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A novel role for CD26/dipeptidyl peptidase IV as a therapeutic target

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1. ABSTRACT

CD26 is a 110 kDa surface glycoprotein with intrinsic dipeptidyl peptidase IV activity that is expressed on numerous cell types and has a multitude of biological functions. The role of CD26 in immune regulation has been extensively characterized, with recent findings elucidating its linkage with signaling pathways and structures involved in T-lymphocyte activation as well as antigen presenting cell-T-cell interaction. In this paper, we will review emerging data on CD26-mediated immune regulation suggesting that CD26 may be an appropriate therapeutic target for the treatment of selected immune disorders as well as Middle East respiratory syndrome coronavirus. Moreover, we have had a long-standing interest in the role of CD26 in cancer biology and its suitability as a novel therapeutic target in selected neoplasms. We reported robust *in vivo* data on the anti-tumor activity of anti-CD26 monoclonal antibody in mouse xenograft models. We herein review significant novel findings and the early clinical development of a CD26-targeted therapy in selected immune disorders and cancers, advances that can lead to a more hopeful future for patients with these intractable diseases.

2. INTRODUCTION

CD26 is a 110-kDa cell surface glycoprotein with known dipeptidyl peptidase IV (DPPIV, EC3.4.1.4.5) activity in its extracellular domain (1-3), capable of cleaving amino-terminal dipeptides with either L-proline or L-alanine at the penultimate position (3). CD26 activity is dependent on the expressing cell type and the microenvironment which influence its multiple biological roles (4-7). CD26 plays an important role in immunology, autoimmunity, diabetes and cancer (8-12). Interacting directly with various other cell surface and intracellular molecules, CD26 can regulate receptor specificity and the function of various interleukins (ILs), cytokines and chemokines via its DPPIV activity (13).

In this review, we summarize our recent work on CD26/DPPIV that elucidated its suitability as a potential therapeutic target in selected immune diseases and cancers. We also discuss our current knowledge of the molecular mechanisms of CD26/DPPIV-mediated T-cell regulation, focusing particularly on CD26/DPPIV role in immune checkpoint pathways and programs associated with human immune regulation. In addition, we describe CD26/DPPIV involvement in cancer immunology.

3. IMMUNE MEDIATED DISORDERS

3.1. Chronic graft-versus-host disease

3.1.1. T cell costimulation in chronic graft-versus-host disease

Graft-versus-host disease (GVHD) is a severe complication and major cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation (alloHSCT) (14). Based on differences in clinical manifestations and histopathology, GVHD can be divided into acute and chronic forms (14). Acute GVHD (aGVHD) and chronic GVHD (cGVHD) are traditionally diagnosed primarily by time of onset, with cGVHD occurring after day 100 of transplantation (15). However, cGVHD has distinct clinicopathologic features and is often diagnosed based on these features regardless of time of onset, being characterized by cutaneous fibrosis, involvement of exocrine glands, hepatic disease, and obliterative bronchiolitis (OB) (16, 17). OB, characterized by airway blockade, peribronchiolar and perivascular lympho-fibroproliferation and obliteration of bronchioles, is a late-stage complication of cGVHD (18). Patients diagnosed with OB have a 5-year survival rate of only 10 to 40%, compared to more than 80% of patients without OB (19, 20). Furthermore, while multiple strategies to control cGVHD involving T cell depletion from the graft or global immunosuppression have been developed, cGVHD is still a common clinical outcome in many alloHSCT patients (17, 21). In addition, immunosuppression potentially abrogates the graft-versus-leukemia (GVL) effect, associated with increased relapses following alloHSCT (22). Novel therapeutic approaches are thus needed to prevent cGVHD without eliminating the GVL effect.

GVHD is initiated when donor-derived T cells are primed by professional antigen presenting cells (APCs) to undergo clonal expansion and maturation (14). Costimulatory pathways are required to induce T cell proliferation, cytokine secretion and effector function following antigen-mediated T cell receptor activation (23), and the important role of costimulatory pathways in transplant biology has been established (24). CD26 is associated with T cell signal transduction processes as a costimulatory molecule, as well as being a marker of T cell activation (1, 25, 26). We previously showed that CD26-mediated costimulation in human CD4 T cells exerts an effect on production of T_H1 type proinflammatory cytokines such as interferon (IFN)-γ (6). Moreover, $CD26^{high}$CD4 T cells respond maximally to recall antigens with a high competence for trafficking to inflammatory tissues and for antibody synthesis by B cells (6, 26). We also showed that CD26-caveolin-1 interaction leads to activation of both CD4 T cells and APCs (27-29). More recently, we demonstrated in *in vitro* experiments that blockade of CD26-mediated T cell costimulation by soluble Fc fusion proteins containing the N-terminal domain of caveolin-1 (Cav-Ig) diminished primary and secondary proliferative responses not only to recall antigen, but also to unrelated allogeneic APC (30). Other investigators recently reported that $CD26^{high}$ T cells contain T_H17 cells, and that $CD26^{high}$ T_H17 cells are enriched in inflamed tissues including rheumatoid arthritis (RA) and inflammatory bowel diseases (IBD) (31). These accumulating data strongly suggest that CD26-mediated costimulation plays an important role in memory response to recall antigens, and that blockade of CD26 costimulation may be an effective therapeutic strategy for immune disorders including GVHD or autoimmune diseases.

3.1.2. Newly established humanized murine model of cGVHD

We previously analyzed a humanized murine aGVHD model involving mice transplanted with human adult peripheral blood lymphocytes (PBL), and showed that liver and skin were predominantly involved as target organs in this model of aGVHD, which was clearly impeded by the administration of humanized anti-CD26 monoclonal antibody (mAb) (32). Our data suggest that $CD26^+$ T cells play an effector role in this aGVHD model. However, since the mice studied in our previous work succumbed to aGVHD around 4 weeks after transplantation of human adult PBL, this early-onset model of aGVHD does not permit the assessment of longer term consequences of interventional therapies, such as their effect on the development of OB, a form of cGVHD of the lung.

In contrast to adult PBL, human umbilical cord blood (HuCB) lymphocytes have been reported to be immature, predominantly consisting of $CD45RA^+$ naïve cells (33, 34). We previously showed that, while all HuCB CD4 T cells constitutively express CD26, CD26-mediated costimulation was considerably attenuated in HuCB CD4 T cells, compared to the robust activation via CD26 costimulation of adult PBL (34). These findings provided further insights into the cellular mechanisms of immature immune response in HuCB. Based on these findings, we hypothesized that HuCB naïve CD4 T cells gradually acquire a xenogeneic response via attenuated stimulatory

signaling with indolent inflammation in the target organs, leading eventually to chronic inflammatory changes. We therefore sought to develop a humanized murine pulmonary cGVHD model utilizing HuCB donor cells, and to overcome the limitations seen in the humanized murine aGVHD model such as vigorous activation of all engrafted T cells and extensive loss of B cell maturation and activation (35, 36).

We first attempted to establish a humanized murine model utilizing NOD/Shi-*scid*IL2rγ^{null} (NOG) mice as recipients and HuCB as donor cells (37). Whole CB transplant mice exhibited clinical signs/symptoms of GVHD as early as 4 weeks post-transplant, and demonstrated significantly decreased survival rate. The lung of whole CB transplant mice showed perivascular and subepithelial inflammation and fibrotic narrowing of the bronchiole. Skin of whole CB transplant mice manifested fat loss, follicular drop-out and sclerosis of the reticular dermis in the presence of apoptosis of the basilar keratinocytes while the liver exhibited portal fibrosis and cholestasis. These findings indicate that whole CB transplant mice develop pulmonary cGVDH as well as concomitant active GVHD in skin and liver. Taken together, our data demonstrate that the lung of whole CB transplant mice exhibits OB as manifestation of pulmonary cGVHD.

3.1.3. IL-26 contributes to the pathophysiology of pulmonary cGVHD

To determine the potential cellular mechanisms involved in the pathogenesis of pulmonary cGVHD, we next analyzed the composition of donor-derived human lymphocytes in the GVHD lung. Utilizing flow cytometric analysis for cell suspension isolated from the lung specimens, donor-derived human CD3$^+$ cells were found to be the predominant cell type observed in the lung of whole CB transplant mice, comprising more than 99% of the lymphocyte population. Moreover, the human CD4 T cell subset was observed to be the predominant cell type compared to CD8 T cells in the lung of whole CB transplant mice. We next analyzed the expression profile of mRNAs of various inflammatory cytokines in human CD4 T cells isolated from the lung of whole CB transplant mice. We found that *IFNG*, *IL17A, IL21* and *IL26* were significantly increased over the course of GVHD development following whole CB transplantation, while *IL2*, *TNF* (TNF-α), *IL4*, *IL6* and *IL10* were decreased. In addition, substantial increases were seen in levels of *IFNG* and *IL26*, with *IL17A* and *IL21* remained at a low level. It has been reported that IFN-γ is produced by T_H1 cells (6), while IL-17A and IL-26 are produced by T_H17 cells (38, 39). Since both T_H1 and T_H17 cells strongly express CD26 (6, 31), we next analyzed the expression level of CD26/DPP4, finding that *DPP4* mRNA expression in human CD4 T cells infiltrating in the lung of mice with OB was significantly increased. These findings regarding mRNA expression levels were further supported by enzyme-linked immunosorbent assay (ELISA) studies examining protein levels in sera of recipient mice. To determine whether these cytokines were produced by the infiltrating human CD26$^+$CD4 T cells, we next conducted flow cytometric analyses of lymphocytes isolated from the lung of the recipient mice. Levels of human IFN-γ^+ or IL-26$^+$CD26$^+$CD4 T cells were significantly increased in whole CB transplant mice. Multicolor-staining flow cytometric studies showed that CD26$^+$CD4 T cells in the lung of whole CB transplant mice predominantly produced IL-26 rather than IFN-γ. In addition, while CD26$^+$IFN-γ^+CD4 cells exclusively expressed IL-26, CD26$^+$IL-26$^+$CD4 cells were predominantly IFN-γ-negative cells, and IL-17A$^+$ cells were exclusively IL-26-negative. These data suggest that CD26$^+$CD4 T cells in the lung of mice with OB express IL-26 as well as IFN-γ but do not belong to the T_H17 cell population.

To further extend the above *in vitro* results to an *in vivo* system, we analyzed the lung of murine alloreactive GVHD using human *IL26* transgenic (Tg) mice. For this purpose, we used mice carrying human *IFNG* and *IL26* transgene (190-*IFNG* Tg mice) or mice carrying human *IFNG* transgene with deleting *IL26* transcription (ΔCNS-77 Tg mice). 190-*IFNG* Tg mice exhibited production of IL-26 by CD4 T cells under T_H1- or T_H17-polarizing conditions, while expression of IL-26 was completely abrogated in ΔCNS-77 Tg mice (38). In addition, production of IFN-γ by T or NK cells was equivalent in both 190-*IFNG* Tg and ΔCNS-77 Tg mice (40). Histologic examinations of the lung of recipient NOG mice deriving from parental C57BL/6 (B6 WT) mice or ΔCNS-77 Tg mice showed peribronchial infiltration and cuffing denoting GVHD, while collagen deposits were not detected by Mallory staining, and IL-26$^+$ cells were not detected. On the other hand, the lung of recipient NOG mice deriving from 190-*IFNG* Tg mice showed peribronchial infiltration and cuffing denoting GVHD with collagen deposition and IL-26$^+$ cell infiltration. These results suggest that human IL-26, but not human IFN-γ, plays a critical role in pulmonary fibrosis associated with lung cGVHD.

3.1.4. IL-26 production via CD26-mediated T cell costimulation

To test whether human CD4 T cells produce IL-26 following CD26 costimulation, we conducted *in vitro* costimulation experiments using HuCB CD4 T cells and analyzed expression of various inflammatory cytokines. We found that levels of *IL26* and *DPP4* were significantly increased following CD26 costimulation compared with CD28 costimulation. We next conducted costimulation experiments evaluating dose and time kinetics using the CD26 costimulatory ligand Cav-Ig as well as anti-CD26 or anti-CD28 mAbs. We showed that production of IL-26 was increased

following CD26 costimulation with Cav-Ig or anti-CD26 mAb in dose- and time-dependent manners, while a slight increase in IL-26 level was observed following CD28 costimulation only at higher doses of mAb and longer stimulation periods. Blocking experiments were then performed for further confirmation, showing that IL-26 production induced by Cav-Ig or anti-CD26 mAb was clearly inhibited by treatment with soluble Cav-Ig in a dose-dependent manner, while no change was observed with CD28 costimulation. These findings strongly suggest that production of IL-26 by HuCB CD4 T cells is regulated via CD26-mediated costimulation. Moreover, since the functional sequences of the N-terminal of caveolin-1 are highly conserved between human and mouse (41) allowing for the capability to bind human CD26 as a costimulatory ligand, it is conceivable that donor HuCB T cells transferred into mice were activated via CD26 costimulation triggered by murine caveolin-1. In fact, using polyclonal antibody recognizing the N-terminal of both human and murine caveolin-1, expression of caveolin-1 was detected in endothelial cells and macrophage-like cells of OB-like lesions in cGVHD lung. Taken together, CD26-mediated IL-26 production triggered by caveolin-1 is identified as a possible therapeutic target in cGVHD using HuCB NOG mice.

3.1.5. Prevention of lung cGVHD development by Cav-Ig administration

Given the role of CD26 costimulation in IL-26 production and IL-26 regulation of collagen production, we therefore sought to determine whether disruption of CD26 costimulation by a blocking reagent, Cav-Ig, prolonged survival of the recipient mice associated with a reduction in the incidence of OB. Recipients treated with Cav-Ig survived for 7 months without any clinical findings of cGVHD. Meanwhile, the survival rate of recipient mice treated with control Ig was significantly reduced, with clinical signs/symptoms of cGVHD. Human cells were engrafted similarly in both groups. Histologic examinations of the lung showed the development of OB in the control Ig cohort, while the lung of Cav-Ig recipient mice displayed normal appearances with none having positive pathology scores. These effects of Cav-Ig were also observed in other GVHD-target organs such as the skin and liver. Moreover, collagen contents in the lung were reduced in Cav-Ig administered-recipients. Taken together, the above results support the notion that Cav-Ig administration prevents the development of pulmonary cGVHD in whole CB transplant mice by decreasing the number of IL-26^{+}CD26^{+}CD4 T cells.

3.1.6. Treatment with Cav-Ig preserves GVL capability

Since GVHD and GVL effect are highly linked immune reactions (42), we evaluated the potential influence of Cav-Ig treatment on GVL effect. For this purpose, cohorts of Cav-Ig or control Ig treated recipient mice of whole CB transplant were irradiated at sublethal doses and then injected intravenously with luciferase-transfected A20 (A20-luc) cells 1 day prior to whole CB transplantation to allow for dissemination of tumor cells. The next day following transplantation, treatment with Cav-Ig or control Ig thrice a week began on day +1 until day +28. Mice inoculated with A20 cells alone all died of tumor progression within 6 weeks. Recipients treated with control Ig exhibited clinical evidence of GVHD such as weight loss and ruffled fur and died of GVHD without tumor progression in 13 weeks. In contrast, recipient mice treated with Cav-Ig displayed significantly prolonged survival without involvement of A20-luc cells. To better characterize the potency of the GVL effect, we repeated these studies with injection of A20-luc cells on day +28 after whole CB transplantation to allow for acquisition of immunosuppression by Cav-Ig treatment. Mice inoculated with A20 cells alone all died of tumor progression within 2 weeks after tumor inoculation. Recipient mice treated with control Ig demonstrated clinical evidence of GVHD such as weight loss and ruffled fur and died of GVHD without tumor progression within 13 weeks after transplantation. In contrast, recipients treated with Cav-Ig exhibited significantly prolonged survival without involvement of A20-luc cells. Collectively, these results demonstrate that Cav-Ig treatment of recipient mice of whole CB transplant was effective in reducing the symptoms of cGVHD without a concomitant loss of the GVL effect.

3.1.7. Role of CD26 in cGVHD

While the human CD26 amino acid (AA) sequence has 85% AA identity with the mouse CD26 (43), the mouse CD26 has different biologic properties from the human CD26, including the fact that the mouse CD26 is not a T cell activation marker, and does not bind to adenosine deaminase (ADA) (43, 44). Therefore, humanized murine models need to be developed to explore the role of CD26-mediated costimulation in cGVHD. With relevance as a costimulatory ligand for human CD26, human caveolin-1 has 95% AA identity with the mouse caveolin-1 (41), and the binding regions of the mouse caveolin-1 for human CD26 are well conserved. Therefore, costimulatory activation of human T cells in NOG mice can occur via CD26-caveolin-1 interaction. Moreover, the N-terminal domain is present in the outer cell surface during the antigen presenting process (27), and caveolin-1 forms homo-dimer or homo-oligomer via its N-terminal domain (41). These collective data suggest that the administered Cav-Ig binds to the N-terminal of caveolin-1 on the cell surface of APCs as well as to CD26 in T cells, leading to suppression of cGVHD in HuCB-NOG mice via blockade of CD26-caveolin-1 interaction. Conclusively, our work

demonstrates that caveolin-1 blockade controls cGVHD by suppressing the immune functions of donor-derived T cells and decreasing IL-26 production. Moreover, IL-26$^+$CD26$^+$CD4 T cell infiltration appears to play a significant role in cGVHD of the lung and skin. While complete suppression of cGVHD with current interventional strategies represents a difficult challenge at the present time, our data demonstrate that control of cGVHD clinical findings can be achieved in a murine experimental system by regulating IL-26$^+$CD26$^+$CD4 T cells with Cav-Ig. Our work also suggests that Cav-Ig treatment may be a novel therapeutic approach for chronic inflammatory diseases, including RA and IBD, in which IL-26 plays an important role.

3.2. Middle East respiratory syndrome coronavirus

3.2.1. Current efforts against Middle East respiratory syndrome coronavirus

Middle East respiratory syndrome coronavirus (MERS-CoV) was first identified in a 60-year-old patient in June 2012 who presented with acute pneumonia, followed by acute respiratory distress syndrome and renal failure with a fatal outcome (45). Between 2012 and August 28, 2017, 2067 laboratory-confirmed cases of MERS-CoV infection were reported to the World Health Organization (WHO), which has notified of at least 720 deaths (around 35% fatality rate) related to MERS-CoV since September 2012 (46). Efforts to develop effective preventive and therapeutic intervention strategies are currently ongoing. Several treatment modalities have been investigated to develop effective therapies against MERS-CoV, including interferon, ribavirin, cyclosporin A, protease inhibitors, convalescent plasma and immunoglobulins. Several promising anti-MERS-CoV therapeutic agents have recently been reviewed extensively (47), while broad spectrum antiviral agents might not be sufficient to treat severe MERS-CoV patients because of its limited effective therapeutic window of opportunity (48).

An alternative approach using prophylactic regimens would be theoretically suitable to limit the spread of MERS-CoV. This scenario includes MERS-CoV vaccine and neutralizing MERS-CoV-specific mAb (48). The MERS-CoV genome encodes for 16 non-structural proteins (nsp1-16) and 4 structural proteins, the spike (S), envelope (E), membrane (M), and nucleocapsid (N) (49). The viral structural proteins, S and N, show the highest immunogenicity (50). While both S and N proteins induce T-cell responses, neutralizing antibodies are almost solely directed against the S protein, with the receptor binding domain (RBD) being the major immunodominant region (51). These great challenges have been extensively reviewed in previously published papers (48, 52).

Recent reports indicated that the spike protein S1 of MERS-CoV is required for viral entry into human host cells (53-55), using CD26/DPPIV as a functional receptor (56). Inhibiting virus entry into host cells could also be achieved by targeting the host receptor CD26/DPPIV. While inhibitors of the CD26/DPPIV enzymatic activity are used clinically to treat type 2 diabetes patients, commercially available DPPIV inhibitors would not serve this purpose since these agents have been shown not to inhibit binding of the RBD of MERS-CoV to CD26/DPPIV (57). We previously showed that human CD26 is a binding protein for ADA (58). Currently, it is known that there are two isoforms of ADA, ADA1 and ADA2 (59). ADA1 is particularly present in lymphocytes and macrophages, while ADA2 is found predominantly in the serum and other body fluids including pleural effusion (59). CD26/DPPIV binds to ADA1, but not ADA2 (58, 60, 61). The crystal structure of CD26/DPPIV and the S protein of MERS-CoV allowed for visualization of the interacting AA in both proteins. However, our *in vitro* experiments showed that blockade of MERS-CoV binding to CD26/DPPIV by ADA1 is incomplete (62). Therefore, mAbs blocking CD26/DPPIV binding to the RBD of MERS-CoV needs to be developed.

3.2.2. CD26/DPPIV is a functional receptor for MERS-CoV entry into host cells

CD26/DPPIV, a host receptor for MERS-CoV, is conserved among different species such as bats and humans, partially explaining the large host range of MERS-CoV (63). In addition to being widely expressed in most cell types including T lymphocytes, bronchial mucosa or the brush border of proximal tubules, CD26/DPPIV exists in systemic circulation as soluble form (13). This distribution of CD26 may play a role in the systemic dissemination of MERS-CoV infection in human (64-66). Therefore, an effective therapy for MERS-CoV is needed not only to block the entry of MERS-CoV into CD26-expressing organs such as the respiratory system, kidney, liver or intestine, but also to eliminate circulating MERS-CoV. In this regard, manipulation of CD26/DPPIV levels or the development of inhibitors that target the interaction between the MERS-CoV S1 domain and its receptor may provide therapeutic opportunities to combat MERS-CoV infection. More recently, the RBD in the S protein was mapped to a 231-AA fragment of MERS-CoV S proteins (residues 358-588) (51).

3.2.3. Identification of specific anti-CD26 mAb clone for blocking MERS-CoV

We have recently mapped MERS-CoV S protein-binding regions in human CD26 molecule and demonstrated that anti-CD26 mAbs, which had been developed in our laboratory, effectively blocked the interaction between the spike protein and

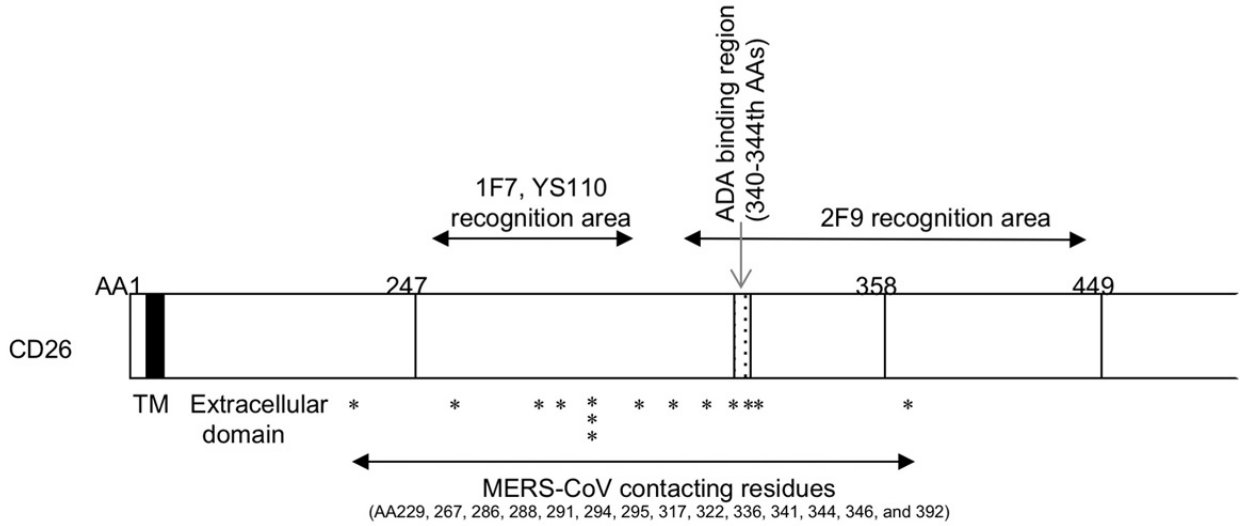

Figure 1. Schematic diagram of human CD26 profiling the predicted contacting areas of anti-CD26 mAbs 2F9, 1F7, YS110 and MERS-CoV S1. 2F9 recognizes between 248-449th AAs including the ADA binding regions, and 1F7 or YS110 recognize between 248-358th AAs excluding the ADA binding regions. MERS-CoV contacting residues of human CD26 are indicated in stars, with available information obtained from recently published data (55, 69). TM indicates the transmembrane region of human CD26 (black box), and the extracellular domain of CD26 is located at the C-terminal residues of TM. This Figure is reprinted with permission from K Ohnuma *et al.*, *J Virol* 87: 13892-13899, 2013 (62).

CD26, thereby neutralizing MERS-CoV infectivity. To determine the specific CD26 domain involved in MERS-CoV infection, we chose six different clones of anti-CD26 mAbs (4G8, 1F7, 2F9, 16D4B, 9C11, 14D10), and the humanized anti-CD26 mAb YS110, which recognize 6 distinct epitopes of the CD26 molecule (67, 68), to conduct MERS-CoV S1-Fc (S1 domain of MERS-CoV fused to the Fc region of human IgG) binding inhibition assays (62). 2F9 inhibited fully binding of MERS-CoV S1-Fc to JKT-hCD26WT (Jurkat cells transfected with full-length human CD26/DPPIV), while other anti-CD26 mAbs demonstrated certain levels of inhibition (1F7, or YS110) or no significant inhibition (4G8, 16D4B, 9C11 or 14D10). These results strongly suggest that the anti-CD26 mAb 2F9 has better therapeutic potential than recombinant MERS-CoV S1-Fc in preventing viral entry into susceptible cells, and that 1F7 or YS110 also block MERS-CoV infection.

Moreover, we have characterized the CD26 epitope involved in MERS-CoV S1-Fc binding to CD26 through the use of various CD26 mutants with deletion in the C-terminal extracellular region, since CD26 is a type II transmembrane protein (9, 29). Our biological experiments on binding regions of CD26 to MERS-CoV using mAbs showed results comparable to those obtained from crystal structure analysis (55, 69), which are summarized in a schematic diagram of human CD26 at 1-449th AAs (Figure 1). Our observations strongly suggest that the main binding regions of CD26 to MERS-CoV appear to be close to the 358th AAs recognized by 2F9, and the regions of CD26 defined by 1F7 and YS110 (248-358th AAs) are also partially involved in MERS-CoV binding.

To determine whether treatment with anti-CD26 mAbs 2F9 as well as 1F7 and YS110 could inhibit MERS-CoV infection, susceptible cells, Huh-7, were pre-incubated with various anti-CD26 mAbs prior to inoculation with the virus (62). In this experimental system, infection was almost completely blocked by 2F9 but not by control IgG or several other anti-CD26 mAbs recognizing other epitopes (4G8, 16D4B, 14D10 or 9C11). Moreover, the anti-CD26 mAbs 1F7 and YS110 considerably inhibited MERS-CoV infection of Huh-7 cells. These results demonstrate that 2F9 inhibits MERS-CoV entry, and therefore can potentially be developed as a preventive or therapeutic agent for MERS-CoV infection in the clinical setting. More importantly, the humanized anti-CD26 mAb YS110 has been evaluated in patients with CD26-expressing cancers in our recent first-in-human (FIH) phase I clinical trial (70). Since no apparent adverse effects of YS110 have been reported besides transient and tolerable injection reactions, YS110 may be an immediate therapeutic candidate for clinical use as potential treatment for MERS-CoV infection.

3.3. Psoriatic pruritus

3.3.1. CD26/DPPIV and psoriasis

Psoriasis (PSO) is one of the most common inflammatory skin diseases, found in about 1-3% of the world general population (71). For a long time, PSO had been considered as a non-pruritic dermatitis. However, within the past 30 years, a number of studies have demonstrated that approximately 60-90% of patients with PSO suffer from pruritus (71-76). Pruritus is an important symptom of PSO. Despite

the fact that several studies have been undertaken to investigate the pathogenesis of pruritus in PSO, many aspects have not yet been studied (71, 77). Therefore, the pathogenesis of this symptom is far from being well-understood and, as a consequence, the therapy of pruritic psoriatic patients still remains a significant challenge for clinicians (78). It has been demonstrated that DPPIV is expressed at high levels on keratinocytes and that DPPIV inhibition suppresses keratinocyte proliferation *in vitro*, and restores partially keratinocyte differentiation *in vivo* (79). Moreover, it has been reported that DPPIV is expressed on keratinocytes and its activity is upregulated in PSO (80, 81), findings which support a potential role for DPPIV enzyme activity in the pathogenesis of PSO. While other investigators have reported a significant improvement in disease severity in PSO patients treated with a DPPIV inhibitor (82, 83), the precise mechanisms involved in DPPIV-mediated regulation of PSO have not been elucidated (84). Recent report showed that T-cell bound expression of CD26/DPPIV in psoriatic skin was explicitly present, albeit in small quantities (81). One hypothesis of potential effect of DPPIV in PSO is that T cell activation mediated by DPPIV is associated with the pathogenesis of PSO (85). Cytokines and chemokines represent the third key player in the psoriatic chronic immune response (86). They are considered as mediators responsible for activation and recruitment of infiltrating leukocytes and therefore play a crucial role in the development and persistence of psoriatic skin lesions (87). DPPIV likely plays a pivotal role in the processing of these molecules (84). The extracellular protease domain of DPPIV (both on keratinocytes and T cells) can cleave dipeptides from the amino terminus of proteins, such as cytokines and chemokines, which are abundantly present in a chronic immune response in PSO, resulting in alterations in receptor specificity and subsequent modifications of biological activity. Taken together, it is conceivable that PSO is a disease involving the complex interplay among activated T cells, keratinocytes and cytokines, and that DPPIV has a key regulatory role in the interactions of these three disease components.

3.3.2. Elevation of sCD26 and DPPIV enzyme in sera of PSO patients

To determine whether serum soluble CD26 (sCD26) and soluble DPPIV (sDPPIV) enzyme play a role in PSO, we evaluated levels of sCD26 and sDPPIV enzyme activity in sera of patients with PSO (88). For this purpose, we performed our in-house capture assay method using anti-human CD26 mAb as a capture antibody for detecting DPPIV enzyme activity specific to sCD26 (89). Since commercially available DPPIV enzyme assay kits measure DPPIV activity in whole serum, but not in captured sCD26 molecules from the samples, it is possible that DPPIV-like peptidase activity other than that possessed by the captured sCD26 molecules was measured, leading to an overestimate of the DPPIV activity in the samples (90). Analyses of serum samples obtained from 18 healthy adult volunteers and 48 PSO patients demonstrated that serum sCD26 concentration of PSO patients was significantly higher than that of healthy adults. Moreover, serum levels of sDPPIV enzyme activity were also significantly higher in patients with PSO compared with healthy adult controls. These data suggest that DPPIV enzyme activity is increased in sera of patients with PSO, which is linked to a concomitant increase in sCD26 in the same patient population. These observations also suggest that DPPIV enzyme plays a role in the pathogenesis of PSO.

3.3.3. Increased pruritus by truncation of substance P, a ligand for CD26/DPPIV

Among various mediators of pruritus investigated in inflammatory skin diseases, substance P (SP) is a key molecule in an itch sensory nerve (91-93), consisting of 11 AA residues with dual DPPIV cleavage sites at its N-terminal position. In fact, DPPIV enzyme digests full-length SP(1-11) resulting in a truncated form of SP(5-11), an activity inhibited by the presence of the DPPIV enzyme inhibitor sitagliptin (88). Moreover, we observed that levels of SP degraded by DPPIV were increased in sera of patients with PSO. Taken together with the above data regarding an increase of sCD26/DPPIV levels in PSO patients, these results also suggest that the increase in DPPIV activity appears to play an important role in PSO by truncating SP.

We next utilized an itchy mouse model by intradermal injection (i.d.) of recombinant SP and quantified scratching behavior in mice to determine an itchy symptom. Mice treated with SP(5-11) i.d. demonstrated a significant increase in scratching behavior, compared with mice receiving control solvent or mice receiving full-length SP(1-11). On the other hand, scratching behavior in SP(1-11) i.d. mice was significantly decreased in mice treated with the DPPIV inhibitor sitagliptin. Furthermore, SP-induced scratching behavior was significantly attenuated in CD26/DPPIV knockout (CD26KO) mice compared with that observed in B6 WT mice. Our data suggest that truncated form of SP cleaved by DPPIV enzyme increases an itch sensation and that SP-induced itch sensation is attenuated by inhibition of the DPPIV activity.

To further determine that DPPIV inhibition affects pruritus, we evaluated scratching behavior utilizing an imiquimod (IMQ)-induced psoriatic itch model (94, 95). Serum levels of truncated form of SP were significantly increased in IMQ-treated mice compared with control cream-treated mice. Moreover, scratching behavior was significantly increased in IMQ-treated mice than control cream-treated mice. These data indicate that IMQ induces psoriatic itchy

skin lesions in mice associated with an increase in the truncation of SP. We next analyzed the frequencies of itch scratching behavior following DPPIV inhibitor administration. IMQ-treated mice receiving sitagliptin showed significant decrease of scratching behavior compared with IMQ-treated mice receiving control solvent. Meanwhile, there was no change in scratching behavior between control cream-treated mice receiving sitagliptin or control saline, with baseline levels of scratching behavior in both cohorts. Taken together, our data suggest that treatment with the DPPIV inhibitor sitagliptin attenuates psoriatic itch sensation via a decrease in the truncated form of SP.

Previous studies have reported that serum levels of SP were decreased in patients with PSO (96-98). Meanwhile, since SP is cleaved by DPPIV enzyme and DPPIV enzyme activity is increased in PSO (88), it is important for a detailed understanding of the role of SP in PSO to precisely measure the truncated form of SP separately from full-length SP. In our recent study, we evaluated full-length SP(1-11) and truncated forms of SP and demonstrated that there was no change in the serum levels of full-length SP(1-11), SP(2-11) and SP(3-11) between PSO and healthy adult controls (88). However, we found that DPPIV enzyme activity and the truncated form of SP were significantly increased in PSO, and that the truncated form of SP(5-11) resulting from DPPIV enzyme activity is associated with an increase in itch sensation. In the IMQ-induced PSO model, the truncated form of SP was significantly increased in sera compared with control mice, and scratching behavior was decreased by administration of sitagliptin. On the other hand, there were no differences in serum levels of DPPIV enzyme activity between IMQ and control cream-treated mice. It is conceivable that the persistent existence of psoriatic skin lesions may be required for the increased serum levels of DPPIV enzyme activity seen in PSO patients, and that SP truncation may result from the increased levels of DPPIV enzyme activity in skin lesions rather than in the circulation (80, 99, 100). Our recent study has conclusively demonstrated that increase in DPPIV enzyme activity exacerbates pruritus in PSO, and that inhibition of DPPIV enzyme reduces severity of itch scratching behavior. Moreover, our results suggest that DPPIV inhibitors are useful as therapeutic agents for pruritus including PSO.

4. CANCERS

4.1. Novel mechanism of CD26/DPPIV in cancer immunology

4.1.1. Anti-tumor effect of CXCL10-mediated CXCR3$^+$ lymphocyte via DPPIV inhibition

CD26/DPPIV regulates the activities of a number of cytokines and chemokines. However, direct *in vivo* evidence for a role for CD26 in tumor biology and its interaction with the tumor microenvironment (TME) has not yet been reported. Recent work has demonstrated clearly the interaction between DPPIV and substrate CXCL10, as well as the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity (101). Preservation of the full length, bioactive CXCL10 by DPPIV inhibition results in increased level of CXCR3$^+$ effector T cells in the TME and subsequent tumor growth reduction. CXCR3 has been shown to be a functional receptor for CXCL10 (102). Importantly, the combination of DPPIV inhibition and checkpoint blockade therapy remarkably augments the efficacy of naturally occurring and immunotherapy-based tumor immunity. These investigators therefore provide the direct evidence of DPPIV as an *in vivo* regulator of CXCL10-mediated T cell trafficking with relevance for tumor immunity and immunotherapy (Figure 2). The TME consists of numerous cell types along with the neoplastic cells. Among them are the effector lymphocytes capable of infiltrating into the tumor sites that are specifically required for anti-cancer immune response (103). CXCL10 is a chemoattractant for immune cells such as monocytes, T cells and NK cells and is secreted from a variety of cells in response to IFN-γ, including monocytes, neutrophils, eosinophils, epithelial cells, endothelial cells, fibroblasts and keratinocytes (104). CXCL10 appears to have a dual role on tumor growth, with its proliferative or anti-proliferative activity being cell-type-dependent as a result of differences in the subtype of its receptor CXCR3 (104). CXCR3 is rapidly induced on naïve T cells following activation and preferentially remains highly expressed on T_H1-type CD4$^+$ cells and CD8$^+$ cytotoxic T lymphocytes (CTL), resulting in enhancement of T cell migration to facilitate tumor immune responses (105). Although strong T_H1 and CTL responses in the TME are beneficial for tumor suppression, these responses are counterbalanced to prevent unwanted tissue damage and immunopathology by disrupting the proinflammatory loop. CXCR3$^+$ T_{reg} has been recently identified (106) , as IFN-γ signaling activates the T_H1 transcription factor *T-bet*, which in turn promotes CXCR3 expression to induce T_H1-specific T_{reg} in the inflammatory sites. Moreover, CXCR3 is a marker of CD8$^+$ IL-10-producing cells with suppressive activity in both mice and human (107). The exact factors determining whether CXCR3$^+$ effector T cells and CXCR3$^+$ regulatory lymphocytes will oppose or cooperate with each other during the tumor growth process *in vivo* remain to be elucidated.

4.1.2. Immune checkpoint mechanism via CD26/DPPIV

Although the cellular and molecular mechanisms involved in CD26-mediated T cell activation have been extensively evaluated by our

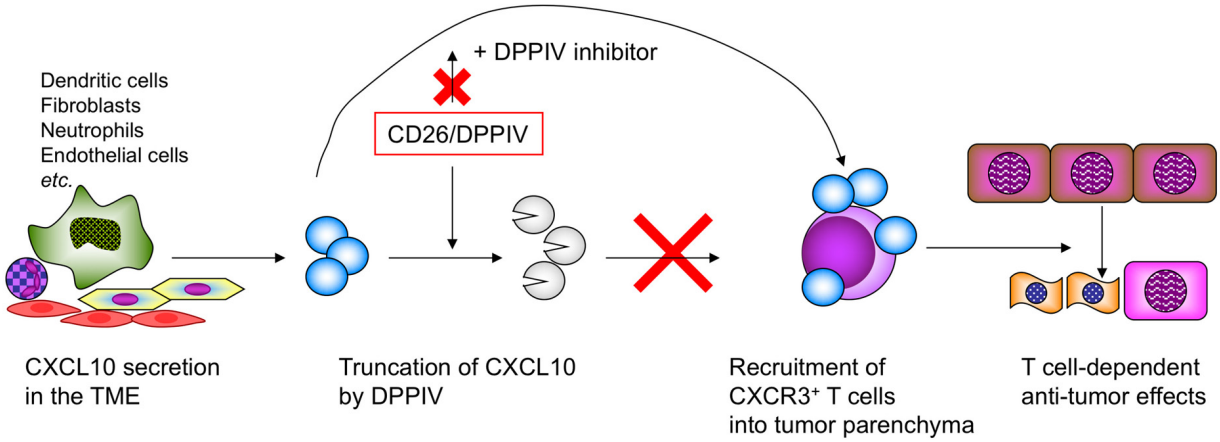

Figure 2. DPP4 inhibition suppresses truncation of its ligand CXCL10, leading to recruitment of CXCR3$^+$ T cells into tumor parenchyma. Through an *in vivo* xenotransplant models, DPPIV inhibitor is found to reduce tumor growth through the preservation of bioactive CXCL10 in the tumor microenvironment (TME). In the normal physiological state, CXCL10 is rapidly degraded by CD26/DPPIV, resulting in decreased recruitment and migration of CXCR3$^+$ T cells into the tumor parenchyma. In contrast, DPPIV inhibition enhances tumor rejection by preserving the full-length biologically active form of CXCL10, leading to increased trafficking of CXCR3$^+$ T cells into the tumor parenchyma. This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy. This Figure is reprinted with permission from K Ohnuma *et al.*, *Nat Immunol* 16: 791-792, 2015 (156).

group and others (4-6, 9, 13, 90), potential negative feedback mechanisms to regulate CD26-mediated activation still remain to be elucidated. Utilizing human PBL, we found that production of IL-10 by CD4$^+$ T cells is preferentially increased following CD26-mediated costimulation compared with CD28-mediated costimulation (108). IL-21 production was also greatly enhanced in the late phase of CD26 costimulation. On the other hand, production of IL-2, IL-5 or TNF-α was much lower following CD26 costimulation than CD28 costimulation. In contrast, no difference in the production of IL-17A, IFN-γ, or IL-4 was observed following CD26 or CD28-mediated costimulation. These data indicate that CD26 and CD28 costimulation of CD4$^+$ T cells results in different cytokine production profiles, with IL-10 production being preferentially enhanced following CD26 costimulation. Furthermore, we found that both the cell surface and intracellular expression of LAG3 (lymphocyte activation gene-3) was clearly enhanced with increasing doses of anti-CD26 mAb, and that CD26-induced enhancement of LAG3 was more pronounced than the effect of CD28-mediated costimulation. On the other hand, both CD26 and CD28-mediated costimulation enhanced the expression of CTLA-4 (cytotoxic T-lymphocyte-associated antigen 4) and FOXP3 (forkhead box protein P3), with no significant difference being detected between these two costimulatory pathways. In contrast with CD28 costimulation, LAP (latency associated protein) complexed with TGF-β1 was hardly induced following CD26 costimulation. We showed that all the CD4$^+$ T cells expressed LAG3 following CD26 or CD28 costimulation, and that no difference was observed in the percentage of LAG3 expressing cells, while the expression intensity of LAG3 after CD26-mediated costimulation was significantly higher than after CD28-mediated costimulation. LAG3 serves as a marker of IL-10 producing T_{reg} (109), and binds to major histocompatibility complex (MHC) class II molecules with higher affinity than CD4, leading to transduction of inhibitory signals for both T cells and APCs (110, 111). Therefore, our data strongly suggest that signaling events via CD26 may induce the development of CD4$^+$ T cells to a Type 1 regulatory T cells (Tr1)-like phenotype. By expression analysis with Western blotting and quantitative real-time polymerase chain reaction (RT-PCR) experiments and by cell functional analysis utilizing chemical inhibitors and small interfering RNA (siRNA) experiments, we showed that co-engagement of CD3 and CD26 induces preferential production of IL-10 in human CD4$^+$ T cells, mediated through NFAT (nuclear factor of activated T cells) and Raf (rapidly accelerated fibrosarcoma)-MEK (mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase)-ERK pathways (108). High level of early growth response 2 (EGR2) is also induced following CD26 costimulation, possibly via NFAT and AP-1 (activator protein-1)-mediated signaling, and knock down of EGR2 leads to decreased IL-10 production. Taken together, these observations strongly suggest that CD26-mediated costimulation of CD4$^+$ T cells results in enhanced NFAT/AP-1-dependent EGR2 expression, which is associated with the preferential production of IL-10. Finally, we demonstrated that CD3/CD26-stimulated CD4$^+$ T cells clearly suppress proliferative activity and effector cytokine production of bystander T cells in an IL-10-dependent manner (108). Collectively, our results above suggest that CD3/CD26 costimulation induces the development of

human Tr1-like cells from $CD4^+$ T cells with high level of IL-10 production and LAG3 expression. Preclinical models showed that antibody-mediated blocking of LAG3 as potential anti-cancer therapy led to enhanced activation of antigen-specific T cells at the tumor sites and disruption of tumor growth (112). Moreover, anti-LAG3/anti-PD-1 (programmed cell death 1) antibody treatment cured most mice of established tumors that were largely resistant to single antibody treatment (112). Taken together, it is conceivable that CD26 itself may function as an inhibitory molecule of an immune checkpoint system in certain disease conditions, similar to LAG3 or PD-1.

4.2. Malignant pleural mesothelioma

4.2.1. FIH phase I clinical trial of humanized anti-CD26 mAb

Our previous work analyzing extracellular matrix (ECM) interactions and intracellular signaling events demonstrated that the malignant mesothelial cell line JMN expresses CD26 (113). Our recent in-depth studies of CD26 expression in malignant pleural mesothelioma (MPM) revealed that CD26 is preferentially expressed in MPM cells but not in normal mesothelial cells (114, 115). These intriguing findings propelled our development of CD26-targeted therapy for MPM. For this purpose, we had developed a novel humanized anti-CD26 mAb, namely YS110. YS110 is a recombinant DNA-derived humanized mAb that selectively binds with high affinity to the extracellular domain of CD26. The antibody is an $IgG_1\kappa$ with a molecular weight of 144 kDa and was humanized via an *in silico* design based on the AA sequence of anti-human CD26 murine mAb (14D10), which inhibited tumor cell growth, migration and invasion, and enhanced survival of mouse xenograft models (116). YS110 is produced by fermentation in CHO (Chinese hamster ovary) mammalian cell suspension culture with the Glutamine Synthetase Expression System. *In vitro* pharmacologic evaluation of YS110 demonstrated its selective binding to human CD26 on a number of human cancer cell lines and tissues, with no apparent effect on immune activation and no inhibition of DPPIV activity, while exhibiting direct cytotoxic effect on certain human CD26-positive cancer cell lines. Moreover, our *in vitro* data indicated that YS110 induces cell lysis of MPM cells via antibody-dependent cellular cytotoxicity (ADCC) in addition to its direct anti-tumor effect via cyclin-dependent kinase inhibitor (CDKI) $p27^{kip1}$ and $p21^{cip1}$ accumulation (68, 117). *In vivo* experiments with mouse xenograft models involving human MPM cells demonstrated that YS110 treatment drastically inhibits tumor growth in tumor-bearing mice and reduces formation of metastases, resulting in enhanced survival (68). Our data strongly suggest that YS110 may have potential clinical use as a novel cancer therapeutic agent for CD26-positive malignant mesothelioma.

In addition to our robust *in vitro* and *in vivo* data on antibody-mediated dose-dependent tumor growth inhibition, YS110 exhibited excellent safety and pharmacological profiles in non-human primate models using single and repeated increasing intravenous doses. Considering the lack of T cell proliferation and cytokine production *in vitro*, YS110 was therefore considered not to have an agonistic nor activating effect on human CD26-positive lymphocytes. Other key safety findings were obtained from studies involving cynomolgus monkeys which express YS110-reactive CD26 molecules, with similar tissue distribution profiles and expression levels to human CD26. We therefore conducted the FIH clinical trial of YS110 for patients with MPM and other CD26-positive solid tumors (70). Thirty-three heavily pre-treated patients with CD26-positive cancers including 22 malignant mesothelioma, 10 renal cell carcinoma (RCC) and 1 urothelial carcinoma underwent YS110 administration. A total of 232 infusions (median 3 (range 1-30)) of YS110 were administered across 6 dose levels ranging from 0.1 to 6.0 mg/kg. Maximum tolerated dose (MTD) was not reached and 2 dose limiting toxicities (DLTs) (1 patient with grade 3 anaphylactic reaction at 1.0 mg/kg and 1 patient with grade 3 allergic reaction at 2.0 mg/kg) were reported with complete resolution following dose omission. Subsequent use of systemic steroid prophylaxis and exclusion of patients with significant allergy histories improved safety profile. No dose-dependent adverse events were observed. Blood exposure pharmacokinetics parameters (AUC and C_{max}) increased in proportion with the dose. Cytokines and immunophenotyping assays indicated CD26 target modulation with no occurrence of infectious nor autoimmune disease complications. These results demonstrated that YS110 is tolerable in human subjects. A secondary objective of this FIH study was to evaluate the potential antitumor activity of YS110 according to RECIST 1.0 (response evaluation criteria in solid tumors) criteria (or modified RECIST criteria for mesothelioma). No objective response was achieved in any of the treated patients. However, stable disease as the best overall response was observed in 13 out of the 26 valuable patients on Day 43 of the first cycle (1 at 0.1 mg/kg, 2 at 0.4 mg/kg, 7 at 2.0 mg/kg, 1 at 4.0 mg/kg and 2 at 6.0 mg/kg). Prolonged stabilization with 26 weeks or more was observed in 7 out of 13 stable disease patients who have received a total of 143 (5 to 30 infusions/patients) infusions with a median PFS (progression-free survival) of 33 weeks (26 to 57 weeks). This FIH study conclusively demonstrated that YS110 exhibits a favorable safety profile and substantial clinical activity in heavily pretreated CD26-positive MPM patients

who had previously progressed on conventional standard chemotherapies. Further clinical trial of YS110 for MPM is in progress worldwide (118).

4.2.2. DPPIV enzyme activity and efficacy of YS110

The FIH clinical study of YS110 revealed that an increase in YS110 infusion dose was associated with decreased serum sCD26 level, particularly in cohorts 4-6 (2.0 to 6.0 mg/kg), with an approximately 80% decrease in sCD26 level (70). Moreover, since sCD26 level reflects DPPIV enzyme activity in sera (119), similar reduction in DPPIV enzyme activity was observed, again particularly in patients in cohorts 4-6. Although DPPIV inhibitors are clinically used as oral hypoglycemic agents (120), hypoglycemia was not observed during YS110 administration. Of note is the fact that greater than 80% inhibition of serum DPPIV activity was obtained 24 hours after oral administration of clinically available DPPIV inhibitors (drug information published by manufacturers of sitagliptin, vildagliptin, saxagliptin and *etc*.), a level of inhibition comparable to that seen in patients treated with YS110. Our current data would therefore indicate that YS110 therapy is tolerable in the clinical setting. As described in the previous section, recent work has demonstrated the functional role of DPPIV-mediated post-translational modification of chemokines in regulating tumor immunity through its interaction with its substrate CXCL10 (101). This anti-tumor response is potentiated in combination with other anti-tumor immunotherapeutic approaches including CpG adjuvant therapy, adoptive T cell transfer therapy and checkpoint blockade therapy (anti-CTLA-4 and anti-PD-1) (101). In view of these recent findings, data from our current trial showing that serum DPPIV activity was decreased following treatment with YS110 in a dose-dependent manner would suggest that anti-tumor activity via DPPIV inhibition may constitute yet another mechanism of action for the anti-tumor activity of YS110.

4.2.3. Mechanisms of action of YS110 for cancer treatment

We previously showed that depletion of CD26 by RNAi results in the loss of adhesive property, suggesting that CD26 is a binding protein to the ECM (68). Moreover, our observations regarding the CD26-CD9-α5β1 integrin complex suggest that CD26 regulates the interaction of MPM cells with the ECM via yet-to-be-determined integrin adhesion molecules (121). Recently, we found that expression of CD26 upregulates periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity (122). Periostin is a secreted cell adhesion protein of approximately 90 kDa, which shares a homology with the insect cell adhesion molecule fasciclin 1 (FAS1) (123). We also demonstrated that the cytoplasmic region of CD26 plays a crucial role in MPM tumor biology through its linkage to somatostatin receptor 4 (SSTR4) and SHP-2 protein tyrosine phosphatase in cell membrane lipid rafts, leading to cytostatic effects in MPM cells without direct association of the ECM to CD26 by anti-CD26 mAb treatment (Figure 3) (124). In view of the findings above, we propose that CD26 forms macromolecular complexes in the cell surface of MPM by connecting periostin and ECM to intracellular signaling events (125); (i) In CD26-negative MPM cells, SSTR4 mediated inhibitory signaling to suppress cell proliferation and motility. In contrast, by locking the signaling domain of SSTR4 with CD26 association, SSTR4-mediated anti-tumor effects were abrogated, leading to increased cell proliferation and motility in CD26-positive MPM cells. (ii) In addition, CD26 regulated ECM-associated tumor cell behavior in association with integrins and periostin-ECM complex. CD9 suppressed cell invasion and migration by inhibiting the formation of CD26-α5β1 integrin complex. Moreover, expression of CD26 upregulated periostin secretion by MPM cells, leading to enhanced MPM cell migratory and invasive activity. (iii) Furthermore, periostin is a secreted cell adhesion protein. The N-terminal region regulates cellular functions by binding to integrins at the plasma membrane of the cells through cell adhesion domain. The C-terminal region of the protein regulates cell–matrix organization and interaction by binding such ECM proteins, leading to increased MPM cell motility. As a result, CD26 molecule in MPM also plays a pivotal role in connecting ECM to intracellular signaling events associated with cell proliferation and motility. It is therefore conceivable that targeting CD26 may be a novel and effective therapeutic approach for MPM.

In addition to the ECM association, our *in vitro* data indicate that YS110 induces cell lysis of MPM cells via ADCC in addition to its direct anti-tumor effect via CDKI $p27^{kip1}$ accumulation (68). More recently, we evaluated the direct anti-tumor effect of YS110 against the MPM cell lines H2452 and JMN, and investigated its effects on cell cycle and on the cell cycle regulator molecules (117). YS110 suppressed the proliferation of H2452 cells by approximately 20% in 48 hours of incubation. Cell cycle analysis demonstrated that the percentage of cells in G2/M phase increased by 8.0.% on average following YS110 treatment. In addition, level of the cell cycle regulator $p21^{cip1}$ was increased and cyclin B1 was decreased after YS110 treatment. Inhibitory phosphorylation of both cdc2 (Tyr15) and cdc25C (Ser216) was elevated. Furthermore, activating phosphorylation of p38 MAPK (Thr180/Tyr182) and ERK1/2 (Thr202/Tyr204) was augmented following 24 hours of YS110 treatment. In addition, we investigated the synergistic effects of YS110 and the anti-tumor agent pemetrexed on selected MPM cell

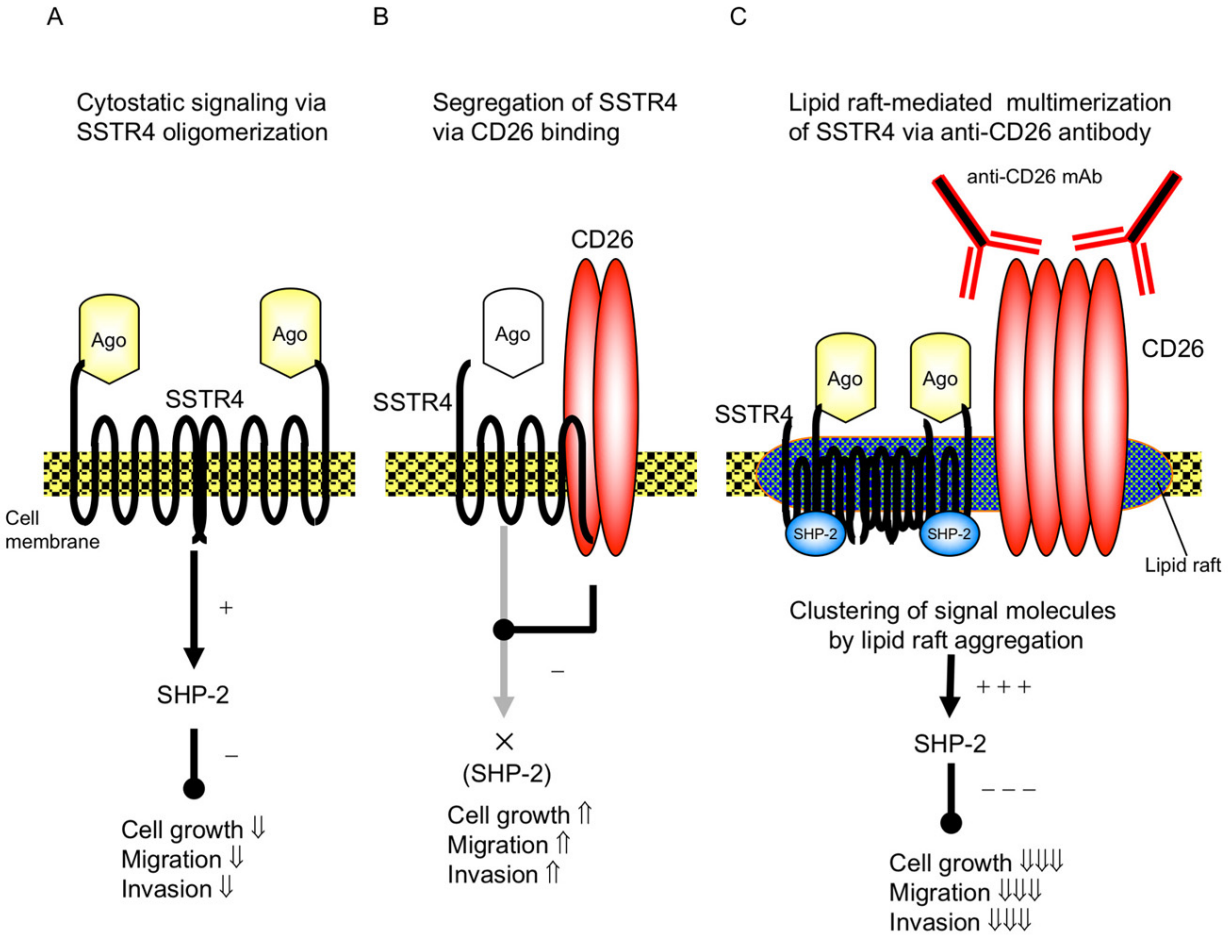

Figure 3. A model for cytostatic signaling mediated by SSTR4 and CD26 coassociation in MPM cells. SSTR4 molecules form homo- or oligo-dimers when stimulated by its agonists (Ago), followed by manifestation of their cytostatic effects via SHP-2 signaling **(A)**. When coassociation of SSTR4 with CD26 occurs, CD26 binds to the C-terminal region of SSTR4, which is necessary to transduce SSTR4 signaling, hence blocking the SSTR4-mediated cytostatic effects **(B)**. Meanwhile, anti-CD26 mAb ligates CD26, leading to dissociation of SSTR4 from CD26 and to recruiting lipid rafts with clustering of SSTR4 molecules **(C)**. As a result, downstream signaling of SSTR4 occurs with activation of SHP-2, leading to the observed cytostatic effects.

lines in both *in vitro* and *in vivo* studies. Pemetrexed rapidly induced CD26 expression on cell surface, and treatment with both YS110 and pemetrexed inhibited *in vivo* tumor growth accompanied by a synergistic reduction in the MIB-1 index (117).

We also demonstrated that treatment with YS110, which inhibited cancer cell growth, induced nuclear translocation of both cell-surface CD26 and YS110 (126, 127). In response to YS110 treatment, CD26 was translocated into the nucleus via caveolin-dependent endocytosis, and interacted with a genomic flanking region of the *POLR2A* gene, a component of RNA polymerase II. This interaction consequently led to transcriptional repression of the *POLR2A* gene, resulting in retarded cancer cell proliferation. Furthermore, impaired nuclear transport of CD26 reversed the *POLR2A* repression induced by YS110 treatment. These findings reveal that nuclear CD26 functions in the regulation of gene expression and tumor growth, and yet another novel mechanism of action of anti-CD26 mAb therapy may involve the regulation of inducible traffic of surface CD26 molecules into the cell nucleus.

4.3. Other cancers

In contrast to our robust findings regarding the role of CD26/DPPIV on MPM, the exact role of CD26/DPPIV in other cancers remains to be elucidated, partly due to its variable expression on these tumors. In general, it is strongly expressed on some cancers, while being absent or present at low levels in others (8). Furthermore, given its multiple biological functions, including its ability to associate with several key proteins and its cleavage of a number of soluble factors to regulate their function, it is likely that the CD26/DPPIV effect on tumor biology is at least partly mediated by the effect of these biological functions on specific tumor types (128).

CD26 has also been shown to be expressed on RCC (129, 130), including the cell lines Caki-1, Caki-2, ACHN, and VMRC-RCW (116). We previously showed that anti-CD26 mAb inhibition of the Caki-2 cell line was associated with G1/S cell cycle arrest, enhanced $p27^{kip1}$ expression, down regulation of cyclin-dependent kinase 2 (CDK2) and dephosphorylation of retinoblastoma substrate (Rb) (116). We also found that anti-CD26 mAb therapy attenuated Akt activity and internalized cell surface CD26 leading to decreased CD26 binding to collagen and fibronectin. Finally, we showed that anti-CD26 mAb inhibited human RCC in a mouse xenograft model (116).

Immunofluorescence analysis revealed expression of CD26/DPPIV on peripheral blood lymphocytes of patients with B chronic lymphocytic leukemia (B-CLL), but not on peripheral B cells from normal donors (131). CD26/DPPIV could also be induced in normal B cells following treatment with IL-4, indicating that expression was regulated at the level of transcription (131). CD26/DPPIV has also been shown to be a marker for aggressive T-large granular lymphocyte (T-LGL) lymphoproliferative disorder. Our work indicated that patients with CD26-positive disease were more likely to require therapies for cytopenia and infections associated with the disease than those with CD26-negative T-LGL (132). Furthermore, CD26-related signaling may be aberrant in T-LGL as compared to T-lymphocytes from normal donors (132). Disease aggressiveness is also correlated with CD26/DPPIV expression in other subsets of T-cell malignancies including T-lymphoblastic lymphoma/ acute lymphoblastic leukemia (LBL/ALL), as those with CD26-positive T-LBL/ALL had a worse clinical outcome compared to patients with CD26-negative tumors (133, 134).

JKT-hCD26WT cells resulted in a greater sensitivity to doxorubicin and etoposide compared to mock transfected cells (135-138). Jurkat cells transfected with a nonfunctional DPPIV catalytic site mutant (Ser630Ala) did not show increased doxorubicin and etoposide sensitivity, suggesting that DPPIV activity is required for chemo-sensitization. A CD26 transfectant with a mutation at the ADA binding site retains DPPIV activity and had a higher level of doxorubicin sensitivity. Surface CD26 expression and DPPIV activity are associated with increased doxorubicin sensitivity and cell cycle arrest in Jurkat cells. Also, there are differences in hyperphosphorylation and inhibition of $p34^{cdc2}$ kinase activity, phosphorylation of cdc25C, and alteration in cyclin B1 expression associated with doxorubicin sensitivity in Jurkat cell lines (136). Therefore, inhibition of CD26 increases cell survival, while increased CD26 expression is associated with decreased drug resistance. The mechanism of this decreased resistance appears to be attributed to enhanced expression of topoisomerase IIα mediated by CD26 – the target for both doxorubicin and etoposide. The increased sensitivity to doxorubicin and etoposide in CD26 expressing tumors may be important in T-cell hematologic malignancies as well as other cancers. Surface expression of CD26 increases topoisomerase IIα level in the B-cell line Jiyoye and increases doxorubicin sensitivity (139). This was demonstrated by using CD26 transfection constructs in the Jiyoye B-cell lymphoma cell line as well as by target specific siRNA inhibition of CD26 in the Karpas-299 T-cell leukemia cell line. Therefore, CD26 has effects on topoisomerase IIα and doxorubicin sensitivity in both B-cell and T-cell lines. Increased CD26/DPPIV levels are associated with increased phosphorylation of p38 and its upstream regulators – MAPK kinase (MAPKK) 3/6 and apoptosis signal-regulating kinase 1 (ASK1). Therefore, the p38 signaling pathway may be involved in the regulation of topoisomerase IIα expression. Doxorubicin treated SCID mice had increased survival in those injected with wild type CD26 compared to vector or DPPIV catalytic site mutant (Ser630Ala) injected mice. CD26/DPPIV levels may be useful predictive markers for doxorubicin treatment of cancer. CD26 level is also associated with etoposide resistance. CD26 mediated changes include hyperphosphorylation of $p34^{cdc2}$, variation in cdc25C level and phosphorylation, and changes in cyclin B1 level. Since CD26/DPPIV cleaves substrates resulting in altered function (140, 141), it is possible that CD26-associated drug sensitivity may therefore be mediated by serum-derived factors. However, our work showed that the increased doxorubicin and etoposide sensitivity of JKT-hCD26WT was independent of serum, data which suggest an effect of CD26 on cell-mediated processes, such as signal transduction, rather than serum-derived factors (137).

Pang *et al.* identified a subpopulation of $CD26^+$ cells uniformly presenting in both primary and metastatic tumors in colorectal cancer (CRC), and showed that $CD26^+$ cancer cells were associated with enhanced invasiveness and chemoresistance (142). These investigators showed that in $CD26^+$ CRC cells, mediators of epithelial to mesenchymal transition (EMT) contribute to the invasive phenotype and metastatic capacity. These results suggest that $CD26^+$ cells are cancer stem cells in CRC, and that CD26/DPPIV can be targeted for metastatic CRC therapy. Recently, other investigators demonstrated in a murine model that lung metastasis of CRC was suppressed by treatment with a DPPIV inhibitor (143). They showed a reduction of EMT markers, suggesting that the EMT status of the murine colon cancer cell line MC38 was at least in part affected by DPPIV inhibition, with a diminution in the growth of metastases. They also showed that DPPIV inhibition decreased the growth of lung metastases of colon cancer by downregulating autophagy, increasing apoptosis and arresting the cell cycle. These data

therefore suggest that DPPIV inhibition may be an effective therapeutic strategy for the treatment of cancers with pulmonary metastases (143).

Yamada *et al.* comprehensively investigated gene expression profiles in surgical samples of untreated gastrointestinal stromal tumors (GIST) of the stomach and small intestine. They found that the disease-free survival of patients with CD26-positive GIST of the stomach was worse than that of patients with CD26-negative GIST (144). Moreover, the postoperative recurrence rate of CD26-negative gastric GIST cases was as low as 2.0.%. They concluded that CD26 is a significant prognostic factor of gastric GIST and may also serve as a therapeutic target (144). Meanwhile, CD26 expression was not associated with clinical outcome of small intestinal GIST.

5. SUMMARY AND PERSPECTIVES

Initially described in 1966 as an enzyme with intrinsic DPPIV activity (145), this activity was subsequently found to be identical to CD26, a 110 kDa extracellular membrane-bound glycoprotein expressed on many tissues including brain, endothelium, heart, intestine, kidney, liver, lung, skeletal muscle, pancreas, placenta, and lymphocytes (26, 146, 147). Originally characterized as a T cell differentiation antigen, CD26 is preferentially expressed on a specific population of T lymphocytes, the subset of $CD4^+CD45RO^+$ memory T cells, and is upregulated following T cell activation (15, 26). Besides being a marker of T cell activation, CD26 is also associated with T cell signal transduction processes as a costimulatory molecule (5-7). CD26 therefore has an important role in T cell biology and overall immune function, and represents a novel therapeutic target for various immune disorders (13, 52, 148-150). CD26 is also expressed on various tumors such as MPM, CRC, RCC, hepatocellular carcinoma, lung cancer, prostate cancer, GIST, thyroid cancer, and hematologic malignancies such as T-anaplastic large cell lymphoma and T-LBL/ALL (10). Moreover, in several human malignancies including CRC, chronic myeloid leukemia, gastric adenocarcinoma and MPM, CD26/DPPIV expression is reported to be a marker of cancer stem cells (142, 151-155). Given the potential role of CD26 surface expression in cancer biology, YS110 therapy may also influence tumor growth through its potential effect on the cancer stem cells of selected tumors. We recently developed novel anti-human CD26 mAbs that can be used as companion diagnostic reagents suitable for immunohistochemical staining of CD26 in formalin-fixed tissue sections with reliable clarity and intensity (155). Since these mAbs display no cross-reactivity with the therapeutic humanized anti-CD26 mAb YS110, they may be suitable for assays analyzing CD26 expression during or following treatment with YS110, with important implications in the clinical setting.

Since CD26/DPPIV has a multitude of biological functions in immune system and human tumor cells, further detailed understanding of the role of this molecule in various clinical settings may lead potentially to novel therapeutic approaches.

6. ACKNOWLEDGEMENT

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Abbreviation: AA, amino acid; ADA, adenosine deaminase; ADCC, antibody-dependent cellular cytotoxicity; aGVHD, acute graft-versus-host disease; alloHSCT, allogeneic hematopoietic stem cell transplantation; AP-1, activator protein-1; APCs, antigen presenting cells; A20-luc, luciferase-transfected A20 cell; B6 WT, parental C57BL/6 mice; Cav-Ig, soluble Fc fusion proteins containing the N-terminal domain of caveolin-1; CB, cord blood; CD26KO, CD26 knockout; cGVHD, chronic graft-versus-host disease; CRC, colorectal cancer; CTL, cytotoxic T lymphocyte; CTLA-4, cytotoxic T-lymphocyte–associated antigen 4; ΔCNS-77 Tg mice, mice carrying human *IFNG* transgene with deleting *IL26* transcription; DPPIV, dipeptidyl peptidase IV; ECM, extracellular matrix; EGR2, early growth response 2; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial to mesenchymal transition; ERK, extracellular signal-regulated kinase; FIH, first-in-human; GIST, gastrointestinal stromal tumors; GVHD,

graft-versus-host disease; GVL, graft-versus-leukemia; HuCB, human umbilical cord blood; IBD, inflammatory bowel diseases; i.d., intradermal injection; IFN, interferon; IL, interleukin; IMQ, imiquimod; JKT-hCD26WT, Jurkat cells transfected with full-length human CD26/DPPIV; LAG3, lymphocyte activation gene-3; LBL/ALL, lymphoblastic lymphoma/acute lymphoblastic leukemia: mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MERS-CoV, Middle East respiratory syndrome coronavirus; MERS-CoV S1-Fc, S1 domain of MERS-CoV fused to the Fc region of human IgG; MPM, malignant pleural mesothelioma; NFAT, nuclear factor of activated T cells; NOG, NOD/Shi-*scid*IL2rγ^{null}; OB, obliterative bronchiolitis; PBL, peripheral blood lymphocyte; PD-1, programmed cell death 1; PSO, psoriasis; RA, rheumatoid arthritis; RBD, receptor binding domain; RCC, renal cell carcinoma; RECIST, response evaluation criteria in solid tumors; sCD26, soluble CD26; sDPPIV, soluble dipeptidyl peptidase IV; siRNA, small interfering RNA; SP, substance P; SSTR4, somatostatin receptor 4; Tg, transgenic; T-LGL, T-large granular lymphocyte; TME, tumor microenvironment; TNF, tumor necrosis factor; Tr1, Type 1 regulatory T cells; WHO, World Health Organization; 190-*IFNG* Tg mice, mice carrying human *IFNG* and *IL26* transgene

Key Words CD26, DPPIV, Caveolin-1, Humanized anti-CD26 monoclonal antibody, Graft-versus-host disease, Malignant pleural mesothelioma, Review

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