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IL-9-producing CD8$^+$ T cells represent a distinctive subset with different transcriptional characteristics from conventional CD8$^+$ T cells, and partially infiltrate breast tumors

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ABSTRACT

Accumulating evidence suggests that IL-9 and IL-9-producing cells exert various roles in antitumor immunity. Our study examined the IL-9 production in CD8$^+$ T cells from breast cancer patients as compared to healthy controls. IL-9 secretion was undetectable in CD8$^+$ T cells *ex vivo*, but could be readily detected following anti-TCR or PMA + ionomycin stimulation, and was higher in breast cancer patients than in healthy controls. The capacity to express IL-9 was not universal to all CD8$^+$ T cells, but was favored in IL-9R^{high} CD8$^+$ T cells, which were also present in breast cancer patients at significantly higher frequency than in healthy controls. Interestingly, exogenous IL-9 could significantly increase the expression of both IL-9 and IL-9R in IL-9R^{high}, but not IL-9R^{low}, CD8$^+$ T cells. IL-9R^{high} CD8$^+$ T cells *ex vivo* presented lower expression of KLRG-1, PD-1, and Tim-3 than IL-9R^{low} CD8$^+$ T cells. Additionally, IL-9R^{high} CD8$^+$ T cells following anti-TCR and PMA + ionomycin stimulation presented higher IL-2 and IL-17 expression, and lower IFN-γ expression, than IL-9R^{low} CD8$^+$ T cells. IL-9-expressing CD8$^+$ T cells could be found in some, but not all, resected breast tumors. IL-9R expression, on the other hand, was readily present in CD8$^+$ T cells, but with high variability from patient to patient. Patients with high intratumoral IL-9 expression also tended to present high IL-9R expression. Together, these data demonstrate that a transcriptionally distinctive IL-9-producing CD8$^+$ T cell subset was elevated in breast cancer patients and could be found inside the tumor, with higher capacity to produce IL-2 and IL-17 and lower expression of inhibitory receptors.

1. Introduction

IL-9 is a pleiotropic cytokine mainly produced by T cells (Goswami and Kaplan, 2011). The production of IL-9 was originally associated with the Th2 subset, but it was later found that other T helper subsets, including the Th17 cells and the natural and the inducible regulatory T (Treg) cells, may also produce IL-9 (Beriou et al., 2010; Lu et al., 2006; Nowak et al., 2009). Later, it is shown that naive CD4$^+$ T cells under the influence of cytokines TGF-β and IL-4 and transcription factors STAT6, PU.1, IRF4, and GATA3 can develop into a specialized IL-9-producing helper cell subset, termed Th9 cells (Kaplan, 2013). This Th9 population has been described *in vivo* in the peripheral blood and the skin of allergy patients and melanoma patients (Jones et al., 2012; Purwar et al., 2012). In breast cancer patients, we showed that a subset CD4$^+$CCR4$^-$CCR6$^-$CXCR3$^-$ T cells, which presented high IL-9 expression without concurrent high IL-17A, IL-4, or IFN-γ expression, could be found in the peripheral blood and the resected tumor (You et al., 2017). In addition, IL-9 production has been found in CD8$^+$ T cells in murine melanoma, graft-versus-host disease, and allergic airway disease models (Lu et al., 2014; Ramadan et al., 2017; Visekruna et al., 2013), as well as in human CD8$^+$ T cells (Ramadan et al., 2017).

Accumulating evidence suggests that IL-9 and IL-9-producing cells exert various roles in antitumor immunity (Végran et al., 2015). IL-9-secreting CD4$^+$ T cells were observed among the tumor-infiltrating cells, and adoptive transfer of Th9 suppressed the growth of melanoma and lung adenocarcinoma in an IL-9-dependent manner (Lu et al., 2012; Purwar et al., 2012; Végran et al., 2014). Mechanistically, IL-9 could induce the local production of CCL20, which then recruits dendritic cells and CD8$^+$ T cells into the tumor (Lu et al., 2012). Additionally, Th9 cells has the capacity to produce IL-21 (Kaplan et al., 2011; Végran et al., 2014), which mediate the differentiation and proliferation of T cells and B cells. In breast cancer, Th9 did not present cytotoxicity

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themselves, but assisted the killing activity of $CD8^+$ T cells against autologous tumor cells (You et al., 2017). Interestingly, IL-9-producing $CD8^+$ cytotoxic T cells (Tc9) presented stronger effector function than the conventional IFN-γ-producing T cytotoxic type 1 (Tc1) cells against murine melanoma, and required IL-9 production for optimal functionality (Lu et al., 2014; Ma et al., 2018). Phenotypical examination demonstrated that Tc9 cells expressed low levels of inhibitory molecules PD-1, LAG-3, KLRG-1, and 2B4, and high levels of IL-7Rα, and was capable of secreting the growth factor IL-2 (Lu et al., 2014). On the other hand, IL-9 may inhibit adaptive T cell response and promote tumor growth (Hoelzinger et al., 2014). Additionally, IL-9 can directly enhance the survival and proliferation of lymphomas and lung cancer (Lv and Wang, 2013; Ye et al., 2012).

Overall, these studies suggest that IL-9 and IL-9-producing cells present complicated roles, which may depend on the specific type of cancer and its developmental stage. We previously demonstrated that Th9 cells promoted antitumor immunity in breast cancer patients via IL-9- and IL-21-mediated effects. To further our understanding of IL-9-mediated effects, we sought to examine the characteristics and functions of Tc9 cells in breast cancer patients.

2. Methods

2.1. Study participants

Thirty all-female breast cancer patients between 33 and 51 years of age were recruited, together with thirty all-female healthy controls between 30 and 50 years of age. All participants were pre-menopausal and not taking hormone replacement therapy. Diagnosis was performed at the Shengli Oilfield Central Hospital using a number of clinical techniques, including physical examination, mammography, ultrasound examination, core biopsy, and lymph node biopsy. Patients included 10 stage II and 20 stage III patients, classified according to the tumor-node-metastasis (TNM) system 7th edition by the American Joint Committee on Cancer (Edge and Compton, 2010). All participants donated peripheral blood samples. In addition, twenty breast cancer patients donated resected tumor samples. This study was approved by the Shengli Oilfield Central Hospital ethics review committee. All participants gave written informed consent. Detailed demographic and clinical information of the patients are presented in Supplementary Table 1.

2.2. Cell collection

Peripheral blood was collected from all healthy controls and breast cancer patients before treatment or surgery. PBMCs were collected using the Ficoll-Paque (GE Healthcare) gradient centrifugation of the whole blood, and then washed twice in sterile phosphate-buffered saline (PBS) + 2% heat-inactivated fetal calf serum (FCS). Resected tumors were dissected into small fragments with a sterile scalpel. The fragments were then digested in a mixture of 2.5 mg/mL collagenase, 20 U/mL DNase, and 1 mg/mL hyaluronidase (Sigma) for 3 h in a 37 °C shaking water bath. The digestion production was filtered using a 70-μm strainer (Falcon), and the lymphocytes were collected using Ficoll-Paque gradient centrifugation.

2.3. Flow cytometry

PBMCs were incubated with anti-human CD3, CD8, and IL-9R (CD129) and/or CD129 isotype control antibodies (Biolegend), as well as Fixable Aqua Dead Cell stain (Thermo Fisher), for 30 min in PBS + 2% FBS. The PBMCs were then washed and acquired using the FACSCanto system (BD), and the results were analyzed in FlowJo software (Tree Star). For sorting of $IL\text{-}9R^{high}$ and $IL\text{-}9R^{low}$ $CD8^+$ T cells, the $CD8^+$ T cells were first isolated from total PBMCs using Human CD8 T Cell Enrichment kit (Stemcell Technologies) with purity greater than 97%. The $CD8^+$ T cells were then stained with anti-human IL-9R antibody, and sorted using the FACSAria system (BD).

2.4. IL-9 ELISA

Freshly isolated whole $CD8^+$ T cells, or $CD8^+$ T cells sorted ex vivo according to $IL\text{-}9R^{high}$ and $IL\text{-}9R^{low}$ expression, were incubated in one of the three following conditions, including pure culture medium (sterile RPMI 1640 supplemented with 1% L-glutamine, 1% Penicillin-Streptomycin, and 10% heat-inactivated fetal bovine serum; all from Gibco) without stimulation, culture medium plus anti-CD3/CD28 antibodies (4 μg/mL each; BioLegend), or culture medium plus PMA (20 ng/mL) and ionomycin (1 μg/mL). After incubation for 24 h under 37 °C der 5% CO_2, the supernatant and the cells were separated via 5-minute centrifugation at 300 *g*. IL-9 ELISA was performed on the supernatant using Human IL-9 ELISA MAX Deluxe (BioLegend).

2.5. Quantification of mRNA

Ex vivo $CD8^+$ T cells, or $CD8^+$ T cells after stimulation and incubation, were treated with the RNeasy Mini kit (Qiagen) to isolate total RNA, onto which the QuantiTect Reverse Transcription Kit (Qiagen) was applied to synthesize cDNA. The TaqMan gene expression assays (Thermo Fisher) were performed for the following human genes: IL-9 (Hs00174125_m1), IL-9R (Hs00174125_m1), KLRG-1 (Hs00195153_m1), PD-1 (Hs01550088_m1), Tim-3 (Hs00958618_m1), LAG-3 (Hs00958444_g1), IL-2 (Hs00174114_m1), IL-17 (Hs00174383_m1), and IFN-γ (Hs00989291_m1). Reactions were carried out using the ABI Prism 7000 System (Thermo Fisher). Expression levels were normalized to the expression of GAPDH using the TaqMan GAPDH Control Reagents kit (Thermo Fisher). The $2^{-\Delta\Delta Ct}$ method was applied.

2.6. Statistical analysis

Data were given as mean ± standard deviation. Statistical difference between two groups was calculated using unpaired *t* test. Correlation between two parameters was examined using Pearson correlation test. Statistical difference between multiple groups was calculated using Two-way ANOVA followed by Sidak's multiple comparisons. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Tc9 cells were undetectable ex vivo but could be found following stimulation, with higher IL-9 production in patients than in controls

Peripheral blood mononuclear cells (PBMCs) were collected from breast cancer patients and age-matched healthy controls. IL-9 production was then examined in purified circulating $CD8^+$ T cells from each participant. In both healthy controls and cancer patients under unstimulated condition, the level of secreted IL-9 was below the detection limit (Fig. 1A). Following anti-TCR stimulation and direct PMA and ionomycin (PMA + Iono) stimulation, IL-9 secretion was readily detected (Fig. 1A). As a group, breast cancer patients presented higher IL-9 secretion than healthy controls, but large individual variation was observed in both groups. Compared to anti-TCR stimulation, PMA + Iono stimulation was more potent at increasing IL-9 secretion ($P = 0.013$ in controls and $P = 0.018$ in patients).

Subsequently, the level of IL-9 mRNA expression was examined in $CD8^+$ T cells directly *ex vivo*, and following anti-TCR stimulation and PMA + Iono stimulation. The IL-9 mRNA expression was then expressed as ratio over GAPDH, the reference gene (Fig. 1B). Directly *ex vivo*, IL-9 mRNA was only detected in approximately half of the controls and patients, with no significant difference between the two groups. Anti-TCR stimulation and PMA + Iono stimulation increased the IL-9 mRNA levels (Fig. 1B). Compared to anti-TCR stimulation, PMA + Iono

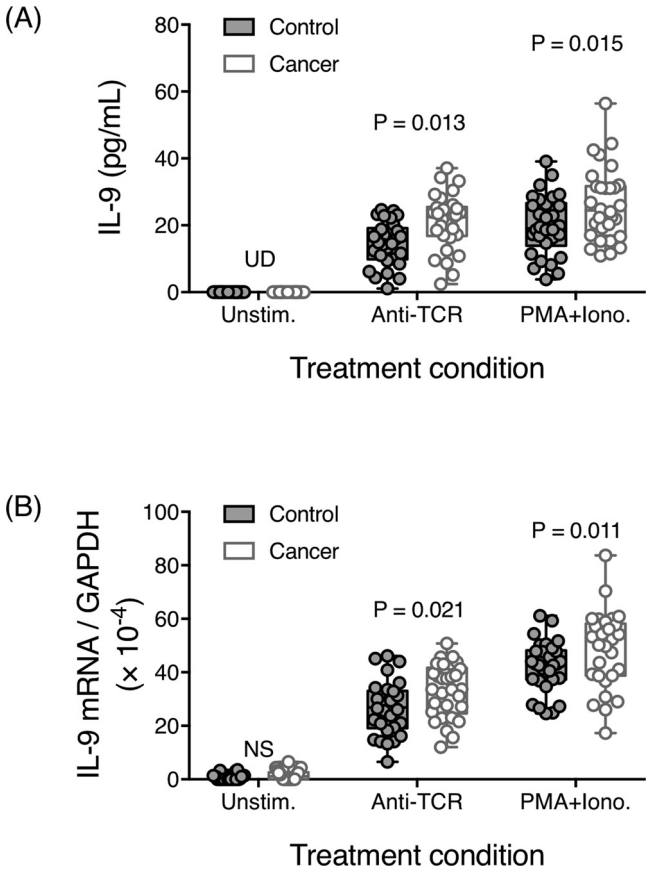

Fig. 1. Expression of IL-9 from circulating CD8$^+$ T cells.
Isolated CD8$^+$ T cells from each of the healthy controls (N = 30) and cancer patients (N = 30) were incubated in complete media without stimulation (Unstim.), with anti-CD3/CD28 stimulation (Anti-TCR), or with PMA and ionomycin stimulation (PMA + Iono.) for 24 h. (A) The secreted IL-9 level in the supernatant at the end of incubation. (B) The IL-9 mRNA level in the CD8$^+$ T cells, relative to the IL-9 mRNA level before stimulation. Two-way ANOVA followed by Sidak's multiple comparisons. UD, undetectable. NS, not significant.

stimulation was more potent at increasing IL-9 secretion ($P < 0.001$ in both controls and patients). Under both anti-TCR and PMA + Iono stimulation, the IL-9 mRNA levels were significantly higher in cancer patients than in healthy controls (Fig. 1B). Again, high variation between individuals in each group was observed.

3.2. *Tc9 cells were enriched in IL-9R^{high} CD8$^+$ T cells*

IL-9 signals through the IL-9 receptor complex, which is composed of an IL-9 receptor (IL-9R) subunit, designated to IL-9, and an IL-2 receptor gamma (IL-2RG) subunit, shared among many cytokines. To examine whether CD8$^+$ T cells could receive IL-9 signaling, we investigated the IL-9R expression in CD8$^+$ T cells. Using flow cytometry, CD8$^+$ T cells were stepwise as live, single, CD3$^+$CD4$^-$CD8$^+$ lymphocytes (Supplementary Fig. 1). In the CD8$^+$ T cells (Fig. 2A), we found that no distinctive IL-9R$^+$ and IL-9R$^-$ populations could be seen in the CD8$^+$ T cells. Rather, a continuous gradient in IL-9R expression was observed (Fig. 2A, right panel). Using isotype control gating (Fig. 2A, left panel) as gating guide, we separated total CD8$^+$ T cells into Left and Right subsets. mRNA examination demonstrated that both the Left and the Right subsets presented IL-9R transcripts, with the Right subset expressed significantly higher level of IL-9R mRNA than the Left subset (Fig. 2B). Hence, we renamed the Left subset as IL-9R^{low}, and the Right subset as IL-9R^{high}. The frequency of IL-9R^{high} CD8$^+$ T cells was slightly higher in cancer patients than in healthy controls (Fig. 2C). Interestingly, the IL-9R^{high} CD8$^+$ T cells also presented higher capacity to express IL-9 than the IL-9R^{low} CD8$^+$ T cells, evident by the discovery that IL-9R^{high}-sorted CD8$^+$ T cells secreted higher level of IL-9 following anti-TCR stimulation and PMA + Iono stimulation, than IL-9R^{low}-sorted CD8$^+$ T cells (Fig. 2D and E).

Subsequently, we investigated the effect of exogenous IL-9 on CD8$^+$ T cells. Total CD8$^+$ T cells were sorted into IL-9R^{high} and IL-9R^{low} subsets as described above. Each subset was then treated with various levels of exogenous recombinant human (rh)IL-9. Following 24 -h incubation, the levels of endogenous IL-9 and IL-9R expression were examined. Interestingly, in IL-9R^{high} CD8$^+$ T cells, the endogenous expression of IL-9 was significantly elevated with increasing levels of exogenous rhIL-9 (Fig. 2F), while in IL-9R^{low} CD8$^+$ T cells, no significant effect was observed. Exogenous rhIL-9 also elevated the expression of IL-9R by IL-9R^{high}, but not IL-9R^{low}, CD8$^+$ T cells, with an increasing trend from 0 to 100 pg/mL, and a plateau from 100 to 1000 pg/mL (Fig. 2G).

3.3. *IL-9R^{high} CD8$^+$ T cells presented lower expression of inhibitory molecules than IL-9R^{low} CD8$^+$ T cells*

Subsequently, we examined the expression of inhibitory molecules by IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells. Compared to IL-9R^{low} CD8$^+$ T cells, IL-9R^{high} CD8$^+$ T cells presented significantly lower expression of KLRG-1, PD-1, and Tim-3 (Fig. 3A–C). However, the expression of LAG-3 was not significantly different between IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells (Fig. 3D). This difference in the expression of inhibitory molecules was observed in both breast cancer patients and in healthy controls. In addition, the IL-9R^{low} CD8$^+$ T cells from healthy controls presented lower KLRG-1, PD-1, and Tim-3 than the IL-9R^{low} CD8$^+$ T cells from cancer patients (Fig. 3A–C). Also, the IL-9R^{high} CD8$^+$ T cells from healthy controls presented lower KLRG-1, and Tim-3 than the IL-9R^{high} CD8$^+$ T cells from cancer patients (Fig. 3A and C).

3.4. *IL-9R^{high} CD8$^+$ T cells presented different cytokine expression profile from IL-9R^{low} CD8$^+$ T cells*

Next, the difference in the expression of cytokines, including IL-2, IL-17, and IFN-γ, was examined between IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells. Directly *ex vivo*, the expression levels of cytokines were very low and often undetectable. No differences between IL-9R^{high} and IL-9R^{low} CD8$^+$ T cells or between healthy controls and cancer patients were observed. Hence, we stimulated the CD8$^+$ T cells using anti-TCR and PMA/Iono stimulations. The expression of IL-2 was significantly higher in IL-9R^{high} CD8$^+$ T cells than in IL-9R^{low} CD8$^+$ T cells (Fig. 4A). Interestingly, the cancer IL-9R^{low} CD8$^+$ T cells presented significantly lower IL-2 secretion than the control IL-9R^{low} CD8$^+$ T cells, while no difference was found between cancer IL-9R^{high} CD8$^+$ T cells and control IL-9R^{high} CD8$^+$ T cells (Fig. 4A). The IL-17 expression was also significantly higher in IL-9R^{high} CD8$^+$ T cells than in IL-9R^{low} CD8$^+$ T cells, but no difference between cancer cells and control cells was observed (Fig. 4B). The IFN-γ expression, however, was significantly higher in IL-9R^{low} CD8$^+$ T cells than in IL-9R^{high} CD8$^+$ T cells (Fig. 4C). The cancer IL-9R^{low} CD8$^+$ T cells presented significantly lower IFN-γ secretion than the control IL-9R^{low} CD8$^+$ T cells, while no significant difference was found between cancer IL-9R^{high} CD8$^+$ T cells and control IL-9R^{high} CD8$^+$ T cells (Fig. 4C).

3.5. *Tc9 cells were detected in a few, but not all, breast tumors*

We wondered whether IL-9-producing and IL-9R-expressing CD8$^+$ T cells could be detected in the breast tumor microenvironment. In 20 resected breast tumor samples, we isolated CD8$^+$ T cells and examined whether they expressed IL-9 and IL-9R mRNA. A few, but not all, breast tumors contained CD8$^+$ T cells that expressed IL-9, and the level of IL-9

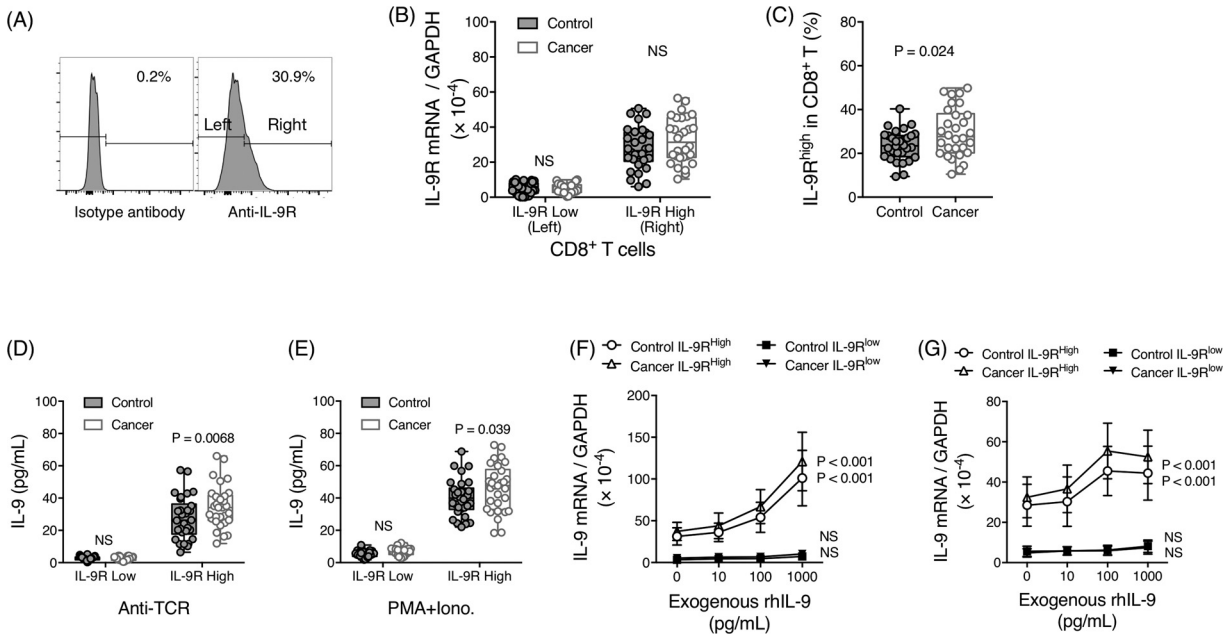

Fig. 2. Expression of IL-9R in circulating CD8$^+$ T cells.
(A) Representative IL-9R vs. isotype control staining in pre-gated CD8$^+$ T cells from one cancer patient. (B) IL-9R mRNA expression in CD8$^+$ T cells, sorted into IL-9R^{high} and IL-9R^{low} subsets using flow cytometry. (C) The frequency of IL-9R^{high} cells in CD8$^+$ T cells from healthy controls and cancer patients. (D) IL-9 secretion by anti-TCR-stimulated IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells. (E) IL-9 secretion by PMA + Iono-stimulated IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells. (F) IL-9 mRNA in anti-TCR-stimulated IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells, with increasing levels of exogenous rhIL-9. N = 30 for healthy controls and cancer patients. (G) IL-9R mRNA in anti-TCR-stimulated IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells, with increasing levels of exogenous rhIL-9. N = 30 for healthy controls and cancer patients. (B), (D) to (G) Two-way ANOVA followed by Sidak's multiple comparisons. (C) Unpaired *t* test. NS, not significant.

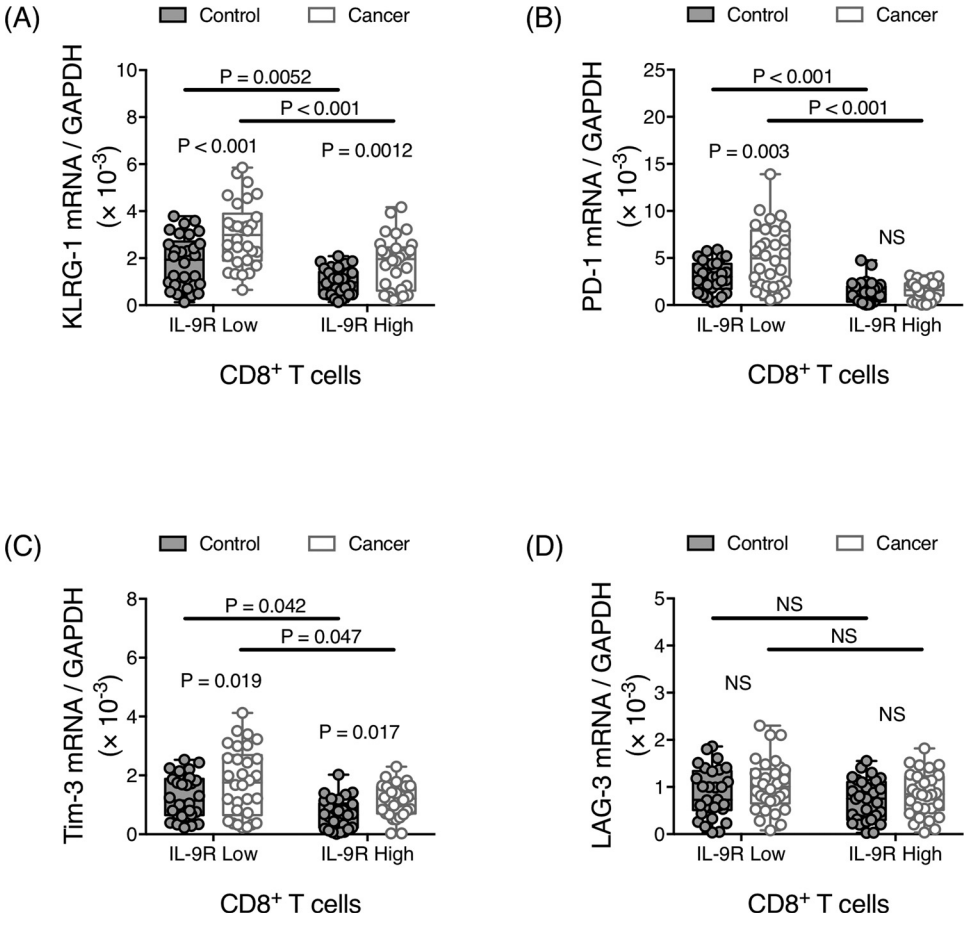

Fig. 3. Expression of inhibitory molecules by IL-9R^{high} and IL-9R^{low} CD8$^+$ T cells.
IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells were sorted using flow cytometry from the PBMCs of healthy controls (N = 30) and cancer patients (N = 30). The mRNA expression of the following inhibitory molecules, including (A) KLRG-1, (B) PD-1, (C) Tim-3, and (D) LAG-3 was examined. Two-way ANOVA followed by Sidak's multiple comparisons. NS, not significant.

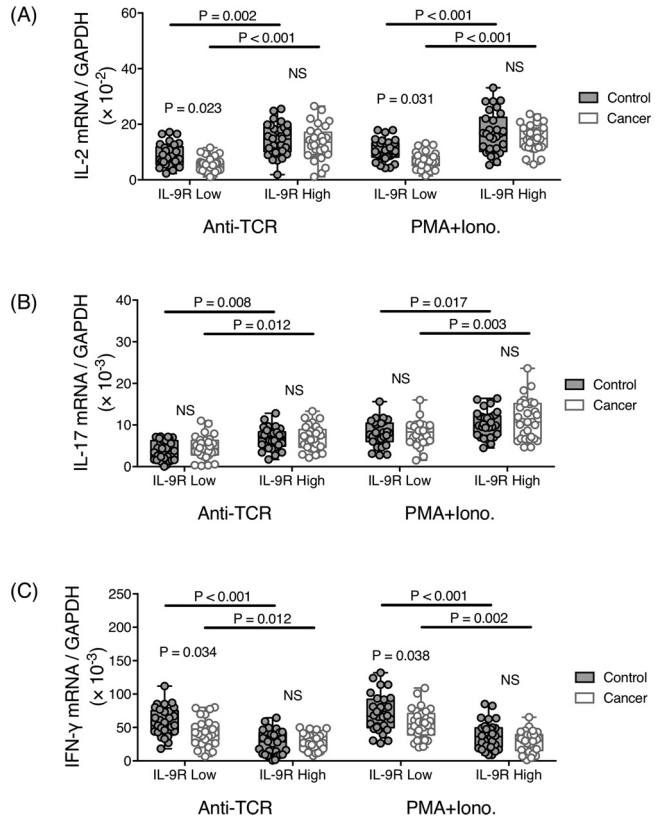

Fig. 4. Expression of cytokines by IL-9R^{high} and IL-9R^{low} CD8$^+$ T cells. IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells were sorted using flow cytometry from the PBMCs of healthy controls (N = 30) and cancer patients (N = 30). The cells were then stimulated using anti-TCR or PMA + Iono for 24 h. The cells were then lysed and the mRNA expression of (A) IL-2, (B) IL-17, and (C) IFN-γ was examined. Two-way ANOVA followed by Sidak's multiple comparisons. NS, not significant.

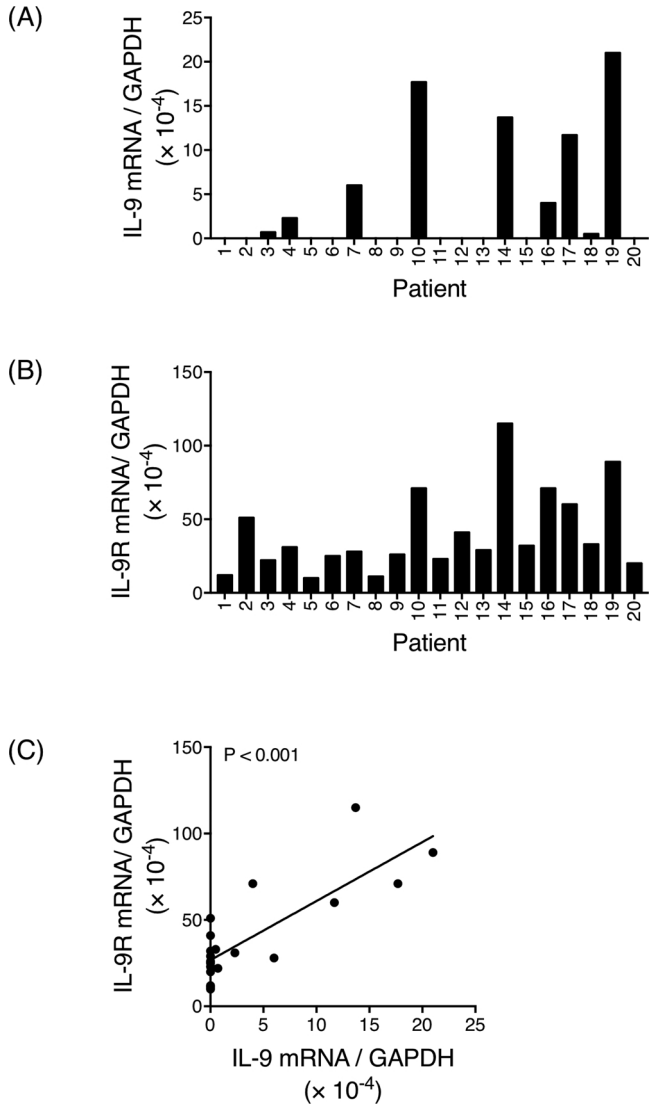

Fig. 5. Expression of IL-9 and IL-9R by tumor-infiltrating CD8$^+$ T cells. Tumor-infiltrating CD8$^+$ T cells from 20 breast cancer subjects were isolated and lysed, and the mRNA levels of (A) IL-9 and (B) IL-9R were examined. (C) The correlation between the level of IL-9 mRNA expression and the level of IL-9R mRNA expression in tumor-infiltrating CD8$^+$ T cells. Pearson correlation test.

expression varied greatly from patient to patient (Fig. 5A). IL-9R expression, on the other hand, could be universally detected in tumor-infiltrating CD8$^+$ T cells from various patients, but also with high variability (Fig. 5B). Interestingly, patients with high intratumoral IL-9 expression also presented high IL-9R expression (Fig. 5C). We also examined whether the IL-9 and IL-9R production by tumor-infiltrating CD8$^+$ T cells was associated with the level of IL-9 and IL-9R expression by circulating CD8$^+$ T cells. No statistically significant association was found between clinical characteristics of the patients, such as tumor size, tumor stage, and status of metastasis, and the levels of IL-9 expression and IL-9R expression in the tumor microenvironment.

4. Discussion

CD8$^+$ T cell-mediated inflammation is a crucial component of the antitumor immunity. Capable of directly mediating tumor cell lysis, CD8$^+$ T cells are also capable of modulating the antitumor immunity by expressing various types of cytokines (Gajewski et al., 2013; Hadrup et al., 2013). The IFN-γ-producing Tc1 cells, thus named due to their analogy with the IFN-γ-producing Th1 cells, are currently the best-studied CD8$^+$ T cell subset in cancer patients, and have been shown to exhibit indispensible function in antitumor immunity (Gerlach et al., 2013). However, several recent studies demonstrated that in murine melanoma models, there existed a transcriptionally and functionally distinctive IL-9-producing Tc9 subset that were involved in tumor-associated inflammation (Lu et al., 2014; Visekruna et al., 2013). This Tc9 subset presented lower IFN-γ but higher perforin and granzyme B expression. Furthermore, unlike canonical tumor-infiltrating CD8$^+$ T cells, which required IL-2 from CD4$^+$ Th cells (Hadrup et al., 2013), this Tc9 cells were found to express IL-2 (Lu et al., 2014). Analogs of this murine Tc9 cell type was also found in human subjects (Ramadan et al., 2017). Here in breast cancer patients, we found that circulating CD8$^+$ T cells were not expressing IL-9 *ex vivo* but was capable of expressing IL-9 when stimulated. This IL-9 expression capacity was biased toward the CD8$^+$ T cell subset with IL-9R^{high} expression. Interestingly, exogenous IL-9 had significantly increased the expression of both IL-9 and IL-9R in IL-9R^{high}, but not IL-9R^{low}, CD8$^+$ T cells, indicating that IL-9 could positively increase the production of itself and its receptor. In resected breast tumor, CD8$^+$ T cell-mediated IL-9 expression was found in some, but not all patients. IL-9R expression was universally found, but exhibited high variability among the patients. Together, these data demonstrate that IL-9-producing CD8$^+$ T cells have the capacity to mediate inflammatory response in tumor patients, but the exact role of these cells in tumor, and their trafficking pattern, still require further studies.

Separating total CD8$^+$ T cells into IL-9R^{high} and IL-9R^{low} subsets, we found that these two subsets exhibited a number of differences.

Compared to the more abundant IL-9R^{low} subset, the IL-9R^{high} subset was characterized by lower KLRG-1, PD-1, and Tim-3 *ex vivo* and lower IFN-γ after stimulation, and higher IL-2 and IL-17 expression after stimulation. Potentially, lower expression of inhibitory receptors may have rendered the IL-9R^{high} CD8$^+$ T cells less susceptible to signaling mediated by inhibitory ligands, thus enabling them to express higher levels of cytokines. Also, IL-9/IL-9R signaling may directly affect the cytokine expression profile of CD8$^+$ T cells, but the molecular mechanisms are unclear and should be further investigated.

A main caveat of this study is that most of the experiments were performed in peripheral blood, but not tumor, samples. It is then justified to ask whether the discoveries on circulating CD8$^+$ T cells could apply to tumor-infiltrating CD8$^+$ T cells. We think that the *ex vivo* activation status and effector molecule expression might be different in circulating CD8$^+$ T cells and tumor-infiltrating CD8$^+$ T cells, given that the antigens and costimulatory/inhibitory molecules in the tumor microenvironment are likely very different from those in the blood. However, some results of mechanistic studies, such as the IL-9 and IL-9R expression in the presence of exogenous IL-9, are likely conserved. The above hypothesis may be independently verified upon availability of new tumor samples. Another caveat is that we focused on the expression of cytokines and inhibitory molecules of Tc9 cells, while the in vivo function of these cells was not examined. Antitumor activity by Tc9 cells has been reported in murine models (Lu et al., 2014; Ma et al., 2018; Mittrücker et al., 2014). Adoptively transferred Tc9 cells presented better antitumor efficiency than Tc1 cells, likely due to its capacity to convert into IFN-γ and granzyme B expressing cells and better persistence. Here, it should be noted that in both healthy controls and breast cancer patients, high individual variations were found, and between the control group and the patient group, the differences in the expression of IL-9 by CD8 T cells were small, though statistically significant. Whether these small differences have clinical significance is still questionable based on available data. In the future, more patients may be recruited and associations between the functional characteristics of the Tc9 cells and the prognosis of the patients should be examined.

Our results have a number of differences from the discoveries made in murine melanoma model. Notably, the Tc9 cells and Tc1 cells in murine melanoma model displayed large differences in the expression of inhibitory molecules and cytokines (Lu et al., 2014). The expression of KLRG-1 and PD-1 in Tc1 cells was over five times that in Tc9 cells, while the expression of LAG-3 in Tc1 cells was three times that in Tc9 cells. The expression of IFN-γ in Tc1 cells was six times that in Tc9 cells. In our study, however, the differences between IL-9R^{high} CD8$^+$ T cells and IL-9R^{low} CD8$^+$ T cells were around two times at maximum. One likely explanation is that IL-9R^{high} was not an exclusive marker of true IL-9-expressing CD8$^+$ T cells. Indeed, the distinction between IL-9R^{high} and IL-9R^{low} was artificially set using an isotype control antibody. Moreover, In CD4$^+$ Th cells, IL-9R expression was not exclusively found in Th9 cells but was present on Th2 cells and Th17 cells as well (Nowak et al., 2009), and whether this extends to CD8$^+$ T cells is yet unclear. Better surface markers for true-IL-9-producing cells are required.

As a recently characterized cell type, Tc9 cells still present many unsolved questions. The IL-9 gene is located downstream of the binding sites for transcription factors PU.1, IRF4, AP1, and NF-κE, and it has been shown that TGF-β and IL-4, and possibly IL-1 and IL-25, could regulate the activation of those transcription factors in Th9 cells (Goswami and Kaplan, 2011). In Tc9 cells, the requirement for IL-9 gene transcription is yet unclear but may involve a similar set of cytokines. In this study, we found that exogenous IL-9 could enhance the transcription of both IL-9 and IL-9R. The role of IL-9 on the expression and binding of PU.1, IRF4, AP1, and NF-κE, should be investigated. Moreover, given that both Th2 cells and Th9 cells could act as sources of IL-9, it should be investigated whether Th2 and Th9 cells are required for Tc9 differentiation. Also, whether Tc9 cells are a committed CD8$^+$ T cell subset, or have plasticity to convert to other subsets, is still unknown, in part due to the lack of a definitive lineage-specific transcription factor. Additionally, whether normal breast tissues themselves could serve as sources of IL-9 or express IL-9R remains unclear. In the literature, it has been shown that IL-9 is present in both the colostrum and the breast milk (Marcuzzi et al., 2013). However, the source of the IL-9 was not identified. In future studies, the tumor, peritumor, and normal breast tissues should be examined for the expression of IL-9 and IL-9R.

It is known that the tumor microenvironment are enriched with an abundance of PD-L1 and other inhibitory ligands, and the low expression of PD-1 on IL-9R^{high} CD8$^+$ T cells may suggest that these cells are less susceptible to inhibitory mechanisms in the tumor microenvironment, enabling them to better exert effector functions. In this study, we did not find associations between the expressions of IL-9 and IL-9R and the clinical characteristics of the breast cancer patients. However, breast cancer is a complicated disease and many factors in the host, including both genetic and environmental factors, could affect the disease severity. Additionally, the differences between healthy controls and cancer patients are often small, despite being statistically significant. This is somewhat expected given the high variability between individual humans. Animal experiments in a more controlled setting, such as CD8$^+$ T cell-specific knockout of IL-9 and IL-9R, are required to elucidate and confirm the role of Tc9 cells and their susceptibility to suppression mechanisms in the tumor, and their capacity to eliminate malignant cells.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.105576.

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