

from samples incubated in 5 % Triton-X or medium alone, respectively. Cytolysis was calculated with the following formula: % lysis = (release in experiment – spontaneous release) \times 100/(maximum release – spontaneous release). The spontaneous release in all assays was less than 20 % of the maximum.

Statistical analyses

Statistical analyses were performed using the paired Student's *t* test, and $p < 0.05$ was considered statistically significant.

Results

Targeting TIM-3 or TIM-4 improves the antitumor effects of cancer vaccines against established B16 melanomas

We examined whether therapeutic manipulation of TIM-3 and/or TIM-4 impacts the antitumor responses induced by cancer vaccines against B16 melanomas. To do so, B16 melanoma cells were inoculated subcutaneously into C57BL/6 mice. After the establishment of tumors ($\sim 25 \text{ mm}^2$), mice were vaccinated with irradiated Flt3L-secreting B16 cells (FVAX) in the presence of isotype-matched control Ig, anti-TIM-3 mAb (RMT3-23) or anti-TIM-4 mAb (RMT4-53). Vaccination with FVAX partially reduced tumor burden when combined with control Ig. On the other hand, co-administration of anti-TIM-3 or anti-TIM-4 mAb with FVAX significantly suppressed tumor growth to a greater extent than FVAX alone (Fig. 1a, b). Monotherapy with anti-TIM-3 or anti-TIM-4 mAb had little impact on B16 melanoma growth (Fig. 1a, b).

The treatment with anti-TIM-3 mAb or anti-TIM-4 mAb also augmented antitumor activities of plasmid DNA encoding melanoma target gp100 or TRP2, suggesting that targeting TIM-3 or TIM-4 improves antitumor activities of other forms of cancer vaccines (data not shown).

These results indicate that treatment with either anti-TIM-3 or TIM-4 mAb improves the antitumor effect of cancer vaccines similarly.

Combined blockade of TIM-3 and TIM-4 elicits potent antitumor responses in cooperation with cancer vaccines

We next examined whether combined blockade of TIM-3 and TIM-4 could further enhance antitumor responses elicited by cancer vaccines against B16 melanoma. Treatment with anti-TIM-3 plus anti-TIM-4 mAbs markedly augmented FVAX to a greater level than with either mAb alone (Fig. 2a). In contrast, the combination of anti-TIM-3 and anti-TIM-4 mAbs had little impact on controlling B16 melanoma growth in the absence of FVAX (Fig. 2a). Furthermore, the combined regimens prevented further tumor growth in the vaccinated mice during the follow-up periods (more than 50 days), while all of the vaccinated group were succumbed to death when treated with anti-TIM-4 mAb or anti-TIM-4 mAb alone (data not shown). The combined treatment with anti-TIM-3 mAb and anti-TIM-4 mAb also augmented antitumor activities of vaccination with plasmid DNA encoding melanoma target gp100 or TRP2 compared to either mAb alone (Fig. 2b and data not shown).

These results indicate that combined administration of anti-TIM-3 and anti-TIM-4 mAbs further augments the antitumor effects of cancer vaccines compared to monotherapy.

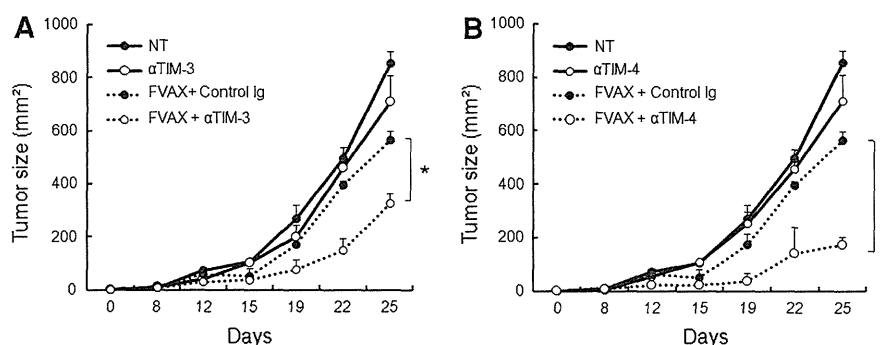


Fig. 1 Blockade of TIM-3 or TIM-4 enhances the therapeutic responses elicited by cancer vaccines. C57BL/6 mice ($n = 3$ per group) were inoculated subcutaneously in the flank with 1×10^5 B16-F10 melanoma cells on day 0. **a, b** The tumor-bearing mice were treated with subcutaneous injection of 1×10^6 irradiated (150 Gy)

flt3L-expressing B16 melanoma cells (FVAX) and intraperitoneal injection of anti-TIM-3 mAb (α TIM-3) (**a**) or anti-TIM-4 mAb (α TIM-4) on days 3, 5 and 7 (**b**). Tumor growth was measured on the indicated days. Similar results were obtained in three independent experiments. * $p < 0.05$

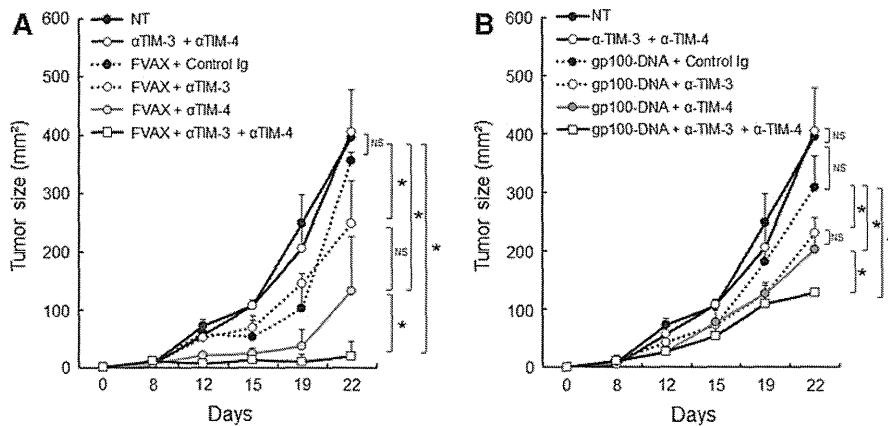
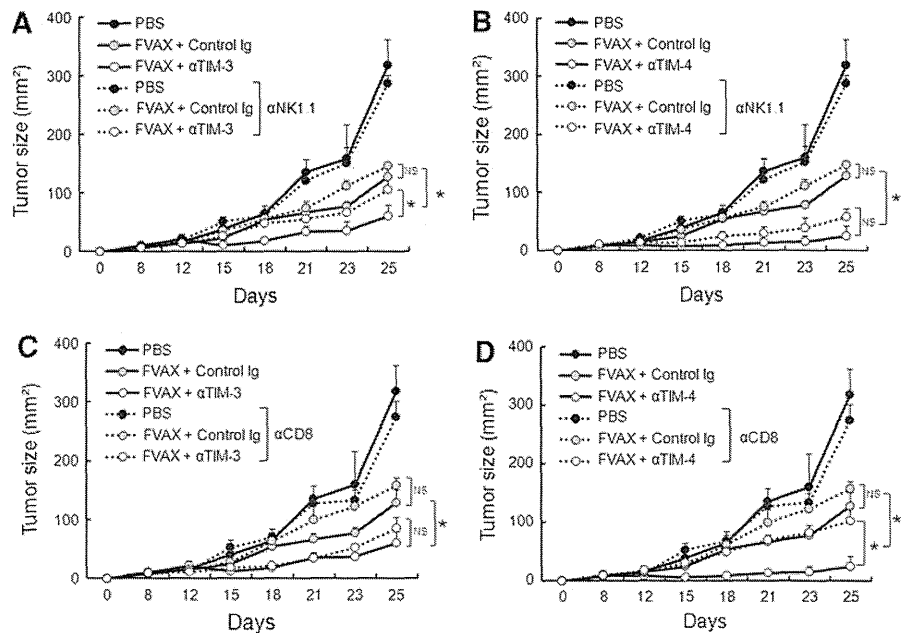


Fig. 2 Combined blockade of TIM-3 and TIM-4 maximizes the antitumor effect of cancer vaccines. **a, b** C57BL/6 mice ($n = 3$ per group) were challenged in the flank with B16-F10 melanoma cells and were treated with subcutaneous injection of irradiated FVAX or plasmid DNA encoding gp100 (**b**), and intraperitoneal injection of

anti-TIM-3 mAb (α TIM-3), anti-TIM-4 mAb (α TIM-4) or both (α TIM-3 + α TIM-4). Tumor growth was measured on the indicated days. Similar results were obtained in three independent experiments. * $p < 0.05$, NS not significant

Fig. 3 Blockade of TIM-3 and TIM-4 enhances the antitumor responses elicited by FVAX via distinct effector mechanisms. C57BL/6 mice ($n = 3$ per group) were treated with depleting Ab for NK1.1 (**a, b**) or CD8 (**c, d**) 2 days prior to B16-F10 melanoma inoculation. The tumor-bearing mice were then treated with FVAX and anti-TIM-3 mAb (α TIM-3; **a, c**) or anti-TIM-4 mAb (α TIM-4; **b, d**) as described in Fig. 1. Tumor growth was measured on the indicated days. Similar results were obtained in two independent experiments. * $p < 0.05$, NS not significant



Blockade of TIM-3 and TIM-4 stimulates distinct antitumor effector mechanisms

We next examined the mechanism by which anti-TIM-3 or anti-TIM-4 mAb triggers effector functions within tumor microenvironments. To do so, we repeated the above experiments in mice that had been treated with depleting mAb for NK1.1 (PK136) or CD8 (53–6.7) 2 days prior to tumor inoculation, as NK cells and CD8⁺ T cells serve as the major innate and adaptive effectors controlling tumor

immunosurveillance, respectively [25, 26]. Treatment with anti-TIM-3 mAb did not augment the antitumor effects elicited by FVAX in mice depleted of NK1.1⁺ cells (Fig. 3a). In contrast, TIM-4 blockade augmented the therapeutic effects of FVAX in mice depleted of NK1.1⁺ cells at a similar level to that in control mice (Fig. 3b).

On the other hand, the depletion of CD8⁺ cells abrogated the immunostimulatory effect of anti-TIM-4 mAb, whereas it had little impact on the effect of anti-TIM-3 mAb (Fig. 3c, d).

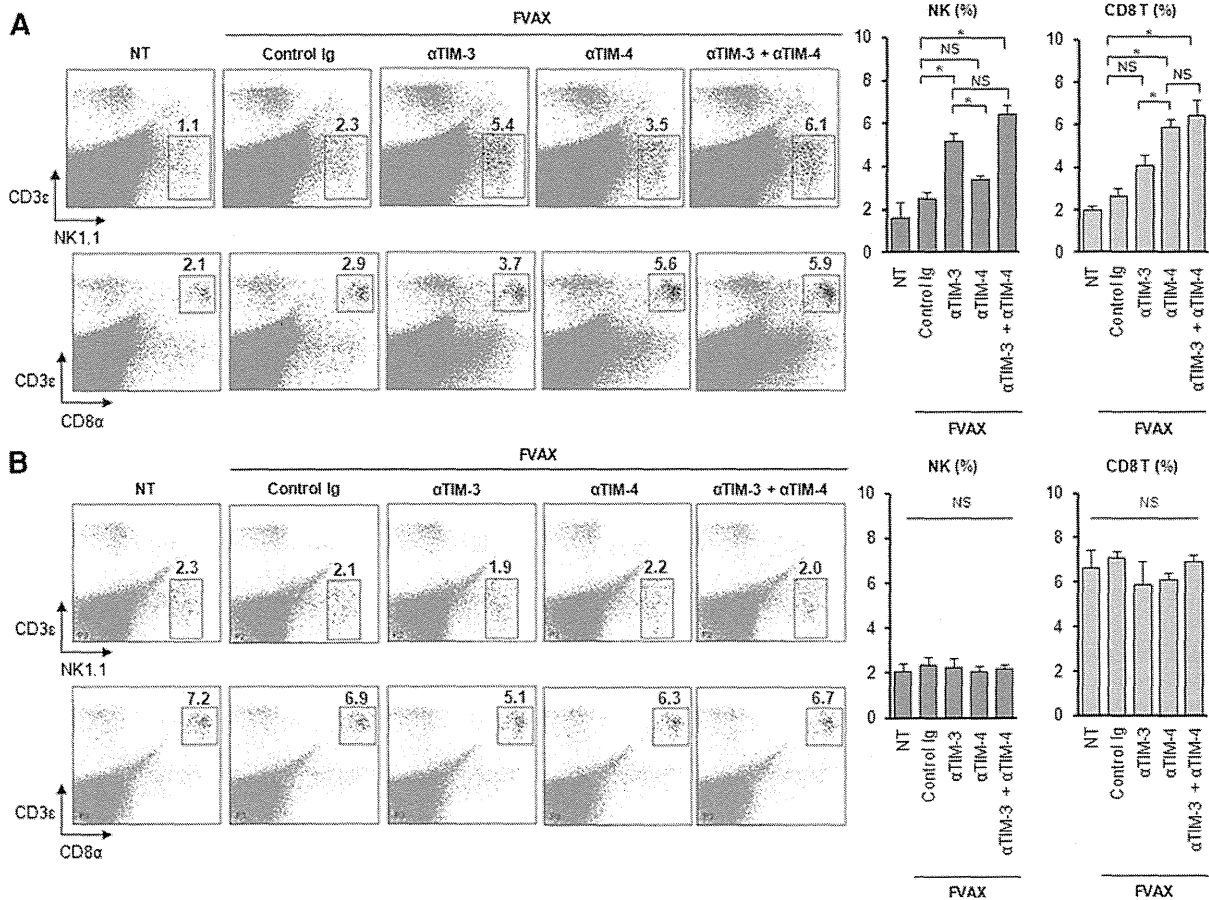


Fig. 4 Anti-TIM-3 and anti-TIM-4 mAb alter the frequency of distinct effector cells in tumors. C57BL/6 mice bearing B16-F10 tumor were treated with FVAX and anti-TIM-3 mAb (α TIM-3), anti-TIM-4 mAb (α TIM-4) or both (α TIM-3 + α TIM-4) on day 3 and 5. Seven days after tumor inoculation, lymphocytes were isolated from

the tumors and spleens of tumor-bearing mice. The frequencies of NK1.1⁺CD3⁻ (NK) cells or CD3⁺CD8⁺ (CD8⁺ T) cells in tumors (a) or spleens (b) were analyzed by flow cytometry. Similar results were obtained in two independent experiments. **p* < 0.05, NS not significant

Together, these results suggest that the blockade of TIM-3 or TIM-4 induces different effector arms and that NK1.1⁺ or CD8⁺ effector cells are responsible for the augmented antitumor effect of FVAX by anti-TIM-3 mAb or anti-TIM-4 mAb, respectively.

Blockade of TIM-3 and TIM-4 activates distinct sets of tumor-infiltrating effector cells

Finally, we examined the mechanisms by which anti-TIM-3 and anti-TIM-4 mAbs modulate the immune responses in tumor microenvironments. To do so, we isolated tumor-infiltrating lymphocytes from established B16 tumors and analyzed the frequencies and activation state of NK cells and CD8⁺ T cells in the tumors and spleens of the treated mice. We found that the frequency of NK1.1⁺ CD3⁻ cells was much higher in mice treated with anti-TIM-3 mAb and

FVAX compared to those treated with FVAX alone, whereas the treatment with anti-TIM-4 mAb and FVAX did not significantly alter the frequency of NK cells (Fig. 4a). In contrast, TIM-4 blockade resulted in a marked increase in CD8⁺ T cells infiltrating the tumors, while the TIM-3 blockade had no significant effect on CD8⁺ T cells (Fig. 4a). Importantly, a combined blockade of TIM-3 and TIM-4 resulted in increased frequencies of both NK cells and CD8⁺ T cells in tumors (Fig. 4a). Treatment with anti-TIM-3 mAb, anti-TIM-4 mAb or both had little impact on the frequencies of NK cells or CD8⁺ T cells in spleens of the same tumor-bearing mice (Fig. 4b).

We also evaluated the activation state of NK cells and CD8⁺ T cells in the tumors and spleens by measuring the levels of CD69 and CD44, respectively. Neither NK1.1⁺ nor CD8⁺ T cells within tumors displayed increased CD69 or CD44 expression in mice treated with FVAX alone as

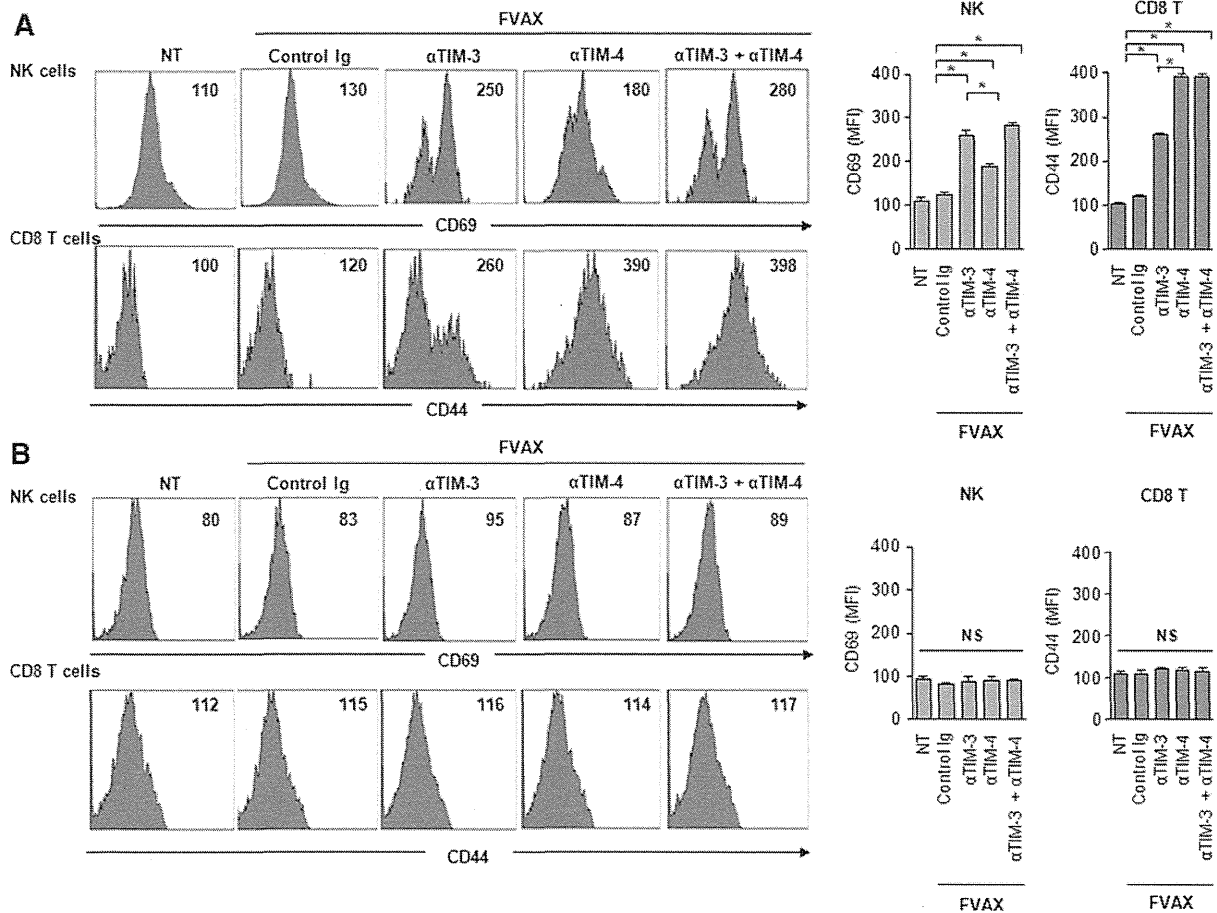


Fig. 5 Anti-TIM-3 and anti-TIM-4 mAbs activate distinct sets of effector cells in tumors. C57BL/6 mice bearing B16-F10 tumor were treated with FVAX and anti-TIM-3 mAb (α TIM-3), anti-TIM-4 mAb (α TIM-4) or both (α TIM-3 + α TIM-4) on days 3 and 5. Seven days after tumor inoculation, lymphocytes were isolated from the tumors and spleens of tumor-bearing mice. NK cells and CD8⁺ T cells were

gated as the NK1.1⁺CD3⁻ and CD3⁺CD8⁺ populations, respectively, and the frequencies of CD69 among NK cells or CD44 among CD8⁺ T cells in tumors (a) or spleens (b) were analyzed by flow cytometry. The mean fluorescence intensity (MFI) was shown in the right-upper panel in the histogram plot. Similar results were obtained in two independent experiments. * $p < 0.05$, NS not significant

compared to those in untreated mice. Treatment with anti-TIM-3 mAb was more effective in increasing CD69 expression on NK cells isolated from the B16 tumor sites of FVAX-immunized mice compared to those treated with anti-TIM-4 mAb (Fig. 5a). On the contrary, TIM-4 blockade increased CD44 expression on tumor-infiltrating CD8⁺ cells to a greater extent than did TIM-3 blockade (Fig. 5a). Importantly, a combined treatment with anti-TIM-3 and anti-TIM-4 mAbs maximized the numbers and activation state of NK1.1⁺ cells and CD8⁺ T cells infiltrating FVAX-immunized B16 tumors (Fig. 5a). Again, treatment with anti-TIM-3 mAb, anti-TIM-4 mAb or both had little impact on the activation status of NK cells or CD8⁺ T cells obtained from the spleens of tumor-bearing mice (Fig. 5b).

To further define whether TIM-3 and TIM-4 distinctly regulate antitumor effector functions, B16-OVA cells and TCR-transgenic mice recognizing H-2K^b-restricted OVA sequence (SIINFEKL: OT-I) were utilized for dissecting tumor antigen-specific responses and antigen-nonspecific innate responses in tumors. In this system, B16-OVA cells were inoculated subcutaneously into C57BL/6 wild-type mice 5 days after intravenous transfer of OVA-specific TCR-V β 5⁺ OT-I cells. The established tumors then received vaccination with FVAX in the presence of anti-TIM-3 mAb, anti-TIM-4 mAb or both. Bulk CD45⁺ lymphocytes isolated from the established tumors or those depleted of NK1.1⁺ cells or OVA-specific TCR-V β 5⁺ populations were used for analyzing OVA-specific cytotoxic activities against B16-OVA cells and OVA-independent cytotoxic activities

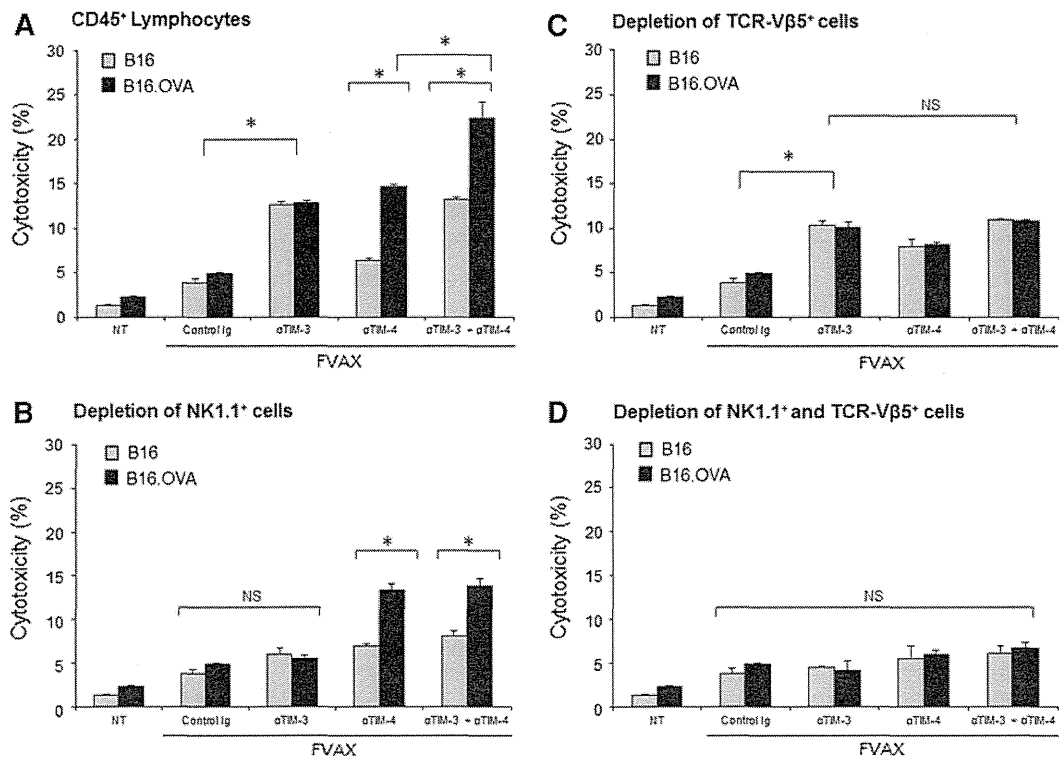


Fig. 6 B16-OVA cells were inoculated subcutaneously into C57BL/6 wild-type mice 5 days after intravenous transfer of OVA-specific TCR-Vβ5⁺ cells isolated from OT-I mice. The established tumors then received vaccination with FVAX in the presence of control Ig, anti-TIM-3 mAb (αTIM-3), anti-TIM-4 mAb (αTIM-4) or both (αTIM-3 + αTIM-4). Four days after final treatment, bulk CD45⁺

lymphocytes isolated from the established tumors (a), those depleted of NK1.1⁺ cells (b), TCR-Vβ5⁺ cells (c) or both (d) were cocultured with B16 or B16-OVA cells at the 100/1 of effector/target ratios for 6 h. The cytotoxic activities were measured by LDH release assay. Similar results were obtained in two independent experiments. **p* < 0.05, NS not significant

against B16 cells. The FVAX alone induced greater cytotoxic activities of intratumor lymphocytes against B16 or B16-OVA targets compared to nontreatment. The treatment with anti-TIM-3 mAb and FVAX enhanced cytotoxic activities of intratumor lymphocytes against either B16 or B16-OVA targets at greater levels than FVAX alone, which were largely abolished by the NK1.1⁺ cell depletion. In marked contrast, the treatment with anti-TIM-4 mAb and FVAX elicited greater cytotoxic responses against B16-OVA targets compared to FVAX alone, which were completely abrogated by the TCR-Vβ5⁺ cell depletion.

Again, the combined blockade of TIM-3 and TIM-4 further increased cytotoxic activities of intratumor lymphocytes against B16 and B16-OVA target cells, and these effects were largely abolished by combined depletion of NK- and TCR-Vβ5⁺-positive cells. Thus, these results further suggest that the therapeutic benefit by combined TIM-3 and TIM-4 blockade relied mainly upon the coordinated actions of OVA-specific responses as well as OVA-independent innate responses from the vaccinated tumors (Fig. 6).

Together, these findings provide a direct proof that the FVAX vaccines combined with TIM-3 blockade trigger NK cell-mediated innate effector responses in a tumor antigen-independent way, whereas TIM-4 blockade induces tumor antigen-specific cytotoxic activities of CD8⁺ T cells in synergy with the autologous tumor cell vaccines in tumor microenvironments.

In summary, these results suggest that the blockade of TIM-3 or TIM-4 induces distinct sets of effector arms within tumor microenvironments, and thus, the combined blockade can exert a synergistic effect.

Discussion

The antitumor efficacy of immunotherapy remains insufficient to achieve durable clinical responses in patients with advanced stage cancers. In this study, we demonstrate that the combined administration of anti-TIM-3 and anti-TIM-4 mAbs augmented the therapeutic efficacies of cancer vaccines by stimulating different effector arms within tumor

microenvironments. These findings provide new evidence that targeting of TIM-3 and TIM-4 may serve as a promising option to improve therapeutic efficacy of cancer immunotherapy.

The antitumor effects exerted by anti-TIM-3 mAb were derived mainly from the activation of NK cell-mediated innate immune systems within tumor microenvironments. These findings contradict a recent report in which the administration of anti-TIM-3 mAb had an inhibitory effect on chemical carcinogenesis through T cell- and IFN- γ -dependent mechanisms [16, 27]. This discrepancy may arise from the unique properties of TIM-3 in modulating distinct sets of immune cells and the related signaling pathways that exist under different microenvironments and treatments. Consistent with this assumption, we recently demonstrated that TIM-3 expressed on tumor-infiltrating dendritic cells was responsible for suppressing the antitumor responses elicited by a chemotherapeutic agent cisplatin and that this effect relied mainly on the impairment of innate immune responses to the nucleic acids released from tumor cells upon chemotherapy [28]. Thus, TIM-3 blockade may serve as a useful strategy for overcoming tolerogenic tumor environments by triggering both innate and adaptive antitumor immunity when appropriate immunogenic adjuvants such as FVAX are co-administered. In this regard, it is of great interest to examine whether TIM-3 inhibition could co-opt other immunotherapies or cytotoxic chemotherapies to create antitumor environments in which endogenous immune systems are capable of antagonizing nascent tumors.

Furthermore, we demonstrated herein that the administration of TIM-4 mAb augmented the antitumor effects of FVAX by CD8⁺ T cell-dependent, but NK cell-independent, mechanisms. Since TIM-4 expression is highly restricted to myeloid cell lineages [20], TIM-4 might modulate the interaction between myeloid cells and antigen-specific cytotoxic T lymphocytes within tumor microenvironments. Indeed, accumulating evidence reveals that tolerogenic microenvironments formed by myeloid cells are critical to the tumor progression and the induction of resistance to anticancer drugs [29, 30]. Thus, it is of great interest to clarify the role of TIM-4 on tumor-associated myeloid cells in the regulation of antitumor immune responses and its affect on the clinical prognosis of cancer patients.

We also demonstrated that the combined administration of anti-TIM-3 and anti-TIM-4 mAbs augments the antitumor responses elicited by cancer vaccines against established B16 melanoma more efficiently than administration of anti-TIM-3 or anti-TIM-4 mAb alone. These findings further imply that the coordinated and/or distinct functions of TIM-3 and TIM-4 are responsible for creating tolerogenic conditions within tumor microenvironments. Since the appropriate stimulation of innate immune signals

determines the direction and quality of adaptive immune responses [31, 32], the concurrent administration of anti-TIM-3 and anti-TIM-4 mAbs may co-opt immunogenic adjuvants to initiate sequential activation of innate and adaptive antitumor immunity. Further clarification of the molecular mechanisms by which TIM-3 and TIM-4 differentially regulate innate and adaptive immune responses in defined microenvironments will improve the therapeutic efficacies of immunotherapies against advanced cancers.

In summary, we have unveiled distinct roles for TIM-3 and TIM-4 in the regulation of innate and adaptive antitumor immunity. The molecular targeting of TIM-3 and TIM-4 provides a new therapeutic strategy, in combination with vaccination protocols or modalities to induce immunogenic cell death [33], to eradicate tumors through the coordinated activation of innate and adaptive antitumor immune responses in tumor microenvironments.

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Conflict of interest The authors declare that they have no conflict of interest.

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