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A distinct subset of FcγRI-expressing Th1 cells exert antibody-mediated cytotoxic activity

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While a high frequency of Th1 cells in tumors is associated with improved cancer prognosis, this benefit has been attributed mainly to support of cytotoxic activity of CD8+ T cells. By attempting to potentiate antibody-driven immunity, we found a remarkable synergy between CD4+ T cells and tumor-binding antibodies. This surprising synergy was mediated by a small subset of tumor-infiltrating CD4+ T cells that express the high-affinity FcγRI receptor for IgG (FcγRI) in both mouse and human patients. These cells efficiently lyse tumor cells coated with antibodies through concomitant crosslinking of their T cell receptor (TCR) and FcγRI. By expressing FcγRI and its signaling chain in conventional CD4+ T cells, we successfully employed this mechanism to treat established solid cancers. Overall, this discovery sheds new light on the biology of this T cell subset, their function during tumor immunity, and the means to utilize their unique killing signals in immunotherapy.

Introduction

Over recent decades, researchers exerted tremendous efforts to dissect the biological and clinical roles of immune-cell populations that infiltrate tumors (1–3). While it is widely accepted that increased prevalence of cytotoxic T cells and Th1 cells in tumors correlates with improved clinical outcomes (4), the role of tumor-specific B cells and tumor-binding antibodies remains unclear (5, 6). A large number of studies suggest that a Th2 signature and tumor-binding antibodies promote tumor escape through various mechanisms, including masking T cell epitopes (7), inducing suppressor mechanisms (8–11), inducing tumor heterogeneity and therapy resistance (12), altering the tumor microenvironment, increasing tumor proliferation, and promoting angiogenesis (13, 14). In contrast, tumor-binding antibodies were shown to inhibit tumor growth through induction of antibody-dependent cell-mediated cytotoxicity (ADCC) (15), activation of DCs (16, 17), and activation of tumor-infiltrating innate cells (18) and were shown to be required for antitumor T cell activity (19, 20). In the clinic, tumor-binding antibodies are widely used as therapeutic agents (21), and when they arise spontaneously, they correlate with a positive prognosis in several cancers (22, 23). Over the past years, it has become clear that a simple assessment of the titer of tumor-binding antibodies is not sufficient to predict their antitumor effect, as their activity is also dependent on their avidity and affinity (24, 25) and patterns of sialylation, which determine their interactions with Fcγ receptor (FcγR) (26, 27).

By studying spontaneous regression of tumor cells in genetically similar allogeneic hosts (MHC-I and -II matched), we discovered that naturally occurring IgG antibodies enable tumor-associated DCs (TADCs) to activate T cells that recognize a wide range of tumor-associated antigens, including neoantigens (28). Through exploiting this principle, we were able to generate a very potent immunotherapy consisting of a combination of tumor-binding antibodies and DC stimuli (16, 28). Despite eliciting a strong T cell immune response, in this as well as in most immunotherapeutic approaches to cancer, most tumors eventually relapse, probably as a result of intratumor heterogeneity and the capacity of tumors to escape immune pressure by editing the antigens that they express. Therefore, there is a need to develop a therapeutic approach capable of addressing tumor evolution and escape mechanisms.

While searching for ways to enhance the efficacy of antibody-driven cancer immunotherapy, we discovered that CD4+ T cells isolated from tumors and tumor-binding antibodies were synergistic in their antitumor effects. This synergy was mediated by a distinct subset of exhausted CD4+ T cells that expressed the high-affinity Fcγ receptor (FcγR) capable of efficiently lysing tumor cells through dual recognition with T cell receptors (TCR) and tumor-binding antibodies. We were able to employ this unique killing mechanism to treat established solid tumors in mouse models, thus diminishing the reliance on the host T cell repertoire.

Results

Adoptive transfer of CD4+ T cells, but not CD8+ T cells, induces potent tumor regression when combined with tumor-binding antibodies. We recently found that treating tumor-bearing mice with tumor-binding antibodies and DC stimuli induces complete tumor regression through induction of systemic T cell immunity (16, 28). This treatment, however, is limited to tumors smaller than 25 mm2 and is largely ineffective once tumors exceed that size. In attempts
However, all mice experienced recurrent tumors after 16 days and needed to be euthanized by day 20 (Figure 1B). Adoptive transfer of CD4+ T cells alone yielded no significant improvement over PBS-treated mice. Addition of DC stimuli increased the potency of injected CD4+ T cells, but all tumors eventually progressed. Strikingly, however, injection of DC stimuli with anti-TRP1 antibodies substantially improved tumor regression induced by adoptive transfer of CD4+ T cells. Furthermore, all mice treated with CD4+ T cells along with DC stimuli and anti-TRP1 experienced sustained tumor regression and remained tumor free for the duration of the experiment (Figure 1C).

Next, we tested to determine whether specificity of the antibodies or T cells for tumor antigens is required for CD4+ T cell-mediated tumor regression. Toward this end, control mice were injected s.c. with B16 melanoma cells. Ten days following implantation, mice were injected intratumorally with antibodies against the melanoma antigen TRP1 (anti-TRP1), anti-CD40, and TNF-α (DC stimuli). After 6 days, effector (CD62LnegCD44+) CD4+ and CD8+ T cells were isolated from the tumors, blood, and draining lymph nodes (DLN) of treated tumor-bearing mice. T cells were expanded for 7 days in vitro using high-dose IL-2 and anti-CD3 antibodies and injected i.v. into tumor-bearing mice alone or in combination with anti-TRP1 antibodies and with or without DC stimuli (the experimental design is illustrated in Figure 1A). Adoptive transfer of CD8+ T cells had only a marginal effect on tumor growth, which was comparable to that in PBS-treated mice. Intratumoral injection of DC stimuli and anti-TRP1 induced marked, yet transient, tumor regression, and most treated mice developed recurrent tumors after 10 to 12 days. Combined injection of DC stimuli with or without anti-TRP1 and with CD8+ T cells induced marked tumor regression.

However, all mice experienced recurrent tumors after 16 days and needed to be euthanized by day 20 (Figure 1B). Adoptive transfer of CD4+ T cells alone yielded no significant improvement over PBS-treated mice. Addition of DC stimuli increased the potency of injected CD4+ T cells, but all tumors eventually progressed. Strikingly, however, injection of DC stimuli with anti-TRP1 antibodies substantially improved tumor regression induced by adoptive transfer of CD4+ T cells. Furthermore, all mice treated with CD4+ T cells along with DC stimuli and anti-TRP1 experienced sustained tumor regression and remained tumor free for the duration of the experiment (Figure 1C).

Next, we tested to determine whether specificity of the antibodies or T cells for tumor antigens is required for CD4+ T cell-mediated tumor regression. Toward this end, control mice were injected s.c. with B16 and tumors were allowed to grow for 10 days. Mice were then injected i.v. with effector CD4+ T cells bearing a single TCR against ovalbumin, which is not expressed on B16, or against the melanoma antigen TRP1. Additionally, mice were injected with DC stimuli and with antibodies against TRP1 or against ovalbumin. Adoptive transfer of effector CD4+ T cells alone yielded tumor growth comparable to that seen in

Figure 1. Adoptive transfer of CD4+ T cells with tumor-binding antibodies induces direct killing of tumor cells. (A) Illustration of experimental outline. (B) B16F10 tumor size (mm2) in WT mice following injection of CD8+ T cells isolated from day 7 tumor-bearing mice with or without antibodies against TRP-1 and DC stimuli (n = 4). (C) B16F10 tumor size (mm2) in WT mice following injection of CD4+ T cells isolated from day 7 tumor-bearing mice with or without antibodies against TRP-1 and DC stimuli (n = 4). The same control groups, PBS and TNF-αCD40LαTRP-1, were used in both B and C. (D) B16F10 tumor size (mm2) following adoptive transfer of CD4+ T clones, with or without DC stimuli and antibodies against TRP-1 and ovalbumin (n = 4). Results are from 1 representative experiment out of at least 3 performed. Statistical significance was calculated using 2-way ANOVA with post hoc Tukey’s test. ***P < 0.001; ****P < 0.0001. P < 0.05 was considered significant.
untreated mice. Similarly, injection of Ova_{323-339}-reactive CD4+ T cells with anti-ovalbumin or anti-TRP1 antibodies had only a marginal effect on tumor progression. In contrast, injection of TRP1-reactive CD4+ T cells along with anti-TRP1, but not anti-ovalbumin, antibodies induced complete and durable tumor regression (Figure 1D). Taken jointly, these results suggest that effector CD4+ T cells, but not CD8+ T cells, synergize with tumor-reactive antibodies to eradicate tumors in a manner that relies upon the antigen specificity of both the antibodies and T cells.
and expanded them in vitro using IL-2 and anti-CD3. T cells from
each organ were injected i.v. into B16 tumor–bearing mice, in com-
bination with DC stimuli and anti-TRP1 antibodies (illustrated in
Figure 2A). Effector CD4+ T cells from blood had only a moderate
effect on tumor burden in these mice compared with in untreated
mice, and all treated mice experienced local tumor recurrence and
had to be sacrificed. In contrast, injection of CD4+ T cells isolated

Figure 3. A small subset of tumor-infiltrating CD4+ T cells expresses Fcγ receptors. (A) Gating strategy and representative FACS analyses of the tumor,
DLN, and blood from day-10 tumor-bearing mice. (B) Mean percentages of Fcγ receptors expressing CD4+ T cells in various organs in day-10 B16 tumor-
bearing mice (n = 4). Statistical significance was calculated using 2-way ANOVA with post hoc Tukey’s test. (C) Confocal microscopy of day-10 B16F10
tumor. Original magnification, ×800. (D) Representative FACS analysis of Fcγ receptors on 4T1 tumor-infiltrating CD4+ T cells. (E) Mean percentages of Fcγ
receptors expressing CD4+ T cells in various organs in day-12 4T1 tumor-bearing mice (n = 4). Statistical significance was calculated using 2-way ANOVA
with post hoc Bonferroni’s test. nDLN, nondraining lymph node. (F) Confocal microscopy of day-12 4T1 tumor sections. Original magnification, ×800. Data
represent mean ± SEM, and results are from 1 representative experiment out of at least 3 performed. *P < 0.05; **P < 0.01; ****P < 0.0001. P < 0.05 was
considered significant.

CD4+ T cells from the tumor and DLN, but not from peripheral
blood, directly kill tumor cells coated with IgG antibodies. Since
the above experiments included effector CD4+ T cells that were
pooled from the blood, tumors, and DLN, we next sought to
determine which of these organs contains the most potent tumor-
reactive CD4+ T cells. We isolated effector CD4+ T cells from the
blood, DLN, and tumors individually following immunotherapy
from the tumor or DLN induced complete tumor regression that was maintained until the experiment was terminated (Figure 2, B and C). We next assessed whether transferred CD4+ T cells kill tumor cells directly or mediate killing by activating other effector T cells. Thus, Rag1-deficient mice (Rag1−/−) were challenged with B16 cells, and tumors were allowed to grow for 10 days. Mice were then treated with 1 × 10⁶ CD4+ T cells derived from tumor-bearing mice treated with immunotherapy along with tumor-binding antibodies and DC stimuli. Interestingly, the efficacy of this treatment in Rag1−/− mice was comparable to that in immune-competent mice, suggesting that tumor lysis is induced directly by the injected CD4+ cells (Figure 2D). Additionally, we isolated CD4+ T cells from the blood, DLN, and tumor of B16 tumor-bearing mice and cocultured them with B16 cells in the presence or absence of anti-TRP1 antibodies. Incubation of CD4+ T cells from all organs with tumor cells at a 1 to 2 ratio, in the absence of antibodies, exhibited minimal effect; about 10% of tumor cell lysis was observed. Consistent with our in vivo observations, incubation of tumor cells coated with antibodies and CD4+ T cells from tumors, and to a lesser extent also from the DLN, but not from the blood, induced tumor cell lysis within 2 days (Figure 2E, F, and Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI112759DS1). Since CD4+ T cells express the C5a and C3b complement receptors, we next tested to determine whether the tumor lysis is mediated by complement deposition. To eliminate any potential inactivated complement, CD4+ T cells were incubated with IgG-coated tumor cells in the absence of serum. Only a minor reduction in tumor cell lysis was observed, suggesting a direct IgG binding by CD4+ T cells (Supplemental Figure 1B).

A small subset of CD4+ T cells in tumors and DLNs express Fcγ receptors. Although it is widely believed that T cells do not express Fcγ receptors (FcγR), in light of our results, we decided to revisit this notion. Toward this end, tumors, DLN, and peripheral blood (PB) were obtained from B16 tumor-bearing mice 10 days following tumor inoculation, and expression patterns of FcγR (FcγRI, FcγRII/III, and FcγRIV) on CD4+ T cells were analyzed. Flow cytometric analysis indicated that between 3% and 5% of the tumor-infiltrating CD4+ T cells expressed all FcγR at levels comparable to those of antigen-presenting myeloid cells (Figure 3, A and B, and Supplemental Figure 2, A and B). Lower yet detectable percentages of CD4+ T cells expressing FcγR were also observed in the DLN, but not in the blood (Figure 3, A and B). To further corroborate these results, histological sections of day-10 B16 tumors were stained for FcγR and T cell markers. Indeed, all FcγRs were detected on a proportion of CD4+ but not CD8+ T cells (Figure 3C and Supplemental Figure 2C).

We then tested to determine whether this FcγR+ subset exists in another tumor model or is limited to B16 melanoma. Female BALB/c mice were injected with 4T1 breast cancer cells into their mammary fat pad, and tumors were allowed to grow for 12 days. Similarly to our results with B16 cells in C57BL/6 mice, all FcγR were expressed on CD4+ T cells in the tumors and DLN of BALB/c mice bearing 4T1 breast carcinoma cells, but not on the T cells in their non-DLNs (Figure 3, D and E). Histological sections of day-12 4T1 tumors confirmed that these receptors are indeed expressed on the membrane of CD4+ T cells (Figure 3F and Supplemental Figure 2D).

Naturally occurring CD4+ T cells expressing FcγRI lyse tumor cells coated with IgG. We next tested to determine whether this population exists in naive mice or is induced exclusively during tumor progression. Various organs were removed from naive mice, and FcγR expression on T cells was analyzed by flow cytometry. Only CD4+ T cells expressing FcγRI and II/III were found in lymph nodes, spleen, and BM, but not in the blood or thymus (Figure 4A and Supplemental Figure 3A). The high-affinity FcγRI was found to be the predominant receptor on T cells, and this population was mostly prevalent in the spleen. Furthermore, histological sections of naïve spleen indicated that they are located at the margins of the T cell zone in the spleen of naïve mice (Figure 4B). Intracellular staining of the Th transcription factor indicated that T cells expressing FcγRI exclusively express the Th1 T-bet in both spleen and tumors (Figure 4C). This population was completely absent in Rag1−/− mice, suggesting that their maturation is dependent on TCR rearrangement (not shown). Expression of FcγRI in these cells was also tested at the transcription level. To this end, splenocytes were subjected to a Ficoll gradient enriched on CD4 magnetic beads, and CD4+CD3+ TCR-β/MHC-IH2-5Mice T cells were sorted into 2 groups based on their and FcγRII expression (Figure 4D). mRNA was extracted from the 2 subsets, and FcγRII transcripts were amplified by PCR. Consistent with our FACS and confocal results, FcγRII-CD3+MHC-IH2-5Mice CD4+ T cells, but not canonical CD4+ T cells, had low yet detectable levels of FcγRII gene transcript (Figure 4E). To further verify that these are indeed T cells, CD4+CD3+ TCR-β/MHC-IH2-5Mice expressing FcγRI were sorted and compared with canonical FcγRI+ T cells by confocal microscopy. The 2 subsets share a similar morphology and size and have identical cell membrane CD4, CD3, and TCR-β staining (Figure 4F, G, and H). Tumor cell lysis was completely abrogated when FcγRI-CD4+ T cells were incubated with anti-TRP1 antibodies induced marked tumor cell lysis (Figure 4, G and H). Tumor cell lysis was completely abrogated when FcγRI-CD4+ T cells were incubated with anti-ovalbumin antibodies, or anti-TRP1 F(ab)2 (Figure 4, G and H, and Supplemental Figure 3D). Tumor lysis was mediated through secretion of lytic granules in an IFN-γ-dependent mechanism (Supplemental Figure 3D). Intriguingly, incubation of FcγRI+CD4+ isolated from OT-II mice with B16 and anti-TRP1 did not induce tumor killing, suggesting that the both MHC-II molecules and the antigen targeted by the antibody should be expressed on the target cells (Figure 4, G and H, and Supplemental Figure 3, E and F). Taken jointly, these data suggest that this specific subset of FcγRI+CD4+ T cells exhibits a unique capacity to lyse tumor cells directly in a manner that is dependent on concomitant TCR and FcγRI crosslinking.

FcγRI is expressed only on a subset of exhausted and nonproliferating CD4+ T cells. To assess the origin of these cells, we tested to determine whether FcγRI CD4+ T cells bear unique TCR, or rather share clones with conventional CD4+ T cells, which do not express FcγRI. 2 × 10⁵ T cells were sorted by FACS, and their TCR-Vα
incubating FcγRI+CD4+ T cells with high-dose IL-2 and immobilized FcγRIIa on T cells from naïve spleens. (E) Agarose gel (1%) electrophoresis of FcγRIIa PCR products performed on cDNA from sorted splenic CD4+ T cells. (F) Confocal microscopy staining of sorted FcγRIIa+ and FcγRIIa- CD4+ T cells. (G) Confocal microscopy of CD4+ T cells from WT and OT-II mice incubated for 48 hours with wasabi-labeled B16 cells, with or without antibodies against TRP1 and ovalbumin. Original magnification, ×400. (H) Viability (measured by fluorescent intensity of wasabi) of B16F10 cells after 48 hours coculture with CD4+ T cells (n = 6). Data represent mean ± SEM, and results are from 1 representative experiment out of at least 3 performed. Statistical significance was determined by 1-way ANOVA with Tukey’s post hoc test. *P < 0.05; **P < 0.01. P < 0.05 was considered significant.

and -Vβ were amplified by PCR and sequenced. We found that CD4+ T cells expressing FcγRI exhibit Vβ segment usage similar to that of conventional CD4+ T cells and are composed of hundreds of different clones with frequency distribution and usage, as well as clonal abundance, similar to those of conventional CD4+ T cells (Figure 5, A and B). Furthermore, our analysis indicated that identical clones can be found in both groups (Supplemental Table 1). Since both subsets undergo similar random VDJ rearrangement, expression of FcγRI on T cells represents an activation state, rather than expansion of selected clones or T cell subsets with restricted Vβ usage, such as NKT cells. Protein production in these cells was also analyzed by mass spectrometry in comparison with canonical splenic CD4+ T cells. The vast majority of detected proteins, including the T markers CD3, CD4, and CD5 as well as the transcription factor NFAT, were expressed at similar levels in both cell populations (Figure 5C and Supplemental Table 2). Several proteins, however, including SYK tyrosine kinase and the chemokine CXCL-4, were detected only in FcγRI+CD4+ T cells. Of particular interest were the high expression levels of lysosome-related enzymes, suggesting that these cells are more active compared with naïve CD4+ T cells (Figure 5C).

To assess the hypothesis that FcγRI expression occurs as a result of activation, canonical CD4+ T cells, which do not express FcγRI, were isolated from spleens of naïve mice and activated overnight with an array of inflammatory mediators, which induce FcγRIIa transcription in myeloid cells (29). None of these stimuli induced FcγRI expression on these T cells (Figure 5D). Since incubating FcγRI+CD4+ T cells with high-dose IL-2 and immobilized anti-CD3 antibodies did not result in their proliferation in vitro (not shown), we speculated that FcγRI may be expressed on exhausted cells. Thus, CD4+ T cells from the spleen and lymph nodes of naïve mice were cultured for 12 days with immobilized anti-CD3 antibodies and IL-2, followed by an additional 48 hours of activation with PMA and ionomycin. Given the high mortality rate of mouse T cells following such long-term culture and activation, special attention was given to exclude dying T cells (our gating strategy is presented in Figure 5E). Indeed, over 40% of long-term activated CD4+ T cells expressed FcγRI, suggesting that its expression is associated with reduced proliferative capacity (Figure 5F). While the majority of long-term activated CD4+ T cells expressed exhausted markers, only about 50% of them also expressed FcγRI. Additionally, the majority of, but not all, CD4+ T cells that expressed FcγRI also express exhausted markers (Figure 5G). While these results strongly support the hypothesis that CD4+ T cells may express FcγRI once they become exhausted and lose their proliferative capacity, the exact underlying mechanism is not fully clear yet.

Canonical T cells equipped with FcγRI exert cytotoxic activities and can be employed to eradicate solid tumors. Since FcγRI+CD4+ T cells could not be expanded to numbers that allow their use for immunotherapy, we tested to determine whether their killing machinery could be transferred to conventional CD4+ T cells. Thus, we transduced conventional splenic CD4+ T cells with TRP1-reactive TCR alone or with FcγRI chain (FcRγ) and with the FcRγK, which is the receptor signaling γ chain (FcRγ). Transduced T cells were sorted by FACS (Supplemental Figure 4A) and incubated overnight with B16 tumor cells. Membrane-bound CD107a was detected in about 10% of CD4+ T cells infected with TRP1-reactive TCR, and they induced about 10% to 15% killing (Figure 6, A and B). Their killing capacities and membrane CD107a levels were not changed by the addition of anti-TRP1 antibodies. Infection with α chain alone was not sufficient to promote B16 lysis and CD107a expression, yet addition of FcRγ substantially potentiated their capacity to lyse antibody-coated tumor cells. Most strikingly, CD4+ T cells transduced with TRP1-reactive TCR, FcγRI, and FcRγ induced substantial killing of tumor cells coated with anti-TRP1 antibodies (Figure 6, A and B, and Supplemental Figure 4, B and C). To determine whether the killing capacities of TRP1-reactive T cells are restricted to anti-TRP1 antibodies, we also co-transduced B16 cells with antibodies against another melanoma antigen, TRP-2. Similarly to our results with anti-TRP1 antibodies, the addition of anti-TRP2 antibodies significantly enhanced the cytotoxic abilities of TRP-1-reactive T cells, suggesting that this killing mechanism is not restricted to a certain antibody (Figure 6C). We then assessed the capacity of these cells to eradicate established melanoma tumors. Mice were injected s.c. with B16 tumor cells, and tumors were allowed to grow for 7 to 9 days, until they reached a palpable size. 0.5 x 10^6 CD4+ T cells were injected i.v., with or without i.p. injection of antibodies. Mice injected with 1 x 10^6 of CD4+ T cells bearing TRP1-reactive TCR served as controls. Consistent with their activity in vitro, combination of TRP1-reactive TCR, FcγRI, and FcRγ T cells and anti-TRP1 antibodies induced tumor eradication in all treated mice that lasted up to 1 month, when the experiment was terminated (Figure 6, D and E). However, many cell types in the tumor microenvironment, such as macrophages and NK cells, express FcγR and can kill tumor cells coated with antibodies. Therefore, we sought to determine whether the synergism between CD4+ T cells and tumor-binding antibodies is mediated through the crosslinking of FcγRI on the T cells or rather in an unrelated manner by activating other effector cells at the tumor site. Toward this end, C57 control mice were injected for 3 days with 30 mg/kg of busulfan and rescued with 15 x 10^4 BM cells from FcRγ KO mice, which lack all FcγR. After 3 weeks, the mice were challenged with B16F10 tumor cells, and tumors were allowed to grow for 7 to 9 days until they reached a palpable size. Chimeric mice were then injected with 1 x 10^6 CD4+ T cells bearing TRP1-reactive TCR FcγRI and FcRγ served and with or without anti-TRP1 antibodies. Consistent with our results in
Figure 5. FcγRI is expressed on exhausted CD4+ T cells and can be utilized to induce tumor regression in mice. (A) Percentages of TCRβ V segment usage and (B) clonal abundance in FcγRI+ and canonical FcγRIN−CD4+ T cell subpopulations from naive spleens. (C) Venn diagram of mass spectrometry analysis of proteins expressed by splenic FcγRIpos and canonical FcγRInegCD4+ T cells. (D) Representative FACS analysis (upper panel) and mean percentages (lower panel) of splenic CD4+ T cells that express FcγRI following 2 days in culture (n = 3). (E) Gating strategy of live splenic CD4+ T cells following 12 days in culture with IL-2 and anti-CD3 antibodies. (F) Representative FACS analysis (upper panel) and mean percentages (lower panel) of splenic CD4+ T cells that express FcγRI following 12 days in culture (n = 3). (G) Representative FACS analysis of exhaustion markers expressed on splenic CD4+ T cells after 12 days in culture. Data represent mean ± SEM, and results are from 1 representative experiment out of at least 3 performed. Statistical significance was calculated using 1-way ANOVA with Dunn’s multiple comparison test (control vs. Iono+PMA200 ng groups, *P < 0.05). P < 0.05 was considered significant.
of tumor-infiltrating CD4+, but not CD8+, T cells, expressing all 3 FcγRs (Figure 7C). Examination of tumor sections under confocal microscopy further indicated that these receptors are expressed on the membrane of CD4+ T cells and that these cells are found in the center of the tumor mass (Figure 7D and Supplemental Figure 5A). In another patient undergoing resection surgery to remove stage III bladder cancer following chemotherapy, we compared the expression patterns of FcγRs on CD4+ T cells in the blood, tumor tissue, and adjacent healthy bladder tissue. Low yet detectable levels of FcγRI and FcγRIII, but not FcγRII, were found in circulating CD4+ T cells (Figure 7E). Importantly, all 3 FcγRs were detected on CD4+ T cells infiltrating the tumor, but not in healthy tissue (Figure 7F and Supplemental Figure 5B). CD4+ T cells expressing all FcγRs were found in the tumor of another melanoma patient, thus demonstrating that these cells preferentially accumulate in cancerous tissues (Figure 7G).

control mice, a significant tumor regression was observed following treatment with both T cells and antibodies, suggesting direct activation of T cells by tumor-binding antibodies (Figure 6F).

FcγRI is expressed by CD4+ T cells that infiltrate to human tumors. We also tested to determine whether CD4+ T cells expressing FcγRI are limited to mice or can also be found in humans. Initially, FcγR expression was tested on T cells from PB of 4 healthy donors. Consistent with our results in mice, we could not detect T cells expressing Fcγ receptors even after their activation for 3 days with PMA and ionomycin (Figure 7A). In contrast to our results in mice, long-term activation with IL-2 and anti-CD3 antibodies followed by 2 days activation with PMA and ionomycin did not induce FcγR expression of blood T cells (Figure 7B). Next, tumor tissues from stage III melanoma patients undergoing primary tumor resection were analyzed by FACS and by histological staining. FACS analysis indicated the presence of tumor-infiltrating CD4+, but not CD8+, T cells, expressing all 3 FcγRs (Figure 7C). Examination of tumor sections under confocal microscopy further indicated that these receptors are expressed on the membrane of CD4+ T cells and that these cells are found in the center of the tumor mass (Figure 7D and Supplemental Figure 5A). In another patient undergoing resection surgery to remove stage III bladder cancer following chemotherapy, we compared the expression patterns of FcγR on CD4+ T cells in the blood, tumor tissue, and adjacent healthy bladder tissue. Low yet detectable levels of FcγRI and FcγRIII, but not FcγRII, were found in circulating CD4+ T cells (Figure 7E). Importantly, all 3 FcγRs were detected on CD4+ T cells infiltrating the tumor, but not in healthy tissue (Figure 7F and Supplemental Figure 5B). CD4+ T cells expressing all FcγRs were found in the tumor of another melanoma patient, thus demonstrating that these cells preferentially accumulate in cancerous tissues (Figure 7G).
Figure 7. FcγR expressing CD4+ T cells are also found in human tumors. (A) Mean percentages of FcγR expression in CD4+ T cells from PB of healthy donors. (B) Mean percentages of FcγRI expression in CD4+ T cells from PB of healthy donors after 28-day culture with IL-2 and anti-CD3 antibodies. (C) FACs analysis of FcγR expression on CD4+ T cells from tumor lesion of a stage Iib melanoma patient. (D) Confocal microscopy of a histological section from the same stage Iib melanoma patient as in C. Original magnification, ×600. (E) FcγR expression in CD4+ T cells from the blood, tumor, and healthy adjacent tissue of a stage III bladder cancer patient. (F) Confocal microscopy staining of FcγR in a histological section of the same bladder cancer patient as in E. Original magnification, ×800. (G) Percentages of FcγR-expressing CD4+ T cells in tumors from 3 patients.

Discussion

Whether tumor-binding IgG promotes or masks T cell immunity is still controversial (5, 6), and several studies have demonstrated that they can inhibit cytotoxic T cell activity by promoting mechanisms of immune suppression (7–11, 13, 14, 30). In contrast, however, tumor-binding antibodies are extensively used in the clinic (21), and a large amount of experimental data compellingly suggest that they synergize and promote T cell immunity (15, 19, 20). Here, we found that tumor-reactive CD4+ T cells and tumor-binding antibodies strongly synergize to mediate tumor regression. This synergy is mediated by a distinct CD4+ T cell population, which expresses the high-affinity FcγRI and directly kills tumor cells coated with antibodies.

Whether or not T cells express Fcγ receptors has been a source of long-lasting controversy (31). While conventional wisdom suggests that T cells do not express them (32), a number of studies have found that activated T cells in the PB of patients with systemic lupus erythematosus (SLE) or with cytomegalovirus infections express the low-affinity FcγRIII. Since the authors could not detect them in the blood of healthy donors, they concluded that the cells were in an activated state caused by exposure to IFN-γ (33). We found that a short-term activation with double-stranded RNA, or with type I or II IFN, was not sufficient to induce FcγRI expression on CD4+ T cells. CD4+, but not CD8+, T cells appear to elevate their FcγRI expression only once they have completely lost their proliferative capacities.

Our findings also suggest a direct cytotoxic activity of tumor-infiltrating CD4+ T cells. While effector Th1 CD4+ T cells are highly associated with increased antitumor immunity and improved clinical outcomes (23, 34), most of the publications attributed the antitumor effects of CD4+ T cells to their capacity to activate other effector cells, in particular, cytotoxic CD8+ T cells (35, 36). Indeed, CD4+ T cells were shown to promote differentiation and clonal expansion of tumor-reactive T cells and are essential for maintaining and reactivating CD8+ memory T cells (37–39). In the clinical setting, Hunder et al. reported a case in which infusion of autologous CD4+ T cell clones against class II-restricted epitopes led to a long-term complete remission of refractory melanoma (40). In a more recent case report, Tran et al. demonstrated transient regression of all metastases following injection of tumor-infiltrating CD4+ T cells that recognize mutated ERBB21P (41). A number of independent studies have shown that CD4+ T cells can mediate the direct killing of melanoma cells. Muranski et al. were the first to demonstrate such a phenomenon through their capacity to secrete perforin and granzyme (42). Alternately, cytotoxic CD4+ T cells that express NKG2A have also been reported in mice (43, 44) and in human melanoma (45). Nonetheless, the relevance of their findings to other tumors remains somewhat questionable, mainly since MHC-II expression on melanoma is diverse and not uniform. Consistently, we found that incubation of tumor cell lines that do not express MHC-II molecules, such as 4T1, with CD4+ T cells expressing FcγRI, is not sufficient to induce tumor cell killing in the presence of tumor-binding antibodies. These results, therefore, strongly support the notion that the killing machinery of such cells is still dependent on TCR specificity.

In that regard, the role of FcγRI-antibody interactions in facilitating killing through TCR could involve stabilizing the low-affinity complexes of TCR and MHC peptides. Presumably, a direct correlation exists between the avidity of the tumor-binding antibodies and T cell killing efficacy.

Overall, this work highlights a surprising synergy between tumor-binding antibodies and CD4+ T cells in lysing tumor cells and positions them as a therapeutic agent—a role that surpasses their traditional one as supporting the cytotoxic activities of other effector cells.

Methods

Micr. WT C57BL/6J and BALB/cOlaHsd mice were obtained from Envigo and from Jackson Laboratory. T cell–deficient mice B6.Cg-Rag1<sup>tm1Hsd</sup> and TCR transgenic mice Tg(Tcrα, Tcrβ)<sup>Rest/J</sup> were purchased from Jackson Laboratory. B6.Cg-Tg (Tcrα,Tcrβ)<sup>245Cbn/J</sup> were purchased from Jackson Laboratory or provided by Ronen Alon (Weizmann Institute, Rehovot, Israel). B6.129P2-FcγRI<sup>ε<sup>−/−</sup>/J</sup> mice were provided by Rony Dahan (Weizmann Institute). Male and female 8– to 12-week-old mice were used in all experiments.

Cell lines. B16F10 cells (CRL-6475) and 4T1 (CRL-2539) cells were purchased from ATCC, and HEK-293FT cells were purchased from Thermo Fisher Scientific, all in January 2017. Cells were cultured in DMEM (Gibco, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Biological Industries), 2 mM l-glutamine, and 100 μg/mL penicillin/streptomycin (Gibco, Thermo Fisher Scientific) under standard conditions. Cells were routinely tested for mycoplasma using an EZ-PCR Mycoplasma Test Kit (Biological Industries) according to the manufacturer’s instructions.

In vivo tumor models. For melanoma tumor studies, 2 × 10<sup>6</sup> B16F10 cells suspended in 50 μL were injected s.c. into C57BL/6 mice above the right flank, and the size of growing tumors was measured twice a week using calipers. Treatment was applied at days 8 and 12 after injection or when tumors reached 20 mm<sup>2</sup> (day 0 and day 4). When tumors reached 120 mm<sup>3</sup>, the mice were sacrificed due to ethical considerations. For a triple-negative breast cancer model, 2 × 10<sup>4</sup> 4T1 cells in 50 μL DMEM were injected into fat pad number 5 of a 12-week-old female BALB/c mouse. At day 12, the mouse was sacrificed and CD4+ T cells from DLN, tumors, and non-DLN were analyzed.

BM chimeric mice. Six-week-old C57BL/6 female mice were injected i.p. for 3 days with 30 mg/kg busulfan. Mice were rescued by i.v. injection of 15 × 10<sup>6</sup> BM cells derived from C57BL/6 or from FcγRI<sup>−/−</sup>deficient mice. Chimeric mice were bred at a specific pathogen–free facility for 3 weeks prior to their challenge with B16F10 tumor cells.

Tumor immunotherapy. Animals were injected intratumorally with 80 μg anti-CD40 (clone FGK4.5; BioXCell) and 10 μg...
Endotoxins, and bacterial contamination. All tissue preparations were performed simultaneously from each individual mouse (after euthanasia, by CO₂ inhalation). For isolation of T cells from lymphoid organs, the spleen, LN, and thymus were removed from euthanized mice and mashed through a 70 μM cell strainer (Gibco, Thermo Fisher Scientific). Cells were then washed by centrifugation at 800 g for 5 minutes at 4–8°C.

For isolation of tumor-infiltrating T cells, tumors were enzymatically digested with 2,000 U/ml of DNase I and 2 mg/mL collagenase IV (both from Sigma-Aldrich, Merck) in HBSS for 30 minutes at 37°C with a magnetic stirrer (400 rpm). Cells were then washed by centrifugation at 800 g for 5 minutes at 4–8°C.

T cells from PB. PB was collected via the posterior vena cava prior to perfusion of the animal and transferred into sodium heparin-coated vacuum tubes prior to 1:4 dilution in FACS buffer (HBSS, 2% FCS, 0.05 mM EDTA). Lymphocytes were enriched on a Ficoll-Paque Premium (Sigma-Aldrich) gradient, and collected PBMCs were washed twice with FACS buffer. For all tissues, cells were then incubated with anti-CD4 or anti-CD8 magnetic beads (MojoSort Nanobeads, BioLegend) according to the manufacturer’s instructions and further sorted by FACSAriaII as FCS⁺SSC⁺TCR-β⁺MHC-II⁺ cells.

T cell culture and expansion. T cells were cultured in RPMI-1640 supplemented with 1% penicillin-streptomycin, 10% heat-inactivated FBS, 1% sodium pyruvate, 1% MEM-Base nonessential amino acids, 1% insulin-transferrin-selenium, and 50 μM β-mercaptoethanol. For T cell expansion, culture dishes were precoated with 0.5 μg/mL anti-CD3 (clone 17A2) and 0.5 μg/ml anti-CD28 (clone 37.51) LEAF antibodies (both purchased from BioLegend) in PBS and were supplemented with 1,000 IU/mL recombinant murine IL-2 (PeproTech). Medium-containing viruses were collected after 24 hours and 48 hours and centrifuged for 1 hour at 100,000 × g. Next, 0.3 mL concentrated retroviruses was added to every group of 2 × 10⁶ CD4⁺ T cells with 10 μg/mL polybrene. Cells were incubated for 30 minutes at 37°C in 5% CO₂ and centrifuged at 37°C, 800 g, for 1 hour. Afterwards, 80% of medium was replaced and T cells were cultured for an additional 3 days in T cell media containing high-dose IL-2.

Adoptive T cell transfer. C57BL/6 mice were injected s.c. with 2 × 10⁶ B16F10 tumor cells. On days 12 and 14, mice were injected intratumorally with 80 μg anti-CD40 (clone FGR4.5; BioXcell) and 1 μg IFN-γ (BioLegend) and 200 μg anti-TRP1. On day 7, mice were euthanized and the tumors and DLNs were removed and dissociated to obtain single-cell suspension. T cells were then enriched on magnetic beads (EasySep, STEMCELL Technologies) and further sorted by FACSAriaII as FCS⁺SSC⁺TCR-β⁺MHC-II⁺ cells. T cells were cultured in T cell medium containing 1,000 IU/mL IL-2 (Peprotech) on culture plates coated with 0.5 μg/mL of anti-CD3. After 9–12 days, T cells were gently collected and a total of 1 × 10⁶ cells was injected intravenously into mice bearing tumors with an average size of 30–50 mm².

Immunochemistry. For frozen sections, mouse and human tissues were fixed in 4% paraformaldehyde for 1 hour and equilibrated in a 20% sucrose solution overnight. Tissues were then embedded in frozen tissue matrix (Scigen O.C.T. Compound Cryostat Embedding Medium, Thermo Fisher Scientific) and frozen at −80°C. The 5μm thick sections were blocked with 5% BSA and stained with 1:100 diluted primary antibodies. For anti-mouse staining, we used anti-CD3 (clone 17A2), anti-CD4 (clone RM4-4), anti-TCR-β (clone H57-597), anti-FcRI (clone X54-5/7.1), anti-FcRII/III (clone 93), and anti-FcRIV (clone 9E9); for the human panel, we used anti-CD3 (clone HIT3a), anti-CD4 (clone RPA-T4), anti-CD8 (clone HIT8a), anti-CD16 (clone 3G8), and anti-CD32 (clone FUN-2), all from BioLegend. Nuclei were counterstained with Hoechst 33342 (Fluka). Microscopy was performed with a Zeiss LSM 800 confocal microscope and analyzed using ZEN software (ZEISS).

Confocal microscopy. B16-Wasabi CD4⁺ T cells were cocultured on glass-bottom confocal plates (Cellvis) in T cell medium without IL-2 and incubated overnight under standard conditions. Cells were further incubated for 1 hour with BV421-conjugated anti-CD107 (BioLegend) at a 1:100 dilution. Images were collected using a Zeiss LSM800 confocal laser scanning microscope and analyzed using ZEN software (ZEISS).

Killing assay. CD4⁺ T cells were cocultured with B16 target cells (50,000 cells per well) at a ratio of 1:2 (T:E) in a round-bottom 96-well plate with or without the following antibodies: anti-chicken ovalbumin (clone TOSGAA1; BioLegend), anti-TRP-1 (clone TA99; BioXcell), or anti-TRP-1 F(ab')₂. After 24 hours and 48 hours, the medium was
replaced with PBS, and the fluorescence intensity of wasabi (excitation, 485 nm; emission, 528 nm) was measured by a Synergy H1M plate reader (BioTek). After 48 hours, cells were stained with annexin V (BioLegend) for 15 minutes and propidium iodide (PI) for 2 minutes on ice, and staining levels were analyzed by flow cytometry.

Preparation of F(ab') fragment. Anti-TRP1 antibody (clone TA99; BioXCell) was dialyzed against 20 mM sodium acetate pH 4.5 and digested with agarose-pepsin beads (GoldBio) for 16 hours in a 37°C incubator with rotation. Next, the sample was centrifuged and supernatant was collected, dialyzed against PBS pH 7.4, and incubated with protein-A agarose beads (Santa Cruz Biotechnology) for 2 hours with rotation. The F(ab')2 fragment was collected after centrifugation and was analyzed by PAGE.

Flow cytometry. Purified T cells were analyzed using flow cytometry (CytoFLEX, Beckman Coulter) and sorted by FACS (BD FACSAria III, BD Biosciences). Data sets were analyzed using FlowJo software (Tree Star). mAbs for anti-TRP1 conjugated to FITC or specific for the following mouse antigens were used: Alexa Fluor 488, CD3 (clone 17A2); phycoerythrin, CD4 (clone RM 4-4); Brilliant Violet 650, CD11c (clone 3.9); Alexa Fluor 647, CD16 (clone 3G8); phycoerythrin/Cy7, CD44 (clone IM7); phycoerythrin/Cy7, CD62L (clone MEL-14); Alexa Fluor 647, FcRIV (clone 9E9); Brilliant Violet 421, TCRb (clone H57-597); allophycocyanin, MHC-II (clone M5/114.15.2); fluorescein, FcRi (clone X54-5/7.1); and phycoerythrin/Cy7, FcRi/III (clone 93). For humans, the following specific antigens were used: Alexa Fluor 488, CD3 (clone HIT3a); Alexa Fluor 594, CD4 (clone RM 4-4); Brilliant Violet 605, CD8 (clone 53-6.7); Alexa Fluor 488, CD11b (clone M1/70); APC/Cy7, CD44 (clone IM7); phycoerythrin/Cy7, CD62L (clone MEL-14); Alexa Fluor 647, FcRIV (clone 9E9); Brilliant Violet 421, TCRb (clone H57-597); allophycocyanin, MHC-II (clone M5/114.15.2); fluorescein, FcRi (clone X54-5/7.1); and phycoerythrin/Cy7, FcRi/III (clone 93). For humans, the following specific antigens were used: Alexa Fluor 488, CD3 (clone HIT3a); Alexa Fluor 594, CD4 (clone RPA-T4); allophycocyanin, CD19 (clone HIB19); Alexa Fluor 647, CD8 (clone HIT8a); Brilliant Violet 650, CD11c (clone 3.9); Alexa Fluor 647, CD16 (clone 3G8); PerCP/Cy5.5, CD32 (clone FUN-2); Brilliant Violet 421, CD45 (clone 10.1); allophycocyanin/Cy7, CD45RO (clone UCHLI); phycoerythrin/Cy7, CD45RA (clone H1100). All Abs were purchased from BioLegend. Cells were suspended in FACS buffer consisting of HBSS with 2% FCS.

TCR repertoire analysis. FcγRI+ and FcγRI+CD4+ T cells were sorted from the spleens of naïve C57BL/6 male mice. Total RNA was immediately extracted from sorted cells using the RNeasy Micro Kit (QIAGEN). Amplification of TCR-α and -β chain cDNA was performed using a SMARTer Mouse TCR α/β Profiling Kit (Takara Bio Inc.) according to the manufacturer’s protocol. Resultant cDNA libraries were sequenced by Illumina MiSeq 250x2 (Kit v2) by Hy Labs Ltd., and data were analyzed with VDJServer (https://vdjserver.org/project).

PCR amplification of CD3 and FcγRI. Total RNA was purified from CD11b+, FcγRI-, and FcγRI-CD4+ sorted cells using the RNeasy Micro Kit (QIAGEN) and was quantified using NanoDrop One (Thermo Fisher Scientific). Reverse transcription was performed using a qScript cDNA Synthesis Kit (Quanta Biosciences) according to the manufacturer’s protocol. cDNA samples were analyzed by PCR for the detection of the FcγRI sequence using AGACACCGCTACACATCTGC and GGAAGTTTGT-GGCCCGATA primers and the CD3c polypeptide sequence using GCATCTGAGAGATTGCCGTG and TGCCCTTGGCCTTCTATTC primers. These were analyzed by agarose gel electrophoresis.

Statistics. Each experiment was performed 3 times. Each experimental group consisted of at least 3 mice. Significance of results was determined using nonparametric 1-way ANOVA when multiple groups were tested. For multiple parameters analysis, 2-way ANOVA was performed with Bonferroni–Šidák post test. For 2-group analysis, 1-way ANOVA with Dunn’s test was performed. For time course data and growth curves, P values were calculated by 2-way ANOVA with Tukey’s post test.

Study approval. All mice were housed in an animal facility accredited by the American Association for the Accreditation of Laboratory Animal Care and were maintained under specific pathogen–free conditions. Animal experiments were approved and conducted in accordance with Tel Aviv University Laboratory Accreditation (01-16-095). The Tel Aviv University Institutional Review Board approved the human subject protocols, and informed consent was obtained from all subjects prior to participation in the study.

Author contributions
DR and NSM conducted the majority of the experiments and helped to design them. LT, AG, and LFY conducted some of the experiments. CS helped with generating the FcγRI constructs and writing the manuscript. NRF made the viral vectors for labeling the tumor cells and has helped with writing the manuscript. YW helped with analyzing the TCR sequencing data. HG, AT, and RB provided biological materials from human cancer patients. EAB assisted with the acquisition of human samples to use for experimentation. PR and YC designed and conducted the experiments and wrote the manuscript.

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