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Original Research

Targeting ALCAM in the cryo-treated tumour microenvironment successfully induces systemic anti-tumour immunity

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Abstract Cryoablative treatment has been widely used for treating cancer. However, the therapeutic efficacies are still controversial. The molecular mechanisms of the cryo-induced immune responses, particularly underlying the ineffectiveness, remain to be fully elucidated. In this study, we identified a new molecular mechanism involved in the cryo failure. We used cryo-ineffective metastatic tumour models that murine melanoma B16-F10 cells were subcutaneously and intravenously implanted into C57BL/6 mice. When the subcutaneous tumours were treated cryoablation on day 7 after tumour implantation, cells expressing activated leucocyte cell adhesion molecule (ALCAM/CD166) were significantly expanded not only locally in the treated tumours but also systemically in spleen and bone marrow of the mice. The cryo-induced ALCAM$^+$ cells including CD45$^-$ mesenchymal stem/stromal cells, CD11b$^+$Gr1$^+$ myeloid-derived suppressor cells, and CD4$^+$Foxp3$^+$ regulatory T cells significantly suppressed interferon γ production and cytotoxicity of tumour-specific CD8$^+$ T cells via ALCAM expressed in these cells. This suggests that systemic expansion of the ALCAM$^+$ cells negatively switches host-immune directivity to the tumour-supportive mode. Intratumoural injection with anti-ALCAM blocking monoclonal antibody (mAb) following the cryo treatment systemically induced tumour-specific CD8$^+$ T cells with higher cytotoxic activities, resulting in suppression of tumour growth and metastasis in the cryo-resistant tumour models. These suggest that expansion of ALCAM$^+$ cells is a determinant of limiting the cryo efficacy. Further combination with an immune checkpoint inhibitor anti-CTLA4 mAb optimized the anti-

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tumour efficacy of the dual-combination therapy. Targeting ALCAM may be a promising strategy for overcoming the cryo ineffectiveness leading to the better practical use of cryoablation in clinical treatment of cancer.

1. Introduction

Cryoablation has been widely used for treating a variety of cancers including melanoma, prostate, liver, breast, colon, and renal cell cancers [1–3]. Spreading of the whole-tumour antigen is a great immunological advantage of the cryo treatment for eliciting potent cytotoxic T lymphocytes (CTLs) against cancer, while cryo efficacy is still controversial in clinical settings [1–3]. A variety of immunomodulatory therapeutics, such as a TLR9 agonist CpG oligodeoxynucleotide [4], a TLR7/TLR8 agonist Imiquimod [5], Bacillus Galmette Guerin (BCG)-stimulated DCs [6], and an immune checkpoint inhibitor anti-CTLA-4 monoclonal antibody (mAb) [7], has been combined with the cryo treatment for enhancing anti-tumour immunity. However, little is known about the precise immunological mechanisms underlying the cryo ineffectiveness.

Recent studies have demonstrated that cancer malignancy depends not only on the tumour properties but also on the microenvironment including mesenchymal stem/stromal cells (MSCs), fibroblasts, vascular cells and immune cells [8]. As well as hypoxia and angiogenesis, immunity is a strong influential factor for promoting tumour progression [9,10]. We have been investigating the interplay between neoplastic lesions and host immunity and recently revealed an unique tumour-aggravative mechanism, in which tumour cells undergoing Snail-induced epithelial-to-mesenchymal transition (EMT) create abnormal immunity via expansion of immunoregulatory cells including $CD4^+Foxp3^+$ regulatory T cells (Tregs), $CD11c^+MHC\ II^{low/-}$ regulatory dendritic cells and $CD45^-$ MSCs, and impaired $CD8^{low}$ T cells [11–14]. Thus, in this study, we attempted to uncover the immunological mechanisms involved in the cryo ineffectiveness for the purpose of better practical use of cryoablation in clinical settings.

2. Materials and methods

2.1. Cell lines and mice

Murine melanoma B16-F10 cells and colon cancer CT26 cells were kindly provided by Cell Resource Center for Biomedical Research at Tohoku University in Japan and were cultured in 10% fetal calf serum-containing DMEM medium (Invitrogen). B16-F10 cells transfected with lentiviral vector encoding *GFP* gene (Biogenova) were used for the *in vivo* study. Five-week-old female C57BL/6 and BALB/c mice were purchased from SLC Inc. (Tokyo, Japan) and were maintained under pathogen-free conditions until use according to the protocols approved by the Animal Care and Use Committee at the Keio University School of Medicine.

2.2. Therapeutic experiments in vivo

C57BL/6 mice were implanted with B16-F10 cells both subcutaneously (s.c.; 5×10^5 cells) to prepare easy accessible sites for cryoablation and intravenously (i.v.; 2×10^5 cells) to analyse systemic induction of anti-tumour immunity ($n = 5–10$ per experiment). The subcutaneous tumours (50–100 mm^3) were frozen by cryoablation (−80 °C for 1 min enough to freeze the whole tumour) using a Cryomaster (Keeler Ltd.) on day 7 after tumour implantation (when lung micrometastasis was already established), and immediately after thawing, anti-ALCAM blocking mAb (50 μg; R&D systems) was directly injected into the cryo-treated tumours. Another anti-ALCAM mAb (LSBio) was also tested, and similar results were observed. In a setting, anti-CTLA4 mAb (200 μg/mouse; Clone 9H10; BioLegend) was intraperitoneally (i.p.) injected into the mice on days 8 and 11. Colon cancer CT26 models were also used for validation of the therapeutic efficacy. Tumour volume (mm^3) was calculated as follows: $0.5 \times length \times width^2$. To determine the effector cells critical for the anti-tumour activity induced by the combination therapy, mice were i.p. injected with mAb (50 μg) specific for CD4 (clone GK1.5), CD8 (clone 2.43) or Gr1 (BD Biosciences) or rat immunoglobulin G as a control (Abcam) on days 6, 9 and 12, and the depletion of the relevant cell population (>90%) was validated by flow cytometry. Seven to 10 d after cryoablation, the subcutaneous tumour, lung, spleen and bone marrow were harvested from the mice for assays. Lungs were fixed with Fekete's solution after perfusion with water, and the number of tumour metastatic nodules was counted.

2.3. Flow cytometric analysis

After Fc blocking, cells were stained with immunofluorescence (FITC, PE, CyChrome, or APC)-conjugated mAbs specific for murine CD45, CD4, CD8, CD11b, Gr1, ALCAM (eBioscience), Foxp3 (eBioscience), a tumour antigen gp70 tetramer (MBL), or the appropriate isotype control antibodies. Antibodies except the