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Lymphocytes are a major source of circulating soluble dipeptidyl peptidase 4

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Summary

Dipeptidyl peptidase 4 (DPP4, CD26) is a serine protease that is expressed constitutively by many haematopoietic and non-haematopoietic tissues. It exists as a membrane-associated protein, as well as in an active, soluble form (herein called sDPP4), present at high concentrations in bodily fluids. Despite the proposed use of sDPP4 as a biomarker for multiple diseases, its cellular sources are not well defined. Here, we report that individuals with congenital lymphocyte immunodeficiency had markedly lower serum concentrations of sDPP4, which were restored upon successful treatment and restoration of lymphocyte haematopoiesis. Using irradiated lymphopenic mice and wild-type to *Dpp4*$^{-/-}$ reciprocal bone marrow chimeric animals, we found that haematopoietic cells were a major source of circulating sDPP4. Furthermore, activation of human and mouse T lymphocytes resulted in increased sDPP4, providing a mechanistic link between immune system activation and sDPP4 concentration. Finally, we observed that acute viral infection induced a transient increase in sDPP4, which correlated with the expansion of antigen-specific CD8^{+} T cell responses. Our study demonstrates that sDPP4 concentrations are determined by the frequency and activation state of lymphocyte populations. Insights from these studies will support the use of sDPP4 concentration as a biomarker for inflammatory and infectious diseases.

Keywords: chemokines, immunodeficiency diseases, T cells

Introduction

Dipeptidyl peptidase 4 (DPP4, CD26, EC 3.4.14.5) is a multi-functional protein involved in the regulation of both physiological and pathological responses. DPP4 has many reported functions, including protease activity, direct interaction with adenosine deaminase (ADA), cell surface co-receptor for viral entry and regulation of intracellular signal transduction in T cells [1–3]. Cell-associated DPP4 has a molecular weight of 110 kDa and is active as a homodimer with an extracellular enzymatic protease domain and a short cytoplasmic region [4]. Both soluble and membrane-bound DPP4 cleave dipeptides from the N-terminus of proteins that contain a proline or alanine in the penultimate position. DPP4 substrates include chemokines, neuropeptides and incretin hormones [5–9]. The functional consequences of DPP4-mediated post-translational modification may vary among substrates, but notably DPP4 cleavage eliminates the biological activity of the incretin hormone glucagon-like protein 1 (GLP-1). This observation led to the development of DPP4 inhibitors that were established successfully as therapeutic agents in type 2 diabetes patients, as they prevent DPP4-mediated inactivation of GLP-1, and thus enhance cellular insulin sensitivity by prolonging the half-life of GLP-1 [10].

Human DPP4 forms a molecular complex with ADA, which is a cell surface enzyme, catalyzing the irreversible deamination of adenosine [11]. ADA–DPP4 interactions act as co-stimulatory signals during T cell receptor signalling, resulting in enhanced secretion of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα). and interleukin 6 (IL-6) [2,12]. Recent studies have shown that DPP4 can also regulate T cell migration negatively through cleavage of proinflammatory chemokines, such as C-X-C motif chemokine 10 (CXCL10) [13–15]. Our previous work demonstrated that inhibition of DPP4 results in the protection of the active, full-length form of CXCL10 in healthy individuals and hepatitis C patients [16]. This mechanism of CXCL10 protection is relevant in the context of preclinical cancer models, as it increases chemokine-mediated T cell migration into the tumour parenchyma, improving tumour immunity and immunotherapy [15,17].

DPP4 is expressed in immune cells such as T lymphocytes, and is also found in non- haematopoietic tissues such as the kidney epithelium, prostate, lung, liver and small intestine [18–20]. Moreover, a soluble active form of DPP4 (sDPP4) is also present in many biological fluids such as plasma, urine, bile and semen [21]. Notably, variations in the concentration and activity of sDPP4 have been associated with many diseases, and these parameters are considered informative biomarkers for solid and haematological cancers, autoimmune disorders and chronic infectious diseases such as hepatitis C virus (HCV), where high plasma levels of sDPP4 are associated positively with liver fibrosis [22–26]. Highlighting its importance in clinical development programmes, sDPP4 is under evaluation as a predictive marker in anti-IL-13 therapeutic trials for the treatment of asthma [27]. Strikingly, despite its apparent utility as a biomarker, the mechanisms mediating DPP4 release from cells are poorly defined, and may involve shedding and/or secretion [28]. Importantly, the cellular origin of sDPP4 is also not well defined.

Herein, we report that lymphocytes are a major source of sDPP4, and that T cell activation accounts for the dynamic changes observed in its plasma concentration. This discovery followed from our hypothesis that patients with ADA deficiency would have perturbed sDPP4 expression, but observed instead that lower levels were found in all types of severe combined immunodeficiency (SCID) patients. In both humans and experimental mouse models, sDPP4 concentration and activity correlated with the number of circulating lymphocytes. Reciprocal bone marrow chimera experiments, using wild-type (WT) and *Dpp4*$^{-/-}$ mice, demonstrated that bone marrow haematopoietic cells were the major source of plasma sDPP4. Finally, mouse and human T cell stimulation, in the context of *in-vivo* infection or *in-vitro* T cell receptor (TCR) cross-linking, respectively, induced release of sDPP4. Together, these findings support that sDPP4 may serve as a surrogate for the number and activation state of lymphocytes in health and disease.

Methods

Human plasma collection and ethical consideration

Human bioresources (serum and plasma samples and associated data such as age and health status) were collected from healthy volunteers of the Investigation Clinique et Accès aux Resources Biologiques (ICAReB) platform (Centre de Recherche Translationnelle, Institut Pasteur, Paris, France) as part of the Diagmicoll protocol, which has been approved by the French Ethical Committee (CPP) Ile-de-France. Peripheral blood from SCID patients diagnosed by genetic analysis was obtained after informed consent on the occasion of other blood testing in the context of a research protocol at Ospedale Pediatrico Bambino Gesù, Roma, Italy. The research protocol was approved by the Institutional Ethical Committee of Ospedale Pediatrico Bambino Gesù and informed consent forms were signed by all subjects' parents or their legal guardian.

ADA–SCID patients and treatment

ADA–SCID patients refer to San Raffaele Hospital or patients' parents signed informed consent on anonymized data collection for research studies conducted at San Raffaele Hospital (Tiget02). A portion of the analyses, shown herein, was performed as a baseline evaluation for patients who subsequently underwent further treatment with pegademase bovine (PEG)–ADA, bone marrow transplantation (BMT) or haematopoietic stem cell gene therapy (HSC-GT). PEG–ADA-treated and ADA–SCID patients received 10–80 U/kg/week Adagen (PEG; Enzon Pharmaceuticals, Piscataway, NJ, USA). BMT ADA–SCID patients received bone marrow transplantation as described previously [29]. Patients with ADA–SCID undergoing HSC-GT were enrolled in Phases I/II clinical protocols approved by the San Raffaele Scientific Institute's Ethical Committee and Italian National Regulatory Authorities. Gene therapy treatment was performed as described previously [30]. Data from patients undergoing HSC-GT were collected from 2008 to 2011. Since April 2012, GlaxoSmithKline has been a sponsor of the ADA–SCID long-term follow-up trial no. 115611 (HSC-GT) conducted at Tiget.

sDPP4 ELISA

DPP4 quantification was performed with the human or mouse DPPIV/CD26 DuoSet ELISA kit (R&D Systems).

Human plasma samples were diluted 1/1000 or 1/2000; mouse plasma sample were diluted 1/100 and 1/500 by serial dilution. Culture supernatants were diluted twofold. All samples were tested in duplicate, following the manufacturer's instructions. Plates were read with a Labsystems Multiskan MS (Thermo Fisher Scientific, Waltham, MA, USA) device set at 450 nm. Eight-point standard curves, using twofold serial dilutions with a high standard of 4000 pg/ml, were performed in duplicate.

DPP4 activity in plasma samples

DPP4 activity was measured with the DPPIV-Glo protease Assay (Promega, Madison, WI, USA). This assay provides a luminogenic DPP substrate, Gly-Pro-aminoluciferin. After cleavage of the proximal two amino acids from the substrate by sDPP4, the aminoluciferin engages luciferase. Briefly, in a white plate (Greiner Bio-One, Monroe, NC, USA), 50 µl of kit reagent containing the luciferase substrate was added to 50 µl of sample diluted in 10 mM Tris-HCl pH 8, 0·1% prionex (Calbiochem, San Diego, CA, USA). Maximal signal is reached within 30 min and is stable for 3 h. Recombinant human DPP4 (Sigma D4943; Sigma Aldrich, St Louis, MO, USA) was used as a reference. A seven-point standard curve, using twofold serial dilutions with a high standard of 125 ng/ml, was performed in duplicate. DPP activity was expressed in units/ml based on supplier information after subtraction of the background signal [phosphate-buffered saline (PBS) or medium only]. Supplier unit definition was: one unit will produce 1·0 micromole of P-nitroaniline from Gly-Pro-P-nitroanilide/min at pH 7·6 at 37°C. Samples were tested in duplicate with the same range of dilutions as reported above for ELISA. Plates were read in a Tristar LB941 device (Berthold Technologies, Bad Wildbad, Germany).

Animal experiments

All experimental protocols were approved by the Comité d'Éthique pour l'Expérimentation Animale (Institut Pasteur, Paris, France), protocol number: B 75 15-06. Experimental animals had access to food and water *ad libitum*. Mice were 7–12 weeks old and were allowed to acclimate for 1 week prior to any manipulation. Mice were anaesthetized with 100 mg/kg ketamine and 5 mg/kg xylazine. WT C57BL/6 CD45.1, *Rag2*$^{-/-}$, *Rag2*$^{-/-}\gamma_c^{-/-}$ and *Dpp4*$^{-/-}$ (CD45.2) mice on a C57BL/6 strain background were bred in the animal facility of Institut Pasteur. Wild-type C57BL/6 CD45.2 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). All mice were specifically pathogen-free; 100 µl of blood was collected by submandibular bleed into tubes containing 10 µl of 100 mM ethylenediamine tetraacetic acid (EDTA) at indicated time-points.

Chimeric mouse experiments: Mice were exposed to a single lethal dose of 9 gray gamma irradiation. To generate chimeric animals, 3×10^6 bone marrow cells were injected intravenously in a volume of 100 µl into recipient mice 5–6 h after irradiation. Mice were bled by submandibular bleed at the indicated time-points. Fifty µl of blood was collected into tubes containing 10 µl of 100 mM EDTA for evaluation of haematopoietic cell reconstitution and 50 µl of blood was collected with heparin-coated capillaries for evaluation of plasma-associated sDPP4 concentration and activity. Mice differing in their congenic CD45 allele (CD45.1 *versus* CD45.2) were used to track host and recipient cells.

Influenza infection: Mice were injected intraperitoneally (i.p.) with 3×10^5 haemagglutinating unit (HAU) of influenza A/PR/8/34 (Charles River Laboratories) resuspended in 100 µl of sterile PBS. As a control, mice were injected i.p. with 100 µl of PBS. Seven days after infection 50 µl of blood was collected from each mouse and circulating influenza virus-specific $CD8^+$ T lymphocytes were quantified by flow cytometry using tetramers specific to nucleoprotein (NP) and polymerase protein (PA) antigens (see below for details).

Flow cytometry of whole blood and absolute cell counts: Fluorochrome-conjugated anti-mouse CD45.1 (clone 20), CD45.2 (clone 104), CD3 (clone145-2C11), CD8 (clone 53- 6.7) and CD4 (clone RM4-5) were from eBiosciences (San Diego, CA, USA) and BD Biosciences (San Jose, CA, USA). Tetrameric complexes of H-2D^b/influenza $PA_{224–233}$ and H-2D^b/influenza $NP_{366–374}$ were made in house. Monomers were prepared using a modified version of that described and tetramerization was performed prior to use with R-phycoerythrin (PE) or allophycocyanin (APC)-conjugated streptavidin (Invitrogen, Carlsbad, CA, USA) [31]. Reagents were combined to assess the total PA and NP-specific T cells present in mice. For determination of absolute cell numbers of immune cells by cytometry, 10 µl of AccuCheck beads (Invitrogen) were added to 10 µl of EDTA-treated whole blood incubated into 200 µl of Fix/Lyse buffer (eBiosciences) prior to acquisition. To identify cell populations, blood was incubated with 1·6% NH_4Cl to lyse red blood cells. Cells were then incubated with fluorescence activated cell sorter (FACS) buffer (PBS, 2% fetal calf serum, 0·01% azide), FcBlock (BD Biosciences) and subsequently stained with specific antibodies. Samples were acquired on a BD LSRFortessa using DIVA software (Becton Dickinson) and data were analysed by FlowJo

software (Tree Star Inc., Ashland, OR, USA).

Peripheral blood mononuclear cell (PBMC) stimulation

Human PBMCs were purified from fresh whole blood [Etablissement Français du Sang (EFS), Paris] by Ficoll–Paque Plus (GE Healthcare, Little Chalfont, UK) density gradient separation. Two million PBMCs were stimulated in a 48-well plate in 500 μl of AIMV medium (Invitrogen). Anti-CD3 antibody (clone HIT3a) (BD Biosciences) was used at a final concentration of 0·5 μg/ml. Superantigen toxic shock syndrome toxin 1 (TSST1), kindly given by the laboratory of Andres Alcover (Institut Pasteur), were used at a final concentration of 0·2 μg/ml. Brefeldin A (BFA; Sigma Aldrich) was used at a final concentration of 5 μg/ml.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 6.0 software. The Mann-Whitney *U*-test was used to determine whether a difference existed between two groups of individuals. The Kruskal–Wallis test, with Dunn's multiple comparison post-test, was used to compare samples in experiments with three or more groups**.** A *P*-value $< 0{\cdot}05$ was considered to be statistically significant. Spearman's correlation (r_s) was determined for analysis in association studies.

Results

SCID patients have reduced levels of sDPP4

It was demonstrated previously that the ADA enzyme (ecto-ADA) binds to DPP4 in human cells [2,32]. These observations led us to hypothesize that a genetic defect in *ADA* might impact DPP4 protein turnover at the surface of DPP4-expressing cells, such as T lymphocytes. To test this prediction, we measured the concentration and activity of sDPP4 in serum samples from ADA-deficient patients. The ADA-deficient cohort was composed of untreated (ADA UT) and treated patients (ADA TR) who were stratified for the analysis. The serum of healthy, aged-matched subjects (healthy) and patients with severe combined immunodeficiency (SCID) caused by genetic defects other than ADA deficiency were analysed as comparator groups. While sDPP4 concentration and enzymatic activity were reduced by 32 and 29%, respectively, in untreated ADA patients compared to healthy controls, the differences were not statistically significant (Fig. 1a,b). Notably, in treated ADA patients, sDPP4 concentrations were restored to levels similar to those observed in healthy donors. We were surprised, however, to observe that sDPP4 concentration and DPP4 activity in non-ADA SCID patients were also significantly lower than in healthy donors, with an absolute reduction of 58 and 47%, respectively. These results suggested that ADA expression did not account for reduced sDPP4 levels in immune-deficient patients. Instead, these data indicated that the presence *versus* relative absence of lymphocytes might determine circulating sDPP4 concentration.

To test this hypothesis, we compared the number of circulating immune cell subsets to sDPP4 levels and enzymatic activity. Within the adult healthy donor group, we observed a positive correlation between the number of circulating lymphocytes and sDPP4 (Fig. 1c,d), but no correlation was observed with circulating monocytes (Fig. 1e,f) or other immune cell subsets (data not shown). A similar result was obtained when analysing samples from ADA patients, as the level of immune reconstitution was correlated positively with sDPP4 concentration and activity (Fig. 1g,h). Of note, sDPP4 concentration and activity was lower in healthy adults compared to healthy children (Fig. 1). Together, these data suggested that plasma-associated sDPP4 might originate from lymphocytes.

Immunodeficient mice have reduced sDPP4 concentration and activity

To extend our observations from human samples we employed experimental mouse models, with the notable caveats that DPP4 does not bind mouse ADA, and that the pattern of DPP4 expression on haematopoetic cells is somewhat different between mouse and human [19]. To assess the impact of lymphocyte deficiency on sDPP4 concentration and corresponding plasma enzymatic activity, we compared WT mice to $Rag2^{-/-}/\gamma_c^{-/-}$ mice, which lack B, T and natural killer (NK) cells [33]. Supporting our findings in human SCID patients, we found a statistically significant difference between WT and $Rag2^{-/-}/\gamma_c^{-/-}$ mice, with the latter showing marked reduction in both sDPP4 concentration and activity (Fig. 2a,b).

Next, we induced immunodeficiency by whole-body irradiation of WT mice to determine the impact of bone marrow depletion on the half-life of plasma sDPP4. Lymphocyte numbers were measured by flow cytometry and the concentration and activity of sDPP4 were analysed in parallel. Three days after irradiation the number of circulating lymphocytes dropped sharply, from a mean frequency of 11 536 cells/μl [standard deviation (s.d.) = 4 146] to a mean frequency of 681 cells/μl (s.d. = 459) (Fig. 2c). Concurrently, the concentration of sDPP4 declined progressively, with a pre-irradiation mean concentration of 84 ng/ml (s.d. = 12), which decreased to 21 ng/ml (s.d. = 16) 6 days post-irradiation (Fig. 2d). Dipeptidyl peptidase activity showed a similar decrease,

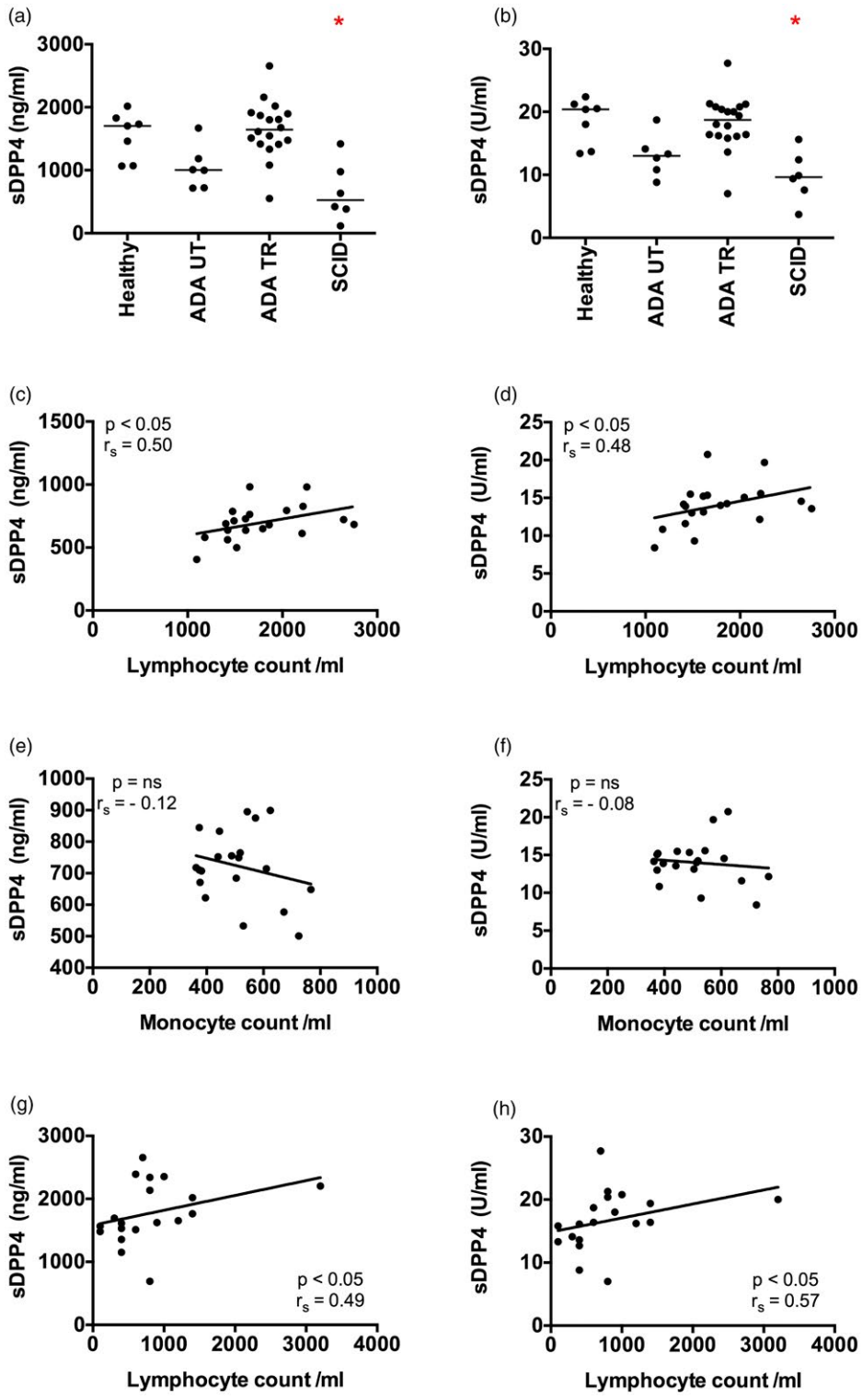

Fig. 1. Patients with severe combined immunodeficiency have low levels of serum soluble dipeptidyl peptidase 4 (sDPP4). (a,b) The concentration (a) and activity level (b) of sDPP4 was measured in the serum of healthy children (healthy), untreated adenosine deaminase (ADA)-deficient patients (ADA UT), treated ADA-deficient patients (ADA TR) and severe combined immunodeficiency (SCID) patients. (c–f) Graphs show circulating lymphocyte or monocyte numbers versus sDPP4 concentration (c,e) or activity (d,f) in the serum of adult healthy donors. (g,h) Graphs show circulating lymphocyte number versus sDPP4 concentration (g) or activity (h) in the serum of treated ADA-deficient patients. Each dot represents the mean value of one healthy donor or patient measured three times. Horizontal lines represent the median of the group. (a,b) Differences between cohorts were tested using the Kruskal–Wallis test followed by Dunn's multiple comparison test. **P* < 0·05. (c–h) Spearman's correlation (r_s) and *P*-value for each analysis are shown.

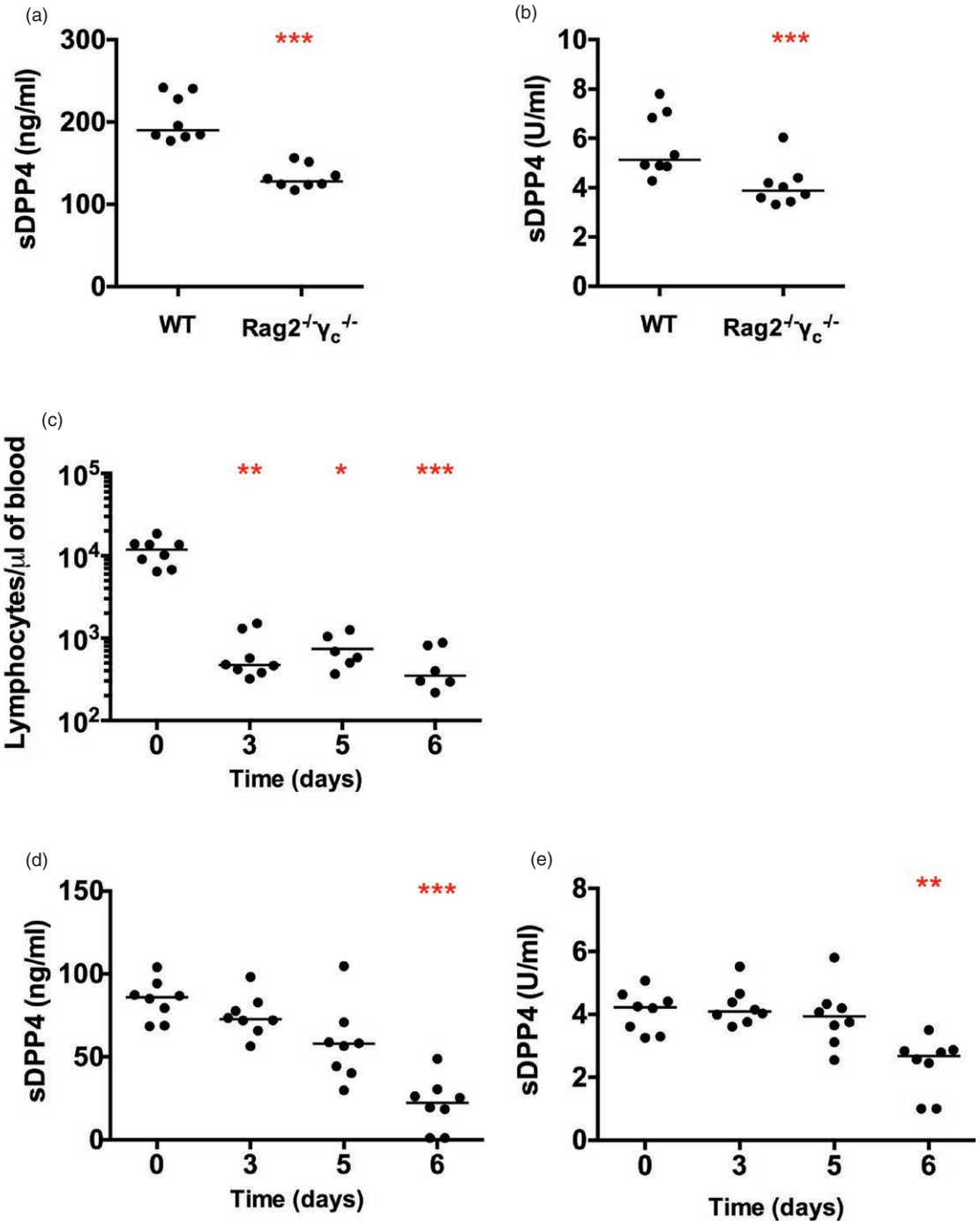

Fig. 2. Soluble dipeptidyl peptidase 4 (sDPP4) level and activity are decreased in immunodeficient mice and after lymphocyte depletion by irradiation. Graphs show (a) sDPP4 concentration and (b) sDPP4 activity in wild-type (WT) and Rag–/– γc–/– mice. (c–e) Mice were exposed to a lethal dose of irradiation. Graphs depict (c) the number of circulating lymphocytes, (d) the sDPP4 concentration and (e) sDPP4 activity in irradiated mice over time. Each dot represents one mouse. The experiment was performed twice. Horizontal lines represent the median. (a,b) Differences between groups were tested for statistical significance using the Mann–Whitney test (c–e). Differences among groups were tested for statistical significance using the Kruskal–Wallis test followed by Dunn's multiple comparison test, comparing all time-points to day 0. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

from a mean activity of 4·1 U/ml (s.d. = 0·6) before irradiation to a post-irradiation activity of 2·1 U/ml (s.d. = 1·3) (Fig. 2e). As the irradiation dose used was lethal, we could not extend our sDPP4 evaluation beyond 6 days. However, based on these findings, approximately 75% of circulating sDPP4 originates from bone marrow-derived cells. Moreover, these data suggest that sDPP4 is produced constitutively, with a plasma half-life of ~3 days. Importantly, we also observed that mouse housing conditions impacted baseline concentrations of sDPP4. In the course of our studies, our colony was relocated to a new, cleaner breeding facility and we observed up to twofold differences in sDPP4 concentration levels in naive WT mice (compare Fig. 2a with 2d). In all experiments, matched controls from the same mouse facility were used.

Bone marrow-derived cells are a significant source of plasma sDPP4

While our human and mouse studies and others supported that lymphocyte numbers correlate with sDPP4 concentration, they did not formally establish a direct source of the enzyme. To investigate the contribution of haematopoietic cells to sDPP4 concentration, we established reciprocal bone marrow chimeras using congenic WT and *Dpp4*$^{-/-}$ mice. We generated four groups of chimeric mice: (1) mice lacking DPP4 on haematopoietic cells (*Dpp4*$^{-/-}$ → WT), (2) mice lacking DPP4 on non-haematopoietic cells (WT → *Dpp4*$^{-/-}$), (3) positive control mice expressing DPP4 on both haematopoietic and non-haematopoietic cells (WT → WT) and (4) negative control mice, deficient for DPP4 in both compartments (*Dpp4*$^{-/-}$ → *Dpp4*$^{-/-}$) (Fig. 3a). To confirm chimerism, we assessed peripheral blood from these animals, tracking congenic markers with specific antibodies. Mice were bled once per week to follow immune cell reconstitution and sDPP4 concentrations in the plasma. Six weeks after bone marrow transfer, > 90% of the haematopoietic cells were donor-derived (Fig. 3b).

In WT → WT control mice, sDPP4 concentration and activity decreased initially to approximately 50% of pre-irradiation levels, and then returned to baseline levels by week 5, consistent with the timing of immune reconstitution. As expected, sDPP4 was not detected at any time in the *Dpp4*$^{-/-}$ → *Dpp4*$^{-/-}$ group (Fig. 3c,d). In the *Dpp4*$^{-/-}$ → WT cohort, sDPP4 decreased rapidly then remained stable at approximately 50% of WT levels (Fig. 3c,d). In WT → *Dpp4*$^{-/-}$ mice sDPP4 was initially absent from the plasma, but reached measurable quantities 1 week after bone marrow transplant, concurrent with the appearance of circulating donor cells (Fig. 3b–d). The concentration and activity levels plateaued at approximately 50% of WT levels 5 weeks post-transplant. Notably, while haematopoietic cells expressed significant levels of DPP4 and served as a robust source of sDPP4, the concentration in WT → *Dpp4*$^{-/-}$ never reached that of WT → WT controls, suggesting that other cellular sources may also contribute to circulating sDPP4 levels. Notably, as observed in patient plasma, there was a positive correlation (r_s = 0·46) between sDPP4 concentration and the number of circulating lymphocytes in reconstituted mice (Fig. 3e).

T cell stimulation results in increased release of sDPP4

Prior studies have suggested a functional relationship between DPP4 and TCR activation (reviewed in Gorrel *et al.* [28]). However, no mechanistic link has been made between T cell activation and the release of lymphocyte-derived sDPP4. We therefore tested whether T cell activation induced sDPP4 release. Using PBMCs derived from healthy donors, we activated lymphocytes *in vitro* using CD3 cross-linking or stimulation with superantigen. Next, we measured sDPP4 concentration and activity in culture supernatants after 20 or 44 h incubation (Fig. 4a,b). Both α-CD3 antibody treatment and *Staphylococcus aureus* TSST1 superantigen stimulation led to induction of sDPP4 (83 and 63% for concentration, 91 and 77% for activity, respectively). Treatment with brefeldin A (BFA) inhibited the release of sDPP4, suggesting that secretory activity is required for the mechanism underlying lymphocyte-derived sDPP4 release.

Finally, we employed *in-vivo* influenza infection to test whether antigen-specific T cell expansion influences sDPP4 concentration. C57Bl/6 mice were injected intraperitoneally with influenza A/PR/8/34 and we sampled blood on days 5, 7 and 9 post-infection, measuring sDPP4 concentration and activity. By day 7 post-infection, we observed a statistically significant increase in the concentration and activity of sDPP4 in influenza-infected mice compared to non-infected animals (Fig. 5a,b). Moreover, influenza-specific CD8$^+$ T cells, enumerated using tetramer analysis (Fig. 5c–g), showed a clear correlation with sDPP4 (Fig. 5h). These results establish a functional relationship between T cell activation and the concentration of sDPP4, providing a rationale for why sDPP4 serves as a prognostic or predictive biomarker in various immune-mediated disease settings.

Discussion

Dipeptidyl peptidase 4 expression is distributed widely among different cell types throughout multiple tissues within the body, participating in multiple biological functions. At least two major forms of the enzyme exist: a membrane-bound homodimeric form located on the surface of cells and a soluble form that is found in multiple

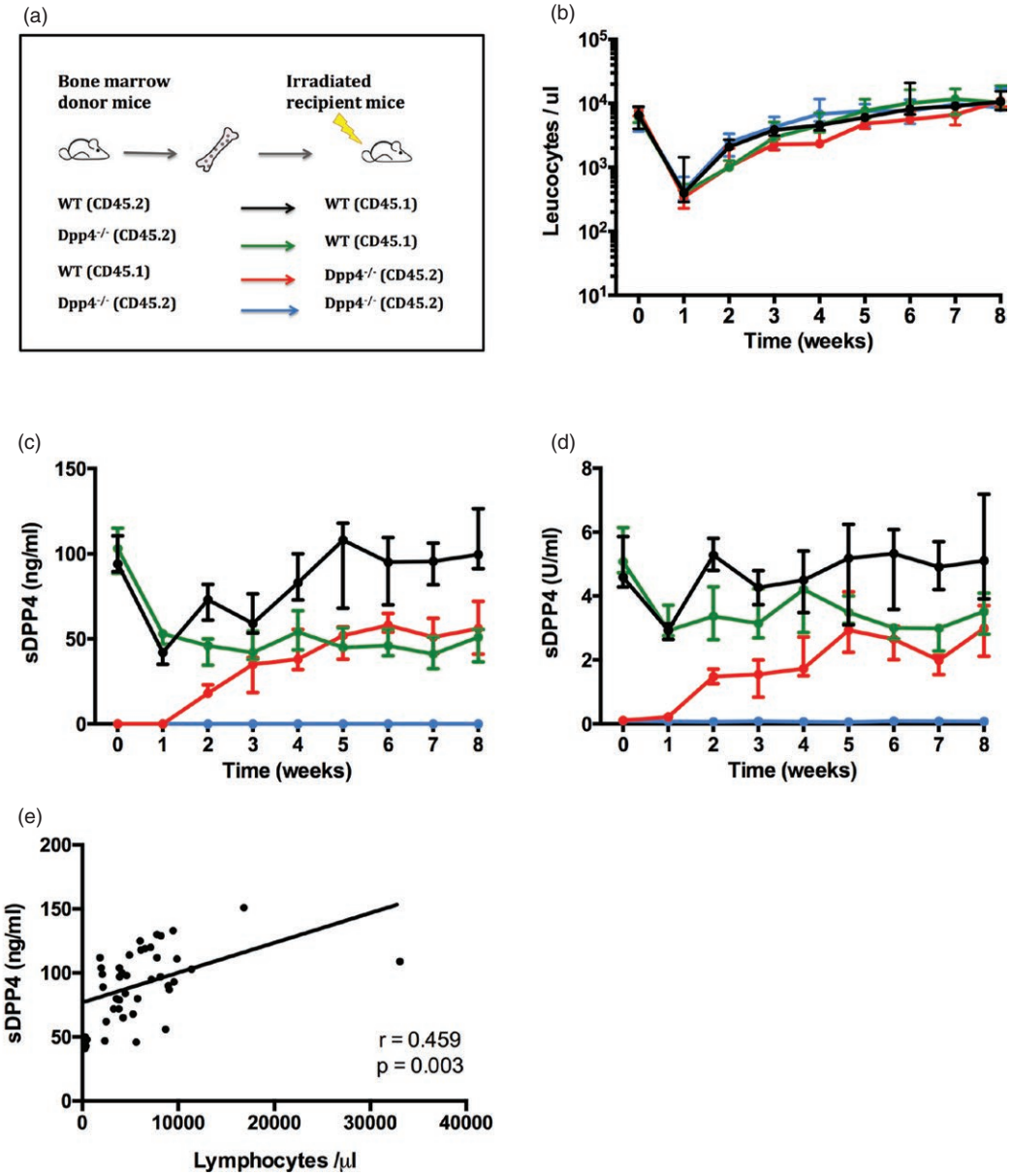

Fig. 3. Bone marrow-derived cells are a major source of soluble dipeptidyl peptidase 4 (sDPP4) in plasma. (a) Schematic representation of the experimental model. (b–e) Mice were lethally irradiated and reconstituted with bone marrow according to the experimental design as illustrated in (a). (b) Graph shows the number of donor leucocytes in circulation in recipient mice. (c) The graphs depict sDPP4 concentration and (d) sDPP4 activity in the plasma of recipient mice. Lines and dots show the mean for each group, with standard deviations. The experiment was performed twice, with three to five mice per group. One representative experiment is shown. (e) Graph shows circulating lymphocyte number versus sDPP4 concentration in [wild-type (WT) → WT] group mice during the experimental follow-up.

bodily fluids. Plasma or serum sDPP4 measurements have been used widely as a biomarker. For example, Cordero *et al.* have reviewed studies pertaining to the origin and altered concentration of sDPP4 in cancer patients [34]. To exploit further the potential of DPP4 as a biomarker, insight into the mechanisms leading to variable sDPP4 concentration is needed. Thus, it is important to establish the tissue or cellular source of sDPP4, as well as the circumstances that provoke altered levels during disease [26].

We report a comprehensive analysis of sDPP4 measurements in a human cohort with primary immune deficiency, before and after treatment, and additional analyses in preclinical experimental models demonstrating that bone marrow-derived cells contribute substantially to circulating sDPP4. Furthermore, we describe a clear

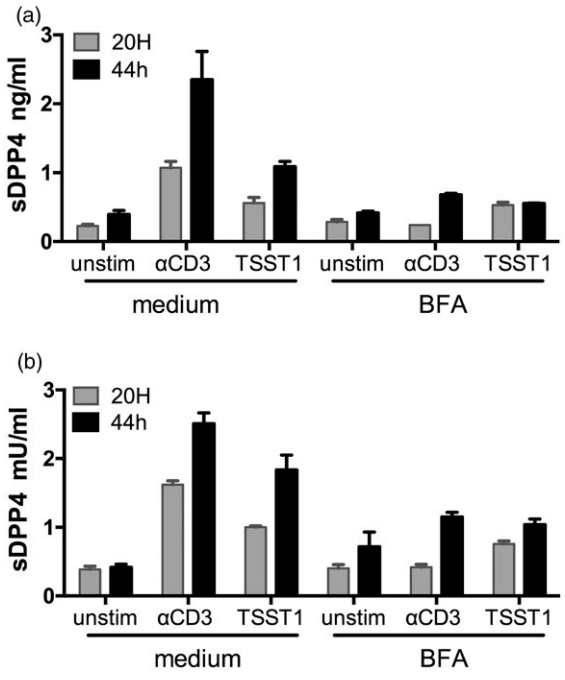

Fig. 4. Peripheral blood mononuclear cell (PBMC) stimulation by anti-CD3 and superantigen toxic shock syndrome toxin 1 (TSST1) induces release of soluble dipeptidyl peptidase 4 (sDPP4). PBMCs were isolated from healthy donors and stimulated as indicated and treated or not with brefeldin A (BFA). (a) sDPP4 concentration and (b) activity were measured in supernatants after 20 or 44 h of culture. Bars represent errors of experimental duplicates. A representative experiment of seven independent experiments with different healthy donors is shown.

correlation between sDPP4 and circulating lymphocyte numbers, establishing a relationship between the secretion of DPP4 and the activation status of lymphocytes. Similar observations in the context of disease, such as rheumatoid arthritis and type 2 diabetes, have been reported [35,36].

In humans, but not in rodents, DPP4 can bind ADA on the surface of T cells and can mediate co-stimulation during T cell activation [37,38]. We hypothesized that patients with an ADA deficiency would have perturbed DPP4 expression and activity. As predicted, we observed lower levels of sDPP4 in these patients, who were restored to normal levels after treatment. However, low levels of sDPP4 could also be detected in non-ADA SCID patients. These data suggested that the number of lymphocytes in circulation, and not ADA expression *per se*, determined plasma sDPP4 levels. Indeed, this interpretation was supported by experiments in irradiated mice in which DPP4 concentration and activity decreased substantially and in chimeric mice, demonstrating that WT bone marrow transplantation into *Dpp4*$^{-/-}$ mice contributed to an appreciable increase in levels of sDPP4. Using a similar approach in rats, others have demonstrated that sDPP4 is derived in part from bone marrow cells [39]. Importantly, however, reconstitution of a *Dpp4*$^{-/-}$ mouse with WT bone marrow did not fully reached sDPP4 concentration of WT mice, suggesting that other cell sources also contribute to the soluble form of this enzyme. It is important to note that DPP4 expression in the haematopoietic compartment differs between human and mouse. Indeed, in humans, DPP4 is expressed mainly by T cells, whereas in mice, dendritic cells, B cells, and NK cells also express DPP4 [40]. Additional studies are needed to determine the inflammatory conditions that lead to increased expression and/or activity and the precise mechanisms of its release.

Both the kidney and liver express high amounts of DPP4, and it has been suggested that these tissues are the source of sDPP4. In the kidney, DPP4 is expressed mainly in the cortex, and not in the medulla, on the cell membranes of glomerular podocytes, but not on the endothelial or mesangial cells [20]. DPP4 is also present on the brush borders of the proximal tubule cells and along the entire proximal tubule [20]. Cleaved sDPP4 may, therefore, accumulate in the urine from this location, accounting for its high expression in this bodily fluid [41]. Consistent with this interpretation, Wang and colleagues showed recently that blood sDPP4 does not originate from the kidney in rats, demonstrated using reciprocal kidney transplantation between DPP4-deficient and WT rats [39]. The hepatobiliary system may also be a source of sDPP4 in the blood circulation. Fukui and colleagues quantified DPP4 in rat liver cells by immunogold localization. In hepatocytes, DPP4 localized mainly to the bile canalicular surface and lysosomal limiting membranes [42]. DPP4 was also observed on the plasma membrane and lysosomal membrane of endothelial and Kupffer cell [42]. Supporting the idea that the liver might be a source of sDPP4, the activity level of this enzyme is elevated in serum from patients with liver diseases, such as hepatitis and cirrhosis, and correlates with liver-associated enzyme concentrations [14,34]. Additionally, aberrant DPP4 expression in human hepatocellular carcinoma and liver cirrhosis has been reported [43,44]. Interestingly, rat bile duct ligation experiments showed that hepatocyte-associated DPP4 is transported in transcytotic vesicles in the bile and serum-associated DPP4 activity is elevated in patients with primary biliary cirrhosis [45,46]. We may speculate, therefore, that in normal physiological conditions, DPP4 would be secreted in the bile and during liver perturbation, DPP4 levels might increase in the blood. However, our experiments support an alternative interpretation, where increased sDPP4 may derive from activated T cells that are the mediators of liver inflammation and liver cell damage.

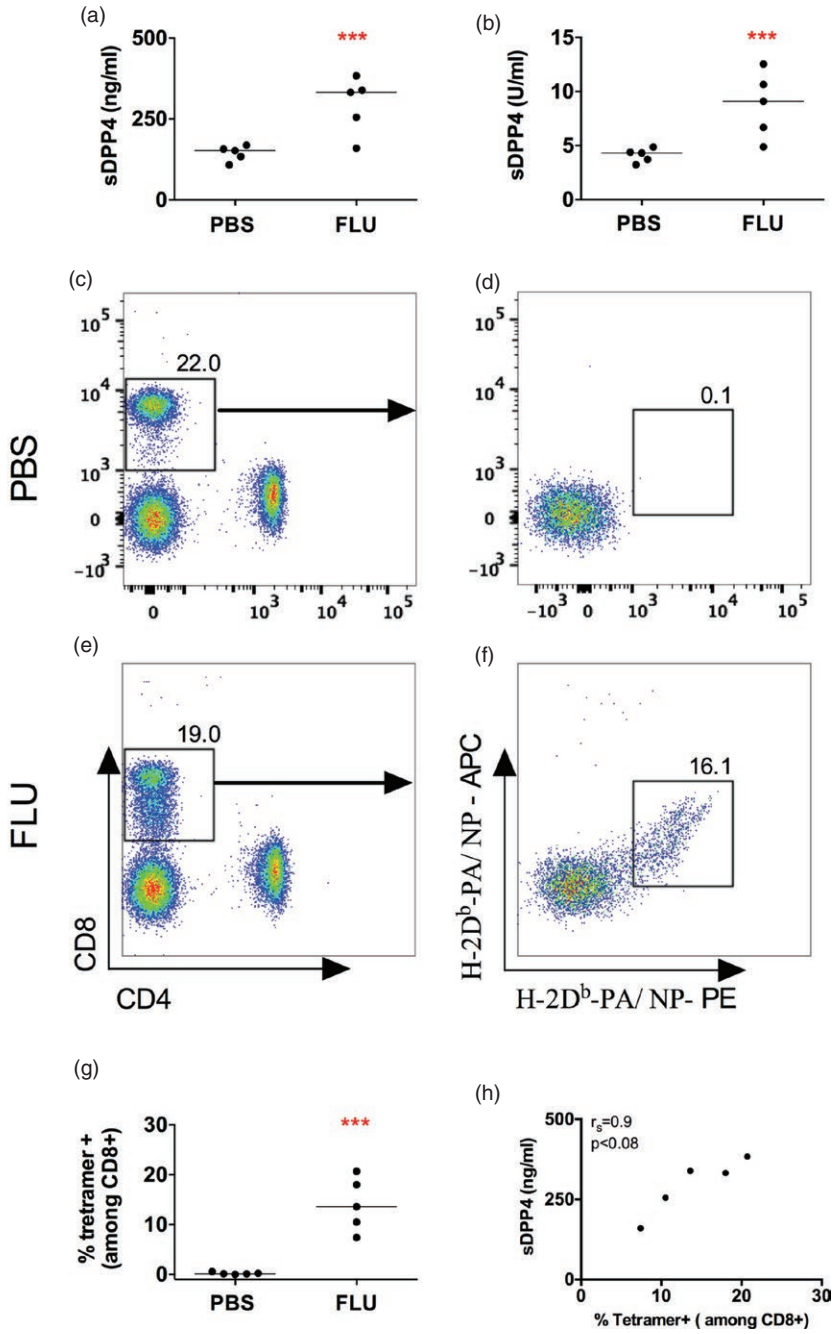

Fig. 5. Induced soluble dipeptidyl peptidase 4 (sDPP4) by influenza virus is correlated with the intensity of tetramer CD8+ T cell-specific responses. Mice were infected with influenza virus PR8 (influenza). (a,b) Graphs show (a) concentration and (b) activity of sDPP4 in blood of mice 7 days after infection. (c–f) Flow cytometry dot-plots show combined tetramer (H-2Db-PA)- and (H-2Db-NP)-specific cells in a representative (c,d) phosphate-buffered saline (PBS)-treated and (e,f) influenza-infected mouse. (g) Graph shows the percentage of tetramer-positive T cells in a group of five PBS-treated and five influenza-infected mice. (h) Graph shows the percentage of circulating tetramer-positive cells versus sDPP4 levels. A representative experiment of three performed is shown. Spearman's correlation (rs) and *P*-value are shown; ***$P < 0{\cdot}001$.

DPP4 was characterized originally as a T cell differentiation antigen and the expression of this enzyme increases following activation (e.g. upon stimulation with antigen, α-CD3 and IL-2, or with mitogens such as phytohaemagglutinin (PHA) [47,48]. Indeed, both the percentage of cells expressing DPP4 and the number of molecules per cell are increased following T cell activation [28,49]. Our results demonstrate that stimulation of human lymphocytes with α-CD3 or superantigen induces DPP4 release *in vitro* and that this release is via a putative

secretory pathway. Furthermore, *in-vivo* T cell activation resulted in increased circulating sDPP4, demonstrated in influenza virus infection. Therefore, activation of lymphocytes is associated directly with higher levels of sDPP4 measured in blood.

The mechanism behind release of sDPP4 from the cell surface is still under investigation. In our experiments, incubation with brefeldin A abolished the release of sDPP4. Brefeldin A causes structural and functional alterations of the trans-Golgi network, suggesting that secretion may facilitate sDPP4 release [50]. Alternatively, brefeldin A may be inhibiting the secretion of a protease necessary for DPP4 shedding. Röhrborn and colleagues described sDPP4 release from adipocytes and smooth muscle cells via shedding by matrix metalloproteinase (MMP)9 and MMP1, MMP2 and MMP14, respectively [51]. However, in their studies, DPP4 secretion from adipocytes and smooth cells was not impacted by brefeldin A treatment. Nargis *et al*. recently reported an alternative mechanism whereby kallikrein-related peptidase 5 (KLK5) was shown to mediate enzymatic cleavage of DPP4 from the surface of IL-17-producing $CD4^+$ T helper type 17 (Th17) cells [52]. Thus, the mechanism of DPP4 secretion may be tissue-dependent and additional biochemical analysis of activated lymphocytes may provide additional insights.

Tanaka and colleagues reported that recombinant sDPP4 enhances proliferative responses of peripheral blood lymphocytes stimulated with soluble antigen [53,54]. More recently, and using various engineered isoforms of DPP4, Gorrel and colleagues demonstrated that sDPP4 enhances human lymphocyte proliferation *in vitro*, independently of both its enzyme activity and adenosine deaminase binding properties [55]. Subsequently, it was demonstrated that sDPP4 induces T cell proliferation through CD86 up-regulation on antigen-presenting cells [56]. As DPP4 plays many biological roles, including the truncation of chemokines that directly impact T cell functions, its influence on lymphocyte activation requires further investigation [57]. Indeed, we have demonstrated previously that DPP4 inhibition enhances lymphocyte trafficking to solid tumours, resulting in increased immunity and delayed tumour growth [15]. Therefore, increased sDPP4 expression may have immunological consequences, as the release of sDPP4 after TCR stimulation would alter the proliferation and migration of lymphocytes.

There is some discrepancy in the literature regarding the usage of sDPP4 as a biomarker in disease [34]. This may be due in part to the contribution of proteins other than DPP4 with DPP4-like activity [58–60]. While a potential caveat, DPP4 contributes to more than 90% of the overall dipeptidyl peptidase activity in the serum and plasma of healthy people [15,57,61]. One important consideration is the use of assays that are optimized for measuring the concentration or activity of DPP4. Throughout our study, we measured protein concentration with an ELISA assay specific for DPP4 and quantified sDPP4 activity using a defined DPP4-selective substrate, demonstrating that activity is well correlated with soluble protein concentrations. To confirm sDPP4 as a biomarker in human disease, it will be necessary to harmonize methods used to measure sDPP4 among various clinical situations. Additional complexity in analysing sDPP4 may arise, as in rheumatoid arthritis and systemic lupus erythematosus, where autoantibodies to DPP4 are present and may interfere with the detection of sDPP4 [62,63].

In sum, our study identified lymphocytes as a major cellular source of sDPP4 and reinforces the critical role of T cell activation in the fluctuation in plasma sDPP4 concentration. Together, a standardized approach for DPP4 measurements and the integration of lymphocyte status for normalization of the biomarker data may help to clarify the role of sDPP4 as a biomarker, and advance it for use in the clinical monitoring of immune-related disorders.

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Disclosures

M. L. A and R. B. d. S. are employees of Genentech. The remaining authors declare no competing financial interests.

Author contributions

A. C. and R. B. d. S. performed the experiments and analysed the data; M. L. A. supervised the experiments; A. S., A. A., M. T. A, S. S. R, ICAReB and M. A. I. provided patient samples and/or critically reviewed the data and manuscript; A. C. and M. L. A. designed the study. A. C., M. A. I. and M. L. A. wrote the manuscript.

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