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Insufficient CD100 shedding contributes to suppression of CD8$^+$ T-cell activity in non-small cell lung cancer

Hong-Min Wang,[1] Xiao-Hong Zhang,[2] Li-Qun Ye,[1] Kai Zhang,[1] Ning-Ning Yang,[1] Shen Geng,[1] Jing Chen,[1] Shun-Xin Zhao,[1] Kang-Li Yang[1] and Fei-Fei Fan[1]

[1]*Department of Respiratory Medicine, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, and* [2]*Department of Respiratory Medicine, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, China*

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Correspondence: Kang-Li Yang and Fei-Fei Fan, Department of Respiratory Medicine, The First Affiliated Hospital of Zhengzhou University, No. 1 Longhu Zhonghuan Rd, Zhengzhou, Henan Province, China.
Emails: wanghongminwhm@yeah.net; fccfanff@zzu.edu.cn
Senior author: Fei-Fei Fan

Summary

CD100 is an immune semaphorin constitutively expressed on T-cells. Matrix metalloproteinase (MMP) is an important mediator of membrane-bound CD100 (mCD100) cleavage to generate soluble CD100 (sCD100), which has immunoregulatory activity in immune cell responses. The aim of the study was to investigate the level and role of sCD100 and mCD100 in modulating CD8$^+$ T-cell function in non-small cell lung cancer (NSCLC). sCD100 and MMP-14 levels in the serum and bronchoalveolar lavage fluid (BALF), and mCD100 expression on peripheral and lung-resident CD8$^+$ T-cells were analysed in NSCLC patients. The ability to induce sCD100 and the effect of MMP-14 on mCD100 shedding for the regulation of non-cytolytic and cytolytic functions of CD8$^+$ T-cells were also analysed in direct and indirect contact co-culture systems. NSCLC patients had lower serum sCD100 and higher mCD100 levels on CD8$^+$ T-cells compared with healthy controls. BALF from the tumour site also had decreased sCD100 and increased mCD100 on CD8$^+$ T-cells compared with the non-tumour site. Recombinant CD100 stimulation enhanced non-cytolytic and cytolytic functions of CD8$^+$ T-cells from NSCLC patients, whereas blockade of CD100 receptor CD72 attenuated CD8$^+$ T-cell activity. NSCLC patients had lower MMP-14 in the serum and in BALF from the tumour site. Recombinant MMP-14 mediated mCD100 shedding from CD8$^+$ T-cell membrane, and led to promotion of CD8$^+$ T-cell response in NSCLC patients. Overall, decreased MMP-14 resulted in insufficient CD100 shedding, leading to suppression of peripheral and lung-resident CD8$^+$ T-cell activity in NSCLC.

Keywords: CD100; CD8$^+$ T-cells; matrix metalloproteinase; non-small cell lung cancer.

Introduction

Lung malignancy is the leading cause of cancer-related deaths worldwide, and can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC).[1] NSCLC accounts for approximately 85% of all cases, with a < 15% 5-year survival rate.[2] The all-cause mortality of NSCLC has been reduced from 20% to 6·7% in the past decade due to the use of low-dose chest computed tomography screening in high-risk individuals as well as the therapeutic involvement of tyrosine kinase inhibitors and immune checkpoint inhibitors.[3] However, the overall

Abbreviations: AC, adenocarcinoma; BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; ELISPOT, enzyme-linked immunospot assay; HBV, hepatitis B virus; HC, healthy control; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN-γ, interferon-γ; LDH, lactate dehydrogenase; mCD100, membrane-bound CD100; MMP, matrix metalloproteinase; NK cells, natural killer cells; NSCLC, non-small cell lung cancer; one-way ANOVA, one-way analysis of variance; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin; SCC, squamous cell carcinoma; sCD100, soluble CD100; SNK-*q* test, Student's–Newman–Keuls-*q* test; TCM, central memory T-cells; TEM, effector memory T-cells; TEMRA, terminally differentiated effector memory T-cells; TNF-α, tumour necrosis factor-α; TNM, tumour-node-metastasis

response rate to treatment is still < 45% even in highly molecularly selected patients.[4] This is partly due to the complicated strategies adopted by cancer cells to escape surveillance and cytotoxicity of the host immune system.[5] Thus, it is still important to better understand the immunopathogenesis and obtain clinically relevant insight for the management of NSCLC.

$CD8^+$ T-cells play an essential role in the prognosis and metastasis of cancers. However, cancer antigens invariably induce dysfunctional or exhausted $CD8^+$ T-cell responses, resulting in hyporesponsiveness and immunological tolerance to host immunity.[6] There are also several important cell surface molecules that characterize the differentiation and activation status of $CD8^+$ T-cells. CD100, also called Sema4D, is the first discovered immune semaphoring, and is involved in several aspects of humoral and cellular immunity in various diseases.[7–9] There are two forms of CD100, membrane-bound CD100 (mCD100) and soluble CD100 (sCD100). mCD100 is preferentially expressed on resting T-cells and natural killer cells (NK cells).[10,11] mCD100 on immune cells is elevated upon activation, which subsequently induces the shedding of the CD100 extracellular domain via proteolytic cleavage by certain factors, such as matrix metalloproteinases (MMPs), to form sCD100 in the serum.[12,13] CD100 functions as a ligand by binding to its receptors, including plexin B1/B2 on non-lymphoid tissues and CD72 on lymphoid tissues.[14] Increasing evidence suggests that CD100 plays a vital role in immune regulation during viral infections and cancer. However, controversy remains as to whether CD100 promotes or inhibits $CD8^+$ T-cell activity in the disease process. A subset of $CD8^+$ T-cells lacking mCD100 were revealed to have decreased functional capacity in human immunodeficiency virus (HIV) infection.[15] Moreover, sCD100 was also shown to enhance the $CD8^+$ T-cell response mainly through CD72 signal transduction in both hepatitis B virus (HBV) and hepatitis C virus (HCV) infection.[13,16] However, whether the change in CD100 levels is associated with and regulates $CD8^+$ T-cell function during NSCLC progression has not been well elucidated. Therefore, we investigated the balance between mCD100 shedding and sCD100 formation in the regulation of $CD8^+$ T-cell responses in patients with NSCLC.

Materials and methods

Enrolled subjects

A total of 53 patients with pathologically diagnosed NSCLC were enrolled in the current study. All patients were hospitalized at the Department of Respiratory Medicine of The First Affiliated Hospital of Zhengzhou University (Henan Province, China) between March 2017 and March 2018. Patients with severe pneumonia or autoimmune diseases were excluded from the study. No patients received surgery, chemotherapy, radiotherapy or immunoregulatory therapy before sampling. The diagnosis of NSCLC was made according to the National Comprehensive Cancer Network Clinical Practice Guideline in Oncology: Non-Small Cell Lung Cancer Version 1.2015. The tumour-node-metastasis (TNM) stages were evaluated according to the American Joint Committee on Cancer/Union for International Cancer Control TNM classification (7th edn). For healthy controls (HCs), 20 sex- and age-matched individuals were also enrolled. The clinical characteristics of all enrolled subjects are listed in Table 1. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. Written informed consent was obtained from each enrolled subject.

Serum and peripheral blood mononuclear cell isolation

Five millilitres of coagulant peripheral blood and 10 ml EDTA-anti-coagulated peripheral blood was collected from each enrolled subject. Serum was isolated from coagulant peripheral blood samples by centrifugation at 12 000 × *g* for 10 min. Peripheral blood mononuclear cells (PBMCs) were isolated from anti-coagulated peripheral blood samples by Ficoll-Hypaque (SolarBio, Beijing, China) density gradient centrifugation.

Bronchoalveolar lavage fluid preparation

Bronchoalveolar lavage fluid (BALF) from NSCLC patients was prepared as previously described.[5] Briefly,

Table 1. Clinical characteristics of enrolled subjects

	NSCLC	HC
Case (*n*)	53	20
Gender (male/female)	41/12	12/8
Age (years)	57·4 ± 12·6	54.9 ± 11·7
COPD history	5	0
Smoking history		
> 10 years	38	10
< 10 years	5	3
None	10	7
Histology		
SCC	28	Not available
AC	25	Not available
Differentiation		
Well	15	Not available
Moderate	25	Not available
Poor	13	Not available
TNM staging		
I	7	Not available
II	21	Not available
III	16	Not available
IV	9	Not available

AC, adenocarcinoma; COPD, chronic obstructive pulmonary disease; HC, healthy control; NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; TNM, tumour-node-metastasis.

the top of the bronchofiberoscope was closely wedged into the opening of the subsegmental bronchus. Fifty millilitres of sterilized saline was rapidly injected through the biopsy hole, and lavage fluid was immediately recovered with 100 mmHg negative pressure with a recovery rate between 40% and 60%. The process was repeated four times. BALF was filtered with double-sterilized gauze and then centrifuged at 1200 × *g* for 10 min at 4°. The supernatants were harvested and stored at −70°, while cell pellets were resuspended and cultured in RPMI 1640 supplemented with 10% fetal bovine serum for subsequent experiments.

$CD8^+$ T-cell purification

Naïve $CD8^+$ T-cells were purified by using a human naïve $CD8^+$ T-Cell Isolation Kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of the enriched cells was more than 95%, as determined by flow cytometry analysis.

Cell stimulation and culture

$CD8^+$ T-cells were pretreated with anti-CD72 (Cat no.: sc-25265; Clone G-5; Santa Cruz, Dallas, TX) for 24 hr. After washing twice, the cells were then stimulated with recombinant human CD100 protein (2 μg/ml; ACRO Biosystems, Beijing, China) for 24 hr in the presence of anti-CD3/CD28 (eBioscience, San Diego, CA) stimulation. In certain experiments, $CD8^+$ T-cells were stimulated with recombinant human MMP-14 (1 μg/ml; CUSABIO, Wuhan, Hubei Province, China) for 48 hr.

Direct and indirect contact co-culture system

A total of 10^5 purified $CD8^+$ T-cells from HLA-A2 restricted individuals were co-cultured in direct or indirect contact with 5×10^5 NCI-1882 lung carcinoma cells, which are also HLA-A2 restricted,[17] as previously described.[5] In the direct contact co-culture system, $CD8^+$ T-cells and NCI-1882 cells were mixed directly, and cultured in the presence of anti-CD3/CD28 (eBioscience) in common cell culture plates. In the indirect contact co-culture system, $CD8^+$ T-cells and NCI-1882 cells were separated by a 0·4-μm pore membrane in a Transwell chamber (Corning, Corning, NY), which only allowed the passage of soluble factors. Anti-CD3/CD28 was added into the upper chamber for maintenance of the $CD8^+$ T-cells. Supernatants were harvested 48 hr post-co-culture.

Enzyme-linked immunosorbent assay

Levels of sCD100, interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and MMP-14 were measured by using commercial enzyme-linked immunosorbent assay (ELISA) kits (CUSABIO) according to the manufacturer's instructions.

Flow cytometry

Cells were stained with fluorescently labelled antibodies (1 μg/ml) for 1 hr at room temperature in the dark and fixed with 2% paraformaldehyde. The antibodies included anti-CD3-APC (Cat no.: 561811, Clone UCTH1; BD Bioscience, San Jose, CA), anti-CD8-APC Cy7 (Cat no.: 561945, Clone SK1; BD Bioscience), anti-CD100-PE (Cat no.: FAB74701P, Clone 758726; R&D Systems, Minneapolis, MN), anti-CD45RA-PE Cy7 (Cat no.: 25-0458-08, Clone HI100; eBioscience, San Diego, CA), anti-CCR7-PerCP Cy5.5 (Cat no.: 45-1971-08, Clone 4B12; eBioscience), anti-plexin B1-Alexa Fluor 700 (Cat no.: FAB37491N, Clone 559830; R&D Systems) and anti-plexin B2-PE (Cat no.: FAB53291P, Clone 537223; R&D Systems). The samples were assayed by using a FACS Aria II Flow Cytometer (BD Bioscience). The data were analysed by using FLOWJO 10.6.1 Software (Tree Star, Ashland, OR).

Enzyme-linked immunospot assay

The numbers of perforin- and granzyme B-secreting $CD8^+$ T-cells were measured by the Human Perforin ELISPOT (enzyme-linked immunospot assay) Kit (Abcam, Cambridge, MA) and Human Granzyme B ELISPOT Kit (Abcam) according to the manufacturer's instructions.

Cytotoxic assay

The cytotoxicity of $CD8^+$ T-cells was determined by calculating the lactate dehydrogenase (LDH) level in the supernatants of co-culture systems as previously described.[5] LDH levels were measured by using a LDH Cytotoxicity Assay Kit (Beyotime, Wuhan, Hubei Province, China) according to the manufacturer's instructions. LDH levels in cultured NCI-1882 cells were used as the low-level control, while LDH levels in Triton X-100-treated NCI-1882 cells were used as the high-level control. The percentage of target cell death was calculated by using the following equation: (experimental value – low control)/(high control – low control) × 100%.[5]

Statistical analysis

All data were analysed by using SPSS version 21.0 for Windows (Chicago, IL). The Shapiro–Wilk test was used for the normal distribution assay. Variables following normal distribution are presented as the mean ± standard deviation, and statistical significance was determined by Student's *t*-test, one-way analysis of variance (ANOVA) or Student's–Newman–Keuls (SNK)-*q* test. Variables

following skewed distribution are presented as the median [Q1, Q3], and statistical significance was determined by Mann–Whitney test, Kruskal–Wallis test or Dunn's Multiple Comparison test. All tests were two-tailed, and a *P*-value < 0·05 was considered statistically significant.

Results

The level of sCD100 was decreased in NSCLC patients

The serum level of sCD100 was investigated in all enrolled subjects. The level of sCD100 in the serum was downregulated in NSCLC patients [6·56 (4·23, 9·67) ng/ml] compared with in HCs [9·95 (6·47, 12·00) ng/ml; Mann–Whitney test, $P = 0{\cdot}026$, Fig. 1a]. There were no significant differences in the serum levels of sCD100 between NSCLC patients with squamous cell carcinoma (SCC) and adenocarcinoma (AC; Fig. S1a), among patients with different differentiation levels (Fig. S1b), or among different TNM stages (Fig. S1c). A total of 31 NSCLC patients underwent bronchoscopy, and BALF was collected from both tumour and non-tumour sites. As expected, the level of sCD100 in BALF from the tumour site was reduced compared with that from the non-tumour site (24·28 ± 6·81 ng/ml versus 30·38 ± 7·27 ng/ml; Student's *t*-test, $P = 0{\cdot}0012$; Fig. 1b). There were no significant differences in the level of sCD100 in BALF from the tumour site between patients with SCC and AC (Fig. S2a), among patients with different differentiation levels (Fig. S2b), or among different TNM stages (Fig. S2c). Among AC patients, 13 (four in stage I, three in stage II and six in stage III) were tested for epidermal growth factor receptor (EGFR) mutations. Five of the 13 patients had EGFR mutations. There were no remarkable differences in the level of sCD100 in either serum or BALF between patients with and without EGFR mutations (Figs S1d and S2d).

mCD100 in $CD8^+$ T-cells was increased in NSCLC patients

The representative flow data analysis for the mCD100 percentage and mean fluorescence intensity (MFI) in peripheral $CD8^+$ and $CD8^-$ T-cells from NSCLC patients and HCs are shown in Fig. 2(a). The percentage of $CD100^+CD8^+$ cells within $CD8^+$ T-cells was elevated in NSCLC patients (78·15 ± 8·29%) compared with in HCs (69·48 ± 9·13%; Student's *t*-test, $P = 0{\cdot}0002$; Fig. 2b). Similarly, the frequency of $CD100^+CD8^+$ cells in BALF was also increased in the tumour site (68·55 ± 7·30%) compared with in the non-tumour site (63·71 ± 6·70%; Student's *t*-test, $P = 0{\cdot}0085$; Fig. 2b). Because CD100 is continuously expressed, the CD100 MFI of $CD8^+$ T-cells was also analysed. As expected, the CD100 MFI of $CD8^+$ T-cells was elevated in NSCLC patients (Student's *t*-test, $P < 0{\cdot}0001$; Fig. 2c) and in the tumour site (Student's *t*-test, $P = 0{\cdot}028$; Fig. 2c). There were no significant differences in $CD100^+CD8^+$ cell percentages in peripheral blood or in BALF between patients with SCC and AC (Figs S3a and S4a), among patients with different differentiation levels (Figs S3b and S4b), among different TNM stages (Figs S3c and S4c), or between patients with and without EGFR mutations (Figs S3d and S4d). Furthermore, the mCD100 percentage and MFI of $CD8^-$ T-cells (mostly $CD4^+$ T-cells) were also investigated. There were no remarkable differences in either the $CD100^+CD8^-$ cell percentage or CD100 MFI in $CD8^-$ cells between NSCLC patients and HCs, or between tumour and non-tumour sites (Student's *t*-tests, $P > 0{\cdot}05$; Fig. 2d,e).

We further investigated the change in CD100 levels in $CD8^+$ T-cell subsets. $CD8^+$ T-cells were divided into the following four different subsets based on the expression of CD45RA and CCR7: naïve $CD8^+$ T-cells ($CD45RA^+$ $CCR7^+$), central memory $CD8^+$ T-cells (TCM; $CD45RA^-$ $CCR7^+$), effector memory $CD8^+$ T-cells (TEM; $CD45RA^-$ $CCR7^-$), and terminally differentiated effector memory $CD8^+$ T-cells (TEMRA;

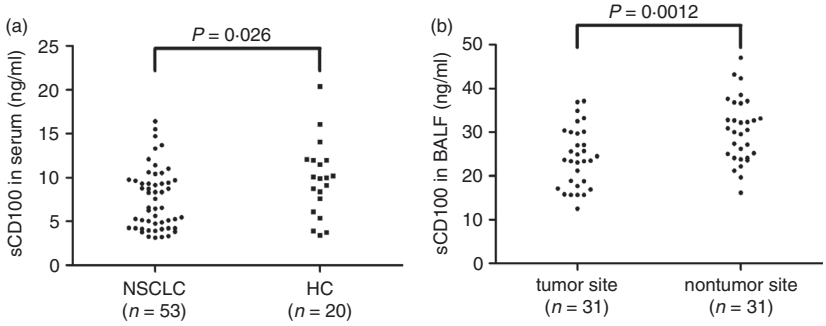

Figure 1. Soluble CD100 (sCD100) levels in the serum and in the bronchoalveolar lavage fluid (BALF) in patients with non-small cell lung cancer (NSCLC). (a) sCD100 levels in the serum were decreased in NSCLC patients ($n = 53$) compared with healthy controls (HCs; $n = 20$). The Mann–Whitney test was used for comparison. (b) The sCD100 level in the BALF from the tumour site was downregulated compared with that from the non-tumour site in patients with NSCLC ($n = 31$). Student's *t*-test was used for the comparison. The individual level of each subject is shown.

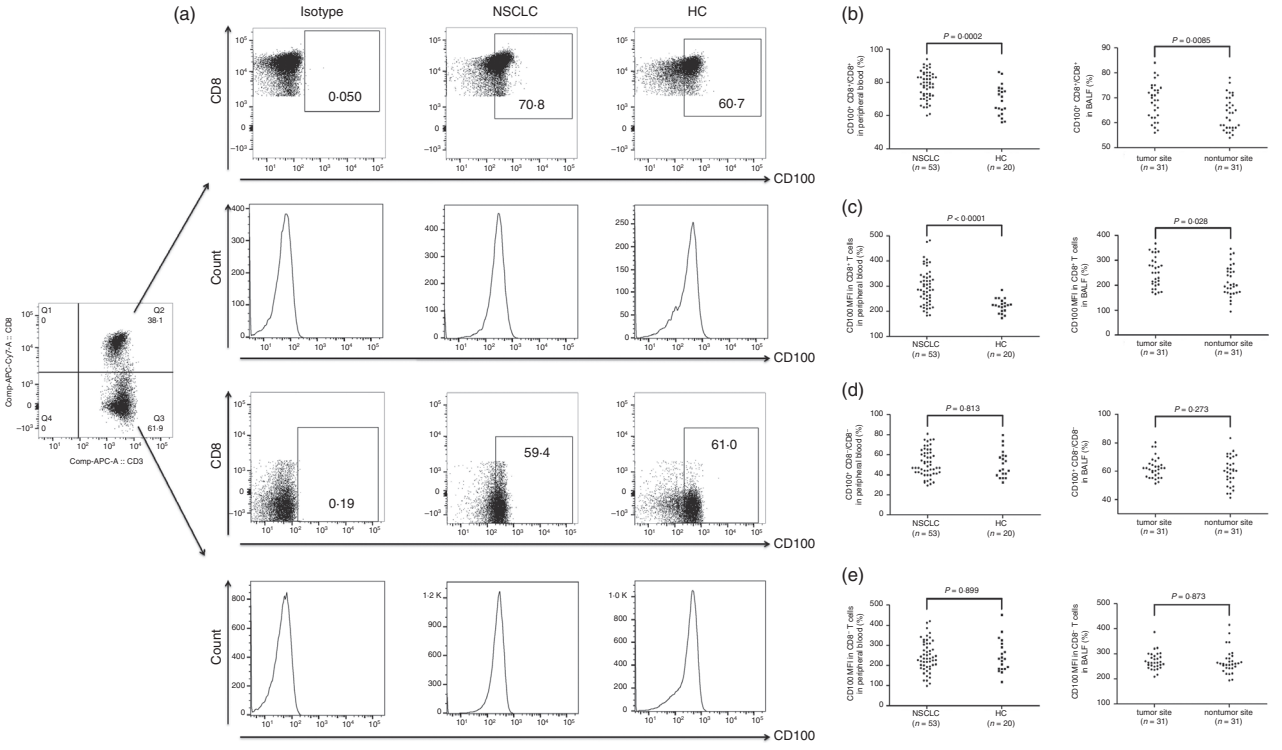

Figure 2. Membrane-bound CD100 (mCD100) expression in $CD8^+$ T-cells from peripheral blood and bronchoalveolar lavage fluid (BALF) in patients with non-small cell lung cancer (NSCLC). (a) Representative flow data analysis for mCD100 percentage and mean fluorescence intensity (MFI) in peripheral $CD8^+$ and $CD8^-$ T-cells from NSCLC patients and healthy controls (HCs) are shown. The isotype control, which was used for the separation of CD100-positive and CD100-negative cells, is also shown. (b) The percentage of peripheral $CD100^+$ $CD8^+$ cells was elevated in NSCLC patients ($n = 53$) compared with HCs ($n = 20$). The percentage of $CD100^+$ $CD8^+$ cells in BALF from the tumour site was increased compared with that from the non-tumour site in patients with NSCLC ($n = 31$). (c) CD100 MFI in $CD8^+$ T-cells in peripheral blood was also elevated in NSCLC patients ($n = 53$) compared with HCs ($n = 20$). CD100 MFI in $CD8^+$ T-cells in BALF from the tumour site was increased compared with that from the non-tumour site in patients with NSCLC ($n = 31$). (d) The percentage of peripheral $CD100^+$ $CD8^-$ cells was comparable between NSCLC patients ($n = 53$) and HCs ($n = 20$). The percentage of $CD100^+CD8^-$ cells in BALF was comparable between tumour and non-tumour sites in patients with NSCLC ($n = 31$). (e) CD100 MFI in $CD8^-$ T-cells in peripheral blood was comparable between NSCLC patients ($n = 53$) and HCs ($n = 20$). CD100 MFI in $CD8^-$ T-cells in BALF was comparable between tumour and non-tumour sites in patients with NSCLC ($n = 31$). Student's *t*-test was used for the comparison. The individual level of each subject was shown.

$CD45RA^+$ $CCR7^-$).[13,16] The representative flow data analyses for mCD100 expression in different $CD8^+$ T-cell subsets from NSCLC patients and HCs are shown in Fig. 3(a). There were no significant differences in naïve $CD8^+$ T-cell percentages in peripheral blood between NSCLC patients and HCs (Student's *t*-test, $P = 0{\cdot}788$; Fig. 3b), or in BALF between samples from the tumour site and the non-tumour site (Student's *t* test, $P = 0{\cdot}103$; Fig. 3c). The percentage of TCM was robustly decreased in the periphery of NSCLC patients (Student's *t*-test, $P < 0{\cdot}0001$; Fig. 3b) and in BALF from the tumour site (Student's *t*-test, $P < 0{\cdot}0001$; Fig. 3c). In contrast, the frequency of TEMRA was notably increased in the periphery of NSCLC patients (Student's *t*-test, $P < 0{\cdot}0001$; Fig. 3b) and in BALF from the tumour site (Student's *t*-test, $P < 0{\cdot}0001$; Fig. 3c). The percentage of TEM in peripheral blood of NSCLC patients was elevated compared with that in HCs ($63{\cdot}71 \pm 7{\cdot}27\%$ versus $60{\cdot}41 \pm 7{\cdot}92\%$), but this difference failed to achieve statistical significance (Student's *t*-test, $P = 0{\cdot}096$; Fig. 3b). However, the frequency of TEM was remarkably higher in BALF from the tumour site than in BALF from the non-tumour site (Student's *t*-test, $P < 0{\cdot}0001$; Fig. 3c). The proportions of CD100-positive cells within naïve $CD8^+$ T-cells, TCM, TEM and TEMRA were all robustly increased in the peripheral blood of NSCLC patients compared with in HCs (Student's *t*-tests, $P < 0{\cdot}0001$; Fig. 3d), and in BALF from the tumour sites compared with non-tumour sites (Student's *t*-tests, $P < 0{\cdot}05$; Fig. 3e).

Recombinant CD100 promoted the non-cytolytic and cytolytic activity of $CD8^+$ T-cells through the CD72 signalling pathway in NSCLC patients

It is well accepted that CD72 is expressed in most immune cells; however, plexin B1/B2 is expressed at very low levels in T-cells.[18] Thus, we first investigated the expression of plexin B1 and plexin B2 on $CD8^+$ T-cells by

flow cytometry. As shown in Fig. S5, there was very little expression of either plexin B1 or plexin B2 on $CD8^+$ T-cells even with anti-CD3/CD28 stimulation. Thus, purified $CD8^+$ T-cells from PBMCs of 10 NSCLC patients were pretreated with anti-CD72 and stimulated with anti-CD3/CD28 in the presence of recombinant CD100 for 24 hr. Phytohaemagglutinin (PHA) stimulation, which activated $CD8^+$ T-cells, induced significant IFN-γ and TNF-α production (Fig. 4a,b). CD72 blockade revealed no remarkable effects on the regulation of cytokine production by $CD8^+$ T-cells in PHA stimulation manners (SNK-*q* test, $P > 0{\cdot}05$; Fig. 4a,b). Recombinant CD100 stimulation also increased the production of IFN-γ and TNF-α by $CD8^+$ T-cells (SNK-*q* test, $P < 0{\cdot}0001$; Fig. 4a, b). CD72 blockade robustly inhibited CD100-induced cytokine production by $CD8^+$ T-cells (SNK-*q* test, $P < 0{\cdot}0001$; Fig. 4a,b). Similarly, blocking CD72 showed a limited effect on perforin and granzyme B secretion by $CD8^+$ T-cells in response to PHA stimulation (SNK-*q* test, $P > 0{\cdot}05$; Fig. 4c,d); however, the effect of CD100-induced perforin and granzyme B secretion by $CD8^+$ T-cells was abolished by CD72 blockade (SNK-*q* test, $P < 0{\cdot}0001$; Fig. 4c,d).

Furthermore, 10^5 purified $CD8^+$ T-cells from nine HLA-A2 restricted NSCLC patients were co-cultured in direct or indirect contact with 5×10^5 NCI-1882 cells for 48 hr. As expected, CD100-activated $CD8^+$ T-cells induced elevated IFN-γ/TNF-α production and increased target cell death in both direct and indirect contact co-culture systems (SNK-*q* test, $P < 0{\cdot}01$; Fig. 5). CD72 blockade suppressed $CD8^+$ T-cell-induced increase in cytokine production (SNK-*q* test, $P < 0{\cdot}05$; Fig. 5a–d) and target cell death (SNK-*q* test, $P < 0{\cdot}01$; Fig. 5e,f) in direct and indirect contact co-culture systems.

MMP-14 mediated CD100 shedding and promoted $CD8^+$ T-cell function in NSCLC patients

We measured the MMP-14 level in all enrolled subjects. Serum MMP-14 levels were decreased in NSCLC patients compared with HCs ($103{\cdot}5 \pm 13{\cdot}11$ pg/ml versus $304{\cdot}7 \pm 61{\cdot}60$ pg/ml; Student's *t*-test, $P < 0{\cdot}0001$; Fig. 6a), and MMP-14 levels in BALF from the tumour site were also reduced compared with those from non-tumour sites [67·29 (56·79, 119·4) pg/ml versus 123·1 (87·34, 199·7) pg/ml; Mann−Whitney test, $P = 0{\cdot}0018$;

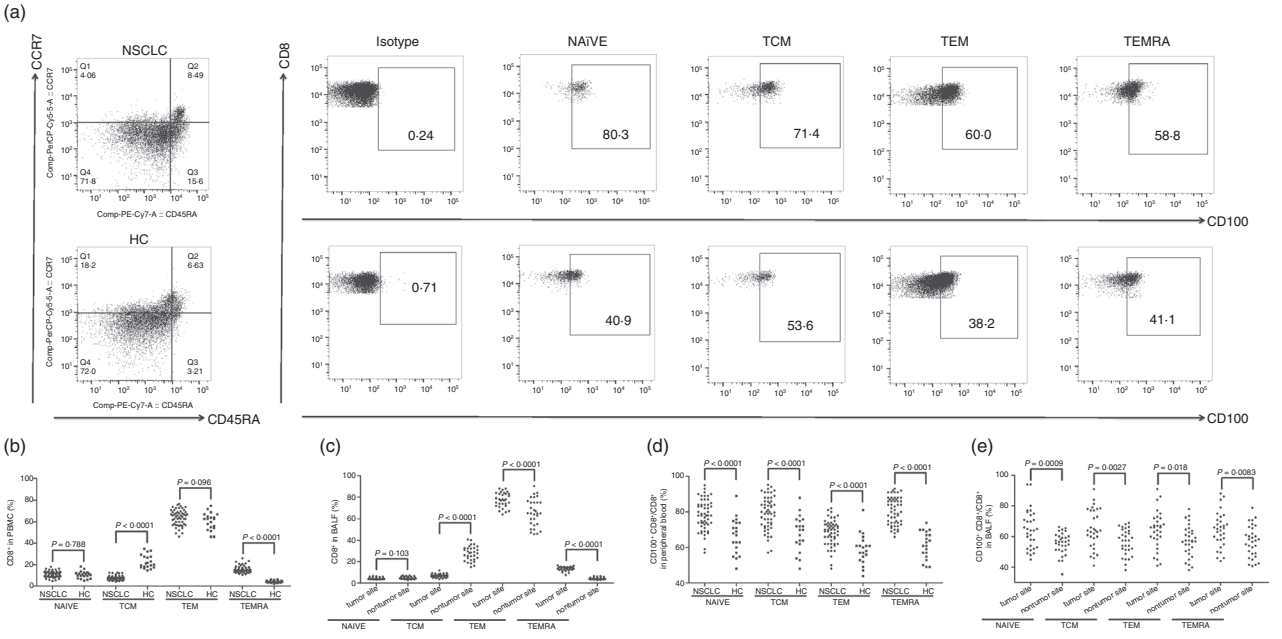

Figure 3. Membrane-bound CD100 (mCD100) expression in different $CD8^+$ T subsets from peripheral blood and bronchoalveolar lavage fluid (BALF) in patients with non-small cell lung cancer (NSCLC). (a) Representative flow data for mCD100 expression in different $CD8^+$ T-cell subsets from NSCLC patients and healthy controls (HCs) are shown. $CD8^+$ T-cells were divided into four different subsets: naïve $CD8^+$ T-cells ($CD45RA^+CCR7^+$); central memory $CD8^+$ T-cells (TCM; $CD45RA^-$ $CCR7^+$); effector memory $CD8^+$ T-cells (TEM; $CD45RA^-$ $CCR7^-$); and terminally differentiated effector memory $CD8^+$ T-cells (TEMRA; $CD45RA^+CCR7^-$). The isotype control, which was used for the separation of CD100 positive and CD100 negative cells, was also shown in different $CD8^+$ T-cell subsets. (b) The percentages of peripheral $CD8^+$ cell subsets were compared between NSCLC patients ($n = 53$) and HCs ($n = 20$). (c) The percentages of $CD8^+$ cell subsets in BALF were compared between tumour sites and non-tumour sites ($n = 31$). (d) The percentages of peripheral $CD100^+$ cells within $CD8^+$ cell subsets were compared between NSCLC patients ($n = 53$) and HCs ($n = 20$). (e) The percentages of $CD100^+$ cells within $CD8^+$ cell subsets in BALF were compared between the tumour sites and non-tumour sites ($n = 31$). Student's *t*-test was used for the comparison. The individual level of each subject is shown.

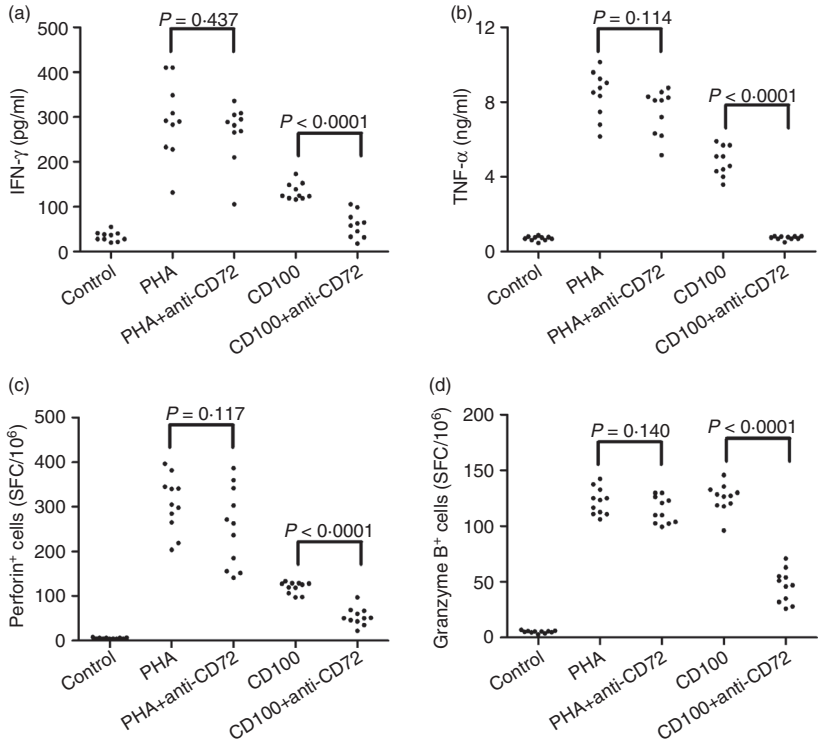

Figure 4. Recombinant CD100 stimulation induces cytokine production and the cytotoxic signalling pathway in $CD8^+$ T-cells from peripheral blood of patients with non-small cell lung cancer (NSCLC; $n = 10$). Purified $CD8^+$ T-cells were pretreated with anti-CD72 for 24 hr. Cells were washed twice and stimulated with anti-CD3/CD28 in the presence of recombinant CD100 or phytohaemagglutinin (PHA) for 24 hr. (a) Interferon-γ (IFN-γ) and (b) tumour necrosis factor-α (TNF-α) levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA), and were compared among groups. (c) Perforin and (d) granzyme B secretion by $CD8^+$ T-cells was measured by enzyme-linked immunospot assay (ELISPOT) and was compared among groups. The results are presented as spot-forming cells (SFC)/10^6. One-way analysis of variance (ANOVA) and Student's–Newman–Keuls-q (SNK-q) test were used for the comparison. The individual level of each subject is shown.

Fig. 6b]. However, there was no significant correlation between MMP-14 and CD100 levels in NSCLC patients (Pearson and Spearman correlation tests, $P > 0{\cdot}05$). $CD8^+$ T-cells from the peripheral blood of 13 NSCLC patients were stimulated with recombinant human MMP-14 for 48 hr. CD100 levels in the culture supernatants and on $CD8^+$ T-cells were then investigated. MMP-14 stimulation enhanced CD100 levels in the culture supernatants ($1{\cdot}08 \pm 0{\cdot}13$ ng/ml versus $0{\cdot}86 \pm 0{\cdot}14$ ng/ml; Student's t-test, $P = 0{\cdot}0003$; Fig. 6c), whereas the percentage of $CD100^+$ $CD8^+$ T-cells was reduced in response to MMP-14 treatment ($56{\cdot}92 \pm 15{\cdot}01\%$ versus $73{\cdot}95 \pm 9{\cdot}33\%$; Student's t-test, $P = 0{\cdot}0020$; Fig. 6d). Moreover, 10^5 purified $CD8^+$ T-cells from six HLA-A2 restricted NSCLC patients stimulated with recombinant MMP-14 were co-cultured in direct or indirect contact with 5×10^5 NCI-1882 cells for 48 hr. MMP-14 stimulation of $CD8^+$ T-cells induced elevated IFN-γ/TNF-α production, and increased target cell death in both the direct and indirect contact co-culture systems (SNK-q test, $P < 0{\cdot}0001$; Fig. 6e–g).

Discussion

Restoration of immune cell exhaustion/dysfunction is currently one of the focuses of therapeutic approaches for NSCLC. The finding in this study indicated that sCD100 expression and mCD100 levels on $CD8^+$ T-cells were affected by NSCLC in both peripheral blood and the lung-resident microenvironment. Upregulation of sCD100 enhanced peripheral and lung-resident $CD8^+$ T-cell responses probably via the interaction between CD100 and CD72 in NSCLC. mCD100 shedding from $CD8^+$ T-cells and sCD100 formation were probably mediated by MMP-14, whereas MMP-14 levels in PBMCs and BALF were also influenced by NSCLC. MMP-14 stimulation increased sCD100 levels but decreased mCD100 expression on $CD8^+$ T-cells, thus further promoting the non-cytolytic and cytolytic activity of $CD8^+$ T-cells in NSCLC. Based on these novel findings of CD100 changes in NSCLC patients, we thus propose a model that during NSCLC progression, decreased MMP-14 levels are insufficient to mediate mCD100 shedding from the surface of

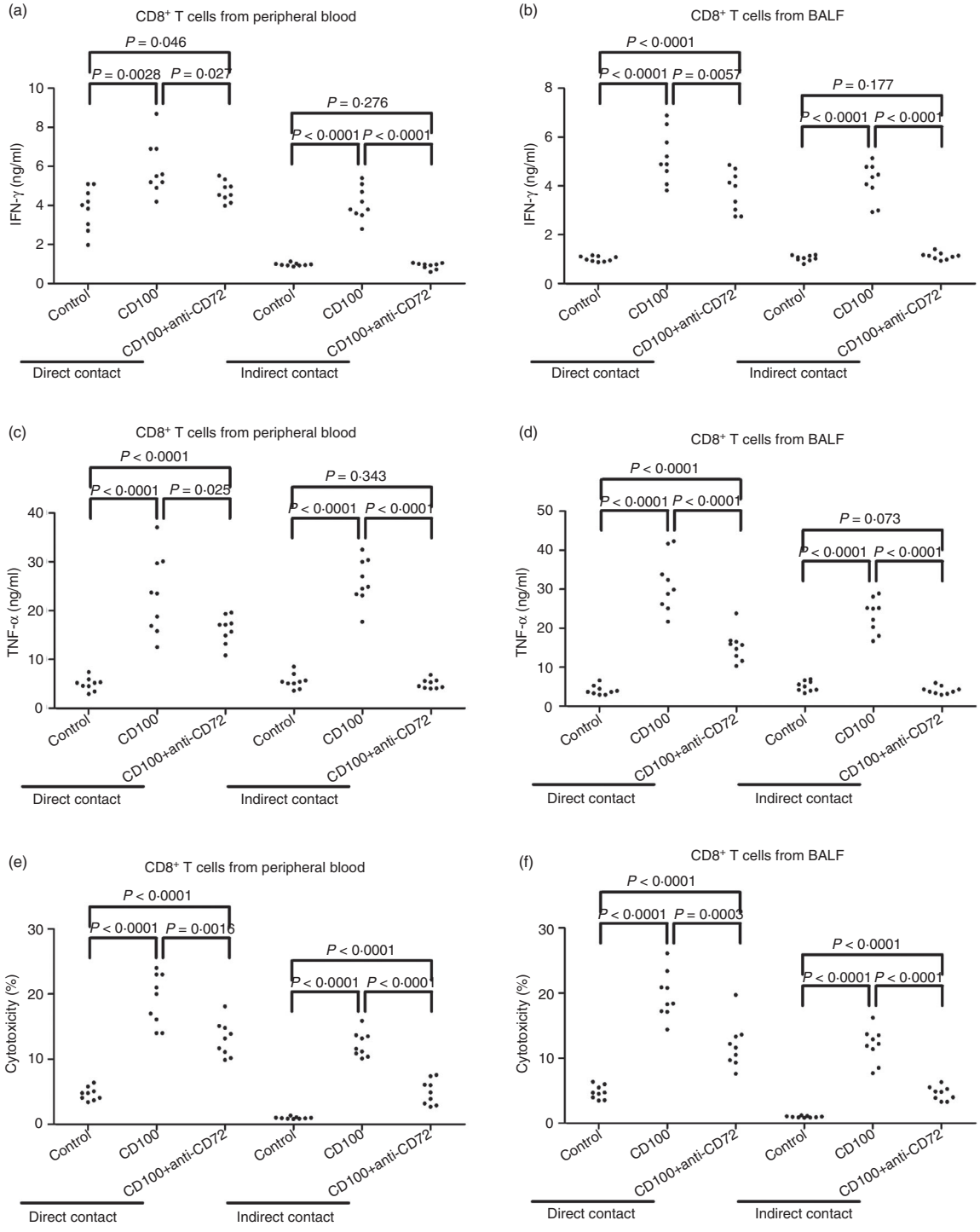

Figure 5. Effect of recombinant CD100 stimulation on the non-cytolytic and cytolytic functions of $CD8^+$ T-cells from HLA-A2 restricted patients with non-small cell lung cancer (NSCLC; $n = 9$). $CD8^+$ T-cells, which were purified from peripheral blood and bronchoalveolar lavage fluid (BALF), were pretreated with anti-CD72 for 24 hr. After washing twice, the cells were then stimulated with recombinant human CD100 protein for 24 hr. A total of 10^5 $CD8^+$ T-cells were co-cultured in direct or indirect contact with 5×10^5 NCI-1882 cells for another 48 hr. (a and b) Interferon-γ (IFN-γ) and (c and d) tumour necrosis factor-α (TNF-α) levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) and were compared among groups. (e and f) The cytotoxicity of $CD8^+$ T-cells towards NCI-1882 cells was calculated by measuring lactate dehydrogenase (LDH) levels in the supernatants. The percentage of target cell death was compared among groups. One-way analysis of variance (ANOVA) and Student's–Newman–Keuls-q (SNK-q) test were used for comparison. The individual level of each subject is shown.

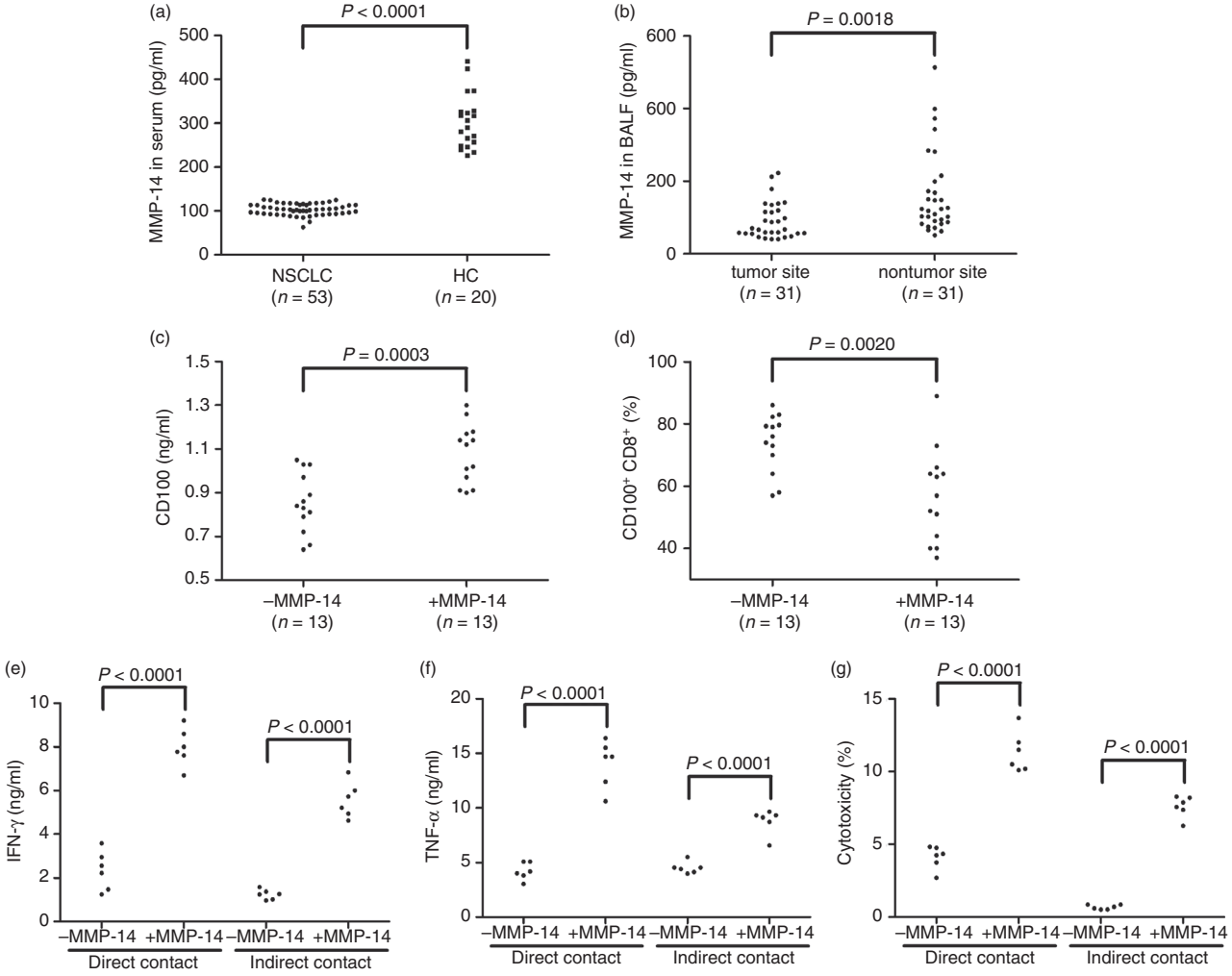

Figure 6. Matrix metalloproteinase-14 (MMP-14) expression and regulation of CD100 shedding and $CD8^+$ T-cell function in patients with non-small cell lung cancer (NSCLC). (a) Serum MMP-14 expression was decreased in NSCLC patients ($n = 53$) compared with healthy controls (HCs; $n = 20$). Student's *t*-test was used for the comparison. (b) MMP-14 levels in bronchoalveolar lavage fluid (BALF) of NSCLC patients ($n = 31$) from the tumour site were reduced compared with those from the non-tumour site. The Mann–Whitney test was used for the comparison. $CD8^+$ T-cells from the peripheral blood of NSCLC patients ($n = 13$) were stimulated with recombinant MMP-14 for 48 hr. (c) CD100 expression in the culture supernatants was increased in response to MMP-14 stimulation. Student's *t*-test was used for the comparison. (d) The percentage of $CD100^+$ $CD8^+$ T-cells was reduced in response to MMP-14 stimulation. Student's *t*-test was used for the comparison. A total of 10^5 purified $CD8^+$ T-cells from HLA-A2 restricted NSCLC patients ($n = 6$), which were stimulated with recombinant MMP-14, were co-cultured in direct or indirect contact with 5×10^5 NCI-1882 cells for 48 hr. (e) Interferon-γ (IFN-γ) and (f) tumour necrosis factor-α (TNF-α) levels in the culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) and were compared among groups. (g) The cytotoxicity of $CD8^+$ T-cells towards NCI-1882 cells was calculated by measuring lactate dehydrogenase (LDH) levels in the supernatants. The percentage of target cell death was compared among groups. One-way analysis of variance (ANOVA) and Student's–Newman–Keuls-*q* (SNK-*q*) test were used for comparison. The individual level of each subject is shown.

$CD8^+$ T-cells, leading to inadequate sCD100 formation and reduced interaction with its receptor CD72, which could not efficiently activate $CD8^+$ T-cells to induce tumour rejection. Thus, MMP-14-mediated CD100 shedding might be important to restore $CD8^+$ T-cell function in NSCLC.

Accumulating evidence has revealed elevation of sCD100 and reduction in mCD100 expression on T-cells in acute viral infection and autoimmune disorder,[13,19–21] indicating that increased sCD100 might mainly result from enhanced CD100 shedding from immune cells in immunoactive status. In contrast, decreased sCD100 levels have been found in breast cancer patients[22] and in chronic HIV-infected patients even following effective antiviral therapy.[23] Our current data indicated imbalanced levels between the two forms of CD100, with decreased sCD100 and increased mCD100 on $CD8^+$ T-cells in both the circulation and tumour

microenvironment in NSCLC patients. This was in line with previous reports on other immunosuppressive conditions, such as persistent HBV infection.[13] However, the mCD100 level on $CD8^-$ T-cells was comparable between NSCLC patients and healthy individuals, indicating that the changes in CD100 might be CD8 specific. Furthermore, the present results were partially in contrast to the findings in several malignancies, including cervical cancer,[24] epithelial ovarian cancer,[25] and even NSCLC.[26] This might be partly because these studies focused on the total CD100 level in tumour tissues, which contained both immune cells and parenchymal cells. Overexpression of total CD100 was also suggested to be associated with the promotion of tumour proliferation, migration, bone metastases and vasculogenic mimicry in breast cancer[27] and lung cancer patients.[26,28,29] However, the role of sCD100/mCD100 in the regulation of immune cell function, especially in $CD8^+$ T-cell activity in NSCLC patients, has never been reported before. The extracellular domain of CD100 retains biological activity after shedding from the cell surface upon activation, leading to the maturation of traditional antigen-presenting cells[18,30] and directly facilitating $CD8^+$ T-cell function during HBV and HCV infection.[13,16] Thus, decreased sCD100 and increased mCD100 suggested insufficient mCD100 cleavage from cell membranes in NSCLC patients, further indicating the consequences of $CD8^+$ T-cell inactivation in NSCLC.

sCD100 induced $CD8^+$ T-cell migration and drove $CD8^+$ T-cell lesional trafficking in oral lichen planus.[31] The cytotoxicity of $CD8^+$ T-cells involved cytolytic (mainly perforin-granzyme pathway) and non-cytolytic (mainly cytokine production) activity on target cells. We found that recombinant human CD100 enhanced both the cytolytic and non-cytolytic function of $CD8^+$ T-cells from NSCLC patients, further revealing the potential immunoregulatory property of sCD100.[32] We then characterized the CD100 signalling pathway involved in regulating the effector $CD8^+$ T-cell response in NSCLC. In line with previous findings in HBV and HCV infection,[13,16] the effect of CD100-induced enhancement of $CD8^+$ T-cell activation was mainly mediated by interacting with the receptor CD72, which was consistent with the finding for the CD100−CD72 interaction and T-cell proliferation.[33] Importantly, $CD8^+$ T-cells could induce target cell death by cell-to-cell contact cytotoxicity and by cytokine production. We thus used an *in vitro* direct and indirect contact co-culture system to independently dissect the cytolytic and non-cytolytic functions of $CD8^+$ T-cells in NSCLC patients. CD100 significantly promoted the cytolytic and non-cytolytic activity of peripheral and lung-resident $CD8^+$ T-cells in NSCLC patients. CD72 blockade suppressed the cytotoxicity of $CD8^+$ T-cells, suggesting that the CD100−CD72 signalling pathway participated in the regulation of $CD8^+$ T-cell function in NSCLC patients. These data revealed that the interaction between CD100 and CD72 participated in the induction of functional $CD8^+$ T-cell responses during the course of NSCLC. Collectively, decreased sCD100 in NSCLC was insufficient for $CD8^+$ T-cell activation, leading to the dysfunction or exhaustion of immune cells in NSCLC patients.

The mechanism of regulation of sCD100 shedding in cancers and other pathological processes remains largely unknown. Hypoxia-inducible factor-1α and microRNAs have been shown to directly regulate CD100 expression by binding to the CD100 promoter.[29,34] However, the processes of mCD100 cleavage and sCD100 formation were only found to be mediated by MMPs.[12,13] Dysregulation of MMPs has been reported in several cancers and is known to be associated with disease progression.[35−37] MMP-14 is highly expressed in most sarcomas and glioblastoma, and regulates tumour invasion, proliferation, angiogenesis and metastasis.[38,39] The MMP-14 protein and mRNA were overexpressed in NSCLC tissue in several studies, and correlated with poor prognosis.[40−42] In contrast, our current findings revealed a decreased level of MMP-14 in the peripheral blood and lung-resident microenvironment. Importantly, MMP-14 is required for the processing and release of CD100 into the soluble form from cancer cell lines, thereby inducing angiogenesis *in vitro* and *in vivo*.[12] Herein, although the current results did not reveal a significant correlation between MMP-14 and CD100, MMP-14 was also a proteolytic enzyme for CD100 cleavage on $CD8^+$ T-cells in NSCLC, which was similar to the role of MMP-9 in oral keratinocytes[31] and HBV infection.[13] Thus, decreased MMP-14 levels were inefficient for CD100 cleavage to form sCD100, resulting in the inadequate activation of $CD8^+$ T-cells in NSCLC.

In summary, decreased MMP-14 levels in the peripheral blood and tumour microenvironment of NSCLC were insufficient for mCD100 cleavage on $CD8^+$ T-cells to form functional sCD100, leading to T-cell exhaustion or dysfunction. MMP-14-mediated CD100 shedding has an important immunoregulatory role in peripheral and lung-resident $CD8^+$ T-cells, which might serve as potential therapeutic targets for NSCLC.

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Disclosures

The authors declare having no competing interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Serum level of soluble CD100 (sCD100) in patients with non-small cell lung cancer (NSCLC).

Figure S2. Level of soluble CD100 (sCD100) in bronchoalveolar lavage fluid (BALF) from tumour site and non-tumour site in patients with non-small cell lung cancer (NSCLC).

Figure S3. Membrane CD100 (mCD100) expression in peripheral $CD8^+$ T-cells in patients with non-small cell lung cancer (NSCLC).

Figure S4. Membrane CD100 (mCD100) expression in peripheral $CD8^+$ T-cells in bronchoalveolar lavage fluid (BALF) from tumour site and non-tumour site in patients with non-small cell lung cancer (NSCLC).

Figure S5. Plexin B1 and plexin B2 expression on $CD8^+$ T-cells in healthy control (HC).

Figure S6. Cytokine production and cytotoxic signalling pathway in $CD8^+$ T-cells from peripheral blood of five healthy individuals.