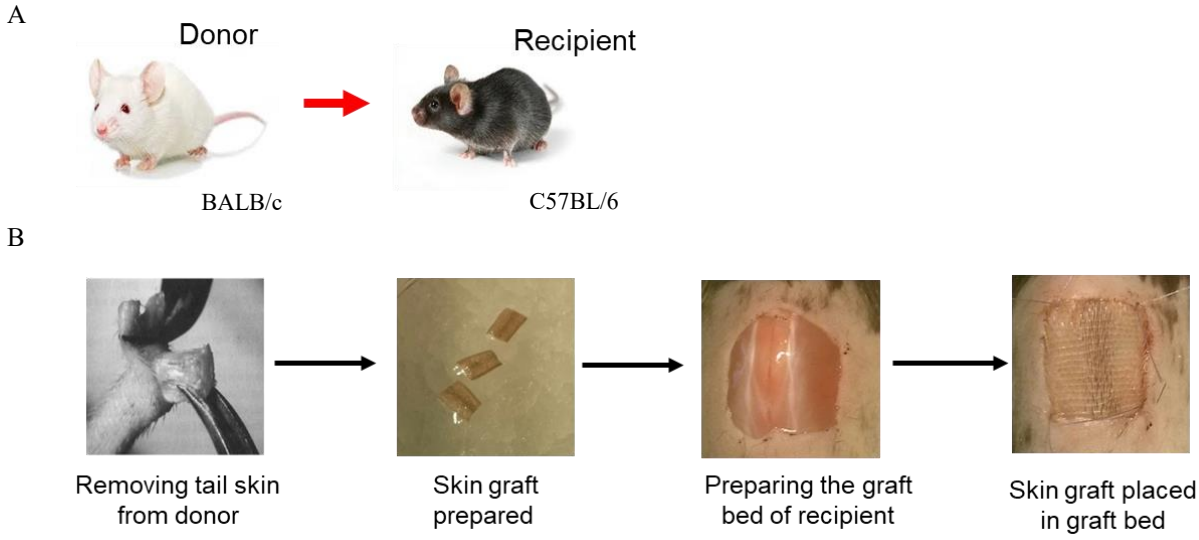

Figure 4.13: Schematic diagram of mouse tail-skin transplantation [128].

(A) Donor mice and recipient mice. (B) A brief illustration of the process of the mice tail-skin transplantation.

4.2.3 Lower necrotic level of allogeneic skin graft in CD26$^{-/-}$ mice

CD26$^{+/+}$ and CD26$^{-/-}$ mice with C57BL/6N background were transplanted with allogeneic grafts from tail-skin of BALB/c (n≥10 per group). Seven days after transplantation, bandages were removed; the necrotic areas of skin grafts (wrinkled skin) were monitored and recorded by photographs daily, up to 15 days post-transplantation (Figure 4.14A). Six different score levels (0–5) were defined according to the necrotic area of the mouse skin grafts after transplantation (Figure 4.14B). Statistical analysis indicated that the necrotic levels of the skin grafts were lower in CD26$^{-/-}$ mice than in CD26$^{+/+}$ mice from day 7 to day 15 (Figure 4.14C). Within 15 days after transplantation, the transplant rejection was delayed in CD26$^{-/-}$ mice in comparison to CD26$^{+/+}$ mice (p<0.001) (Figure 4.14D), indicating that CD26-deficiency may be an important factor for graft survival. The graft rejection of CD26$^{-/-}$ mice is significantly slower and necrotic score levels were markedly lower in comparison to that of CD26$^{+/+}$ mice, suggesting an involvement of CD26 in graft rejection.

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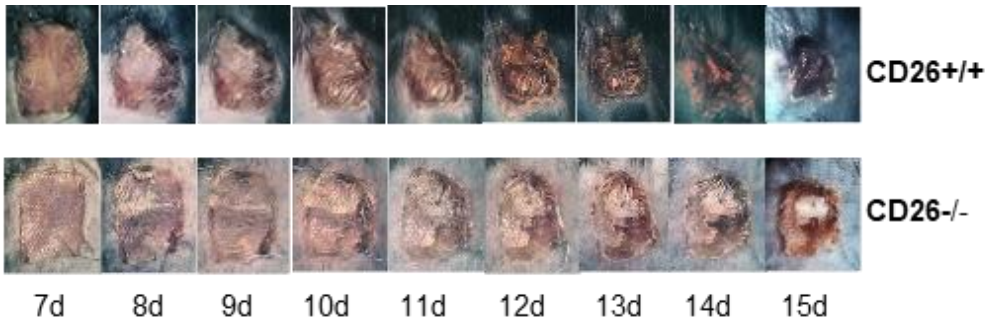
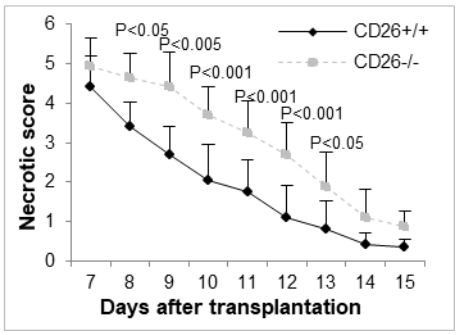


B

Score	Necrotic area
5	0-20%
4	20%-40%
3	40%-60%
2	60%-80%
1	80%-100%
0	Graft Removed

C



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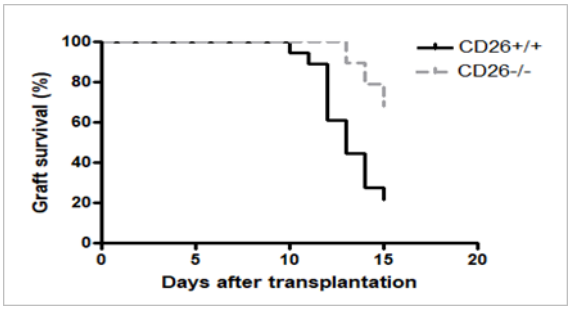


Figure 4.14: Allograft rejection of CD26$^{+/+}$ and CD26$^{-/-}$ mice.

(A) Skin allograft appearance of CD26$^{+/+}$ mice (upper) and CD26$^{-/-}$ mice (lower panel) from day 7 to day 15 post-transplantation. (B) Different score levels indicate different necrotic areas of the mice skin allografts. (C) Statistical analysis of the necrotic levels of grafts from day 7 to day 15 post-transplantation. (D) Graft survival in CD26$^{+/+}$ mice and CD26$^{-/-}$ mice within 15 days post-transplantation. The values of (C) and (D) represent the mean ± SD of 10 mice at each time point. Results shown are representative of three independent experiments. The data of graft survival (D) was analyzed with GraphPad 6; the *p*-value was calculated with Chi-square test.

4.2.4 Less IgG, IgG1 and IgG2a in serum of CD26$^{-/-}$ mice after allogeneic skin transplantation

In order to understand the underlying molecular mechanisms of CD26 in allogeneic graft rejection, the productions of IgG as well as IgG1 and IgG2a in mice serum at different time points were measured by ELISA after skin transplantation. The productions of these antibodies in mice serum were elevated rapidly until day 15 post-transplantation (Figure 4.15). The production levels of IgG, IgG1 and IgG2a reached a maximum level on day 11 or day 15 post-transplantation. However, the concentration levels of serum IgG, IgG1 and IgG2a were significantly lower in CD26$^{-/-}$ mice than in CD26$^{+/+}$ mice until 15 days post-transplantation. On day 7 and 11 post-transplantation, the concentrations of IgG were 1.7-fold and 2-fold higher in CD26$^{+/+}$ mice than in CD26$^{-/-}$ mice (6640 *vs.* 3868 μg/mL, $p<0.05$ and 12027 *vs.* 6912 μg/mL, $p<0.05$), respectively (Figure 4.15A). Further analysis demonstrated that the concentrations of both IgG isotypes IgG1 and IgG2a were also remarkably lower in CD26$^{-/-}$ mice, especially the levels of IgG1 on day 7 and day 11 in serum of CD26$^{-/-}$ mice were about 44% of that of CD26$^{+/+}$ mice (1054 *vs.* 2355 μg/mL, 1247 *vs.* 2868 μg/mL, respectively) (Figure 4.15B and C). These results suggest a delayed and insufficient immune response of CD26-deficient mice to the allogeneic transplantation.

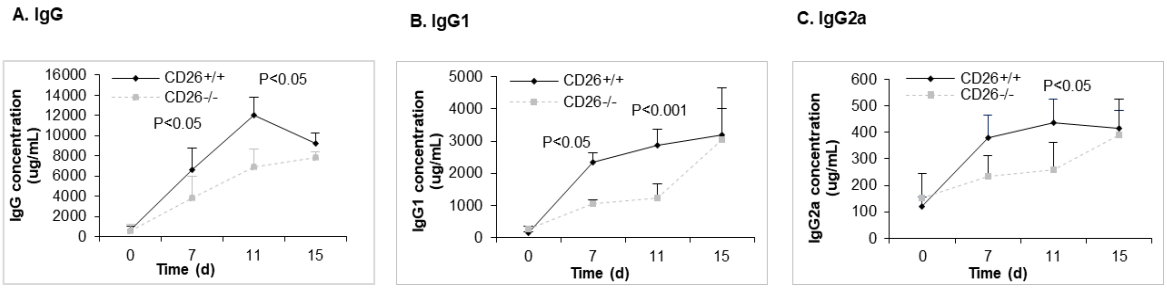


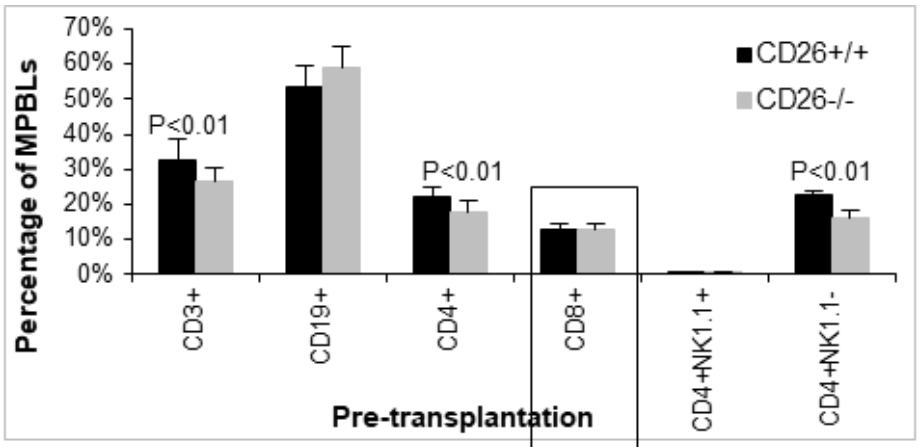
Figure 4.15: Levels of IgG, IgG1 and IgG2a in the serum of mice after skin transplantation

Blood was taken at indicated time points after skin transplantation (day 0 represents the day before transplantation). Serum concentrations of IgG, IgG1 and IgG2a were measured by ELISA. Values represented the mean ± SD of at least 8 mice at each time point. Results shown are representative of three independent experiments.

4.2.5 Reduced percentage of $CD8^+$ cells in the MPBLs of $CD26^{-/-}$ mice after allogeneic skin transplantation

In order to clarify why $CD26^{-/-}$ mice produced lower IgGs after skin transplantation, the proliferation and differentiation of lymphocytes in both kinds of mice were investigated. The percentages of different lymphocyte subpopulations in MPBLs and MSLs of $CD26^{+/+}$ mice and $CD26^{-/-}$ mice on days 0 (before transplantation) and 7 after skin transplantation were measured. As shown in Figure 4.16A, before transplantation, the percentages of $CD3^+$ and $CD4^+$ cells in $CD26^{-/-}$ MPBLs were both lower than that in $CD26^{+/+}$ mice, while the percentages of $CD8^+$ and $CD19^+$ cells did not differ significantly in MPBLs between two mice types. However, in comparison to pre-transplantation, the percentage of $CD3^+$ cells of MPBLs was increased, while the percentage of $CD19^+$ cells was decreased in both mice types on day 7 after transplantation (Figure 4.16B), indicating a proliferation of CD3 T lymphocytes after skin transplantation. Nevertheless, no significant changes were detected in the percentages of $CD4^+$ and NKT ($CD4^+NK1.1^+$) cells between pre- and post-transplantation. Interestingly, the percentage of $CD8^+$ cells in MPBLs showed no significant difference between $CD26^{+/+}$ and $CD26^{-/-}$ mice before skin transplantation, however, at day 7 post-transplantation, the percentages of $CD8^+$ cells in MPBLs of both kinds of mice were increased. In addition, $CD8^+$ cells in $CD26^{-/-}$ MPBLs was 11% less than that in $CD26^{+/+}$ MPBLs (15.27% *vs.* 17.10%, $p<0.05$), indicating a reduced activation and proliferation of $CD8^+$ cells in $CD26^{-/-}$ mice in response to allogeneic transplantation.

A



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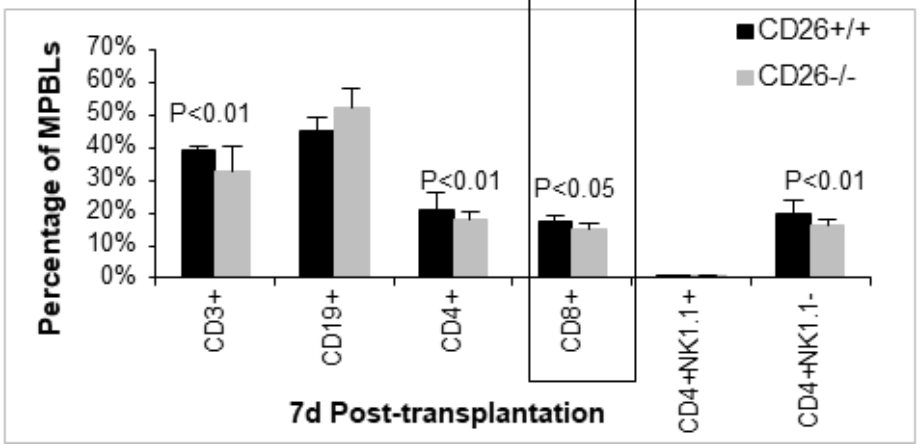

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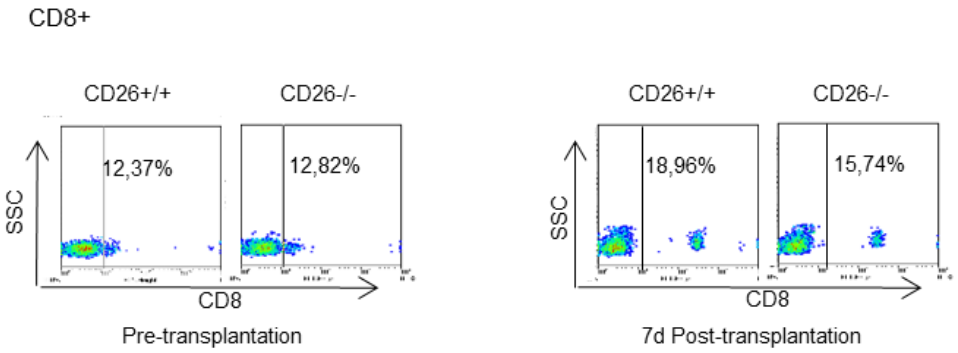


Figure 4.16: Percentages of MPBL subpopulations in CD26$^{+/+}$ and CD26$^{-/-}$ mice. Lymphocytes were prepared and analyzed by flow cytometry.

(A) Percentages of MPBL subpopulations before transplantation. (B) Percentages of MPBL subpopulations on day 7 post-transplantation. (C) FACS dot plot of CD8^{+} cells in MBPLs of CD26$^{+/+}$ and CD26$^{-/-}$ mice pre- and on day 7 post-transplantation. The values of A and B were represented as the mean ± SD of 7 mice. Results shown are representative of three independent experiments.

4.2.6 Lower percentage of $CD4^+$ cells in MSLs of $CD26^{-/-}$ mice

According to the data shown in Figure 4.17, the percentage of $CD3^+$ cells in MSLs was 17% lower in MSLs of $CD26^{-/-}$ mice than that of $CD26^{+/+}$ mice before transplantation (30.12% *vs.* 35.44%). Further analysis indicated that the diminishing $CD3^+$ cells in MSLs of $CD26^{-/-}$ mice were mainly $CD4^+$ cells. The percentage of $CD4^+$ cells of MSLs was 14% lower in $CD26^{-/-}$ mice than that in $CD26^{+/+}$ mice (22.47% *vs.* 25.7%, $p<0.005$) before transplantation. The percentage of $CD4^+NK1.1^-$ of MSLs was 20% lower in $CD26^{-/-}$ mice than that in $CD26^{+/+}$ mice before transplantation (15.49% *vs.* 18.64%). However, no obvious difference of percentages of $CD3^+$ and $CD4^+NK1.1^-$ cells between $CD26^{-/-}$ and $CD26^{+/+}$ mice was observed on day 7 post-transplantation. While the percentage of $CD4^+$ was still lower in $CD26^{-/-}$ mice in comparison to that in $CD26^{+/+}$ mice post-transplantation.

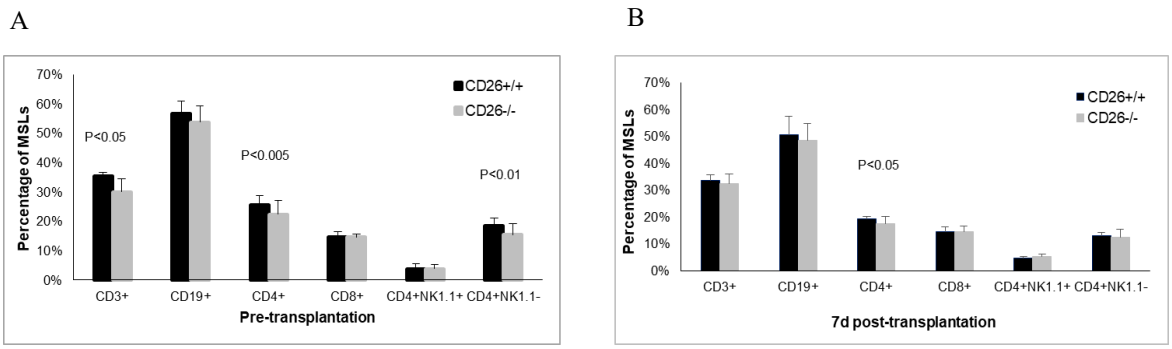


Figure 4.17: Percentages of MSL subpopulations in $CD26^{+/+}$ mice and $CD26^{-/-}$ mice on day 0 and day 7 post-transplantation.

MSLs were separated and analyzed by flow cytometry. (A) Percentages of MSL subpopulations before transplantation. (B) Percentages of MSL subpopulations on day 7 post-transplantation. The values represented the mean ± SD of a minimum 7 of mice at each time point.

4.2.7 Lower levels of IL-2, IFN-γ, IL-6, IL-4, and IL-13, while higher level of IL-10 in serum of $CD26^{-/-}$ mice after skin transplantation

As shown, $CD26^{-/-}$ mice presented a lower percentage of $CD4^+$ cells in both MPBLs and MSLs before and after skin transplantation, and a lower percentage of $CD8^+$ cells in MPBLs after skin transplantation. Further investigation was necessary to clarify the differentiation and effects of lymphocyte subpopulations of $CD26^{-/-}$ mice after skin allogeneic transplantation. For this purpose, the cytokine secretions, which serve as

specific signals for lymphocyte differentiation and effects, were determined after allogeneic skin transplantation.

The cytokine levels in serum at different time points were analyzed by ELISA. Figure 4.18 shows that all of the measured cytokines were secreted after skin transplantation. The concentrations of these cytokines peaked on day 7 post-transplantation, except IL-13 which peaked on day 4 post-transplantation. It is notable that the levels of Th1 cytokines IFN-γ and IL-2 (Figure 4.18A and B) in the serum of $CD26^{-/-}$ mice were much lower than that in the serum of $CD26^{+/+}$ mice. On day 7 post-transplantation, the level of IFN-γ in serum of $CD26^{-/-}$ mice was only one third of that in the serum of $CD26^{+/+}$ mice (3.54 pg/mL *vs.* 10.45 pg/mL) and the level of IL-2 in serum of $CD26^{-/-}$ mice was about 60% of that in serum of $CD26^{+/+}$ mice (11.88 pg/mL *vs.* 19.85 pg/mL). These suggest a reduced differentiation and functions of Th1 cells in $CD26^{-/-}$ mice after skin allogeneic transplantation.

Interestingly, levels of different Th2 cytokines in serum of $CD26^{-/-}$ mice were different in comparison to $CD26^{+/+}$ mice. The levels of IL-4 (Figure 4.18C) and IL-13 (Figure 4.18E) in the serum of $CD26^{-/-}$ mice were both significantly lower than that in the serum of $CD26^{+/+}$ mice post-transplantation. The level of IL-4 in the serum of $CD26^{-/-}$ mice was only 57% of that in the serum of $CD26^{+/+}$ mice on day 7 post-transplantation (15.26 pg/mL *vs.* 26.01 pg/mL). The level of IL-13 in the serum of $CD26^{-/-}$ mice was 70% of that of $CD26^{+/+}$ mice on day 4 post-transplantation (119.24 pg/mL *vs.* 167.38 pg/mL). In contrast, the concentration of IL-10 in the serum of $CD26^{-/-}$ mice at day 7 post-transplantation was 38% (268 ± 58 pg/mL) more than that in the serum of $CD26^{+/+}$ mice (195 ± 34 pg/mL) (Figure 4.18G). It is notable that IL-6 levels in the serum of $CD26^{-/-}$ mice from day 1 after skin transplantation was significantly lower than that in the serum of $CD26^{+/+}$ mice. On day 7 post-transplantation, the concentration of IL-6 in the serum of $CD26^{-/-}$ mice showed only one-third of that in $CD26^{+/+}$ mice (42.74 pg/mL *vs.* 122.32 pg/mL) (Figure 4.18D). These data suggest a difference of cytokine levels and immune response of Th2 cells in $CD26^{-/-}$ mice to allogeneic skin transplantation.

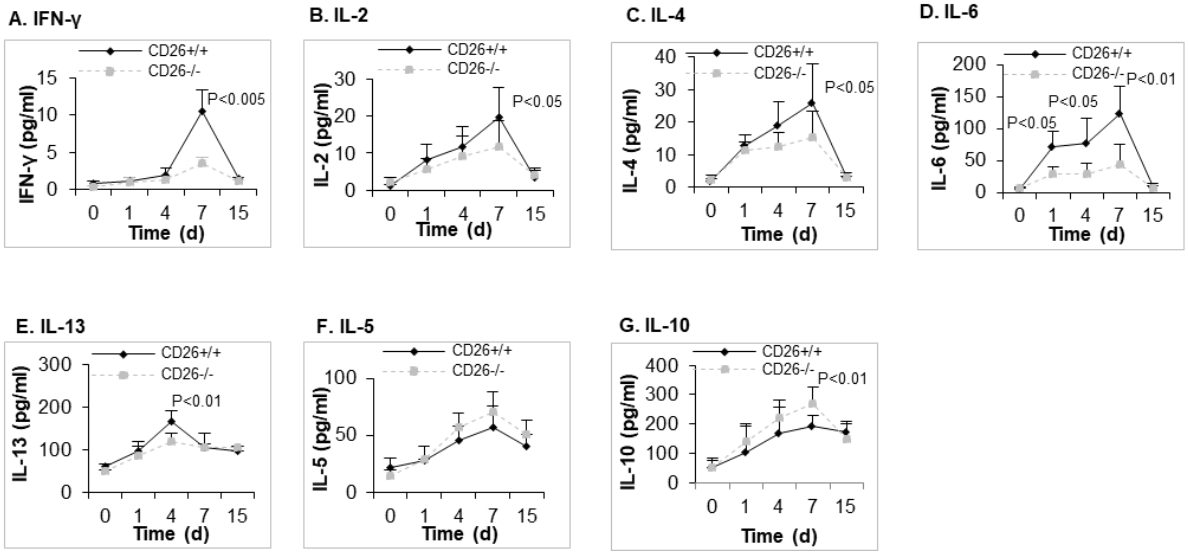


Figure 4.18: Determination of cytokine secretions in the serum of CD26$^{+/+}$ and CD26$^{-/-}$ mice by ELISA
Blood was taken at indicated time points before and after allogeneic skin transplantation (0 days represented the day before transplantation). The values represented the mean ± SD of a minimum 7 of mice at each time point.

4.2.8 Higher level of IL-4 in the graft tissues of CD26$^{-/-}$ mice after skin transplantation

The mice graft tissues were collected at indicated time points. The graft tissues were homogenized in lysing buffer and then centrifuged. After centrifugation, the supernatant of the lysate was transferred into new tubes. The protein concentration in the lysate was determined by Bicinchoninic Acid Assay (see section 3.2.14). The levels of cytokines in graft tissues were analyzed through the measurement of the lysate with the same amount of proteins by ELISA. As shown in Figure 4.19, the concentrations of IL-2, IFN-γ, IL-6, IL-4, IL-13, IL-10, and IL-5 were all elevated after transplantation and peaked on day 7 after skin transplantation. The secretion level of IL-4 was 27% higher in CD26$^{-/-}$ mice than that in CD26$^{+/+}$ mice on day 7 post-transplantation (73.72% *vs.* 46.89%). However, the secretion levels of other cytokines had no significant differences in skin grafts between CD26$^{-/-}$ mice and CD26$^{+/+}$ mice after skin transplantation.

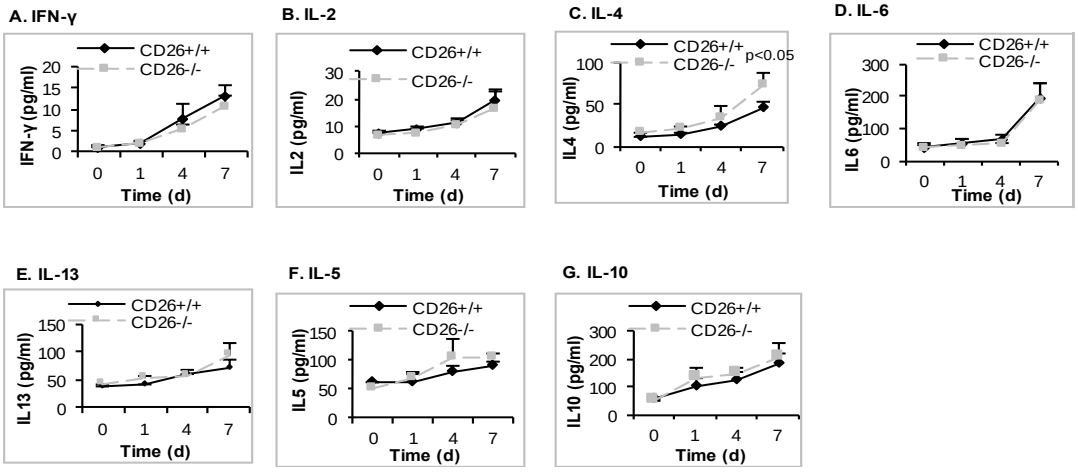


Figure 4.19: Determination of cytokine secretions in graft tissues of CD26$^{+/+}$ and CD26$^{-/-}$ mice by ELISA. Graft tissues were taken at indicated time points before and after allogeneic skin transplantation (0 days represented the day before transplantation). The lysate with the 40 μg proteins was analyzed by ELISA to determine the secretion levels of different cytokines in graft tissues. The values represented the mean ± SD of a minimum 6 of mice at each time point.

4.2.9 Decreased Th17 lymphocytes and increased Tregs in CD26$^{-/-}$ mice after skin transplantation

In view that IL-6 is essential for the differentiation of CD4^{+} cells to Th17 subpopulation and Th17 subpopulation plays an important role during inflammation, the amount of Th17 cells in peripheral blood was determined by measurement of Th17 typical cytokine IL-17 and the percentage of cells producing IL-17. It was observed that the release of IL-17 in the serum of both two mice types was increased after skin transplantation and peaked on day 7 after transplantation (Figure 4.20A). The secretion level of IL-17 in serum was significantly lower in CD26$^{-/-}$ mice than that in CD26$^{+/+}$ mice. On day 4 post-transplantation the concentration of IL-17 in the serum of CD26$^{-/-}$ mice was only 80% of that of CD26$^{+/+}$ mice (385 pg/mL *vs.* 477 pg/mL). On day 7 post-transplantation, the concentration of IL-17 in the serum of CD26$^{-/-}$ mice was reduced to 76% of that in CD26$^{+/+}$ mice (455 pg/mL *vs.* 597 pg/mL). The percentage of cells producing IL-17 (Th17 cells) in CD4^{+} cells of MPBLs was no difference between two mice types before transplantation, while the percentage of Th17 cells in CD4^{+} cells of CD26$^{-/-}$ mice was only 47% of that in CD26$^{+/+}$ mice (22.77% *vs.* 48.88%, $p<0.01$) on day 7 after transplantation (Figure 4.20B and Figure 4.20C).

Given that the balance between Th17 cells and Tregs is critical for allograft rejection and immunological tolerance, the percentages of biomarkers of Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$) were analyzed by flow cytometry. The percentage of $CD4^+CD25^+Foxp3^+$ in spleen and peripheral blood were found not to exhibit any difference between $CD26^{+/+}$ mice and $CD26^{-/-}$ mice before transplantation (pre-transplantation) (Left panel of Figure 4.21A and C). However, on day 15 post-transplantation, the percentage of $CD25^+$ cells in $CD4^+$ cells of $CD26^{-/-}$ MSLs was almost 2-fold of that of $CD26^{+/+}$ mice (10.62% versus 5.42%); and the percentage of $CD25^+$ in $CD4^+$ cells in MPBLs of $CD26^{-/-}$ was 48% higher than that of $CD26^{+/+}$ mice (10.39% versus 7.00%) (Right panel of Figure 4.21A and C). Consistent with this data, the percentage of $CD25^+Foxp3^+$ cells in $CD4^+$ cells of $CD26^{-/-}$ MSLs was 30% higher than that of $CD26^{+/+}$ MSLs (4.62% *vs.* 3.64%), while the percentage of $CD25^+Foxp3^+$ cells in $CD4^+$ subpopulation of $CD26^{-/-}$ MPBLs was 50% higher than that of $CD26^{+/+}$ MPBLs (3.18% *vs.* 2.02%). These data suggest that the deficiency of CD26 resulted in the reduced differentiation of $CD4^+$ cells to Th17 cells while increasing the differentiation of $CD4^+$ cells to Tregs, which could contribute to the immune tolerance and retarded graft rejection in $CD26^{-/-}$ mice after allogeneic skin transplantation.

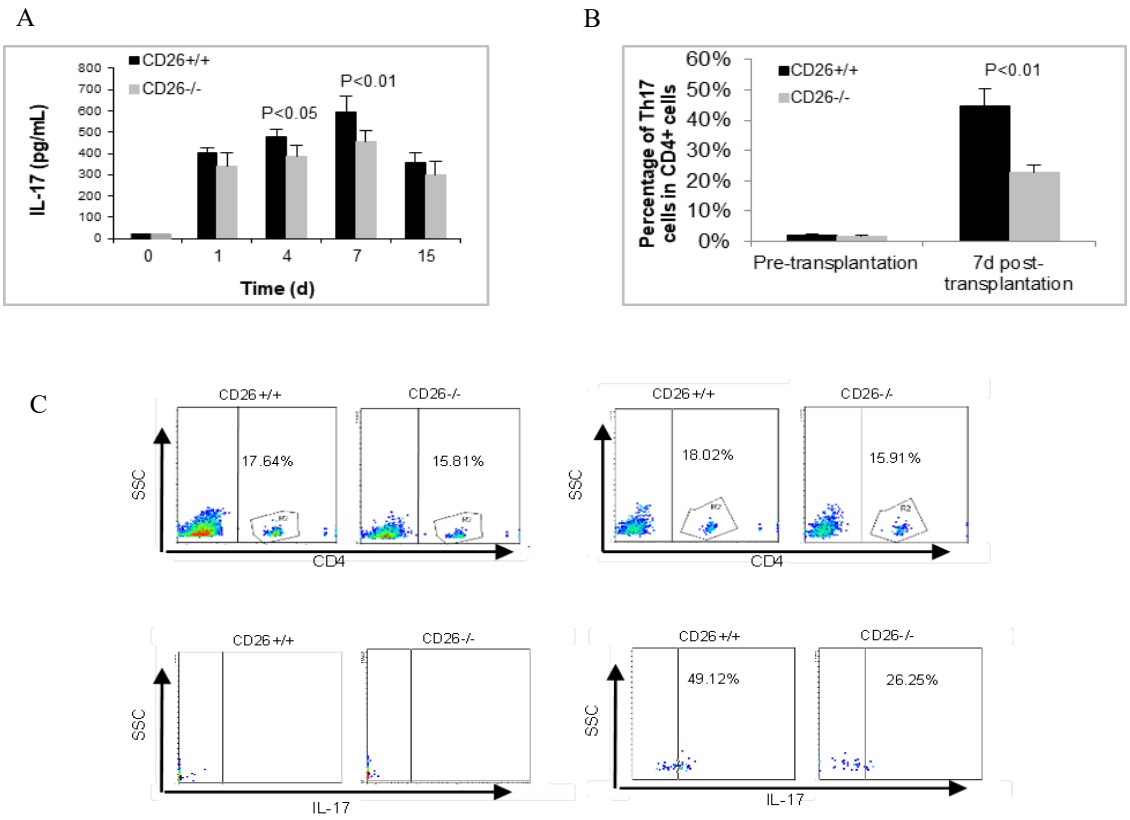


Figure 4.20: Analysis of IL-17 secretion and percentage of Th17 cells in $CD4^+$ cells of MPBLs in $CD26^{+/+}$ and $CD26^{-/-}$ mice

Blood was taken at indicated time points before and after allogeneic skin transplantation (day 0 represents the day before transplantation). The secretion of IL-17 in serum was analyzed by ELISA. The percentage of Th17 cells in $CD4^+$ cells were stained with anti-IL-17 after cell permeability and analyzed by flow cytometry. (A) The secretion of IL-17 in the serum of $CD26^{+/+}$ and $CD26^{-/-}$ mice. (B) Percentage of Th17 cells in $CD4^+$ cells of $CD26^{+/+}$ and $CD26^{-/-}$ mice on the day before transplantation and on day 7 after transplantation. (C) FACS dot plot showing the percentage of Th17 cells in $CD4^+$ cells. The values represent the mean ± SD of at least 7 mice at each time point.

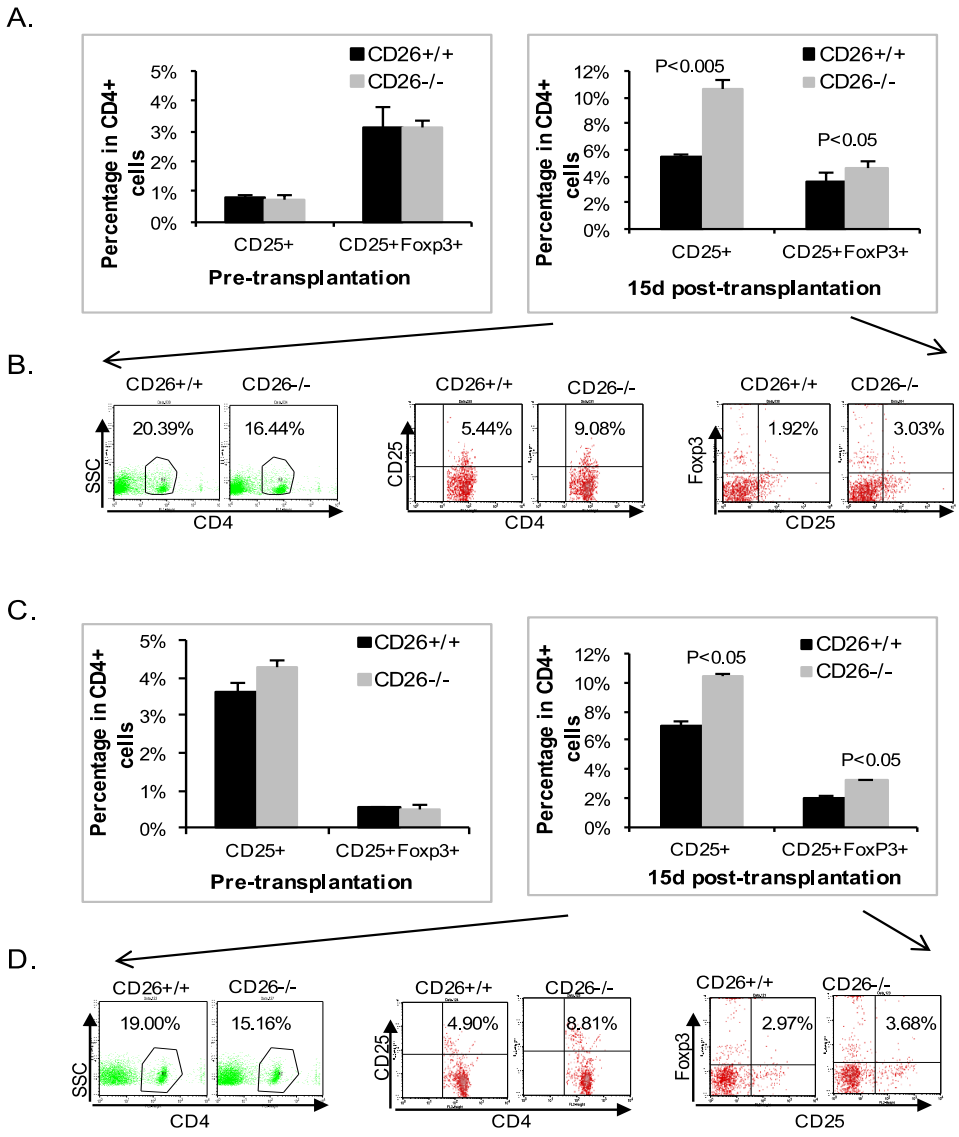
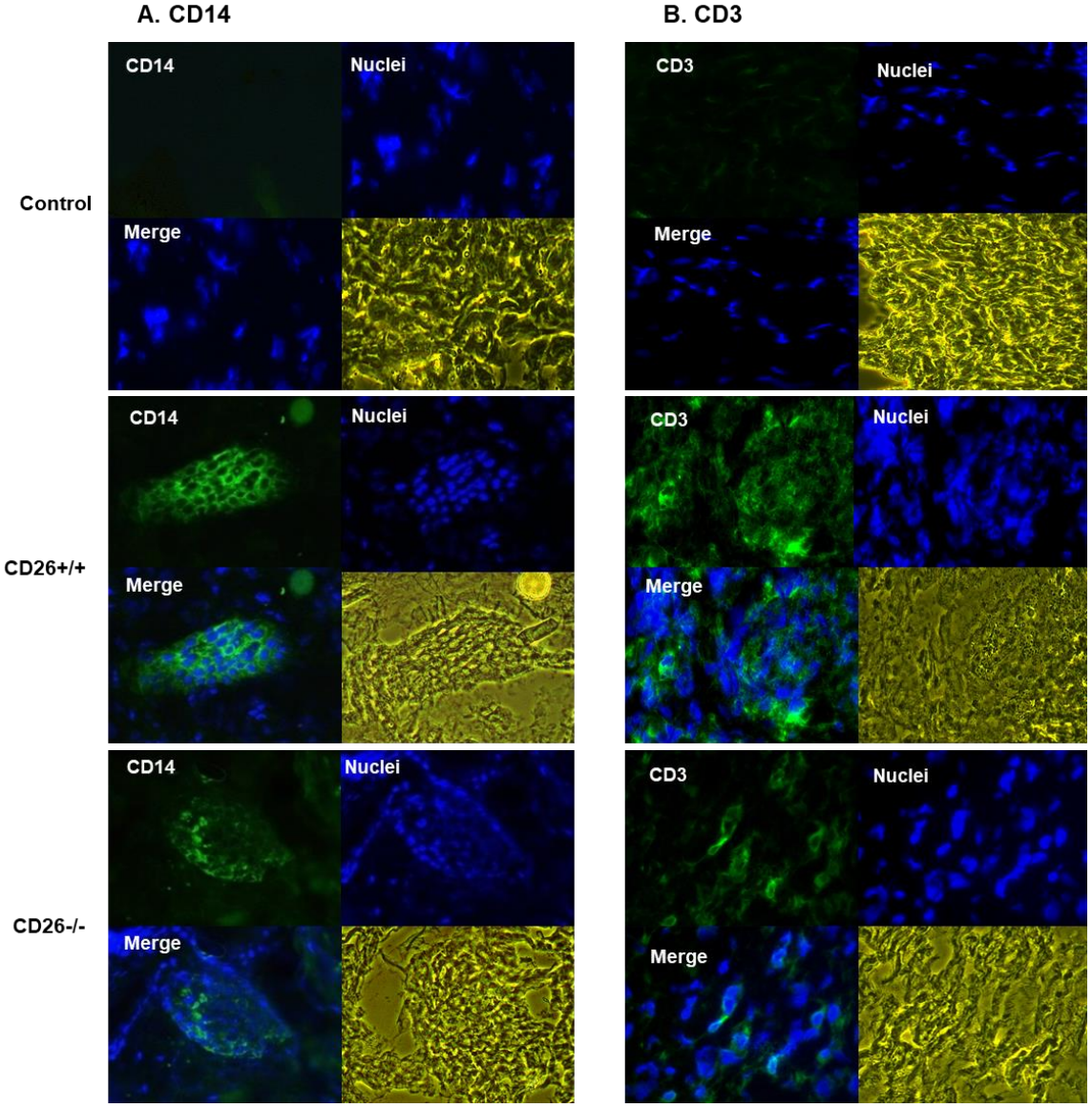


Figure 4.21: Percentage of Tregs in $CD4^+$ lymphocytes of $CD26^{+/+}$ and $CD26^{-/-}$ mice. Lymphocytes of MSLs and MPBLs were separated pre- and on day 15 post-transplantation

The percentage of Tregs ($CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$) in $CD4^+$ cells of MSLs and MPBLs was analyzed by flow cytometry. (A) Percentage of Tregs in $CD4^+$ cells of MSLs. (B) Dot plot of $CD4^+$ cells of MSLs, $CD25^+$ and $CD25^+Foxp3^+$ in $CD4^+$ cells of MSLs in $CD26^{+/+}$ and $CD26^{-/-}$ mice on day 15 after allogeneic skin transplantation. (C) Percentage of Tregs in $CD4^+$ cells of MPBLs. (D) Dot plot of $CD4^+$ cells of MBPLs, $CD25^+$ and $CD25^+Foxp3^+$ in $CD4^+$ cells of MPBLs in $CD26^{+/+}$ and $CD26^{-/-}$ mice on day 15 after allogeneic transplantation.

4.2.10 Reduction of infiltration of macrophages and T lymphocytes in the grafts of $CD26^{-/-}$ mice

The infiltration of macrophages ($CD14^+$) and T cells ($CD3^+$, $CD4^+$, and $CD8^+$) in the graft tissues of $CD26^{+/+}$ and $CD26^{-/-}$ mice were determined by immunohistofluorescence analysis. In the graft tissues of both $CD26^{+/+}$ and $CD26^{-/-}$ mice, clusters of $CD14^+$ macrophages were detected on day 7 post-transplantation (Figure 4.22A). However, the numbers of these clusters in the grafts of $CD26^{-/-}$ mice were obviously fewer and the area of these clusters in the grafts of $CD26^{-/-}$ mice was relatively smaller than that in the grafts of $CD26^{+/+}$ mice, suggesting a reduced infiltration of macrophages in the grafts of $CD26^{-/-}$ mice (Figure 4.22A). Infiltration of T lymphocytes ($CD3^+$) was also found in the grafts of both kinds of mice on day 7 post-transplantation (Figure 4.22B). The number of infiltrated $CD3^+$ cells in $CD26^{-/-}$ mice was obviously less than that in $CD26^{+/+}$ mice on day 7 after skin transplantation (Figure 4.22B). Further analysis found that T cell subsets $CD4^+$ cells and $CD8^+$ cells were both infiltrated in the graft tissues of the two mice types after skin transplantation. The numbers of infiltrated $CD4^+$ cells and $CD8^+$ cells were both less obvious in $CD26^{-/-}$ mice than that in $CD26^{+/+}$ mice (Figure 4.22C and 4.22D). However, no obvious infiltration of B lymphocytes ($CD19^+$) was detected in two kinds of mice after skin transplantation (data not shown).


A. CD14
B. CD3
Control
CD26+/+
CD26-/-
CD14
Nuclei
Merge
CD3
Nuclei
Merge

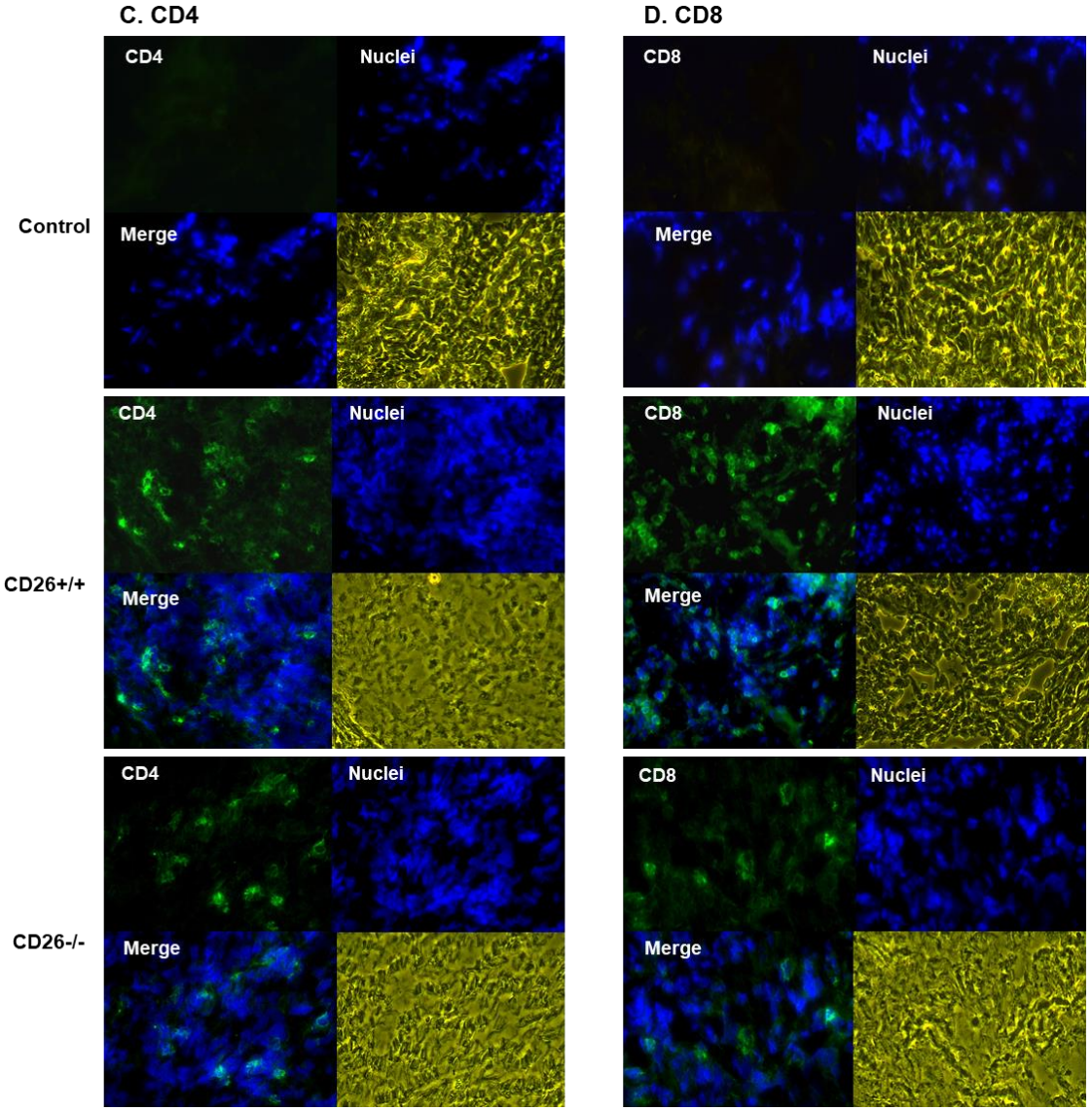


Figure 4.22: Determination of infiltrated macrophages and T lymphocytes in the grafts of $CD26^{+/+}$ and $CD26^{-/-}$ mice after allogeneic skin transplantation

The tail-skin before transplantation was collected as control, graft tissues from $CD26^{+/+}$ mice and $CD26^{-/-}$ mice were obtained on day 7 post-transplantation. The frozen sections of skin grafts were stained with monoclonal antibody against mouse CD14, CD3, CD4, or CD8, and then the nucleus was counterstained with Hoechst 33342. A, B, C, D represents the immunofluorescence analysis of infiltrated $CD14^{+}$, $CD3^{+}$, $CD4^{+}$, or $CD8^{+}$ cells in the grafts of $CD26^{+/+}$ and $CD26^{-/-}$ mice, respectively. Photos were shown at 400x magnification.

5. Discussion

5.1 The role of CD26 in the proliferation and differentiation of T cells

5.1.1 As a co-stimulator of T cell activation

T cell activation requires two simultaneous signals. The first one is provided by the stimulation of CD3/T cell receptor (TCR) complex, which plays the central role in T cell activation. However, in general, peptide antigen alone against TCR complex cannot induce T cell activation and proliferation. T cell activation needs a second co-stimulatory signal delivered by accessory molecules expressed on T cell surfaces. CD26 is one of such molecule expressed on T cell surfaces [2]. Many studies showed that certain anti-CD26 monoclonal antibodies provided co-stimulatory activity in the CD3/TCR-driven T cell activation *in vitro*. Later studies found that the signal transduction of the co-stimulatory of CD26 for T cell activation could be mediated by the interaction between CD26 and ecto-adenosine deaminase (ADA), tyrosine phosphatase CD45, CARMA1 or caveolin-1 [129]. In the present work, just like previous study [126], immobilized anti-CD3 mAb (OKT3, IgG2a) (Thermo Fisher Scientific, USA) was used for antigen stimulation to investigate the role of CD26 in T cell activation and differentiation. Three days after immobilized anti-CD3 stimulation, the activation of lymphocytes was determined through the measurement of the expression of lymphocyte activation markers CD69, CD71, CD25 and CD26. 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 [130]. CD71 (the transferrin receptor) and CD25 (the IL-2 receptor) are other two molecular surface markers of T cell activation and proliferation [131]. From Figure 4.2A, it could be seen that the percentage of $CD69^+$, $CD71^+$ or $CD25^+$ cells increased in the stimulated group. This indicated that part of the lymphocytes were activated after stimulation. Acting as one of the T cell activation markers, CD26 expression is also up-regulated after stimulation. This is consistent with previous studies [2]. However, the association of CD26 with the differentiation of T cell subpopulations needs further investigation.

5.1.2 CD26 expression on the surfaces of different lymphocyte subpopulations

After determining lymphocyte activation (Figure 4.2), the proliferation of lymphocytes was analyzed next after stimulation. It was found that lymphocytes proliferated up to five generations in the stimulated group while the lymphocytes of PBS treatment group did not proliferate (Figure 4.3). Further analysis showed that the percentage of $CD4^+$ cells was increased by 12% after stimulation, while the percentages of $CD8^+$ and $CD19^+$ cells did not change significantly. The up-regulated percentage of $CD4^+$ cells suggests that the immobilized anti-CD3 mAb can trigger the proliferation of human $CD4^+$ lymphocytes, which is consistent with previous study [132]. Since the stimulation of T cells in the present study is driven by immobilized anti-CD3 mAb as antigen stimulation which is specific for T cell activation, it is expected that CD19 expression was not changed after stimulation. Cell survival analysis indicates that there were no dead lymphocytes after stimulation, which means that T cytotoxic cells ($CD8^+$ cells) did not die after stimulation. The reason for the unchanged percentage of $CD8^+$ cells after stimulation might be explained by that $CD8^+$ cells may not proliferate or the proliferation rate of $CD8^+$ cells is much slower than that of $CD4^+$ cells after stimulation. In addition, the co-expression level of CD26 with $CD4^+$ or $CD8^+$ was increased after stimulation. About 70% of $CD4^+$ cells and 40% of $CD8^+$ cells co-expressed with CD26 (Figure 4.4), respectively. CD26 acts as a co-stimulator of T cell activation as its expression was strongly up-regulated following T cell activation [133]. The increased percentage of cells co-expressing CD26 with $CD4^+$ or $CD8^+$ indicate that the activation of $CD4^+$ cells and $CD8^+$ cells may be both related to CD26 expression. 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 molecular [41, 134]. Blockade of CD26-mediated T cell co-stimulation with soluble caveolin-1 induces anergy in $CD4^+$ cells [135]. Based on the results obtained in the present work, the influence of CD26 on the activation of $CD4^+$ cells was exhibited by its co-expression with CD26 after stimulation. Although only 40% of $CD8^+$ cells co-expressed CD26 after stimulation, it nevertheless demonstrates an involvement of CD26 during the cytotoxic T cell activation. Some previous study found that CD26 mediated the co-stimulation of not only $CD4^+$ T cells but also $CD8^+$ T cells [136], which is consistent with the results of the present work.

In order to further investigate the role of CD26 on the activation and differentiation of $CD4^+$ cells and $CD8^+$ cells, $CD26^{high\text{-}expressing}$ cells ($CD26^{high}$) were separated from $CD26^{low\text{-}expressing}$ cells ($CD26^{low}$) (see section 3.2.6). It was found that the percentage of $CD4^+$ cells was significantly higher in the $CD26^{high}$ group than in the $CD26^{low}$ group. Co-expression analysis shows that the percentage of $CD4^+CD26^+$ cells in the $CD26^{high}$ group is significantly higher than that of $CD26^{low}$ group (Figure 4.7). These data suggest that the activation of $CD4^+$ cells is associated with CD26 expression. $CD4^+$ cells are T helper cells and they can secret different cytokines upon T cell activation and the cytokines play a crucial role in the activation and/or proliferation of other effector cells, such as B cells, cytotoxic T cells and macrophages. Besides studies on the involvement of CD26 in the activation and proliferation of $CD4^+$ T cells *in vitro* [6, 41, 137], *in vivo* investigation using CD26 knockout mice presented a decreased percentage of $CD4^+$ cells [43]. The higher percentage of $CD4^+$ cells in $CD26^{high}$ group observed in the present work further confirms and implies that CD26 expression is involved in the activation, differentiation and functioning of $CD4^+$ cells. Interestingly, in the present work, it was found that the percentage of $CD8^+$ cells was significantly lower in the $CD26^{high}$ group than in the $CD26^{low}$ group (Figure 4.7). However, the percentage of cells co-expressing $CD8^+$ and $CD26^+$ is obviously higher in the $CD26^{high}$ group than in the $CD26^{low}$ group. This suggests that the activation of $CD8^+$ cells is also associated with CD26 expression. $CD8^+$ cells are T cytotoxic cells and are one of the most important effector cells in the cell-mediated immunity. T cytotoxic cells can kill cancer cells, infected cells or damaged cells by releasing cytotoxins perforin, granzymes and granulysin to induce cell apoptosis. 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 [138]. A later study indicated that $CD26^+CD8^+$ cells belong to the early effector memory T cell subsets [139]. *In vivo,* it was found that CD26-deficiency impaired the development of $CD4^+$ cells but did not affect the development of $CD8^+$ cells [43]. CD26 is a co-stimulator not only for the activation of $CD4^+$ cells but also for $CD8^+$ cells, CD26 mediated co-stimulation of $CD8^+$ cells provokes effector function via granzyme B, tumor necrosis factor-α, IFN-γ and Fas ligand [136, 139]. In the present study, only small percentage of CD8 cells was found to express CD26,

the role of CD26 in the differentiation and function of $CD8^+$ cells needs further investigation.

5.1.3 Association of CD26 expression with the differentiation and functions of Th1 and Th2 lymphocytes

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 [140]. 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 [141]. To further understand the role of CD26 on T lymphocyte differentiation, the profiles of the cytokines released by different T cell subpopulations were investigated. The secretion level of cytokines IL-2, IFN-γ, IL-6, IL-4, or IL-13 was found to be significantly higher after stimulation (Figure 4.4). After cell sorting of CD26-expressing cells, the percentage of cells secreting each of Th1-typical cytokines IFN-γ or IL-2 in the $CD26^{high}$ group was significantly higher than that in the $CD26^{low}$ group (Figure 4.8A). Moreover, the percentages of cells co-expressing CD26 and IFN-γ or IL-2 were significantly higher in the $CD26^{high}$ group in comparison to that in the $CD26^{low}$ group (Figure 4.9). In the previous study, the up-regulation of CD26 expression on $CD4^+$ cell surfaces was identified to be related to the production of Th1 cytokines [6]. 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 [132]. 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 [42, 142]. Supporting these findings, the results of the present work show 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 4.7B) benefits the differentiation of $CD4^+$ cells into a Th1 subset.

Interestingly, the percentage of cells secreting Th2-typical cytokines IL-4 or IL-13 was not different between the $CD26^{low}$ and $CD26^{high}$ groups (Figure 4.8A). As one of the main subpopulations of T helper cells, Th2 subset is often recognized as an opposite of Th1

cells as Th2 cytokines may suppress the activity and proliferation of Th1 cells during immune responses [143]. Supporting results obtained in the current study, it was found that Th2-like clones display lower expression of CD26 in comparison to Th1-like clones of allergen-specific T cells [6], indicating that CD26 expression is not related to the differentiation of $CD4^+$ cells into Th2 subset after stimulation.

Association of CD26 with the differentiation of Th1 and Th2 after stimulation	
In $CD26^{high}$ group in comparison to $CD26^{low}$ group	
Th1	**Th2**
IL-2 (higher)	IL-4 (no difference)
IFN-γ (higher)	IL-13 (no difference)

Table 5.1: Association of CD26 expressing with the secretion of Th1 or Th2 cytokines *in vitro* after immobilized anti-CD3 mAb stimulation.

5.1.4 Association of CD26 expression with the differentiation and functions of Th17 and regulatory lymphocytes

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 [44] which is distinct from classic Th1 and Th2 subsets. These cells originate from naive $CD4^+$ precursor cells mainly in the presence of TGF-β and IL-6, and their differentiation requires IL-23 [144]. 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. The high expression of CD26 in $CD4^+$ cells (Figure 4.4 and Figure 4.7) and the high relation between CD26 expression and Th1 differentiation, as well as the higher secretion level of IL-6 (Figure 4.5) which was indicated to be a critical molecule for the balance between Th17 and Tregs [116], prompted the examination of the influence of CD26 on the differentiation of Th17 subset. After cell sorting, the percentage of cells secreting Th17 typical cytokines (IL-17 or IL-22), or expressing Th17 molecular markers (IL-23R, CD161 or CD196) were found to be significantly higher in the $CD26^{high}$ group than in the $CD26^{low}$ group (Figure 4.8). Moreover, the percentage of CD26 positive cells co-expressed with Th17 typical cytokines

(IL-17 or IL-22) or Th17 molecular biomarkers (IL-23R) was significantly higher in the $CD26^{high}$ group than in the $CD26^{low}$ group (Figure 4.9). This indicated an involvement of CD26 in the differentiation of $CD4^{+}$ cells into Th17 subset. In support of the obtained results, 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 [53]. 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 [145]. A recent study indicated the involvement of CD26 in organ transplantation in which IL-17 plays an important role [51]. It was found that the inhibition of IL-17 could inhibit Th17 and Th1 immunity by affecting the initial recruitment of immune cells to the inflammation sites thereby modulating the innate and adaptive immune responses in allograft rejection [146]. Moreover, the inhibition of DPPIV enzyme activity was reported to induce the reduction of IL-17 expression and increased the allograft acceptance [147]. The role of CD26 in different clinical pathologies caused by Th17 cells have not been explained completely. However, the high association between CD26 expression and Th17 differentiation observed in the present study provides more insight into future research about the role of CD26 in the function of Th17 cells and related diseases.

Association of CD26 with the differentiation of Th17 and Tregs after stimulation	
In $CD26^{high}$ group in comparison to $CD26^{low}$ group	
Th17	**Tregs**
IL-6 (higher)	CD25+FoxP3+ (no difference)
IL-17 (higher)	CD4+FoxP3+ (no difference)
IL-22 (higher)	
IL-23R (higher)	
CD196 (higher)	
CD161 (higher)	

Table 5.2: Association of CD26 expressing with the cytokines secretion or biomarkers expression *in vitro* after stimulation.

Considering that the balance between Th17 cells and Tregs plays a prominent role in immune responses, the expression profile of Tregs in $CD26^{high}$ and $CD26^{low}$ groups were further analyzed. Results showed that the percentage of Tregs was very low in both groups (Figure 4.8), and no obvious difference was found of the Tregs´ percentages in the $CD26^{high}$ group and $CD26^{low}$ group. The function of Tregs is to restrain excessive response of other effector T cells as they are essential for maintaining peripheral tolerance and preventing autoimmune diseases. In allograft transplantation, Tregs are involved in alloantigen-specific immunosuppression and then induce immune tolerance [148]. In view that CD26 acts as a positive marker for Th17 cells, it is reported to be used as a negative selection marker for Tregs [59]. In the present study, no obvious differences in the percentage of Tregs were detected in both $CD26^{high}$ and $CD26^{low}$ groups which indicates that the expression of CD26 is not related to the differentiation of Tregs after immobilized anti-CD3 mAb stimulation. The exact mechanism of immunosuppression of Tregs in clinical disease is yet to be completely defined, especially in allogeneic transplantation. Th17 cells, Tregs, and the balance between them play important roles for graft survival after organ transplantation. The association of CD26 with Th17 and Tregs observed in the present study provides more insight for future research about the role of CD26 in the functions of Th17 cells, Tregs and related diseases, especially in organ transplantation.

5.1.5 Influence of CD26 on the expression of cell adhesion molecules

CD26 has been reported to be involved in cell adhesion and migration through its interaction with extracellular matrix proteins, such as fibronectin and collagen [149-151]. Cell adhesion is essential for the invasion of immune cells to the inflammation sites which are infected by bacteria. Immune cells should be attached to endothelial cells of vessel walls through the mediation of adhesion molecules, and then migrate into the inflammation sites to destroy the foreign cells. Examples of adhesion proteins include integrins, selectins, syndecans and cadherin [152]. CD11 is the alpha component of various integrin which contains three distinct subunits (CD11a, CD11b, and CD11c). The three subunits share a common beta component CD18. CD11a/CD18, also known as leukocyte-function-associated antigen-1 (LFA-1), is expressed on all leukocytes. It is involved in immune response through mediating the adhesion of T cells, B cells or NK cells to endothelial cells [153]. CD11b/CD18, also termed macrophage-1 antigen (Mac-1), is a human cell surface receptor found on leukocytes, NK cells and macrophages. It is capable of recognizing and binding to some molecules on the surface of bacteria invading cells, such as CD54 and fibrinogen. Both T cells and B cells express CD54, however, B cells show a uniform distribution of CD54 while CD54 is highly mobile on the surface of activated $CD4^+$ cells. When activated $CD4^+$ cells co-cultured with B cells, the majority of CD54 on the surfaces of $CD4^+$ cells was found at or immediately adjacent to the point of attachment to B cells and interacted with its ligands. These findings suggest that CD54 plays a critical role in T cell – B cell collaboration during the immune response [154]. In addition, CD54 was in low concentrations in resting leukocytes while its expression was greatly increased on $CD4^+$ T cells after antigen stimulation [154]. In the present study, the percentage of $CD54^+$ cells was observed to increase significantly after stimulation (Figure 4.5). Moreover, the higher expression of CD54 is related to the high expression of CD26 (Figure 4.11) indicating an association of CD26 with the CD54 expression. Furthermore, it was found that higher expression of CD11b is also associated with high CD26-expression (Figure 4.11). CD11b is not only present on NK cells, monocytes and neutrophils, but is also expressed on lymphocytes, mainly on $CD8^+$ cells. CD11b was reported to be a marker for the activation of memory $CD8^+$ cells during virus infection [155]. The higher CD11b expression in $CD26^{high}$ group observed in the present study (Figure 4.11) might contribute

to the adhesion of cytotoxic cells to their targets during immune responses. This provides more insight into the role of CD26 in lymphocytes adhesion.

In conclusion, the results of the *in vitro* part of the current study show that CD26 is involved in the activation and differentiation of lymphocytes after stimulation, especially in the differentiation of Th1 and Th17 lymphocyte subsets. Moreover, the high-expression of CD26 may increase the expression of adhesion molecules CD11b and CD54, indicating that CD26 may be involved in the cell-cell interaction of immune cells. Altogether, the findings provide a further insight into the role of CD26 in immune response and immune regulation.

5.2 The role of CD26 in allogeneic graft rejection

Finding an effective way to inhibit immune rejections is one of the most important strategies to support clinical transplantations. Recently, some studies associated with organ transplantation have demonstrated that the use of DPPIV inhibitor or anti-CD26 monoclonal antibody increased the engraftment of donor organs [71, 147] or decreased the acute graft-versus-host disease (GVHD) [156], pointed to CD26 as a novel target for therapeutic intervention in organ transplantation. In order to clarify the role of CD26 in allogeneic graft rejection, CD26 knockout mice were used in an allogeneic skin transplantation study. Results show that $CD26^{-/-}$ mice represented a lower necrotic degree of grafts and delayed allograft rejection (Figure 4.14).

5.2.1 Influence of CD26-deficiency on the allogeneic skin graft rejection

Allograft skin transplantation is one of the most effective ways to treat large-area skin burn or wound injury although the long-term survival of the allogeneic or xenogeneic skin grafts is difficult to achieve in clinical therapy because of the immune rejection. Many studies have indicated an involvement of CD26/DPPIV in organ transplantation. Previous work found that the inhibition of CD26/DPPIV improved lung transplant function [147]. Moreover, the graft-versus-host disease was decreased by the application of anti-CD26 monoclonal antibody [125]. Nevertheless, the molecular mechanisms of the role of CD26 during immune rejection are not completely elucidated.

Many investigations have suggested that skin allograft rejection requires the activation of T helper cells and T cytotoxic cells specific for graft alloantigen [157]. After skin transplantation, the dendritic cells (DCs) of the donor skin migrate out of the graft and present the donor antigens which could be recognized by the recipient T cells. Following allorecognition, the recipient T cells become activated, proliferated and then could secrete proinflammatory cytokines [107]. Cytokines secreted by different T cell subsets play a crucial role in the activation of other effector immune cells, such as macrophages. The inflammatory stage initiates the effector T cells and macrophages arrive at the graft sites to destroy the grafts [117]. Especially Th17 cells, which secrete proinflammatory cytokine IL-17, was found to play an important role in skin transplantation [51]. In addition, it is becoming clear that $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) play a primary central role in the induction of allograft tolerance [158]. In the *in vitro* part of the present work, results suggest that CD26 may be involved in the activation and differentiation of Th1 and Th17 cells. The lower necrotic degree of grafts and delayed allograft rejection in CD26-deficiency mice observed (Figure 4.14) prompted further examination of the molecular mechanisms of the role of CD26 in the immune rejection *in vivo* in the current study.

5.2.2 Influence of CD26-deficiency on the proliferation and differentiation of lymphocytes after allogeneic skin transplantation

CD26 is an activation marker of T cells. Blockade of CD26-mediated T cell co-stimulation induced anergy in $CD4^+$ T cells [135]. In the present work, lower percentages of $CD3^+$ and $CD4^+$ cells were found in MPBLs and MSLs of $CD26^{-/-}$ mice both pre- and post-skin transplantation (Figure 4.16 and Figure 4.17). In addition, the infiltration of $CD3^+$ and $CD4^+$ cells was less detected in the graft tissues of $CD26^{-/-}$ mice than in $CD26^{+/+}$ mice after skin transplantation (Figure 4.22B and Figure 4.22C). This indicates that the deficiency of CD26 results in an impaired development and activation of $CD3^+$ cells and $CD4^+$ cells, which corresponds to our previous findings [43]. Interestingly, the percentage of $CD8^+$ cells in MPBLs of $CD26^{-/-}$ mice was the same as in $CD26^{+/+}$ mice before transplantation. However, it was significantly lower in $CD26^{-/-}$ mice than in $CD26^{+/+}$ mice after skin transplantation (Figure 4.16). The infiltration number of $CD8^+$ cells in skin grafts was obviously lower in $CD26^{-/-}$ mice than in $CD26^{+/+}$ mice after skin transplantation (Figure 4.22D). These findings suggest a reduced proliferation and activity of $CD8^+$ cells

in $CD26^{-/-}$ mice in response to allogeneic antigens. Consistently, *in vitro*, it was observed that CD26 was involved in the activation of $CD4^+$ and $CD8^+$ T cells during antigen stimulation. Intensive investigations indicated that skin allograft rejection is not the exclusive function of a particular T cell, the interactions between distinct T cell subsets are essential for the graft rejection, especially between the activated T helper cells ($CD4^+$) and T cytotoxic cells ($CD8^+$) [157]. The cytokines secreted by T helper cells play a crucial role in the cytotoxic T cell activation, such as IL-2 and IFN-γ [159]. The lower percentage of $CD4^+$ cells in CD26-deficient mice may reduce the interaction between T helper cells and T cytotoxic cells thereby reduce the $CD8^+$ cell response during the skin transplantation. In addition, results in the present work found that adhesion molecules CD11b and CD54 presented a lower expression in the $CD26^{low}$ group *in vitro* (Figure 4.11), and CD11 and CD54 mediate the cell – cell adhesion during immune response [154]. The deficiency of CD26 in $CD26^{-/-}$ mice may influence the expression of adhesion molecules; thereby reduce the cell – cell adhesion which may reduce the migration and infiltration of effector cells (macrophages and T lymphocytes) into graft tissues of $CD26^{-/-}$ mice. However, the influence of CD26-deficiency on the adhesion molecular expression during the allogeneic skin transplantation needs further investigation. $CD8^+$ T cells are a prominent component of the allogeneic T-cell repertoire induced after allogeneic transplantation in mice and their cytotoxic activity is directed to donor MHC class I peptides [160]. The reduced percentage of $CD8^+$ cells in $CD26^{-/-}$ mice after allogeneic transplantation (Figure 4.16) may partly contribute to the reduced necrotic degree of grafts and delayed allograft rejection.

Additionally, the percentages of $CD3^+$ and $CD4^+$ cells were lower of MSLs in $CD26^{-/-}$ mice than in $CD26^{+/+}$ mice before transplantation, but the difference was in MSLs between both kinds of mice reduced after transplantation (Figure 4.17). As it is well known, the spleen is the largest single peripheral lymphoid organ in mammals, contributing to both innate and adaptive immune response as well as lymphocytes homing [161]. It has been reported that the inhibition of CD26 enzyme activity increased the donor cell homing and improves the allogeneic engraftment [122]. The decrease in the percentage difference of $CD3^+$ and $CD4^+$ cells of MSLs between both kinds of mice after transplantation may be probably due to the increased homing of these cells to the spleens in $CD26^{-/-}$ mice.

5.2.3 Influence of CD26-deficiency on the secretion of Th1- and Th2-cytokines after allogeneic skin transplantation

Allograft rejection is primarily driven by the ability of host T cells. While all components of the innate and adaptive immune systems participate in the graft rejection, T lymphocytes, particularly $CD4^+$ cells, are of paramount importance in this process [162]. Once activated, $CD4^+$ T cells primarily direct the progression of the response by secreting cytokines which activate, expand, and/or recruit other effector cells, such as macrophages, $CD8^+$ T cells, and B cells [163, 164]. Through further analysis of cytokine levels in both types of mice, markedly reduced secretion of IL-2, IFN-γ, IL-6, IL-17, IL-4, or IL-13 was found in the serum of $CD26^{-/-}$ mice after allogeneic transplantation (Figure 4.18 and Figure 4.20). It may have been caused by a lower number of $CD4^+$ cells in $CD26^{-/-}$ mice before transplantation; on the other hand, it appears to be more important to consider an reduced activation and proliferation and impaired differentiation and functions of $CD4^+$ cells in response to the allogeneic antigen in $CD26^{-/-}$ mice. The lower serum levels of IL-2, IFN-γ and IL-6 indicate a defective differentiation and function of Th1 cells, while the lower levels of IL-4 and IL-13 indicate an insufficient differentiation and function of Th2 cells in $CD26^{-/-}$ mice.

Being one of the key cytokines, IFN-γ has diverse and potentially contradictory effects on organ allograft rejection [112]. IL-2 is another Th1-associated cytokine which also has complex effects on allograft rejection [165]. Both IFN-γ and IL-2 are pleiotropic cytokines and play an important role in the proliferation of T- and B-cells during the inflammatory reaction. They first act as the molecules initiating T cell growth and survival during the immune response, and then reinforce Th1 response with a positive feedback [112, 165]. In an acute rejection, Th1 cells predominantly infiltrate into grafts, in where IL-2 and IFN-γ can induce the activation of natural killer cells and macrophages that are the strong weapons to destroy allografts [112, 165]. Furthermore, IFN-γ induces the expression of class II major histocompatibility complex (MHC) molecules and the secretion of IgG2a and IgG3 from activated B cells. In the acute rejection model, IFN-$\gamma^{-/-}$ mice showed delayed skin graft rejection [166]. Several studies have reported that the tolerance in allograft rejection is mediated at least in part by IL-4, a typical cytokine of Th2 cells, through promoting IL-10 and IgG1 production [167]. Other studies have provided conflicting results that the administration of Th2 inhibitor prolongs the cardiac allograft

survival [114]. IL-13 that has similar effects as IL-4 is another cytokine associated with Th2 response. It shares a receptor chain (IL-4R α-chain) with IL-4 but differs in the target cells involved, which result in a series of different biological events [113]. More and more studies have demonstrated that the cytokines of both Th1 and Th2 cells are capable of supporting B cell clonal expansion and antibody syntheses, such as IL-2, IFN-γ and IL-4 [163]. The deficiency of CD26 results in an impaired differentiation and function of Th1 and Th2 cells in $CD26^{-/-}$ mice. The lower levels of Th1 cytokines IFN-γ and IL-2 in $CD26^{-/-}$ mice (Figure 4.18) may result in the decrease of the activation and proliferation of $CD8^{+}$ cells (Figure 4.16) and activation of macrophages, thereby reduce the infiltration of $CD8^{+}$ cells and macrophages into grafts (Figure 4.22) during the allograft rejection. On the other hand, the lower levels of IFN-γ, IL-2 and IL-4 in $CD26^{-/-}$ mice may impair the activation and differentiation of B cells, leading to the reduction of antibody production (Figure 4.15) after allogeneic transplantation.

5.2.4 Influence of CD26-deficiency on the immunoglobulin production after allogeneic skin transplantation

Immune rejection is a complex process that involves cellular as well as humoral immune response which is characterized by the production of antibodies by B lymphocytes. IgG, the main component of seral immunoglobulins, usually as a pathogenic antibody in patients with transplant rejection, plays the indispensable role in damaging the grafts during transplant rejection [168]. Besides the main function of degradation of allograft, immunoglobulin has been shown to protect graft rejection after transplantation. In skin transplantation model, the administration of IgG prolonged the survival of skin grafts, it reduced the number of pro-inflammatory cells and increased the anti-inflammatory factors [169]. It seems that the administration of exogenous immunoglobulin during the immune rejection may reduce the host effective cells. However, the antibodies produced by the host plasma cells are required to destroy the alloantigen.

In the present work, lower production of IgG and its subsets IgG1 and IgG2a were detected in the serum of $CD26^{-/-}$ mice (Figure 4.15). This corresponds to our previous findings which showed that the antibody production was obviously less in $CD26^{-/-}$ mice than that in $CD26^{+/+}$ mice either after pokeweed mitogen (PWM)-stimulation or after

ovalbumin (OVA)-immunization [43, 102]. The implication is that the differentiation of B cells may have been impaired by CD26-deficiency. On the T cell-dependent B cell activation, the interaction between B cells and T helper cells and some Th1 or Th2 cytokines, are required to support B cell clonal expansion and antibody synthesis. In $CD26^{-/-}$ mice, the lower percentage of T help cells ($CD4^{+}$) (Figure 4.16) and the less production of IL-2 and IL-4 (Figure 4.18) could be responsible for impaired activation and differentiation of B cells; hence resulting in a reduced IgG production. IgG is a major component that mediates the allorecognition between exogenous antigens and recipient $CD8^{+}$ cells during graft rejection [160, 170]. The lower IgG production in $CD26^{-/-}$ mice should, therefore, result in the reduction of the grafts attack by effector cells.

5.2.5 Influence of CD26-deficiency on the differentiation and functions of Th17 and Tregs after allogeneic skin transplantation

Interestingly, the secretion level of IL-10 was higher in $CD26^{-/-}$ mice after allogeneic skin transplantation (Figure 4.18G). Supporting the results of the current study, CD26/DPPIV blockade has been shown to improve lung allograft transplant and increase the expression of IL-10 [147]. IL-10 is known to be an anti-inflammatory cytokine and it has been reported to downregulate the expression of Th1 and Th17 cytokines in the inflammation process, especially in the regulatory T cell signaling process [171]. Various cell types produce IL-10, including Th2, macrophages and regulatory T cells [172]. In $CD26^{-/-}$ mice, the higher secretion level of IL-10 (Figure 4.18G) might be the result of the higher percentage of Tregs (Figure 4.21). It is notable that, in response to allogeneic transplantation, high level of IL-6 was detected in the serum of $CD26^{+/+}$ mice from the first day and peaked on day 7 post-transplantation. However, only a small amount of IL-6 was detected in the serum of $CD26^{-/-}$ mice until the day 7 post-transplantation (Figure 4.18D). Recent studies have demonstrated that IL-6 plays a very important role in regulating the balance between Th17 cells and Tregs [116]. Thus, the lower level of IL-17 and a higher percentage of Tregs in $CD26^{-/-}$ mice found in the present study (Figure 4.20 and Figure 4.21) could have resulted partly from the reduced IL-6 and higher IL-10 levels.

Furthermore, it has been reported that human Th17 cells are characterized by a high expression of CD26 [53] and CD26 is a negative selection marker for human Tregs [59]. It

was found that CD26 may be involved in the differentiation and function of Th17 cells but is not related to Tregs *in vitro* of the present study (Figure 4.8). It is therefore not surprising that an impaired balance of differentiation of Th17 and Tregs was found in $CD26^{-/-}$ mice (Figure 4.20 and Figure 4.21). Although allograft rejection was traditionally associated with Th1 differentiation, many recent studies have shown that Th17 cells and IL-17 are closely associated with allograft rejection [51, 146]. Th17 cells are a more recent addition to T cell paradigm, while IL-17, one of the key Th17 cytokines, is a pro-inflammatory factor [44]. Accumulating evidence suggests that Th17 cells play a role in the development of chronic allograft injury in transplantation of various organs. The hallmark of Th17 cell-mediated allograft rejection is IL-17´s ability to recruit neutrophils, which are one of the first inflammatory effector cells capable to infiltrate into the allografts after transplantation and then cause allograft damage [173].

Conversely, Tregs are considered to be an essential factor for peripheral tolerance by maintaining immune homeostasis. They play a crucial role in the immune tolerance and negative control of various immune responses through the suppression or down-regulation of other effector T cells during the immune rejection [174]. It was reported that the inhibition of CD26/DPPIV promotes the secretion of TGF-β1, which is essential for the differentiation of Tregs [175]. For the development and function of $CD4^{+}CD25^{+}$ regulatory T cells, forkhead transcription factor (Foxp3) is required [55, 176]. IL-10 is reported to be an important factor in maintaining FoxP3 expression [177]. The higher level of IL-10 in $CD26^{-/-}$ mice (Figure 4.18G) may benefit the expression of FoxP3. The reduced Th17 activity and increased Tregs´ percentage in MSLs and MPBLs of $CD26^{-/-}$ mice after allogeneic transplantation may be the most important reason for the reduced necrosis of the graft and delayed allograft rejection in $CD26^{-/-}$ mice.

5.2.6 Influence of CD26-deficiency on the activity of macrophage after allogeneic skin transplantation

Macrophages are prominent inflammatory cells, having many functions in promoting inflammation, inducing tissues damage and secreting inflammatory mediators in organ transplantation [119]. They play an important role in both cellular and antibody-mediated rejection. Some studies showed that the depletion of macrophages significantly attenuated

graft damage during the acute allograft rejection [178]. Additionally, the intravascular macrophage accumulated in cardiac allograft undergoing antibody-mediated rejection [179]. In porcine skin transplantation model, macrophages were one of the most important effector cells infiltrated in the allogenic grafts [106]. Cytokines are the main factors for the activation of macrophages; some of them such as IL-2 and IFN-γ induce the infiltration of macrophages into grafts in allogeneic transplantation [112, 165]. In the present study, the infiltration number of macrophages ($CD14^+$ cells) in the grafts of CD26-deficiency mice (Figure 4.22) was much less than in $CD26^{+/+}$ mice. The lower levels of Th1 cytokines IFN-γ and IL-2 in $CD26^{-/-}$ mice might reduce the activation of macrophages after allogeneic transplantation leading to their reduced infiltration into grafts (Figure 4.22).

5.2.7 The role of CD26 in the wound healing

CD26 is a multifunctional protein with its enzymatic activity as well as by its interaction with different molecules. Some recent studies reported an involvement of CD26/DPPIV in the cutaneous wound healing. A better rate of wound closure, revascularization and cell proliferation were revealed in $CD26^{-/-}$ mice and DPPIV inhibitor showed a potential benefit in the process of wound healing [180]. It was found that CD26/DPPIV gene knockout mice had fewer $CD4^+$ cells, IgD and $CD21^+$ intrahepatic lymphocytes in carbon tetrachloride (CCL4)-induced liver fibrosis [181]. This suggested a pro-fibrotic role of DPPIV which involved in energy metabolism. Another study also found the inhibition of DPPIV induced diminishing cutaneous scarring [182]. In case of skin transplantation, CD26 is involved not only in immune rejection but also in the wound healing process, playing a Janus role between engraftment and rejection [183]. In the present work, the necrotic levels of grafts, the values of IgG and related cytokines in serum of $CD26^{-/-}$ mice were markedly lower than those of $CD26^{+/+}$ mice. However, from 13 days post-transplantation, it was observed that the wound healed quickly and the grafts were removed in $CD26^{-/-}$ mice. The reduction of allograft rejection in $CD26^{-/-}$ mice as observed in the present work may counteract partly by the enhancement of wound healing, in which both processes CD26 involved.

In conclusion, the results of the present work indicate an involvement of CD26 in the allogeneic graft rejection. The deficiency of CD26 resulted in a partially impaired

differentiation of Th1, Th2 and Th17 subpopulations, but increased the percentage of Tregs. The reduced functions of Th1, Th2 and Th17 in turn affected the differentiation of B cells and decreased activity of $CD8^+$ cells and macrophages, leading to the lower productions of IgGs and delayed allograft rejection in $CD26^{-/-}$ mice.

5.3 Outlook

CD26 plays an important role in differentiation and function of T lymphocytes. In the present work *in vitro*, it was found that CD26 is associated with Th1 and Th17 differentiation and influences on the expression of adhesion molecules. However, the role of CD26 in the activation, differentiation and functions of B lymphocytes and other immune cells need further investigation. *In vivo* obtained from the current work demonstrated that the deficiency of CD26 could result in delayed allograft rejection in allogeneic skin transplantation. The underlying mechanisms to be further investigated and elucidated should include: the activation and differentiation of B cells and macrophages after allogeneic transplantation in $CD26^{-/-}$ mice, the influence of CD26-deficient on activities of some chemokines as well as on the infiltration of macrophages and neutrophils, and the mechanism that drives Tregs in $CD26^{-/-}$ mice. Furthermore, in view that CD26 plays important roles in both skin transplantation and wound healing, the extent to which wound healing influences graft survival/rejection in $CD26^{-/-}$ mice after allogeneic skin transplantation should also be clarified.

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7. Appendix

7.1 Summary

CD26, also known as dipeptidyl peptidase IV (DPPIV) is a multifunctional protein and is involved in many biological processes. As a co-stimulator of T cell activation, CD26 plays an important role in the immune system, especially in T cell activation and differentiation. However, the role of CD26 in the differentiation of new paradigms of T cells, such as Th17, is not fully clarified. Moreover, CD26 is associated with many diseases and the mechanisms of CD26 in some of these diseases such as in immune rejection of organ transplantation have not been fully elucidated.

In the present study, the role of CD26 in T cell activation and differentiation was investigated *in vitro*. CD26 expression was analyzed in the different subsets of human peripheral blood T lymphocytes after antigen stimulation. It was found that the percentage of $CD4^+$ cells, $CD26^+CD4^+$ cells and $CD26^+CD8^+$ cells were all increased after stimulation. After magnetic cell sorting of CD26-expressing cells, two groups of cells were obtained; CD26 low-expressing group ($CD26^{low}$) and CD26 high-expressing group ($CD26^{high}$). The percentage of $CD4^+$ cells was found to be higher while the percentage of $CD8^+$ cells was lower in $CD26^{high}$ group in comparison to $CD26^{low}$ group. However, the percentages of $CD4^+CD26^+$ cells and $CD8^+CD26^+$ cells were both significantly higher in $CD26^{high}$ group. Furthermore, the percentages of cells secreting Th1-typical cytokines (IL-2 or IFN-γ), or Th17-typical cytokines (IL-6, IL-17, or IL-22), or expressing Th17-typical biomarkers (IL-23R, CD161, or CD196) were significantly higher in the $CD26^{high}$ group. Besides, a co-expression of CD26 with IL-2, IFN-γ, IL-17, IL-22, or IL-23R in lymphocytes by fluorescence microscopy was demonstrated. Moreover, the co-expression percentages of $CD26^+$ cells with IL-2, IFN-γ, IL-6, IL-17, IL-22, or IL-23R were significantly higher in the $CD26^{high}$ group than that in the $CD26^{low}$ group, respectively. These data indicate that the high expression of CD26 correlates with the differentiation of Th1- and Th17-subsets.

In order to investigate the potential role of CD26/DPPIV in organ transplantation, the animal model of allogeneic tail-skin transplantation was established using CD26-deficient

mice. After allogeneic tail-skin transplantation, in comparison to wild-type ($CD26^{+/+}$) mice, $CD26^{-/-}$ mice showed reduced necrosis of grafts and delayed graft rejection. The production levels of IgG, including its subclasses IgG1 and IgG2a were much lower in the serum of $CD26^{-/-}$ mice. Moreover, the secretion levels of cytokines IFN-γ, IL-2, IL-6, IL-4, and IL-13 were significantly lower while the level of the cytokine IL-10 was higher in the serum of $CD26^{-/-}$ mice than in $CD26^{+/+}$ mice. In addition, the concentration of IL-17 in serum and the percentage of cells secreting IL-17 in MPBLs were both significantly lower, while the percentage of regulatory T cells (Tregs) in MPBLs was significantly higher in $CD26^{-/-}$ mice than in $CD26^{+/+}$ mice. Furthermore, a lower percentage of $CD8^{+}$ cells in mouse peripheral blood lymphocytes (MPBLs) was found after skin transplantation. Less infiltrated macrophages and T cells were detected in the graft tissues of $CD26^{-/-}$ mice during graft rejection. These results indicate that CD26 is involved in allogeneic skin graft rejection and suggests a potential role of CD26-deficiency in repressing the immune rejection in clinical organ transplantation.

7.2 Zusammenfassung

CD26, auch bekannt als Dipeptidylpeptidase 4 (DPPIV), ist ein multifunktionales Protein. Als ein Co-Stimulator der T-Zell-Aktivierung spielt CD26 eine wichtige Rolle im Immunsystem, besonders bei der Aktivierung und Differenzierung der T-Lymphozyten. Der Einfluss von CD26 auf die Differenzierung neuer T-Zell-Typen, wie beispielsweise Th17, ist noch nicht vollständig geklärt. Außerdem steht CD26 mit vielen Erkrankungen in Verbindung, und die Mechanismen von CD26 bei einigen dieser Erkrankungen, wie bei der Immunabstoßung nach Organtransplantationen, sind nicht vollständig geklärt worden.

In der vorliegenden Arbeit wurde die Rolle von CD26 bei der T-Zell-Aktivierung und Differenzierung *in vitro* untersucht. Hierfür wurde die CD26-Expression in verschiedenen humanen T-Zell-Subpopulationen von peripheren Blut-T-Lymphozyten nach der Antigenstimulation analysiert. Es konnte gezeigt werden, dass die prozentualen Anteile von $CD4^+$, $CD26^+CD4^+$ und $CD26^+CD8^+$ Zellen nach der Antigenstimulation jeweils gestiegen waren. Bei der magnetischen Zellsortierung von CD26-exprimierenden Zellen fanden sich zwei Gruppen: eine CD26 niedrig exprimierende Gruppe ($CD26^{low}$) und eine CD26 hoch exprimierende Gruppe ($CD26^{high}$). Dabei war der Anteil an $CD4^+$ Zellen höher und der Anteil der $CD8^+$ Zellen niedriger in der $CD26^{high}$ Gruppe, verglichen mit der $CD26^{low}$ Gruppe. Jedoch war der Anteil sowohl von $CD4^+CD26^+$ als auch $CD8^+CD26^+$ Zellen signifikant größer in der $CD26^{high}$ Gruppe. Außerdem zeigte sich, dass die Anteile der Zellen, die Th1-typische Zytokine (IL-2 oder IFN-γ) oder Th17-typische Zytokine (IL-6, IL-17 oder IL-22) sezernieren, sowie Th17-typische Biomarker (IL-23R, CD161 oder CD196) exprimieren, signifikant höher in der $CD26^{high}$ Gruppe waren. Zudem wurde eine Ko-Expression von CD26 mit IL-2, IFN-γ, IL-17, IL-22 oder IL-23R in Lymphozyten durch Fluoreszenzmikroskopie nachgewiesen. Darüber hinaus waren die prozentualen Anteile von $CD26^+$ Zellen mit der Expression von IL-2, IFN-γ, IL-6, IL-17, IL-22 oder IL-23 jeweils signifikant höher in der $CD26^{high}$ Gruppe als in der $CD26^{low}$ Gruppe. Diese Daten zeigen, dass die starke Expression von CD26 mit der Differenzierung der Th1- und Th17 Untergruppen korreliert ist.

Um die potentielle Rolle von CD26/DPPIV bei Organtransplantationen zu untersuchen, wurde das Tiermodel allogener Schwanzhaut-Transplantationen bei CD26-defizienten Mäusen entwickelt. Nach einer allogenen Transplantation der Schwanzhaut zeigten $CD26^{-/-}$ Mäuse eine im Vergleich zum Wildtyp ($CD26^{+/+}$) reduzierte Nekrose der Transplantate und eine verzögerte Transplantatabstoßung. Die gebildete Menge von IgG, einschließlich der Subklassen IgG1 und IgG2, war im Serum bei $CD26^{-/-}$ Mäusen deutlich geringer. Gleichzeitig konnte gezeigt werden, dass im Serum der $CD26^{-/-}$ Mäuse die Sekretionsniveaus der Zytokine IFN-γ, IL-2, IL-6, IL-4 und IL-13 signifikant niedriger waren, und die Konzentration des Zytokines IL-10 höher war als bei $CD26^{+/+}$ Mäusen. Außerdem war die Konzentration von IL-17 im Serum und der prozentuale Anteil von IL-17 sezernierenden Zellen in den peripheren Blut-Lymphozyten der Mäuse (MPBLs) signifikant niedriger bei gleichzeitig signifikant höherem prozentualen Anteil der regulatorischen T-Zellen (Tregs) bei $CD26^{-/-}$ Mäusen. Weiterhin wurde ein geringerer prozentualer Gehalt von $CD8^{+}$ Zellen in MPBLs nach der Hauttransplantation gefunden. Es wurden weniger infiltrierte Makrophagen und T-Zellen in den Transplantatgeweben von $CD26^{-/-}$ Mäusen während der Transplantatabstoßung nachgewiesen. Diese Ergebnisse zeigen, dass CD26 an der allogenen Hauttransplantats-Abstoßung beteiligt ist, und legen eine mögliche Rolle einer CD26-Defizienz zur Unterdrückung der Immunabstoßung bei klinischen Organtransplantationen nahe.

8. Abbreviations

ADA	Adenosine deaminase
BSA	Bovine serum albumin
CD	Cluster differentiation
CTL	Cytotoxic T lymphocytes
DPPIV	Dipeptidyl peptidase IV
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
TAE	Tris base, acetic acid and EDTA
IFN	Interferon
Ig	Immune globulin
IL	Interleukin
kD	Kilo dalton
mAb	Monoclonal antibody
MACS	Magnetic cell sorting
MPBL	Mouse peripheral blood lymphocytes
MSL	Mouse spleen lymphocytes
NK	Natural killer cells
PBS	Phosphate buffered saline
rpm	rotations per minute

9. Publications and manuscripts under preparation

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3. Kai Zhang, Yanfang Liu, **Xiangli Zhao,** Qingjiu Tang, Jens Dernedde1, Jingsong Zhang, Hua Fan. Anti-inflammatory properties of GLPss58, a sulfated polysaccharide from Ganoderma lucidum. Int J Biol Macromol 2017. ***IF=3.096***

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✦ **Xiangli Zhao,** Wenhan Wang, Kai Zhang, Jingya Yang, Hua Fan. **Involvement of CD26 in the differentiation of Th1- and Th17- subpopulations of T lymphocytes.** *under preparation*

10. Curriculum Vitae

For reasons of data protection, the curriculum vitae is not published in the electronic version.

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