Immunoglobulin Fc Fragment Tagging Allows Strong Activation of Endogenous CD4 T Cells To Reshape the Tumor Milieu and Enhance the Antitumor Effect of Lentivector Immunization

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Immunoglobulin Fc Fragment Tagging Allows Strong Activation of Endogenous CD4 T Cells To Reshape the Tumor Milieu and Enhance the Antitumor Effect of Lentivector Immunization

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A major problem with current cancer vaccines is that the induction of CD8 immune responses is rarely associated with antitumor benefits, mainly owing to multiple suppressions in established tumor lesions. In this study, we investigated if and how activation of endogenous CD4 T cells could be achieved to influence the suppressive tumor milieu and antitumor effect. We engineered a lentivector (lv) to express a nominal fusion Ag composed of hepatitis B surface protein and IgG2a Fc fragment (HBS-Fc-lv) to increase the magnitude of CD8 response but, more importantly, to induce effective coactivation of CD4 T cells. We found that, remarkably, immunization with HBS-Fc-lv caused significant regression of established tumors. Immunologic analysis revealed that, compared with HBS-lv without Fc fragment, immunization with HBS-Fc-lv markedly increased the number of functional CD8 and CD4 T cells and the level of Th1/Tc1-like cytokines in the tumor while substantially decreasing the regulatory T cell ratio. The favorable immunologic changes in tumor lesions and the improvement of antitumor effects from HBS-Fc-lv immunization were dependent on the CD4 activation, which was Fc receptor mediated. Adoptive transfer of CD4 T cells from the HBS-Fc-lv–immunized mice could activate endogenous CD8 T cells in an IFN-γ–dependent manner. We conclude that endogenous CD4 T cells can be activated by lv expressing Fc-tagged Ag to provide another layer of help—that is, creating a Th1/Tc1-like proinflammatory milieu within the tumor lesion to boost the effector phase of immune responses in enhancing the antitumor effect. The Journal of Immunology, 2012, 188: 4819–4827.

CD4 T cells is much more difficult (16), especially when the production of effector cytokines by CD4 T cells is used as a criterion of activation. We hypothesize that one possible reason for the lack of significant antitumor benefit in treating established tumors is due to ineffective coactivation of endogenous CD4 T cells by current cancer vaccines. As a helper T cell, CD4 is widely recognized for its role in assisting the induction of CD8 responses by possibly “licensing” DCs (17–21). In addition, the “postlicensing” role of CD4 T cells at the effector phase is becoming increasingly appreciated (22). Recent studies showed that the recruitment, proliferation, and effector functions of CD8 T cells inside tumors or infected lesions could be greatly enhanced by cotransfer of CD4 T cells (23–25). Furthermore, several recent reports showed that some CD4 T cells could directly kill tumor cells (26–29). However, in most, if not all, of these studies, adoptive transfer of a large number of highly selected TCR transgenic (Tg) CD4 T cells was used. At present, it is not clear if the rare endogenous CD4 T cells can exercise similar functions because most of the cancer vaccines, especially those that are gene based, are not effective at activating CD4 T cells. Although proven to be very effective at stimulating CD8 responses, lv immunization has limited ability to activate endogenous CD4 T cells. Collins and colleagues (12) demonstrated that CD4 T cells could be activated by lv immunization only in the scenario in which adoptive transfer of a high number of exogenous TCR Tg OT-II CD4 T cells was used. In another study, Goold et al. (30) demonstrated that a limited activation of endogenous OVA-specific CD4 T cells was achieved after lv immunization when OVA Ag was channeled to an MHC II–restricted pathway. To improve CD4 activation, we recently studied various methods and Ags for activating endogenous CD4 T cells and found that

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Abbreviations used in this article: DC, dendritic cell; FcRγ, FcR γ-chain; GrB, granzyme B; HBsAg, hepatitis B virus surface Ag; HBS-Fc, HBsAg and mouse IgG2a Fc fragment fusion Ag; HBS-Fc-lv, lentivector expressing HBS-Fc fusion Ag; KO, knockout; lv, lentivector; qRT-PCR, quantitative RT-PCR; Tg, transgenic; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell; WT, wild-type.
tagging HBsAg with Ig Fc fragment in the lv immunization platform could potently activate endogenous CD4 T cells to produce IFN-γ and to enhance CD8 responses (31). In this report, we studied the questions of if and how activation of endogenous CD4 T cells could be achieved to modulate the tumor milieu and to improve the antitumor effect of lv immunization. We investigated the antitumor effect of lv immunization and the immunologic changes in the tumor lesions with or without CD4 coactivation. We found that immunization with lv expressing Fc-tagged HBsAg effectively activated both CD8 and CD4 responses and caused regression of established HBsAg+ B16 (B16-S) tumors. Immunologic analysis revealed a significant increase of CD8 and CD4 T cells and preservation of their effector function in tumor lesions when CD4 cells were activated. The level of Th1/Th1-like cytokines and chemokines was also markedly increased in tumor lesions in the presence of CD4 activation. In contrast, the regulatory T cell (Treg) ratio was substantially decreased in the immunized tumor when CD4 cells were activated. These favorable immunologic changes in the tumor lesions following lv immunization were dependent on CD4 activation, which was mediated by FcγR. Using the adoptive transfer approach, we further discovered that the vaccine-activated CD4 T cells could effectively activate endogenous CD8 T cells in an IFN-γ-dependent pathway. We conclude that tagging tumor Ag with an Fc fragment offers an effective way to activate endogenous CD4 and that the activated CD4 T cells can improve the tumor milieu by increasing Th1/Th1-like proinflammatory cytokines and chemokines, reducing the Treg ratio, and maintaining the effector function of tumor-infiltrating lymphocytes (TILs), which together result in an enhanced antitumor effect.

Materials and Methods

Cell lines, tumors, and mice

B16-F10 and 293T cells were obtained from American Type Culture Collection. B16-S tumor cells were kindly provided by Dr. Jorg Reinmann (32). IFN-γ knockout (KO; IFN-γ−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). FeR common γ-chain (FcγR) KO mice were purchased from Taconic Farms (Germantown, NY). The common γ-chain is associated with FcγR I, III, and FcεRI, which is essential for FcγR assembly, signaling, and expression on the cell surface. C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD). All the mice were housed under specific pathogen-free conditions in the Laboratory Animal Services of Georgia Health Sciences University. Animal care protocols were approved by the Institutional Animal Care and Use Committee of Georgia Health Sciences University.

To establish tumors, B16-S (5 × 10^5) or B16-F10 (2 × 10^5) cells were inoculated s.c. into the shaved flank of C57BL/6 mice. Tumor growth was monitored by measuring the perpendicular diameters three times per week. The tumor mass was weighed at the end of experiments.

Lv and immunization

HBs-lv (expressing the HBsAg without Fc tagging) and HBs-Fc-lv (expressing the HBsAg and mouse IgG2a Fc fragment fusion Ag [HBs-Fc]) were constructed by replacing the TRP1 gene in TRP1-lv (9) with the small S full gene of hepatitis B virus (ayw serotype) or the HBsAg-fusion gene (HBs-Fc) that contained the HBsAg and CH2-CH3 domains (Fc fragment) of the mouse IgG2a H chain. The entire amino acid sequences of HBs-Fc protein were provided in Supplemental Fig. 1. Lv preparation, concentration, and titration were conducted as previously described (7). For immunization, 2.5 × 10^7 transaction units of HBs-lv or HBs-Fc-lv was injected in the footpad. For tumor treatment, all immunizations were started on day 5, when the tumor lesions were clearly visible.

Preparation of single-cell suspension from tumor lesions

The mice were sacrificed; tumors were collected and weighed. Tumor single-cell suspensions were prepared as previously reported (9). Briefly, 20–100 mg of each tumor was cut into small pieces and incubated at 37°C for 0.5 h in RPMI 1640 containing 1 mg/ml collagenase, 1 mg hyaluronidase, and 100 U DNase I. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). Cells were then stained with various cocktails of indicated Abs.

Analysis of tumor-infiltrating lymphocytes by flow cytometry

The Abs used in this study—αCD45, αCD90, αCD8, αCD4, αCD40L, αCD107a, anti-granzyme B (GrB), anti-Foxp3, anti-IFN-γ, and anti–TNF-α—were purchased from BD Biosciences (San Diego, CA), BioLegend (San Diego, CA), and eBioscience (San Diego, CA).

To measure cytokines, single-cell suspensions from peripheral blood, spleen, or tumor were ex vivo stimulated for 4 h with 1 μg/ml HBsAg peptide S190–197, identified previously by Schirmbeck et al. (33) (Genesis, Picataway, NJ), or 5 μg/ml whole HBsAg (Propsect. East Brunswick, NJ) in the presence of GolgiStop (BD Biosciences). In some experiments, the CD4 T cells were stimulated with PMA/Ionomycin (leukocyte activation mixture; BD Biosciences). Intracellular staining of IFN-γ and TNF-α or GrB was performed (7). Alternatively, to measure degranulation, Ab against CD107a was added to the ex vivo cell culture, as described previously (34). After staining, the cell events were collected using a FACScan cytometer (BD Biosciences). Data were analyzed using the FCS Express V3 software (De Novo Software, Ontario, Canada).

Quantitative RT-PCR

Tumor tissue total RNA was extracted using the RNA extraction kit from Qagen (Valencia, CA). The expression level of chemokines was determined using the Mouse Chemokines and Receptors RT2 Profiler PCR Array from SABiosciences (Frederick, MD), which can detect 89 different chemokines and receptors based on the manufacturer’s recommendation. Those chemokines with significant changes were further verified with quantitative RT-PCR (qRT-PCR), using primers derived from previous reports (35). To determine cytokine expression in the tumor lesions, we also used qRT-PCR. To determine levels of Foxp3, GrB, perforin, TNF-α, and IFN-γ, qRT-PCR was performed as described by Kalthemeyer et al. (36). qRT-PCR primers for IL-17, IL-21, RORyt, and GADPH were derived from a previous report by Das et al. (37) and Tsujita et al. (38). qRT-PCR primers for other cytokines or transcription factors were also derived from previous publications: T-bet (39), TGF-β (40), IL-6, IL-1β (41), IL-7 (42), IL-12 (43), and IL-15 (44). Primers for IL-2 are designed and synthesized as follows: IL-2 upstream: 5′-CCCCCGCTAATCACTGCCCTCA-3′; IL-2 downstream: 5′-GAGCCCTCTGGATGGGCTCA-3′.

Adoptive transfer

Wild-type (WT) C57BL/6 mice (Thy1.1) or IFN-γ−/− KO mice (Thy1.2 congenic) were immunized with HBS-Fc-lv or HBS-lv. Two weeks after immunization, total CD8 and CD4 T cells were isolated using anti-CD4 and anti-CD8 magnetic microbeads, as described by the manufacturer (Miltenyi Biotec, Auburn, CA). Purified T cells were then injected into the irradiated (low dose, 5 Gy) mice bearing 5–10 B16-S tumors.

Statistical analysis

Data were analyzed using Student unpaired t test or ANOVA with the Prism software (GraphPad Prism, La Jolla, CA).

Results

Fc tagging increases the CD8 as well as CD4 immune responses of lv immunization that causes regression of established tumors

Ag–Ab immune complex (45–47) and recombinant DNA expressing Fc-tagged Ag (48, 49) were previously reported to enhance Ag-specific T cell immune responses, possibly because they could be more effectively captured by APCs via Fc receptors (50). We recently found that lv expressing the HBS-Fc fusion Ag could effectively activate endogenous CD4 T cells in addition to enhancing CD8 responses (31). To determine whether the Fc tag is indeed required to enhance CD8 responses, and, more importantly, to induce activation of endogenous CD4 T cells, we compared the magnitude of CD8 and CD4 responses of HBs-lv and HBs-Fc-lv immunization. Two weeks after immunization, peripheral blood cells were restimulated ex vivo with either HBs190 peptide or whole HBsAg protein for 4 h before measuring the IFN-γ level by in-
tracellular staining. We found that, compared with HBS-lv, HBS-Fc-lv immunization not only significantly increased the magnitude of CD8 responses but also, more importantly, induced potent CD4 responses (Fig. 1). In contrast, HBS-lv (without Fc tag) immunization stimulated no measurable CD4 responses. Therefore, we conclude that tagging the lv-encoded Ag with Fc fragment induces CD4 activation.

To find out whether the enhanced Ag-specific CD8 and CD4 immune responses are correlated with a better antitumor effect of lv immunization, mice bearing established B16-S tumors of sizes 10–15 mm² were treated with HBS-Fc-lv or HBS-lv immunization (Fig. 2A). As shown in Fig. 2B, immunization with both HBS-lv and HBS-Fc-lv could strongly inhibit B16-S tumor growth in treated mice, compared with untreated controls. However, only the tumors receiving HBS-Fc-lv immunization experienced substantial regression and even complete eradication. During the peak of the immune response period, the majority of B16-S tumors in the group of mice treated with HBS-Fc-lv underwent regression. Some of the tumors were completely eradicated (Fig. 2B). In a summary of four experiments, ~70–80% of well-established B16-S tumors experienced shrinkage after HBS-Fc-lv immunization, and complete regression was found in 5 of 20 tumor-bearing mice. The tumor-free mice from HBS-Fc-lv treatment resisted further challenge by not only B16-S tumor cells but also B16-F10 tumor cells, strongly suggesting that the antitumor immune responses had spread to other tumor-associated Ags. In contrast, even though B16-S tumor growth was inhibited by HBS-lv immunization, no tumor regression was observed. All mice in the HBS-lv–treated group eventually succumbed to tumor growth. Thus, in the lv immunization platform, Fc tagging not only increases the magnitude of CD8 responses but also induces potent CD4 responses, which may contribute to the tumor regression observed in HBS-Fc-lv–treated tumors.

Fc tagging increases the ability of lv immunization to stimulate a proinflammatory milieu within tumor lesions

Tumor lesions are characterized as indolent chronic inflammation that can promote tumor growth (51). However, recent studies demonstrate that Th1 cytokines in tumor lesions may make the chronic tumor-promoting inflammation become immune stimulating (52). Although cancer vaccines have been proved to stimulate antitumor immune responses, it is not clear how immunization with cancer vaccines will affect inflammation status in tumor lesions. The remarkable regression of established tumors by HBS-Fc-lv immunization provides a strong rationale for analyzing the inflammatory changes in tumor lesions. Using qRT-PCR, we compared the levels of proinflammatory cytokines and chemokines in tumor lesions after immunization with HBS-lv and HBS-Fc-lv. We found that, compared with tumors without treatment, those treated with HBS-Fc-lv had a 50- to 100-fold increase in mRNA levels of proinflammatory cytokines such as IL-1β and IL-6. At the same time, the Th1/Tc1 cytokines of IFN-γ, perforin, granzyme, TNF-α, and transcription factor T-bet in the tumor lesions of HBS-Fc-lv–immunized mice increased by 50- to 200-fold. In addition, cytokines for CD8 T cell survival, such as IL-2 and IL-7, were significantly increased in HBS-Fc-lv–immunized tumors. But IL-15 was not obviously changed. Without Fc tagging, HBS-lv immunization also increased the amount of proinflammatory and Th1/Tc1 cytokines in the tumor lesions, but to a significantly lesser extent. In contrast, the RNA levels of Foxp3, IL-17, and RORγt increased only slightly or remained unchanged (Fig. 3). Thus, our data demonstrate that Fc tagging significantly increases the effect of lv immunization on converting tumor lesions into a Th1/Tc1-like immune stimulatory microenvironment.

Consistent with the Th1/Tc1-like cytokine changes, the chemokines responsible for attracting NK, DCs, Th1, and Tc1 cells in the tumor lesions of HBS-Fc-lv–immunized mice increased as much as 150-fold, compared with untreated tumor. Again, the lv expressing the HBS-Fc fusion Ag demonstrated a much more significant effect (Fig. 3). In contrast, the chemokine CCL22 (53) for Treg recruitment was only slightly increased. These data strongly indicate that HBS-Fc-lv immunization alters chemokine levels such that innate immune effectors and T effectors, but not Tregs, can be effectively recruited into tumor lesions, which may
play an important role in reshaping the tumor milieu to become less immune suppressive and more Th1/Tc1-like and immune stimulating.

**Fc tagging markedly increases tumor infiltration of functional CD8 and CD4 T cells of lv immunization**

The increase of chemokines in the tumor milieu following HBS-Fc-lv immunization suggests that more T effectors can be recruited to the tumor lesions. To test this hypothesis, we analyzed the cellular immune components in the B16-S tumor lesions after different treatments. First, we counted the absolute number of CD4 and CD8 TILs (Supplemental Fig. 2, Fig. 4). We found that HBS-Fc-lv immunization induced significantly more CD8 T cell infiltration compared with HBS-lv immunization (Fig. 4). More importantly, only HBS-Fc-lv immunization significantly increased the number of CD4 TILs in the tumor lesions, which is consistent with data showing that only HBS-Fc-lv immunization could effectively activate CD4 T cells (Fig. 1). When the Treg ratio was analyzed in the tumor lesions, we found that HBS-Fc-lv reduced the Treg ratio in tumor lesions more significantly than did HBS-lv immunization (Supplemental Fig. 2, Fig. 4). Thus, we concluded that, in the lv platform, Fc tagging significantly increases infiltration of CD4 and CD8 T cells into tumor lesions and at the same time reduces the Treg ratio. More specifically, the marked increase of CD4 TILs and reduction of Treg ratio were observed only in the HBS-Fc-lv–immunized tumor lesions.

In the next study, the effector function of CD8 and CD4 TILs following HBS-Fc-lv and HBS-lv immunization was measured. As demonstrated in Fig. 5, when compared with HBS-lv immunization, HBS-Fc-lv immunization stimulated more CD8 TILs to produce IFN-γ in the tumor lesions (Fig. 5A). In addition, more CD4 TILs produced IFN-γ in the tumors treated with HBS-Fc-lv (Fig. 5B). To examine the cytolytic function of CD8 TILs, we stained for CD107a, the degranulation marker in response to Ag stimulation, which can be used as a surrogate test of cytolytic function (34). We found that similar to the results of IFN-γ staining, when compared with HBS-lv immunization, HBS-Fc-lv immunization caused more CD8 TILs to be CD107a positive (Fig. 5C). Because the absolute number (Fig. 4) and effector function (Fig. 5) of CD8 and CD4 TILs were significantly increased in the HBS-Fc-lv–immunized tumors, the total number of functional CD8 and CD4 TILs in the HBS-Fc-lv–immunized tumors should be much higher. These data suggest that Fc tagging markedly increases the number and function of CD4 and CD8 effector T cells in the tumor lesions.

The immunologic changes in the tumor lesions and the antitumor effect of HBS-Fc-lv immunization are dependent on CD4 activation

Using the two lv (HBS-lv and HBS-Fc-lv) that could differentially activate CD4 T cells, we demonstrated in the above studies that effective activation of CD4 T cells by HBS-Fc-lv immunization may play an important role in increasing Th1/Tc1-like proinflammatory cytokines and functional effector T cell infiltration and decreasing Treg ratio in the tumor lesions. However, in those studies, the differences in the magnitude of CD8 responses may also contribute to immunologic changes in the tumor lesions. To examine the role of CD4 in immunologic changes in the tumor milieu, it would be a good approach if only the activated effector CD4 T cells could be selectively depleted in vivo. However, no such

![FIGURE 4.](http://www.jimmunol.org/Downloaded_from/http://www.jimmunol.org/)

HBS-Fc-lv immunization markedly increases tumor infiltration of CD4 and CD8 T cells and decreases the Treg ratio in the tumor lesions. Mice bearing 5-d tumors were immunized with either HBS-lv or HBS-Fc-lv, or left untreated. The tumor lesions were collected on days 17–20 after immunization and analyzed for tumor-infiltrating CD8 and CD4 T cells and the Treg ratio. The absolute numbers of CD8 and CD4 T cells and the Treg ratios in the tumor lesions of control and treated mice from a cohort of two studies are summarized.
strategy is available at present, and the simultaneous depletion of Tregs with anti-CD4 Ab will confound the analysis of the role of effector CD4 T cells. To circumvent this problem, we used FcRγ KO mice because activation of CD4 T cells following HBS-Fc-lv immunization was severely compromised, whereas the CD8 response was not affected (31) (Fig. 6A), creating a scenario that allowed us to study immunologic changes in the tumor milieu in the absence of CD4 activation. The data showed that, following HBS-Fc-lv immunization, the Treg ratio in the tumors of FcRγ KO mice was significantly higher than that in WT mice (Fig. 6B). Furthermore, increase of Th1 cytokines in the tumor milieu after lv immunization was significantly compromised in FcRγ KO mice (Fig. 6C). Consistent with the Foxp3 staining data (Fig. 6B), the level of Foxp3 mRNA was significantly higher in the FcRγ KO tumor (Fig. 6C). Concurrently, the antitumor effect of HBS-Fc-lv immunization in FcRγ KO mice was also compromised (Fig. 6D). These data suggest that CD4 activation has an important part in increasing the Th1/Tc1 cytokines and decreasing the Treg ratio in tumors and in enhancing the antitumor effect of HBS-Fc-lv immunization.

Adoptive transfer of CD4 T cells can activate endogenous CD8 TILs in the tumors and generate antitumor effects

To further examine the antitumor effect of CD4 and CD8 T cells and their effect on endogenous CD8 T cells in the tumor lesions, we conducted adoptive transfer experiments using vaccine-activated

FIGURE 5. CD4 and CD8 TILs in the tumors treated with HBS-Fc-lv possess better effector function. (A) The effector function of CD8 TILs was measured by intracellular staining of IFN-γ after brief ex vivo peptide stimulation. Representative dot plots of CD8 TILs from HBS-lv- or HBS-Fc-lv–immunized tumors are shown. Dot plots from control tumors are not shown because only few CD8 TILs could be collected. Only the CD8 T cells were gated and shown. Data of 5 mice are summarized. (B) Effector cytokine production by CD4 TILs is shown after stimulation with PMA/IONOMYCIN. Only the CD4 T cells were gated and shown. These experiments examining the TIL and their effector functions were repeated three times with similar results. (C) Degranulation of CD8 TILs was measured by CD107a staining. A summary of data from five tumors in each group is presented. This experiment was repeated twice, with similar observations.
CD4 and CD8 T cells. CD4 and CD8 T cells were isolated from HBS-Fc-lv–immunized Thy1.1 congenic C57BL/6 mice and adoptively transferred into irradiated B16-S tumor-bearing Thy1.2 congenic mice (Fig. 7A). Two weeks later, the effector function of TILs was analyzed. We found that adoptive transfer of activated CD4 T cells could effectively activate endogenous CD8 TILs to express GrzB (Fig. 7B) and IFN-γ (data not shown). In addition, more endogenous CD8 T cells were recruited into tumor lesions in the presence of activated CD4 T cells. In contrast, transfer of activated CD8 T cells did not increase granzyme expression of the endogenous CD8 TILs (Fig. 7B). The adoptive exogenous CD8 as well as CD4 T cells were found to be capable of expressing GrzB in the tumor lesions (Fig. 7C). Adoptive transfer of CD4 or CD8 T cells could effectively inhibit tumor growth, but low-dose irradiation alone did not significantly affect tumor growth (Fig. 7D). Thus, adoptive transfer of both activated CD4 and CD8 T cells achieved similar antitumor effects in tumor-bearing mice, but the mechanisms were different. The antitumor effect of CD4 was mediated indirectly by activating endogenous CD8 T cells, whereas exogenous CD8 T cells may directly kill tumor cells.

Then, we compared the antitumor activity of the CD4 T cells from mice immunized with HBS-Fc-lv and HBS-lv, as the level of CD4 activation was drastically different. We observed that, in agreement with the finding that more CD4 T cells were activated in the HBS-Fc-lv–immunized mice (Fig. 1), adoptive transfer of the preactivated CD4 T cells from HBS-Fc-lv–immunized mice could result in more infiltration of endogenous CD8 TILs that possessed better effector function (Supplemental Fig. 3B), resulting in stronger antitumor effect (Supplemental Fig. 3A).

**IFN-γ expression by CD4 T cells plays a critical role in the CD4-mediated antitumor effect**

With use of TCR Tg CD4 T cells, it was reported that IFN-γ produced by CD4 T cells played a critical role in helping recruit CD8 T cells to virally infected lesions (25) and tumor lesions (23, 54). To assess the role of IFN-γ in the CD4-mediated antitumor effect, we examined the antitumor effect of CD4 T cells from WT and IFN-γ KO mice. First, the WT and IFN-γ KO mice were immunized with HBS-Fc-lv. Then, CD4 T cells from immunized WT and IFN-γ KO mice (Thy1.2) were isolated and adoptively transferred into tumor-bearing mice (Thy1.1) to monitor their antitumor effects and their activation of endogenous CD8 T cells (Fig. 8A). On the basis of the TNF-α expression, a comparable number of activated CD4 T cells were found in the WT and IFN-γ KO mice after HBS-Fc-lv immunization (Fig. 8B). The data from the transfer experiment showed that, compared with WT CD4 T cells, adoptive transfer of CD4 T cells from the IFN-γ KO mice was incapable of activating endogenous CD8 T cells in the tumor lesions (Fig. 8C). Furthermore, tumor infiltration of endogenous CD8 T cells was also significantly decreased in mice treated with adoptive transfer of CD4 T cells from IFN-γ KO mice (Fig. 8C).

In agreement with the data, adoptive transfer of CD4 T cells from IFN-γ KO mice had severely compromised antitumor effect (Fig. 8D). These data suggest that the expression of IFN-γ by vaccine-induced CD4 T cells plays a vital part in initiating endogenous CD8 TILs and in mediating antitumor effect.

**Discussion**

Cancer vaccine research, thus far, demonstrates that mere induction of CD8 T cell response has little correlation with success in achieving antitumor effect. The lack of antitumor effect may be in part due to the inability of most current cancer vaccines to simultaneously coactivate CD4 T cells. In this study, we asked whether activation of endogenous CD4 T cells by lv immunization would influence the tumor milieu and antitumor effect of cancer vaccines and how it would accomplish this. Remarkably, we found that strong activation of endogenous CD4 T cells and enhancement of CD8 responses could be achieved by tagging the Ag with the Fc fragment, which increased the antitumor effect of lv immunization, including regression of established tumors. This
potent antitumor effect of Fc tagging in the lv platform is associated with improved immunologic changes in the tumor milieu, from substantial increase of TIL number and conversion of the tumor milieu into a Th1/Tc1-like proinflammatory microenvironment to significant decrease of the Treg ratio. These favorable changes in the tumor microenvironment and remarkable antitumor effects of lv immunization were dependent on the activation of endogenous CD4 T cells. IFN-γ production plays a critical role in CD4-mediated immunologic changes in the tumor milieu. Thus, in addition to the well-recognized CD4 role in priming adaptive immune responses, our findings suggest that activation of endogenous CD4 helps the effector phase of antitumor immunity by modulating the tumor milieu.

How does activation of endogenous CD4 T cells change the tumor microenvironment? First, activation of CD4 may help effector T cell migration into tumor lesions and license DCs to reabsorb CD8 TILs. We found that IFN-γ production by activated CD4 T cells has a function in mediating tumor infiltration of effector T cells into tumor lesions, as the CD8 infiltration was markedly decreased after adoptive transfer of CD4 T cells from IFN-γ KO mice (Fig. 8C). These data are in agreement with previous findings, which show that adoptive transfer of TCR Tg CD4 T cells can help migration of CD8 T cell to infection site (25) and tumor lesion (23) in an IFN-γ–dependent manner. Therefore, it is likely that, following activation by cancer vaccines, the activated endogenous CD4 T cells may enter tumors and secrete IFN-γ to mediate chemokine production in the tumor stroma to attract more CD8 T cells. After migrating into tumor lesions, CD8 T cells that are activated in the lymphoid tissues need reactivation to execute their effector function in the tumor milieu. In this process, the CD40L on the activated CD4 T cells may license DCs to reactivate CD8 TILs. We found that IFN-γ production by activated CD4 T cells has a function in mediating tumor infiltration of effector T cells into tumor lesions, as the CD8 infiltration was markedly decreased after adoptive transfer of CD4 T cells from IFN-γ KO mice (Fig. 8C). These data are in agreement with previous findings, which show that adoptive transfer of TCR Tg CD4 T cells can help migration of CD8 T cell to infection site (25) and tumor lesion (23) in an IFN-γ–dependent manner. Therefore, it is likely that, following activation by cancer vaccines, the activated endogenous CD4 T cells may enter tumors and secrete IFN-γ to mediate chemokine production in the tumor stroma to attract more CD8 T cells. After migrating into tumor lesions, CD8 T cells that are activated in the lymphoid tissues need reactivation to execute their effector function in the tumor milieu. In this process, the CD40L on the activated CD4 T cells may license DCs to reactivate CD8 T cells in the tumor milieu to produce more IFN-γ. The substantial increase of chemokines responsible for effector T cell recruitment into the tumor lesions after lv immunization supports this argument. Then, the CD4 and CD8 TILs secrete more cytokines and turn the suppressive tumor lesion into a Th1/Tc1-like immune stimulatory microenvironment.

Second, activation of CD4 T cells may help reduce the Treg ratio in tumor lesions. Tregs are a key component of immune suppression in tumor lesions (55) and have been shown to inhibit effector function. They are also associated with poor clinical outcome (56, 57). The T effector/Treg ratio correlates with the outcome of tumor immunotherapy (58, 59). Thus, reduction of Tregs can be an effective way to increase the antitumor efficacy of immunization (60). In our study, we found that activation of endogenous CD4 T cells by lv immunization could markedly decrease the Treg ratio in tumor lesions. It is not clear how such activation of endogenous CD4 T cells can accomplish this decrease. But, two recent studies showed that Th1/Th2 T cells could inhibit peripheral Treg induction in vitro and in vivo (61) in an IFN-γ–dependent manner (62). Addition of exogenous IFN-γ markedly decreased Treg generation in vitro. Thus, it is possible that induction of endogenous CD4 T cells to become Th1 cells following HBS-Fc-lv immunization will reciprocally inhibit expansion of Tregs. Another possible mechanism for the decreased Treg ratio following CD4 activation is the induction of Treg apoptosis via the Fas–FasL pathway (63). However, we were unable to detect increased apoptosis of Tregs in the tumor after lv immunization (data not shown).

Recently, DCs were found to take up Ab–Ag immune complexes efficiently via the FcγR to prime CD8 T cell responses (47, 50). In our study, we found that even though covalent Fc tagging could significantly enhance CD8 T cell immune responses, FcγR may not be critical in lv immunization because a similar magnitude of CD8 responses could be induced in FcγR KO mice (Fig. 6A). A possible explanation is that, following lv immunization, direct presentation of Ag synthesized endogenously in the DCs is the main mechanism for priming CD8 T cells (5, 6, 64). The increase of CD8 responses by Fc tagging may result mainly from an increase in Ag production (31). The reuptake of secreted Ag by DCs in an autocrine or paracrine fashion may play only a minor role in CD8 T cell activation. Thus, activation of CD8 T cells following lv immunization is less dependent on FcγR-mediated cross-presentation. In contrast, CD4 activation predominantly relies on the reuptake of foreign Ag to enter the MHC II-mediated Ag processing and presentation pathway and thus depends on the FcγR-mediated Ag reuptake. In agreement with this argument, a classic paper by Celis and Chang (65) nearly 30 y ago showed that in vitro proliferation of HBsAg-specific CD4 T cell clone induced by HBsAg could be markedly enhanced by addition of HbsAb. Although in our study, the role of humoral immunity is not examined, the increase of humoral response following immuniza-
tion, as we recently observed (31), may also contribute to the enhanced antitumor effect by targeting and opsonizing the tumor cells (66).

In this study, using an HBsAg-expressing B16 tumor model, we demonstrated that activation of endogenous CD4 T cells by active immunization could markedly relieve immune suppression in tumor lesions and substantially increase the antitumor effect. However, the expression of foreign HBsAg by tumor cells per se in the absence of immunization did not reduce the Treg ratio in the tumor lesions, suggesting that expression of foreign Ag is not sufficient to significantly change the tumor microenvironment.

More importantly, HBsAg immunization incapable of activating CD4 T cells did not significantly change the tumor milieu and resulted in no regression of B16-S tumor. Thus, improvements of the tumor milieu—that is, conversion of the tumor milieu into a Th1/Th1-like environment and reduction of the Treg ratio in the tumor lesions by HBsAg-Ⅲ immunization—are related to the vaccine’s ability to activate CD4 T cells. Whether these findings made in the B16-S tumor model have general implications requires further studies using other Ags and even self tumor Ags. Such studies will be critical for the rational design of tumor vaccines to effectively treat tumors not only by increasing effector T cells but also by improving the tumor microenvironment through activation of endogenous CD4 T cells.

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Disclosures

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References


