

CD4⁺ T-Cell Help in the Tumor Milieu Is Required for Recruitment and Cytolytic Function of CD8⁺ T Lymphocytes

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Abstract

CD4 help for CD8⁺ T lymphocytes prevents tolerance and promotes the survival of effector and memory CD8⁺ T cells. Here, we describe additional helper functions that require CD4⁺ T cells within the tumor environment. CD8⁺ T-cell recruitment, proliferation, and effector function within the tumor were greatly enhanced by tumor-specific CD4⁺ T cells. Recruitment of CD8⁺ T cells was accelerated by IFN- γ -dependent production of chemokines. Production of interleukin-2 by tumor resident CD4⁺ T cells enhanced CD8⁺ T-cell proliferation and upregulated expression of granzyme B. These results highlight a novel role for tumor-specific CD4⁺ T cells in promoting CD8⁺ T-cell recruitment and cytolytic function, two previously unappreciated aspects of tumor-specific CD4 help. *Cancer Res*; 70(21); 8368–77. ©2010 AACR.

Introduction

The requirements for successful cancer immunotherapy are not fully understood. Tumor vaccines that successfully result in the stimulation of large numbers of tumor-specific CTL do not necessarily result in tumor destruction (1–5). Several factors may constrain tumor eradication by specific effector CD8⁺ T cells. One is the relatively low affinity of the T-cell repertoire specific for self/tumor antigens caused by mechanisms of tolerance that delete and inactivate T cells with high affinity for self-antigen (6–8). Also, unlike inflammatory sites initiated by an infectious agent, the tumor milieu is an immunosuppressive environment that prevents the recruitment, survival, and function of tumor-specific effector cells (9, 10). Furthermore, the tumor vasculature can be inhibitory to migration of immune effector cells (11, 12).

Using a tumor model, in which pancreatic neuroendocrine tumors that express influenza hemagglutinin (HA) as a tumor antigen develop (13), we have shown that CD8⁺ T cells expressing an HA-specific TCR obtained from mice that express HA as a self-antigen (Clone-1; ref. 14) are unable to eradicate tumor, even when activated by a potent viral vaccine. Co-transfer of HA-specific SFE CD4⁺ T cells greatly enhanced the accumulation of Clone-1 cells in the tumor milieu and promoted tumor destruction (14, 15). The provision of non-tumor-specific CD4 help during CD8 priming had no such effect, suggesting that the benefit of CD4 help was

accrued in the tumor milieu and was not due to the programming of CD8⁺ T cells during initial priming (15).

Previous studies have shown the importance of CD4⁺ T cells in preventing the tolerance of CD8⁺ T cells in the face of persistent antigen produced by self, tumor, or persistently infected tissue (16–22). However, tumor-specific CD4⁺ T cells may afford additional benefits that assist in tumor eradication. We hypothesized that CD4⁺ T cells may promote recruitment, proliferation, survival, and effector function of CD8 effectors within the tumor milieu. Here, we have independently assessed each of these parameters and have identified the cytokines required for such enhanced activities.

Materials and Methods

Mice

B10.D2 rat insulin promotor (RIP)-Tag2-HA mice have been previously described (13) and were used at 8 to 9 weeks of age. B10.D2 Clone-1 TCR transgenic mice, which express a TCR specific for HA₅₁₈₋₅₂₆ (IYSTVASSL) in the context of HA-2K^d, and SFE and SFE interleukin-2^{-/-} (IL-2^{-/-}) TCR transgenic mice, which express a TCR that recognizes HA₁₁₀₋₁₁₉ (SFERFEIFPK) in the context of I-E^d, were bred with the congenic markers Thy1.1 and CD45.1, respectively. B10.D2 DO11.10 TCR transgenic mice express a TCR that recognizes OVA₃₂₃₋₃₃₉ in the context of I-A^d. All mice were bred in our facility. All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee of the Scripps Research Institute.

Adoptive transfer of naïve transgenic T cells and peptide immunization

Lymph nodes were collected and purified by magnetic cell sorting using CD8⁺/CD4⁺ T-cell enrichment sets (BD Bioscience). Purified lymphocytes (0.3×10^6 or 1×10^5) were injected into RIP-Tag2-HA mice i.v. Recipient mice were immunized with 10 μ g HA₅₁₈₋₅₂₆-K^d peptide, 50 μ g SFE₁₁₀₋₁₁₉

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or OVA₃₂₃₋₃₃₉ peptide, and 200 µg poly(inosinic-cytidylic acid) [poly(I:C); EMD Biosciences] in incomplete Freund's adjuvant (IFA; Difco Laboratories) s.c. in the right flank. Glucose levels in the blood were measured as described before (15).

***In vitro* analysis of lymphocytes**

The pancreas was minced in medium containing 2 mg/mL collagenase P (Roche Diagnostics) and 2 µg/mL DNase (Sigma-Aldrich). Enzymatic digestion was allowed for 20 minutes at 37°C. Cells were washed with ice-cold complete RPMI (Life Technologies), and lymphocytes were purified by density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich). Cells were stained for fluorescence-activated cell sorting (FACS) analysis in HBSS containing 1% FCS and 2 mmol/L EDTA. For intracellular staining of IFN-γ, cells were stimulated overnight with 1 µg/mL HA₅₁₈₋₅₂₆ peptide in the presence of 1 µL/mL GolgiPlug. Antibodies for FACS were used from eBioscience, BD Biosciences, and Alexis Biochemicals (Bim_{S/EL/L}). Intracellular stainings were performed according to the manufacturer's instructions using the Cytotfix/Cytoperm plus kit (BD Biosciences).

***In vivo* cytokine production**

Mice received 0.3×10^6 SFE cells i.v. and were immunized with 50 µg SFE peptide and 200 µg poly(I:C) in IFA s.c. At day 4, 250 µg of Brefeldin A (Sigma-Aldrich) were injected i.p., and after 15 hours, spleens and pancreata were isolated and analyzed by FACS.

Cytokine array

Mice were immunized with 50 µg SFE peptide and 200 µg poly(I:C) in IFA s.c. in the right flank with or without the injection of 0.3×10^6 purified SFE CD4⁺ cells i.v. Pancreata were isolated 6 days later, and after density gradient centrifugation with Histopaque-1077, cells were resuspended in PBS with protease inhibitors. Cell lysates were prepared according to the manufacturer's instructions, and cytokines were analyzed with Mouse Cytokine Array Panel A (R&D Systems).

***In vitro* generation of effector Clone-1 cells and *in vivo* recruitment**

Lymph node cells from Clone-1 mice were cultured in complete RPMI (Life Technologies) with 1 µg/mL HA-K^d peptide, 20 ng/mL human IL-2, and splenocytes. After 3 days, live cells were purified by Ficoll and cultured for 3 days with 20 ng/mL IL-2. Activated Clone-1 cells (1×10^6) were injected i.v. into RIP-Tag2-HA mice that were immunized 6 days earlier with 50 µg SFE peptide and 200 µg poly(I:C) in IFA s.c. with or without the injection of 0.3×10^6 purified SFE CD4⁺ T cells i.v. Pancreata and spleens were isolated after 40 hours, and the number of recruited Clone-1 cells was analyzed by FACS. Neutralizing antibodies against IFN-γ (500 µg/mouse, Clone R4-6A2 BioXcell), CXCL10, CCL2, CCL3, CCL5 (R&D Systems), and CXCL9 (kindly provided by Dr. R. Schreiber, Washington University School of Medicine) were injected 6 hours before and 18 hours after injection of Clone-1 cells (80 µg/mouse per antibody).

Statistical analysis

Differences between group means were determined by a Mann-Whitney test. Data are presented as means ± SEM.

Results

CD4 help within the tumor milieu is required for accumulation of tumor-specific CD8⁺ T cells

Tumor-bearing RIP-Tag2-HA mice received HA-specific Clone-1 cells, either alone or with HA-specific SFE cells, and were immunized with a vaccine containing cognate peptides and poly(I:C) injected s.c. in IFA. As previously shown using a viral vaccine, the presence of SFE cells during priming did not significantly increase the expansion of Clone-1 cells in the blood or spleen, and no differences were observed in expression levels of CD25, CD44, and CD62L on Clone-1 cells in the spleen or lymph nodes draining the vaccination site (ref. 15; Fig. 1A and data not shown) and Clone-1 cells developed equally well into effector cells and produced similar levels of IFN-γ and granzyme B (ref. 15; Fig. 1B). However, in accordance with our earlier studies, tumor-specific CD4⁺ T cells were required to promote tumor eradication by Clone-1 cells, as evidenced by elevated blood glucose that occurs when the majority of normal and transformed pancreatic islet β cells are destroyed (Fig. 1C). Successful tumor eradication was paralleled by a 6-fold to 10-fold enhancement in the numbers of Clone-1 cells found within the tumors 6 days after immunization (Fig. 2A).

To determine whether this difference was due to CD4 help provided during priming and/or the presence of CD4⁺ T cells within the tumor environment, we applied two different strategies. First, we immunized mice that received Clone-1 cells and non-tumor-specific, OVA-specific DO11.10 CD4⁺ T cells, with a vaccine containing cognate HA and OVA peptides. This provided CD4 help during priming, but not in the tumor milieu. Such non-tumor-specific CD4 help during priming did not increase the numbers of Clone-1 cells in the tumor (Fig. 2A) and had no effect on tumor growth (Fig. 2B). Thus, help within the tumor environment was critical for tumor eradication. Second, the sites of activation of tumor-specific SFE and Clone-1 cells were spatially separated in opposite flanks, so no CD4 help was present during priming of Clone-1 cells. This resulted in antigen-specific CD4 help for Clone-1 cells only at the site of the tumor (Supplementary Fig. S1; CTL peptide right flank/SFE peptide left flank), which was found to be sufficient for enhanced accumulation of Clone-1 cells in the pancreas and resulted in an effective anti-tumor response [Fig. 2A and B; Clone-1 + SFE (tumor)]. Thus, as reported previously using virus immunization (15), CD4 help within the tumor milieu greatly enhances the accumulation of tumor-specific CD8⁺ T cells and promotes tumor eradication.

CD4⁺ T cells enhance recruitment of CD8⁺ T cells by an IFN-γ-dependent mechanism

To determine the extent to which enhanced accumulation of CD8⁺ T cells in the tumor was due to recruitment, RIP-Tag2-HA mice received SFE cells and cognate peptide

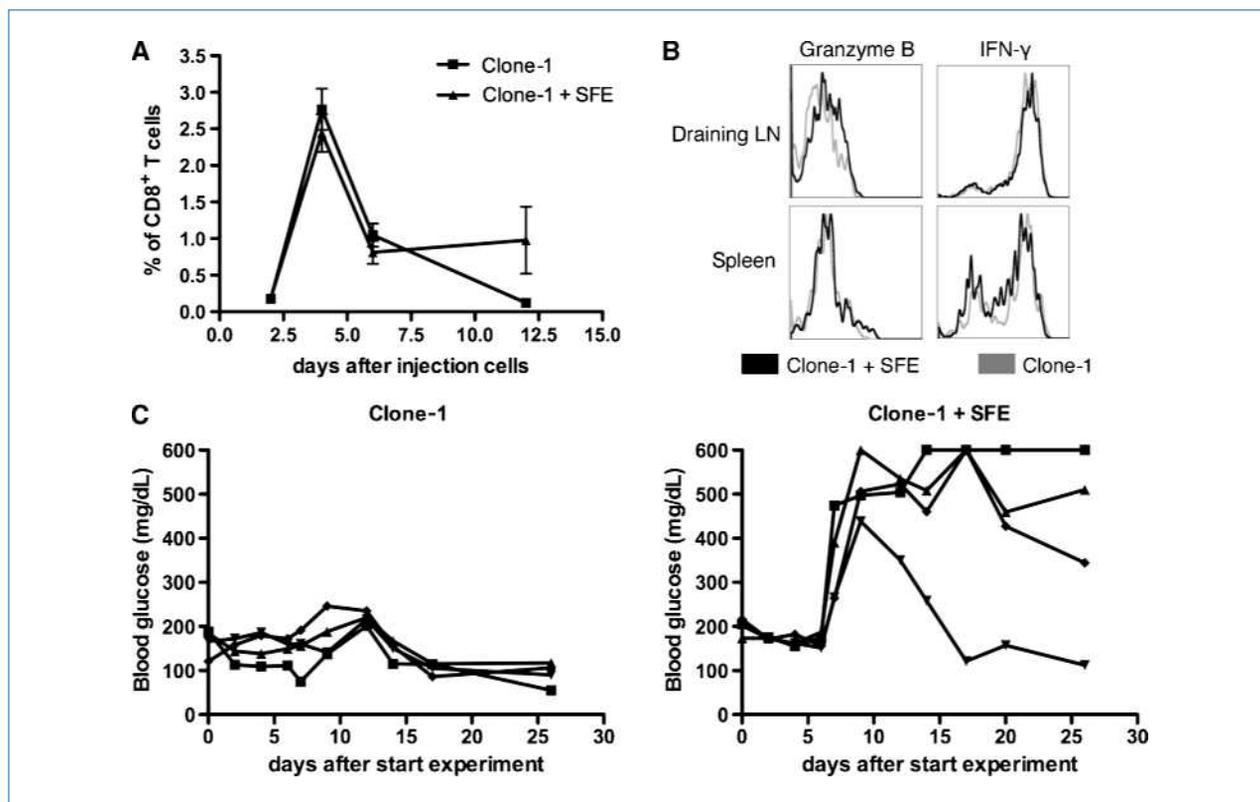


Figure 1. Antitumor efficacy of Clone-1 CD8⁺ T cells. RIP-Tag2-HA mice were immunized and Clone-1 cells (0.3×10^6) with or without 0.3×10^6 SFE cells were injected i.v. A, on the indicated days, peripheral blood lymphocytes were analyzed by FACS for Thy1.1⁺ cells. B, effector function of Clone-1 cells was measured in the spleen and draining lymph node of the vaccination site at day 6 by intracellular staining of granzyme B and IFN- γ . C, glucose levels in the blood were measured at the indicated time points, and each line represents one mouse. Data are representative of two independent experiments with four mice per group.

vaccine. Mice were rested (6 days) to allow for accumulation of tumor-specific CD4⁺ T cells in the pancreas and then received a bolus of *in vitro* activated Clone-1 CD8⁺ T cells. Forty hours later, significant numbers of Clone-1 cells were found only in mice that received SFE cells, suggesting that tumor-specific CD4⁺ T cells were required for the recruitment of Clone-1 (Fig. 3A). To verify that increased numbers of Clone-1 cells in the tumor were due to recruitment and not proliferation, Clone-1 cells were labeled with CFSE to detect division. No proliferation of Clone-1 cells was observed indicating that the increased numbers of Clone-1 cells were due to recruitment by SFE cells (Supplementary Fig. S2).

We hypothesized that SFE cells enhance recruitment of immune cells to the tumor through their production of inflammatory mediators. One of the major effector functions of CD4⁺ T cells is the secretion of IFN- γ , which induces the secretion of inflammatory mediators, including chemokines (23–26). We confirmed that SFE cells become activated within the tumor to produce IFN- γ (Supplementary Fig. S3). To assess the role of IFN- γ in the recruitment of Clone-1 cells, mice that received SFE cells and vaccine were given an IFN- γ neutralizing antibody on days 6 and 7. This effectively blocked the recruitment of Clone-1 cells to the tumors (Fig. 3A). To identify the cytokines and chemokines produced

in the presence of SFE cells, we examined total cell lysates of pancreata from RIP-Tag2-HA mice 6 days after they received SFE cells and vaccine (Fig. 3B). The presence of SFE cells induced the production of numerous chemoattractants, all of which were suppressed by IFN- γ neutralizing antibody (Fig. 3B). The expression of CXCL10 (Fig. 3A–C) was verified by immunohistochemistry. These data confirm that the expression of this chemoattractant in the pancreatic islets requires SFE cells (Supplementary Fig. S4).

To test the role of specific chemokines in promoting recruitment, neutralizing antibodies were injected before transfer of activated Clone-1 cells. Whereas anti-CXCL9 and anti-CXCL10 antibodies alone had only a minor effect, coinjection of these antibodies resulted in a significant inhibition of the recruitment of Clone-1 cells to the site of the tumor (Fig. 3A). Also a mixture of anti-CCL2, anti-CCL3, and anti-CCL5 antibodies inhibited recruitment, indicating that multiple chemoattractants acting through distinct chemokine receptors contributed to recruitment of Clone-1 cells to the tumor.

CD4⁺ T cells enhance proliferation of Clone-1 cells in the tumor milieu

CD4⁺ T cells could also increase the number of Clone-1 cells within the tumor by enhancing their proliferation. Division

was tested using a marker specific for proliferating cells, ki-67. RIP-Tag2-HA mice received either Clone-1 cells or both Clone-1 and SFE cells and were immunized as in Fig. 1. The presence of SFE cells made no difference in the proliferation of Clone-1 cells in the spleen (Supplementary Fig. S5A). However, in the absence of SFE cells, less division of Clone-1 cells occurred in the pancreas as assessed 6 days after immunization (60% versus 80%, Fig. 4A). This stimulatory effect was the result of CD4 help at the site of the tumor rather than during priming as DO11.10 cells, which help only at the site of priming, did not enhance proliferation of the Clone-1 cells infiltrating the tumor, whereas tumor-specific help, available at the site of the tumor and not during priming, increased the percentage of ki-67⁺ Clone-1 cells (Fig. 4A).

CD4⁺ T cells may also promote survival of CD8⁺ T cells in the tumor milieu. Attempts to detect apoptosis of Clone-1 cells in the pancreas by Annexin staining were unsuccessful (data not shown). We therefore examined expression of Bim, a proapoptotic Bcl-2 family member, which plays a key role in T-cell death *in vivo* (27). No differences were observed in the spleen (Supplementary Fig. S5B); however, SFE cells significantly reduced Bim expression by Clone-1 cells in the

pancreas. The presence of DO11.10 cells did not reduce Bim expression, suggesting that CD4 help in the tumor milieu rather than during priming improves T-cell survival (Fig. 4B).

IL-2 is critical for Clone-1 proliferation in the tumor

Production of IL-2 by CD4⁺ T cells promotes expansion and survival of CD8⁺ T cells (28, 29). To determine the effect of IL-2 on the accumulation of Clone-1 cells in the tumor, we compared the numbers of Clone-1 cells infiltrating the pancreas in mice that received SFE cells or IL-2-deficient SFE cells. Because IL-2 deficiency (and neutralization of IFN- γ) reduced the number of CD4⁺ T cells in the pancreas, we injected a larger number of CD4⁺ T cells in these groups of mice to compensate for this difference (Supplementary Fig. S6). The lack of IL-2 production by SFE cells greatly reduced the number of Clone-1 cells found in the pancreas 6 days after immunization (Fig. 5A). We also found decreased numbers of infiltrating Clone-1 cells after blocking of IFN- γ , confirming the role of IFN- γ on recruitment (Fig. 5A). Comparison of ki-67 expressed by Clone-1 cells in the presence of IL-2-deficient and IL-2-sufficient SFE cells indicated that IL-2 was necessary to increase proliferation of Clone-1 cells

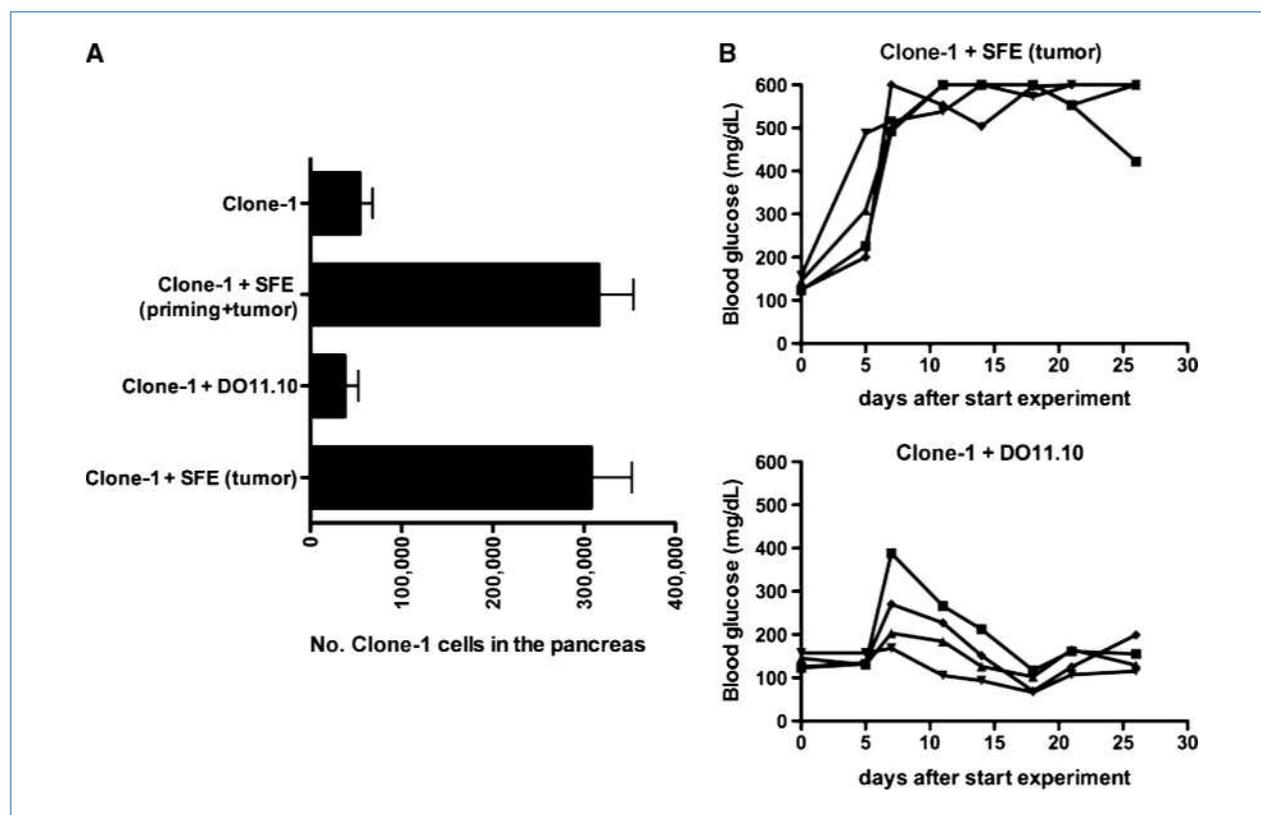


Figure 2. CD4⁺ T cells are required in the tumor environment for accumulation of Clone-1 cells and antitumor immunity. A, RIP-Tag2-HA mice were immunized, and Clone-1 cells (0.3×10^6) with or without 0.3×10^6 SFE or DO11.10 cells were injected i.v. One group of mice received HA-K² peptide and 200 μ g poly(I:C) in IFA s.c. in the left flank and SFE peptide and 200 μ g poly(I:C) in IFA s.c. in the right flank. Pancreata were analyzed by FACS at day 6. Data are cumulative over four experiments. B, glucose levels in the blood were measured at the indicated time points, and each line represents one mouse. Data are representative of two independent experiments with four mice per group.

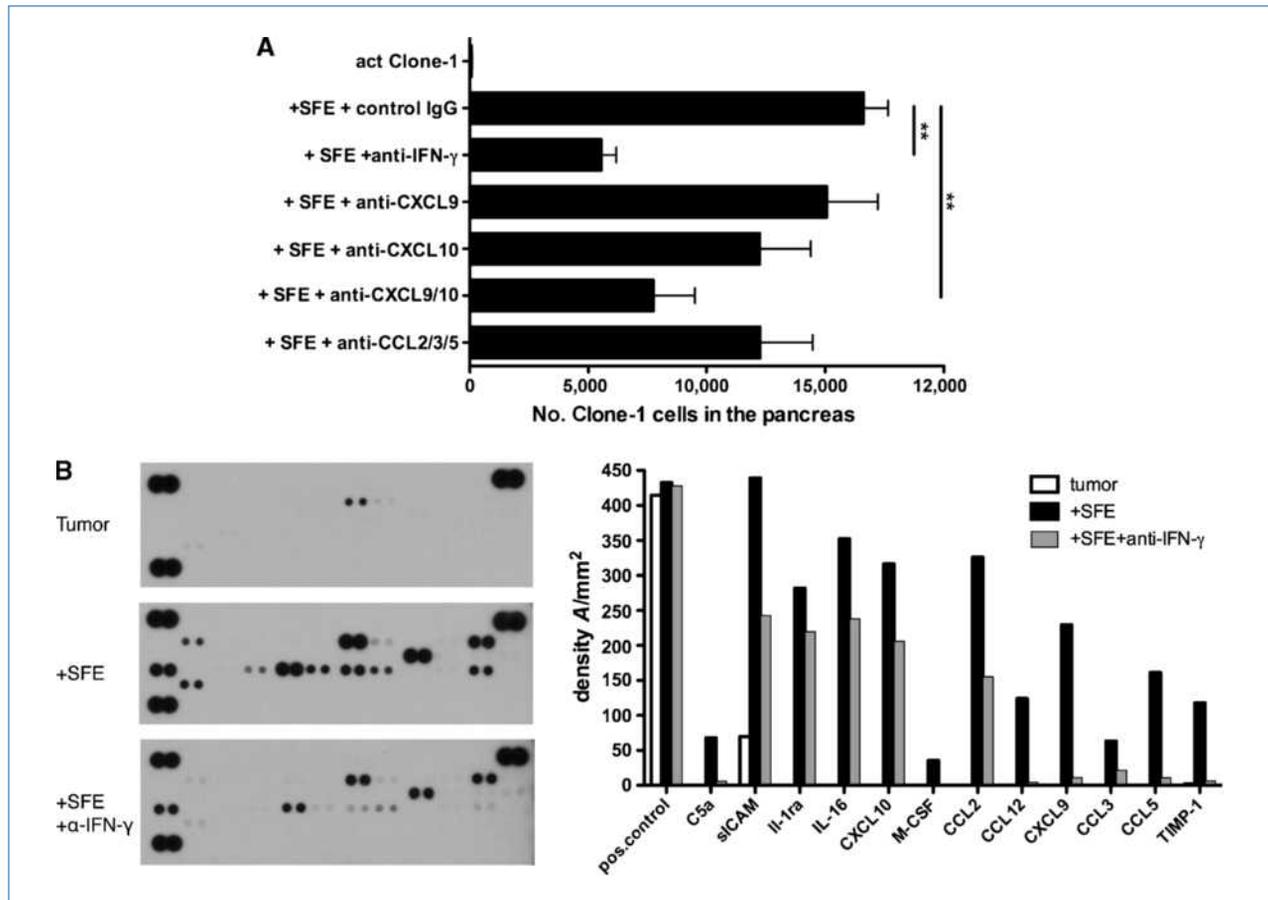


Figure 3. Tumor-specific CD4⁺ T cells induce an inflammatory environment that promotes recruitment of Clone-1 cells. **A**, RIP-Tag2-HA mice were immunized with or without injection of 0.3×10^6 SFE cells. At day 6, 1×10^6 *in vitro* activated Clone-1 cells were injected, and recruitment of Clone-1 cells to the pancreas was analyzed 40 h later. The indicated neutralizing antibodies were injected at days 6 and 7. Data are cumulative over three experiments. **, $P < 0.005$. **B**, RIP-Tag2-HA mice were immunized with or without injection of SFE cells (0.3×10^6) 6 d before isolation of pancreata, and one group was injected with IFN- γ neutralizing antibodies at days 4 and 5. Cell lysate (200 μ g) was used for a cytokine array. Array images are from 30-min exposures to X-ray film and quantitated data show relative changes between conditions. Data are representative of two independent experiments.

(Fig. 5B). In contrast, blocking IFN- γ had no effect on cell division. We also assessed whether IL-2 expression by SFE cells was required for downregulation of Bim expression by Clone-1 cells. IL-2-deficient SFE cells were almost as effective as IL-2-sufficient cells in promoting Bim downregulation (Fig. 5C). There was an increase in Bim expression by Clone-1 cells in mice in which IFN- γ was blocked, but this was found to be variable and not statistically significant.

To determine whether IL-2 also affected the recruitment of CD8⁺ T cells, previously activated Clone-1 cells were injected into RIP-Tag2-HA mice that received IL-2-deficient SFE cells and vaccine 6 days earlier. IL-2 deficiency did not affect the numbers of Clone-1 cells recruited to the tumor (Fig. 5D).

CD4⁺ T cells in the tumor milieu enhance granzyme B expression by Clone-1 cells through an IL-2-dependent mechanism

By day 6 following transfer, significant numbers of Clone-1 cells could be found in the tumor even in the absence of transferred CD4⁺ T cells (Fig. 2A). Nevertheless, as shown

in Figs. 1 and 2, this did not result in tumor destruction. This observation prompted us to examine the effector function of Clone-1 cells in the pancreas. The presence of SFE cells did not significantly enhance production of IFN- γ after *in vitro* peptide restimulation (data not shown; ref. 15). However, we did observe a higher frequency of granzyme B⁺ Clone-1 cells in the pancreas of mice that received SFE cells (Fig. 6A). This difference was detectable in the pancreas and not in the spleen (Supplementary Fig. S5C). In accordance with the antitumor efficacy of Clone-1 cells shown in Fig. 1, CD4 help by DO11.10 cells did not enhance granzyme B expression, and help was only required at the site of the tumor and not during priming (Fig. 6A). The enhanced frequency of granzyme B⁺ Clone-1 cells observed in the presence of SFE cells was dependent on expression of IL-2 but did not require IFN- γ (Fig. 6A).

The role of IL-2 and IFN- γ in tumor eradication

To determine which of these mechanisms of CD4 help contributed to tumor eradication, we tested the antitumor

efficacy of Clone-1 cells in RIP-Tag2-HA mice that received SFE cells and neutralizing IFN- γ antibodies and/or IL-2-deficient SFE cells (Fig. 6B). When tumor-bearing mice received Clone-1 cells and IL-2-deficient SFE cells, tumor eradication was greatly reduced. Blocking IFN- γ had less of an effect on tumor eradication, as most of the mice showed evidence of tumor destruction, although in most mice, this was less effective than when IFN- γ was present. When both cytokines were absent, CD4 help was completely abrogated as indicated by a lack of tumor killing.

Discussion

In the present study, we examined the molecular mechanisms of CD4 help for tumor-specific CD8⁺ T cells within the tumor environment. It was reported that the innate immune activator poly(I:C) can substitute for CD4 help during priming by upregulating dendritic cell expression of CD70 (30, 31). Consistent with those results, we found that immunization with poly(I:C) obviates the need for CD4 help with respect to the numbers and function of the resultant CD8 effectors in the periphery. However, as previously reported using virus as a tumor vaccine, this was not sufficient to either promote the accumulation of Clone-1 cells in the pancreas or to achieve tumor eradication (ref. 15 and this study). Furthermore, this was not improved by providing CD4 help during the priming of CD8⁺ T cells in the form of non-tumor-specific DO11.10 CD4⁺ T cells, yet was improved when tumor-specific help was provided. Thus, tumor-specific CD4⁺ T cells play a

unique role postpriming in promoting tumor eradication. Here, we have identified the benefits of CD4 help that are exclusive to the tumor milieu and that are required for effective immunotherapy.

Whereas the numbers of Clone-1 cells in the blood were comparable in the absence or presence of SFE cells, far greater numbers of Clone-1 cells were found in the pancreas of mice that received SFE cells. Our study shows that enhanced intratumoral accumulation of tumor-specific CD8⁺ T cells is the result of multiple effects by tumor-specific CD4⁺ T cells involving the recruitment, proliferation, and possibly survival of the CD8⁺ T cells.

A strong inflammatory environment that is induced by the presence of SFE cells relies mainly on the production of IFN- γ and facilitates early recruitment to the tumor. In the presence of SFE cells, large numbers of Clone-1 cells were found in the pancreas 2 days after injection, and a significant reduction was observed when IFN- γ was blocked. Furthermore, blocking of several chemokines that are induced by IFN- γ (CXCL10, CXCL9, CCL2, CCL3, and CCL5) decreased the numbers of infiltrating Clone-1 cells. We have shown that CD4⁺ T cells can produce IFN- γ in the tumor milieu (Supplementary Fig. S3); however, this does not exclude the role of other cell types, such as natural killer cells and macrophages, in contributing to production of this cytokine. Others have also shown the importance of an IFN- γ -dependent increase in the expression of Th1 chemokines within the tumor environment (9), and these chemokines have been described to be preferentially expressed in human tumors that contain T cells (32). A direct role for production of IFN- γ by CD4⁺

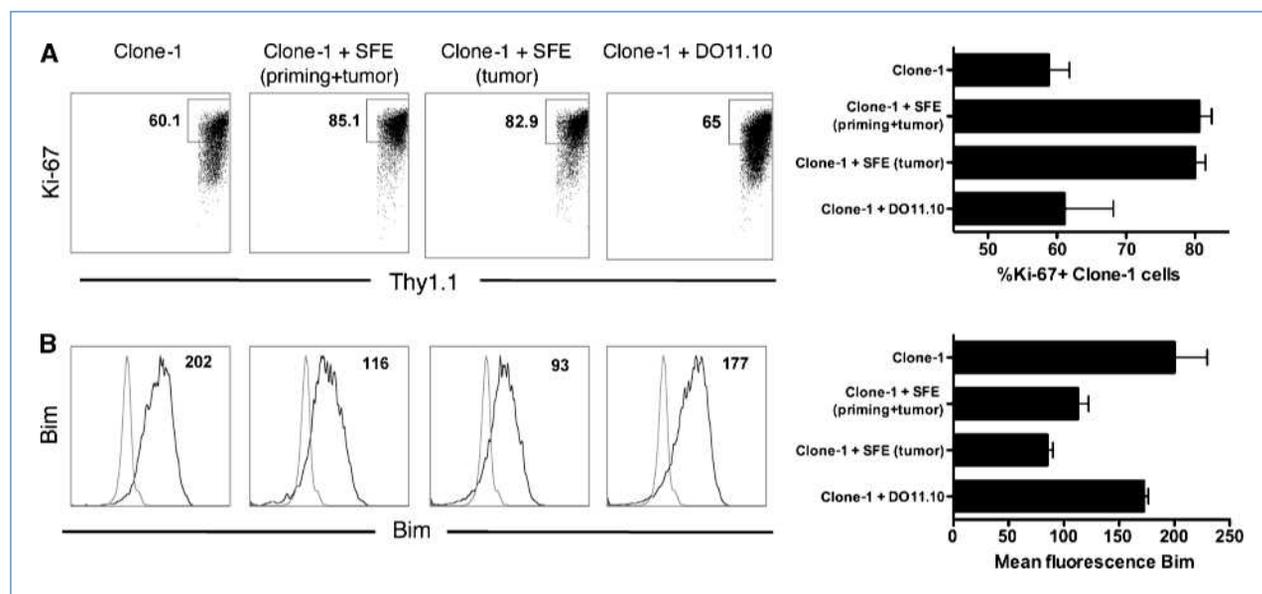


Figure 4. CD4⁺ T cells in the tumor environment stimulate proliferation and survival. RIP-Tag2-HA mice were immunized, and Clone-1 cells (0.3×10^6) with or without 0.3×10^6 SFE or DO11.10 cells were injected i.v. Tumor-infiltrating Clone-1 cells were analyzed at day 6 by FACS for proliferation (A; Ki-67) and Bim expression (B). The gate of the Ki-67 staining was based on the Thy1.1⁺ Ki-67⁺ cells in the pancreas. Control stainings in the histograms show staining with the Bim antibody of Bim^{-/-} splenocytes. Dot plots and histograms are representative examples of each condition, and bar graphs depict cumulative data of three experiments with two to four mice per group.

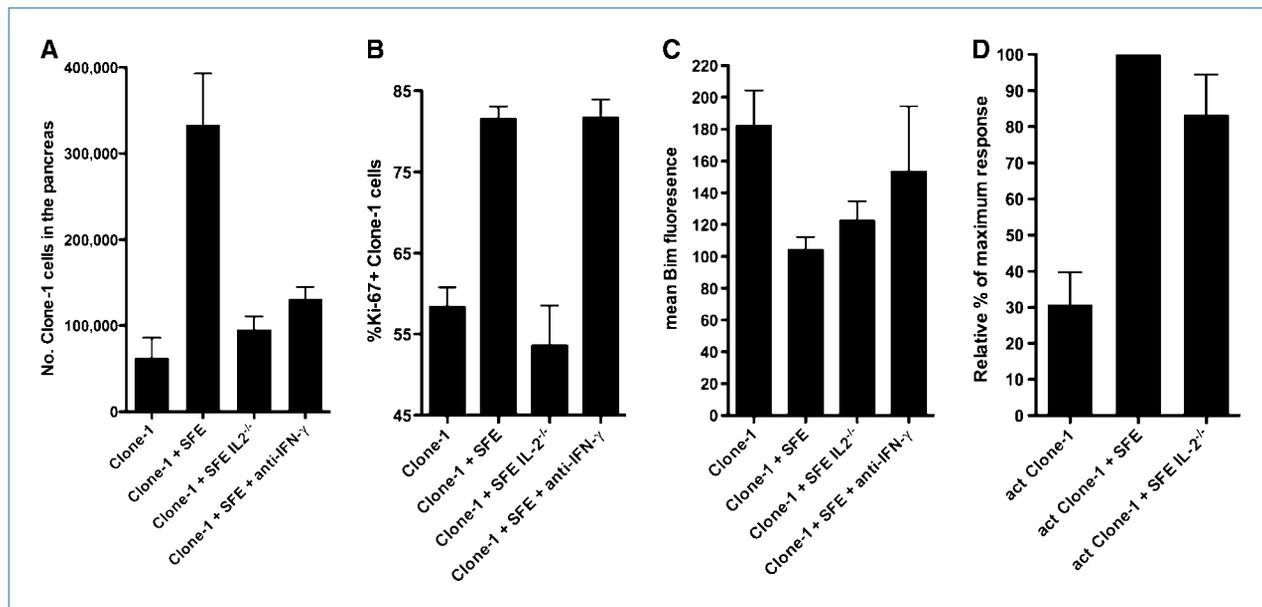


Figure 5. The role of IL-2 production by CD4⁺ T cells. A, RIP-Tag2-HA mice were immunized, and Clone-1 cells (0.3×10^6) with or without 0.3×10^6 SFE, 2×10^6 IL-2^{-/-} SFE, or 2×10^6 SFE cells were injected i.v. The latter group was injected with IFN- γ -neutralizing antibodies at days 4 and 5. Pancreata were analyzed by FACS at day 6. Data are cumulative over two experiments with seven mice per group. B, mice were injected as described in A. Expression of markers for proliferation (B; Ki-67) and survival (C; Bim) on Clone-1 cells were analyzed by FACS at day 6. Data are cumulative over three experiments with nine mice per group. D, RIP-Tag2-HA mice were immunized with or without i.v. injection of 0.3×10^6 SFE or 2×10^6 SFE IL-2^{-/-}. At day 6, 1×10^6 *in vitro* activated Clone-1 cells were injected and recruitment of Clone-1 cells to the pancreas was analyzed 4 d later. Data are cumulative over three experiments with nine mice per group.

T cells in the recruitment of virus specific CD8⁺ T cells was recently reported in a model of herpes virus-infected vaginal tissue (33). These results highlight the importance of CD4⁺ T cells for recruitment of CD8⁺ T cells in a variety of different tissues that are not able to directly recruit effector CD8⁺ T cells. This brings up the interesting question of why CD4⁺ T cells, but not CD8⁺ T cells, are effectively recruited into the transformed islets. The underlying basis for such differences in recruitment of CD4⁺ and CD8⁺ T cells is an unexplored area of investigation.

Our data indicate that recruitment of effector CD8⁺ T cells by CD4⁺ T cells in the tumor milieu is not sufficient to explain tumor eradication. Another major effector function of CD4⁺ T cells, the production of IL-2, was required to optimize tumor eradication by infiltrating CD8⁺ T cells. Although a number of studies have shown a critical role for IL-2 during CD8 priming, (34–36), there has been less emphasis on the importance of IL-2 in sustaining a CD8 response within infected parenchymal tissue or in the tumor tissue. D'Souza and Lefrancois compared the numbers of CD8⁺ T cells sufficient or deficient in the high-affinity IL-2 receptor (CD25) following viral infection. They observed comparable numbers of cells in lymphoid tissue; however, the numbers of CD25-deficient cells were greatly reduced in the lamina propria (37). Shrikant and colleagues examined the role of IL-2 in sustaining tumor immunity. In their model, delivery of IL-2 to tumor-bearing mice on days 4 and 5 following injection of tumor-specific CD8⁺ T cells was required to prevent anergy of tumor-specific CD8⁺ T cells (38). However, they saw no dif-

ference in effector function in the presence or absence of IL-2 (39). Our experiments highlight the important role for IL-2 production by CD4⁺ T cells in the tumor environment and further show that IL-2 affects both CD8⁺ T-cell proliferation and effector function. Importantly, SFE cells deficient in IL-2 did not support tumor eradication by Clone-1 CD8⁺ T cells. Whether this is due to a need for proliferation or enhanced cytolytic function is not directly distinguished by these studies. However, previous experiments in which 10-fold larger numbers of Clone-1 cells were transferred to RIP-Tag2HA mice in the absence of CD4 help did not result in tumor eradication, despite the fact that the very significant numbers of Clone-1 infiltrates were observed in the islets (14). In addition, most Clone-1 cells undergo at least some division in the tumor even in the absence of IL-2. IL-2 has been shown to promote the induction of granzyme B via signal transducers and activators of transcription 5 activation (40, 41) as well as upregulating expression of perforin (42). Taken together, these observations suggest that the main function of IL-2 in this model is most likely the upregulated cytolytic activity, as indicated by enhanced granzyme B. It is striking that, even in the presence of CD4 help, granzyme B levels are low in the periphery but show highly increased levels (3-fold to 4-fold increase) at the site of the tumor, emphasizing the importance of the local effects of IL-2. Other approaches to deliver cytokines like IL-2 and/or IFN- γ into the tumor environment that improved antitumor responses have also been described (43–46). Considering our results, it will be of great interest to determine whether the local

delivery of IL-2 and IFN- γ into the tumor environment may replace CD4 help in our model.

An important outstanding question is how CD4 help is delivered to CD8⁺ T cells in the tissue. As the tumor cells do not themselves express MHC class II, it is likely that an antigen-presenting dendritic cell is required for stimulation of CD4⁺ T cells in the tumor. MHC class II positive cells were observed in the absence of CD4⁺ SFE cells, but this number was greatly increased when CD4⁺ SFE cells were present (Supplementary Fig. S7). This increase could be due to the recruitment of MHC class II positive cells to the site of the tumor or upregulation of MHC class II on resident cells. CD4⁺ T cells are able to activate dendritic cells in tumors by CD40-CD40L interactions, a process often called licensing, which may be important for preventing CD8⁺ T-cell tolerance (22). However, such "licensed" dendritic cells would not provide the IL-2 and IFN- γ required for tumor eradication in our

model. Whether in our model the CD8⁺ T cells also require dendritic cells for their stimulation in the tissue is not known.

These data clearly illustrate the importance of CD4 help at the site of the tumor and the different mechanisms by which CD4⁺ T cells increase the number and function of tumor-specific CD8⁺ T cells activated by a tumor vaccine. Of interest, we also find that previously activated CD8⁺ T cells, such as those used for adoptive transfer in tumor immunotherapy, also greatly benefit from the presence of tumor-specific CD4⁺ T cells, which enhances CD8 recruitment to the tumor milieu. Taken together, these results provide strong evidence that tumor-specific CD4⁺ T cells uniquely support tumor-specific CD8⁺ T cells activated by either tumor vaccines or adoptive immunotherapy. Whether the numerous functions provided by tumor-specific CD4⁺ T cells can be provided by alternative means remains to be determined.

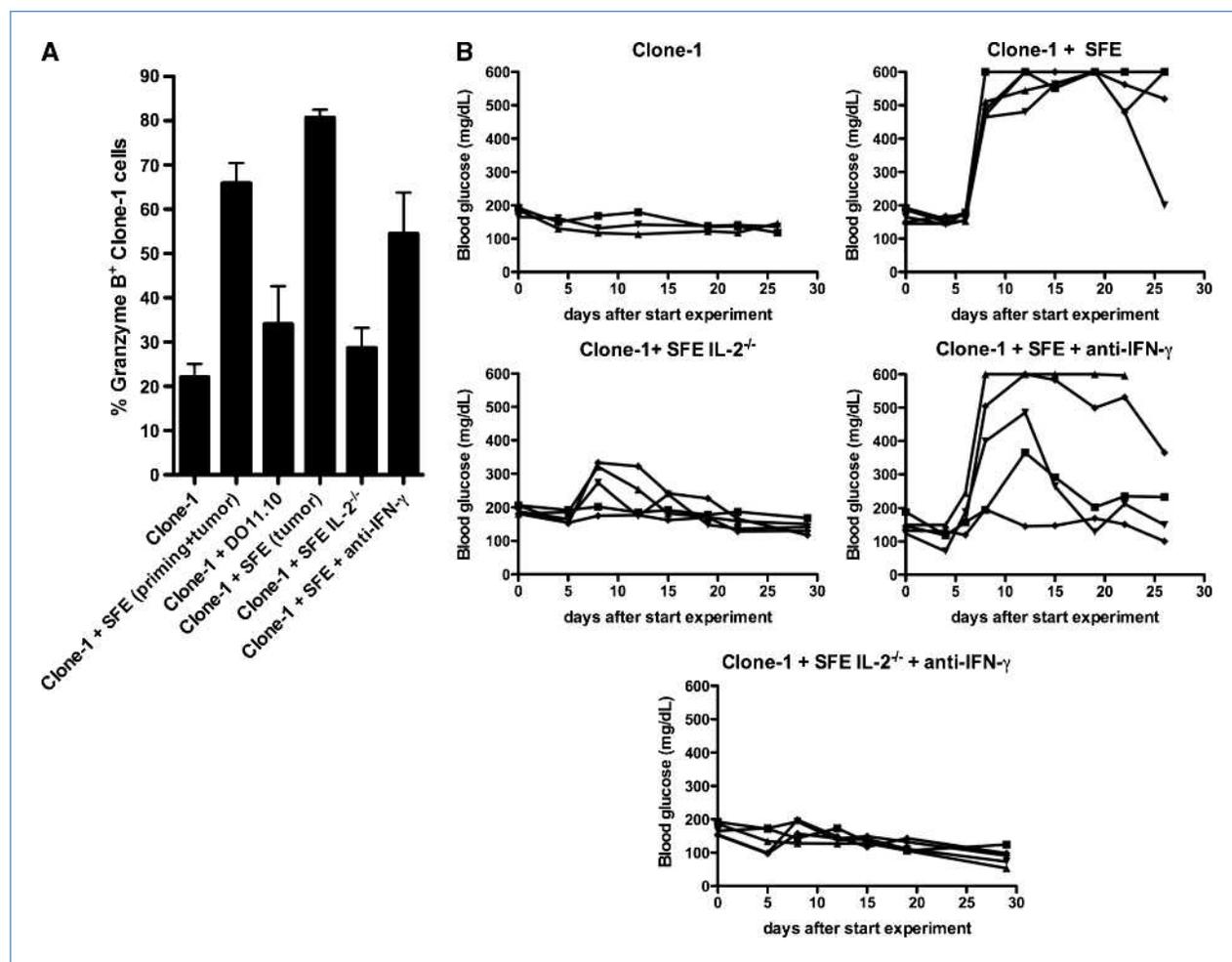


Figure 6. IL-2 production by CD4⁺ T cells is critical for increased effector function and antitumor efficacy of Clone-1 cells. A, RIP-Tag2-HA mice were immunized, and Clone-1 cells (0.3×10^6) with or without 0.3×10^6 SFE, 0.3×10^6 DO11.10, 2×10^6 IL-2^{-/-} SFE, or 2×10^6 SFE cells were injected i.v. The latter group was injected with IFN- γ neutralizing antibodies at days 4 and 5. Pancreata were isolated at day 6. Data are cumulative over three experiments with nine mice per group. B, RIP-Tag2-HA mice were immunized, and Clone-1 cells (1×10^5) with or without 1×10^5 SFE, 7×10^5 IL-2^{-/-} SFE, or 7×10^5 SFE cells were injected i.v. The latter group was injected with IFN- γ -neutralizing antibodies daily starting at days 4 through 13 followed by injections every 2 d. Glucose levels in the blood were measured at the indicated time points. Data are representative of two independent experiments with three to five mice per group. Each line represents one mouse.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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CD4⁺ T-Cell Help in the Tumor Milieu Is Required for Recruitment and Cytolytic Function of CD8⁺ T Lymphocytes

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