Clinical Cancer Research

Manipulating the Tumor Microenvironment *Ex Vivo* for Enhanced Expansion of Tumor-Infiltrating Lymphocytes for Adoptive Cell Therapy

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Abstract

Purpose: Cultured tumor fragments from melanoma metastases have been used for years as a source of tumor-infiltrating lymphocytes (TIL) for adoptive cell therapy (ACT). The expansion of tumor-reactive $CD8^+$ T cells with interleukin-2 (IL2) in these early cultures is critical in generating clinically active TIL infusion products, with a population of activated 4-1BB $CD8^+$ T cells recently found to constitute the majority of tumor-specific T cells.

Experimental Design: We used an agonistic anti–4-1BB antibody added during the initial tumor fragment cultures to provide *in situ* 4-1BB costimulation.

Results: We found that addition of an agonistic anti–4-1BB antibody could activate 4-1BB signaling within early cultured tumor fragments and accelerated the rate of memory $CD8^+$ TIL outgrowth that were highly enriched for melanoma antigen specificity. This was associated with NFκB activation and the induction of T-cell survival and memory genes, as well as enhanced IL2 responsiveness, in the $CD8^+$ T cells in the fragments and emerging from the fragments. Early provision of 4-1BB costimulation also affected the dendritic cells (DC) by activating NFκB in DC and promoting their maturation inside the tumor fragments. Blocking HLA class I prevented the enhanced outgrowth of $CD8^+$ T cells with anti–4-1BB, suggesting that an ongoing HLA class I–mediated antigen presentation in early tumor fragment cultures plays a role in mediating tumor-specific $CD8^+$ TIL outgrowth.

Conclusions: Our results highlight a previously unrecognized concept in TIL ACT that the tumor microenvironment can be dynamically regulated in the initial tumor fragment cultures to regulate the types of T cells expanded and their functional characteristics. *Clin Cancer Res; 21(3); 611–21. ©2014 AACR.*

Introduction

Adoptive transfer using tumor-infiltrating lymphocytes (TIL) has emerged as a powerful immunotherapy against solid malignancies, especially metastatic melanoma (1, 2). Adoptive cell therapy (ACT) involves the initial outgrowth of TILs using IL2 from 4 to 6 mm^2 cut fragments from metastatic melanoma surgical resections (1–3). Initial tumor fragment cultures take about 4 to 5 weeks to yield enough TIL for secondary expansion. A non-myeloablative lymphodepleting chemotherapy regimen before adoptive transfer of autologous TIL has boosted clinical response rates to 45% to 50% (4, 5).

$CD8^+$ TIL are critical in mediating tumor regression in a number of phase II TIL trials. Current efforts are aimed to increase the expansion of $CD8^+$ T cells from tumor tissue, and increase the antitumor activity of the final TIL infusion product to improve persistence after adoptive transfer (2, 3, 6–9). Accelerating the rate of TIL expansion from the initial tumor fragment cultures has become priority (6, 10–12). Metastatic melanomas contain a population of $CD8^+$ T cells expressing immunomodulatory markers, such as PD-1 and 4-1BB, indicating a recent history of antigenic stimulation in the tumor microenvironment *in vivo* (13, 14). $CD8^+$ TIL expressing 4-1BB appear to represent the most highly enriched tumor-specific subpopulation of T cells in melanoma (13). Protocols are being developed to purify 4-$1BB^+$ $CD8^+$ T cells from melanoma tissues and expand these selected cells for infusion. Although this approach is promising, it has caveats, including the need to prepare single-cell suspensions from tumor tissues, the small sizes of tumor tissue that can be available yielding few cells after enzymatic or mechanical disaggregation, and the possibility that not all tumor-specific $CD8^+$ T cells may be in an activated (4-$1BB^+$) state at the time the tumor is processed.

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Translational Relevance

The success of adoptive cell therapy (ACT) using autologous tumor-infiltrating lymphocytes (TIL) depends on the expansion of effector-memory T cells (especially $CD8^+$ cells) capable of recognizing tumor antigens. In this study, we developed a novel approach at inducing a more rapid outgrowth of TIL from tumor fragments of melanoma metastases highly enriched for tumor specificity. We capitalized on the specific expression of the 4-1BB costimulatory molecule on a subset of tumor-resident $CD8^+$ T cells enriched for tumor specificity. Using an agonistic GMP-grade anti–4-1BB antibody added directly to the initial tumor fragment cultures to provide *in situ* 4-1BB costimulation, we enhanced the rate of $CD8^+$ T-cell outgrowth, NFκB survival gene activation, and tumor specificity of the expanded TIL. This method offers a practical way to improve the quality of TIL products for ACT by reducing the culture time needed, while ensuring high tumor-specificity and maintenance of effector-memory properties of the cells.

An alternative approach is to directly manipulate costimulatory pathways within the initial melanoma tumor fragment cultures. This approach capitalizes on the *de novo* expression of costimulatory molecules due to previous antigenic stimulation on resident $CD8^+$ T cells that can accelerate the rate of TIL expansion out of the tumor fragments. Tumor fragments have been used for years to expand TIL by adding exogenous IL2, but the inclusion of other immunomodulators in tumor fragment cultures to affect TIL expansion and phenotype has not been investigated.

In this study, we hypothesized that the activation of the 4-1BB costimulatory pathway in melanoma tumor fragments enhances $CD8^+$ T-cell output, TIL tumor reactivity, and memory properties. This question is distinct from our previous studies, where the effects of 4-1BB agonists were tested at much later stages of *ex vivo* TIL expansion and where 4-1BB costimulation improved output and function of T cells in the rapid expansion protocol (REP) and the survival of the post-REP TIL (15, 16). We tested an agonistic anti–4-1BB antibody, added during the initiation of individual tumor fragment cultures (at the start of the whole TIL expansion process), and found that this increased the rate of $CD8^+$ TIL expansion as well as the tumor reactivity of the expanded product. 4-1BB costimulation during these early tumor fragment cultures induced the expression of survival signaling pathways (NFκB) in $CD8^+$ TIL, and the expression of antiapoptotic and T-cell memory genes. We examined potential mechanisms of action and found that resident dendritic cells (DC) in the tumor fragments survive for considerable periods of time and express 4-1BB. These tumor fragment resident DC also activate NFκB, and upregulate certain maturation markers in combination with 4-1BB agonism. We examined whether ongoing HLA class I antigen presentation occurs in the early tumor fragment cultures that may enhance the output of $CD8^+$ TIL. Addition of a blocking anti-HLA class I antibody reduced the output of $CD8^+$ TIL, suggesting that continual antigen presentation occurs *ex vivo* in these early tumor fragment cultures that was not considered before. Our results indicate that tumor fragments placed in culture to expand TIL for ACT are not static material, but small, dynamic tumor microenvironments that can be manipulated to alter the yield and phenotype of TIL being expanded for cell therapy as well as enrich for tumor reactivity and improved memory phenotype. The use of 4-1BB costimulation in this system can be the first of many ways to manipulate these *ex vivo* tumor microenvironments to develop protocols to expand optimally enhanced TIL for ACT.

Materials and Methods

Agonistic anti–4-1BB antibody

A fully human IgG4 monoclonal agonistic anti–4-1BB antibody (mAb;BMS-663513 Lot 6A20383/1187261) was provided by Bristol-Myers Squibb. The anti–4-1BB antibody was added at day 0 of the fragment set up and each time the TIL were fed. Tumor fragments were cultured in the presence of 10 μg/mL anti-human PD-1 antibody (BMS-936558), anti-human PD-L1 antibody (cloneM1H1; eBioscience), or 10 μg/mL anti-human CTLA-4 antibody (Ipilimumab; Bristol-Myers Squibb) in some experiments.

TIL isolation and culture from melanoma tumors

Studies were performed under approved Institutional Review Board (IRB) laboratory protocols at the MD Anderson Cancer Center (MDACC; Houston, TX) and the H. Lee Moffitt Cancer Center (Tampa, FL). Melanoma tumors were obtained from patients from a tumor bank under MDA laboratory protocol LAB06–0755. Melanoma tumors were surgically resected from patients with stage IIIc–IV melanoma at MDACC and H. Lee Moffitt Cancer Center as part of ongoing TIL ACT preclinical and clinical trials. The tumor was cut into 4 to 6 mm^2 fragments and the fragments were placed in a 24-well plate with TIL culture media (TIL-CM) and 6,000 IU/mL interleukin-2 (IL2). The TIL-CM contained RPMI-1640 with GlutaMax (Gibco/Invitrogen), 1× Pen–Strep (Gibco/Invitrogen), 50 μmol/L 2-mercaptoethanol (Gibco/Invitrogen), 20 μg/mL Gentamicin (Gibco/Invitrogen), and 1 mmol/L pyruvate (Gibco/Invitrogen). For our control cultures, the fragments were placed in 6,000 IU/mL IL2 with a fully human IgG4 Isotype control (Eureka therapeutics ET904) in our experiments. The control cultures are referred to as "IL2."

Flow cytometry staining for NFκB and IκBα

Tumors were cut up into multiple fragments and placed in culture with TIL-CM, IL2, with or without the anti-4-1BB antibody. After 1 week, the fragments and the cells that migrated out of the tumor fragments were harvested. The fragments were mechanically disaggregated and filtered. The fragments and the cells that migrated out of the fragment were washed with FACS Wash Buffer (FWB), and stained for CD3 (BD Biosciences) and CD8 (BD Pharmingen) on ice for 25 minutes. The cells were washed with FWB, and fixation buffer (BD Bioscience) was added to each tube. The tubes were placed at room temperature for 20 minutes. The cells were washed with FWB for 5 minutes at 1,400 rpm, 4°C and Perm Buffer III (BD Bioscience) was added. The tubes were placed on ice for 30 minutes and washed in FWB for 5 minutes, 1,400 rpm, 4°C and then stained using anti-NFκB (p65) Alexa Fluor 647 or anti-IκBα Alexa Fluor 647 (BD Pharmingen) and left in the dark at room temperature for 1 hour. The cells were washed with FWB for 5 minutes, 1,400 rpm, 4°C, then resuspended in FWB and samples were subsequently acquired.

Staining for IFNγ and degranulation

Melanoma tumors were cut into multiple fragments, and placed in culture with TIL media, IL2, with or without anti–4-1BB antibody. After 3 weeks, the TIL were set up at a 1:1 ratio with HLA-matched melanoma tumor lines in a 96-well plate for 1 hour

at 37°C. GolgiStop (BD Biosciences) was added to each well and the cultures were left at 37°C for a 5-hour period. During this time, CD107a antibody (BD Biosciences) was also added to each condition. The cells were harvested, washed with FWB, and stained for CD3 and CD8 for 25 minutes at 4°C. The cells were washed with FWB, resuspended in fixation buffer, and incubated for 15 minutes at room temperature, then washed with FWB, and resuspended in Cytofix/Cytoperm buffer and incubated at room temperature for 20 minutes. The cells were washed with FWB and stained for IFNγ for 25 minutes in 4°C, washed, and the samples were acquired.

Cytospin and immunocytochemistry staining for surface and nuclear markers

The tumor fragments were set up with or without anti–4-1BB antibody. After 1 week in culture, the cells were collected and washed in 1× D-PBS for 5 minutes at 1,400 rpm then resuspended in 1 mL 4% paraformaldehyde for 20 minutes at room temperature. The cells were washed in D-PBS and placed at 4°C, then cytospun onto glass slides at 750 rpm for 3 minutes. The samples for Ki67 (Dako) and NFκB (p65; BD transduction laboratories) were placed in PBS, 0.2% Triton X-100 solution for 10 minutes at room temperature, and washed in 1× D-PBS. Samples were placed in 3% H_2O_2/methanol for 10 minutes at room temperature. The samples were washed in D-PBS and 2.5% normal horse serum was added to all slides for 30 minutes in a humid chamber at room temperature followed by addition of the primary antibody at 4°C overnight. Thereafter, a biotinylated secondary antibody was added to the samples for 30 minutes at room temperature (Vectastain Universal Elite ABC Kit). The slides were then washed in D-PBS and peroxidase-conjugated avidin biotin complex ABC Reagent (Vectastain Universal Elite ABC Kit) was added for 30 minutes at room temperature. The samples were washed with D-PBS and 3,3-diaminobenzidine (DAB) was added. The samples were counterstained and covered. Analysis was done using Leica Application Suite (LAS) V4.2 software (Leica Microsystems). For NFκB (p65) staining, analysis was also done using the LAS V4.2 software, and additional analysis was conducted using Vectra Intelligent Slide Analysis System (Vectra; Perkin Elmer) Nuance software 3.0.1.2, using composite coloring style "Fluorescence."

Fresh fragment flow cytometry staining

Melanoma fragments were mechanically disaggregated using glass slides, filtered, resuspended in FWB, washed, and stained for CD3 (BD Biosciences; cat no: 555342), CD8 (BD Pharmingen; cat no: 558207), 4-1BB (BioLegend; cat no: 309817; Lot B147320), and AmCyan Aqua Live/Dead dye (Molecular Probes by Life Technologies; Lot 1413034). One week after the fragments were set up with or without the anti–4-1BB antibody, the fragments were mechanically disaggregated, filtered, and washed with FWB for 5 minutes at 1,400 rpm. The cells were stained for CD11c, CD80, CD86, 4-1BB, HLA-DR (eBioscience, BD Biosciences, and BD Pharmingen) and AmCyan Aqua Live/Dead fixable dye for 25 minutes on ice. The cells were then washed in FWB for 5 minutes at 1,400 rpm and fixed. The cells were then acquired using a BD FACSCanto II flow cytometer machine.

TIL staining using flow cytometry after 3 weeks in culture

Fragments were set up with or without the addition of agonistic anti–4-1BB antibody. After 3 weeks, the TIL were harvested and stained for CD3, CD4, CD25, CD27, CD28, and CD8 (eBiosciences, BD Pharmingen, and BD Biosciences). Regulatory T cells (Tregs) were stained for CD4, CD25, and Foxp3 using a Treg staining kit from eBiosciences. The cells were stained for 25 minutes on ice, then washed with FWB and BD Cytofix/Cytoperm Fixation and Permeabilization Solution (BD Biosciences; cat no: 554722; used for Granzyme B, bcl-2, and EOMES) or Perm Buffer III (BD Biosciences; cat no: 558050) was used for bcl-6. The buffers were added to the samples for 20 minutes at room temperature in the dark. The cells were washed with 1× BD Perm/Wash Buffer for 5 minutes at 1,400 rpm, followed by intracellular staining for Foxp3, Granzyme B, bcl-2, EOMES, and bcl-6 (eBiosciences, BD Biosciences) in 1× Fixation Buffer for 25 minutes on ice. The samples were washed with FWB for 5 minutes at 1,400 rpm and fixed, followed by acquisition using a BD FACSCanto II flow cytometer machine.

Measurement of tumor reactivity using IFNγ ELISA

After 3 weeks, the TIL were isolated, washed, and cocultured overnight in 96-well plates at a 1:1 ratio with tumor target cells from autologous or HLA-A–matched melanoma tumor cell lines. The supernatants were collected after 24 hours and IFNγ secretion was measured using a human IFN-gamma ELISA kit (Thermo Scientific KB132422). A 96-well ELISA plate reader (ELx808; Bio-Tek Instruments Inc.) was used to read the plate.

Statistical analysis

Statistical analysis for comparison of the two groups was done using an unpaired, nonparametric, Mann–Whitney test to determine the statistical values for all the figures, with the test using biologic relevance occurring when $P < 0.05$. Statistical analysis was done using GraphPad Prism.

Results

4-1BB expression in melanoma TILs

We previously found that some $CD3^+CD8^+$ TIL expanded from tumor fragments expressed 4-1BB (16) and wanted to determine whether 4-1BB was expressed on the freshly isolated TIL from melanoma tumors used to derive tumor fragment cultures. Tumors were surgically resected from patients with metastatic melanoma and single-cell suspensions were prepared and stained for 4-1BB on the $CD3^+CD8^+$ subset. In 18 independent freshly isolated TIL samples, we found that 4-1BB was mainly expressed on $CD8^+$ TIL (Supplementary Fig. S1A). We also looked at TIL emerging from tumor fragments 7 days after tumor fragment culture initiation from 7 patients and observed that a considerable frequency of TIL expressed 4-1BB (Supplementary Fig. S1B).

Agonistic anti–4-1BB antibody increases TIL expansion *in vitro*

The detection of $4\text{-}1BB^+$ $CD8^+$ T cells in the isolated tumor fragments prompted us to ask whether costimulation of 4-1BB in these early cultures could affect the outcome of TIL outgrowth. We tested a costimulatory anti–4-1BB antibody from Bristol-Myers Squibb (through a Materials Transfer Agreement) we previously used in later-stage TIL cultures that protected expanded $CD8^+$ TIL from activation-induced cell death (15, 16). Melanoma tumors were surgically resected from patients and cut into four 3 to 5 mm^2 fragments per condition and cultured over a 3-week period. We conducted dose titration experiments with the anti–4-1BB added on day 0 of culture to determine the optimal concentration of antibody that would increase the yield of $CD8^+$ T cells from the

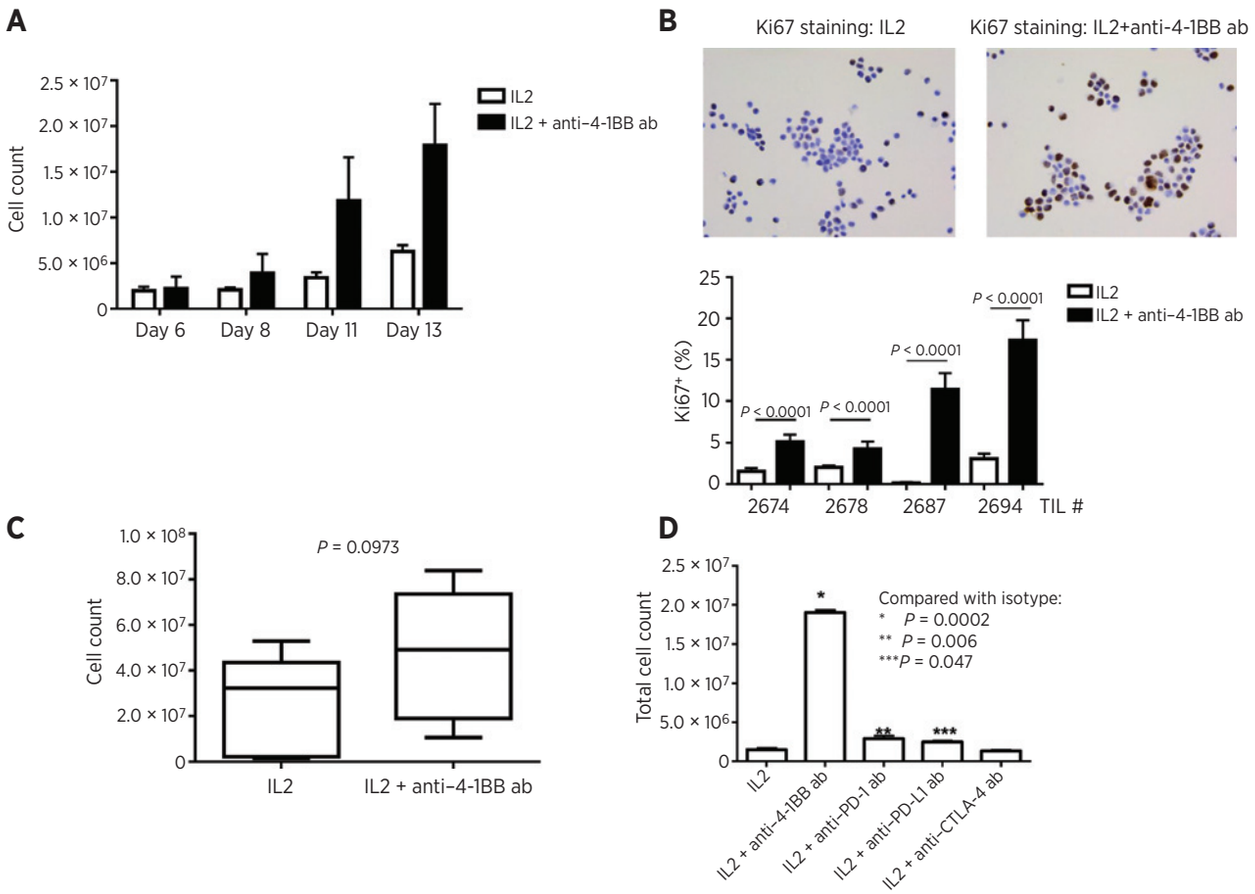

Figure 1.
Anti–4-1BB agonistic antibody increases TIL expansion *in vitro*. Melanoma tumors were surgically resected from patients. The tumors were then cut into multiple fragments and placed in culture with IL2 with or without the anti–4-1BB antibody. Cell counts were conducted from days 6 to 13 using a hemocytometer and Trypan Blue. At these early time points, the 4-1BB–activated TIL grew faster compared with the IL2 control, as demonstrated in 4 representative TIL patient samples each having multiple fragments in culture (A). When determining the proliferation of the TIL at early time points, we found an increased expression of Ki67 proliferation marker in the 4-1BB activated TIL as compared with the control as demonstrated using immunocytochemistry (B, top) and further confirmed and quantified the immunocytochemistry in 4 independent TIL lines (representative of 16 fragments; B, bottom). The melanoma tumors were cut into multiple fragments and placed in culture with IL2 with or without the anti–4-1BB antibody over a 3-week period. Using a hemocytometer and Trypan Blue, we determined viable cell counts in the different conditions after the TIL were in culture for 3 weeks. We found that in seven TIL samples (28 separate tumor fragments; 4 pooled fragments per patient sample), the TIL expanded with IL2 and the anti–4-1BB antibody expanded better than the IL2 control (C). After the fragments were in culture for 3 weeks with IL2 alone or with IL2 and different antibodies (anti–4-1BB, anti–PD-1, anti–PD-L1, and anti–CTLA-4), cell counts were conducted using a hemocytometer after Trypan Blue staining. As demonstrated in one representative TIL sample (4 separate fragments), the TIL grown with the agonistic anti–4-1BB antibody exhibited the most TIL expansion as compared with the other antibodies (D). An unpaired, nonparametric, Mann–Whitney test was conducted to determine the statistical values for all the figures.

fragments (Supplementary Fig. S2). As shown in Supplementary Fig. S2, represented in 3 different melanoma TIL patient samples, we found that 10 μg/mL anti–4-1BB antibody produced the most consistent result in terms of enhancing total TIL and $CD8^+$ TIL outgrowth. In subsequent experiments, this dose was used each time the TIL were sub-cultured with fresh media and IL2.

We tested how anti–4-1BB affects TIL yield and the rate of TIL outgrowth from the tumor fragments and found that addition of 4-1BB accelerated the rate of TIL outgrowth over the first 2 weeks of culture fragments from four representative melanoma TIL patients (Fig. 1A). The error bars indicate the standard deviation cell counts from 4 independent patients with melanoma. The accelerated rate of TIL outgrowth in the 4-1BB cultures was associated with a marked increase in proliferating cells emanating from the fragments during the first 7 days in culture, as shown by the increased nuclear Ki67 staining of isolated cells surrounding the tumor fragments after cytospin (Fig. 1B; four independent experiments and melanoma TIL samples). We performed experiments on an additional 7 melanoma patient samples, four fragments per melanoma patient samples (28 separate tumor fragments) and found that this enhanced outgrowth of TIL after 3 weeks was consistently observed in a larger sampling of patient tumors (Fig. 1C). We determined how anti–4-1BB facilitates TIL outgrowth compared with other immunomodulatory (checkpoint) antibodies, anti–PD-1, anti–PD-L1, and anti–CTLA-4, currently being used in clinical trials and standard of care for melanoma (anti–CTLA-4). As shown in Fig. 1D, anti–4-1BB antibody was superior in inducing TIL outgrowth from the tumor

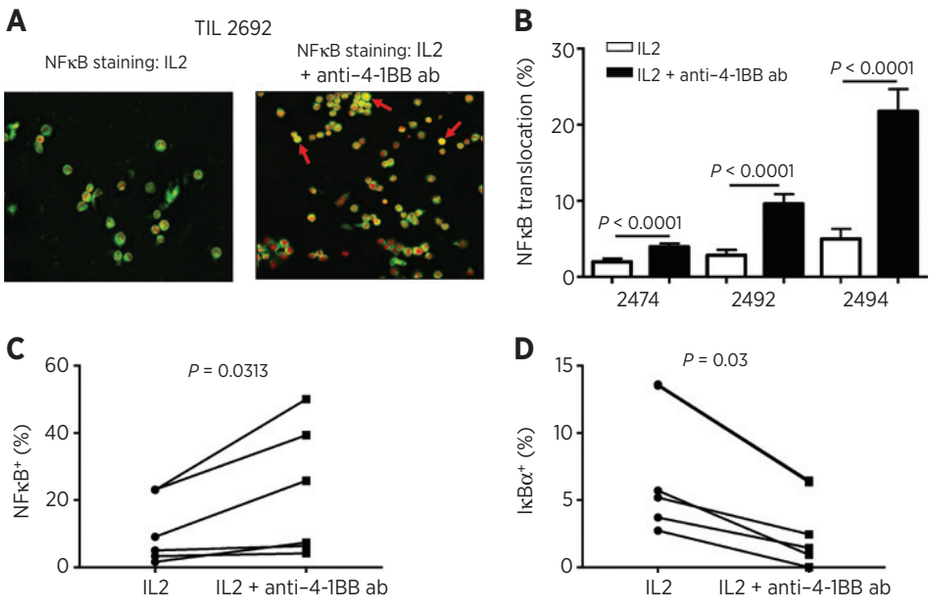

Figure 2.
Increased NFκB translocation in TIL expanded with the anti–4-1BB antibody. Melanoma tumors were cut into multiple fragments and placed in culture with IL2 alone or IL2 with or without an anti–4-1BB antibody. After 1 week, the cells were collected and cytospun onto a glass slide, followed by staining using immunocytochemistry. Staining for NFκB (p65) revealed more translocation in the nucleus of TIL grown with IL2 and the anti–4-1BB antibody compared with TIL grown with IL2 alone. We used Vectra Intelligent slide analysis system (Nuance software; A) to observe the translocation of NFκB (red is nucleus; green is NFκB; and yellow is overlay translocation). The red arrow indicates one area where translocation occurred. Quantification of NFκB translocation in 10 different areas per sample is demonstrated in three independent TIL samples (12 fragments; 4 pooled fragments per patient sample; B). In addition to immunocytochemistry, we also measured the percentage of NFκB and IκBα expression in the $CD8^+CD3^+$ TIL within the fragment after 1 week in culture using flow cytometry in 6 patient samples (24 representative fragments; 4 pooled fragments per patient; C). The fragments were set up with IL2 with or without the addition of anti–4-1BB antibody. After 1 week, the fragments were collected (4 fragments per patient sample) and mechanically disaggregated using glass slides. The cells were filtered and stained using flow cytometry. The TIL were gated on live, $CD3^+$, $CD8^+$ TIL and the percentage of NFκB (C) and IκBα (D) was measured. We found that the TIL cultured with IL2 plus anti–4-1BB antibody exhibited an increase NFκB (C) with a paralleled decrease in IκBα (D) as demonstrated in six independent TIL samples (24 representative fragments). An unpaired, nonparametric, Mann–Whitney test was used to determine the statistical values for B–D.

fragments compared with IL2 alone or IL2 plus these other checkpoint antibodies.

Addition of anti–4-1BB increased NFκB nuclear translocation in TIL

We determined the effect of 4-1BB costimulation on the TIL within the tumor microenvironment at the start of the tumor fragment cultures as well as on TIL that had migrated out from the fragments. One of the key signaling pathways activated by 4-1BB costimulation is the classical NFκB (p65/cREL) transcription factor pathway. 4-1BB costimulation results in TRAF recruitment that activates IKKαβ complexes. This results in degradation of IκBα and subsequent translocation of NFκB to the nucleus (17). Melanoma tumor fragments (4 fragments per condition for each patient) were established in culture with or without addition of anti–4-1BB. After 1 week, the TIL that had migrated out of the tumor fragment supernatant were collected, cytospun onto glass slides, fixed, and stained for NFκB (p65). We observed that the TIL grown with IL2 and anti–4-1BB antibody had markedly more NFκB (green) translocation in the nucleus (red) as compared with the TIL grown in IL2 (Fig. 2A; arrows and yellow color indicate NFκB nuclear translocation). Staining for NFκB in TIL in cytospins from 3 different patient tumor samples found a consistent increase in translocated p65 in cultures treated with anti–4-1BB, after quantifying the staining using a digital imaging system (Fig. 2B). We also isolated fragments 1 week after culture initiation and stained for NFκB (p65) and IκBα in the TIL using flow cytometry after mechanically disaggregating the isolated fragments into single-cell suspensions. After gating on viable $CD3^+CD8^+$ T cells, we detected an increase in NFκB (p65) staining (Fig. 2C) together with a decrease in IκBα expression (Fig. 2D), indicative of NFκB activation, in the anti–4-1BB antibody group compared with the IL2 alone control group, demonstrated in 6 independent melanoma patient samples. These results indicate that addition of agonist anti–4-1BB antibody to melanoma tumor fragments can activate NFκB in the T cells inside and outside the tumor fragments.

Activating the 4-1BB pathway increases the $CD8^+$ TIL expansion

We determined whether the cells that were growing out of the fragment were enriched for $CD8^+$ T cells and also measured the yield of $CD4^+$ T cells and $CD4^+$ $CD25^+Foxp3^+$ Tregs. Melanoma fragments from patient tumors were set up with or without the addition of anti–4-1BB antibody. We conducted CD8 staining of cytospun cells that were isolated from outside the tumor fragments in early cultures (after 1 week). As shown in one representative patient sample, 4-1BB costimulation increased the number of CD8-stained cells versus IL2 alone (Fig. 3A). In 14 patient tumor samples (representing 56 independent tumor fragments), we found that addition of anti–4-1BB increased the percentage of

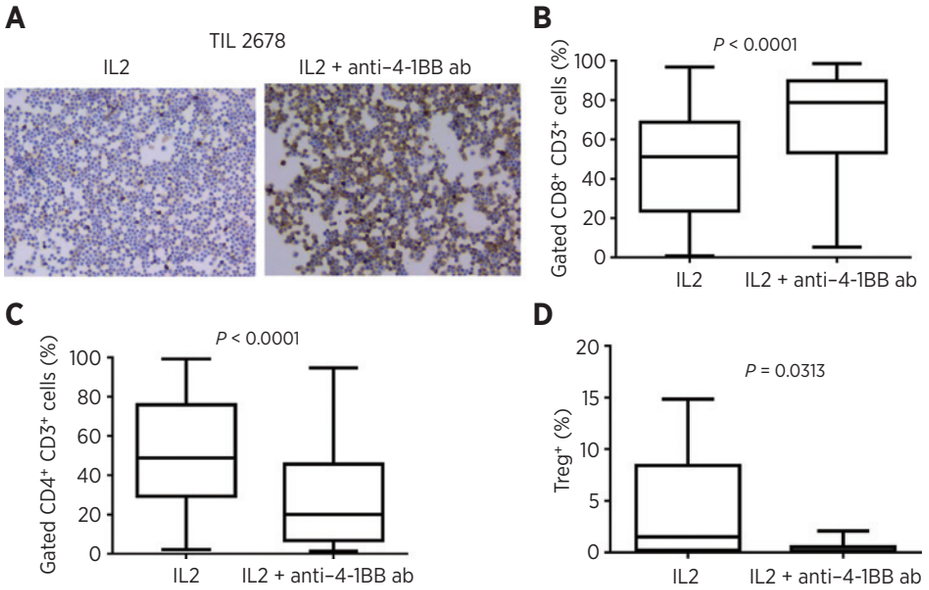

Figure 3.
$CD8^+$ TIL percentage is increased with the addition of anti–4-1BB antibody to TIL cultures. The melanoma tumors were surgically resected from the patients, cut into multiple fragments, and placed in culture with IL2 alone or with IL2 and anti–4-1BB antibody. After 1 week in culture, we collected the cells, cytospun them onto a glass slide, conducted Immunocytochemistry, and found that the TIL expanded with IL2 and the anti–4-1BB antibody exhibited an increase in $CD8^+$ cells, as shown in one representative TIL line (4 pooled fragments; A). After the melanoma fragments were cultured with IL2 alone or IL2 and anti–4-1BB antibody for 3 weeks, the TIL were stained for the expression of CD3, CD8, and CD4 using flow cytometry. We observed that in 56 independent TIL lines grown with IL2 and anti–4-1BB antibody exhibited an increase in the percentage of $CD8^+$ TIL in the CD3 subset compared with the IL2 control (B). When we examined the $CD4^+$ expression in 56 independent TIL samples, we found that the TIL expanded with IL2 alone exhibited an increase in $CD4^+$, compared with TIL grown with IL2 with an anti–4-1BB antibody (C). We went on to determine the percentage of $CD3^+CD4^+CD25^+FoxP3^+$ Tregs. Cells were stained for Tregs with anti-Foxp3, anti-CD4, and anti-CD25 using the Foxp3 staining kit from eBioscience. The control TIL (IL2 alone) exhibited an increased percentage of Tregs than the TIL expanded in anti–4-1BB antibody (D) as demonstrated in seven TIL samples (28 representative fragments). An unpaired, nonparametric, Mann–Whitney test was used to determine the statistical values for figures B–D.

$CD8^+$ T cells expanded from the tumor fragments after 3 weeks (Fig. 3B), whereas the percentage of $CD4^+$ T cells decreased (Fig. 3C). Staining for Tregs after 3 weeks in experiments on 7 patient tumor samples (representing 28 independent tumor fragments) found that 4-1BB costimulation in these early fragment cultures reduced their frequency (Fig. 3D).

Modulation of TIL effector phenotype by activating the 4-1BB pathway

Melanoma tumor fragments were cultured with or without anti–4-1BB antibody. After 3 weeks, the TIL were stained using flow cytometry for effector-memory markers (CD28 and CD27). In 48 independent tumor fragment lines (representing 12 different melanoma patient samples), we found that CD28 expression was unaltered (Fig. 4A), but CD27 expression decreased somewhat (Fig. 4B). We tested whether TIL that grew out of tumor fragments over the 3-week period with anti–4-1BB costimulation could further expand after restimulation through the T-cell receptor (TCR) in a REP. In 6 independent patient melanoma samples, 4-1BB costimulation during the initial TIL outgrowth did not affect the fold-expansion of TIL after TCR restimulation in the REP (data not shown) or the phenotype of the post-REP cells in terms of CD8, CD28, and CD27 expression (data not shown).

We investigated the effect of anti–4-1BB on the expression of molecules associated with effector (Granzyme B and Eomes), memory (bcl-6 and Eomes), and survival (bcl-2 and bcl-6) functions of cytotoxic T lymphocyte (CTL). Granzyme B is a cytolytic granule molecule in $CD8^+$ T cells, while Eomes is a T-box transcription factor helping drive Granzyme B expression as well as being involved in memory T-cell maintenance (18–20). Bcl-2 and bcl-6 are cell survival molecules in memory T cells (19–22). We found an increased intracellular expression of the Granzyme B (Fig. 4C) and Eomes (Fig. 4D) in $CD8^+$ TIL expanded from fragments with 4-1BB costimulation. We observed an increase in the mean fluorescence intensity (MFI; data not shown) and percentage of Granzyme B in the $CD8^+$ subset in 12 independent TIL samples (representative of 48 melanoma patient fragments) and an increase in Eomes in 19 independent melanoma patient samples. The MFI (data not shown) and percentage of bcl-2 and bcl-6 in the $CD8^+$ subset was also increased (Fig. 4E and F). Bcl-2 was increased in 13 independent TIL samples, while we observed an increase in eight independent TIL samples. Each melanoma patient sample contained four fragments that were pooled together before staining.

Because of the accelerated outgrowth of $CD8^+$ T cells from the tumor fragments after addition of anti–4-1BB, we also surmised that IL2 signaling may be altered. Because there have been STAT5-binding sites identified in 4-1BB promoter region, and 4-1BB has its expression modulated by IL2R signaling through activation of STAT5 (23), we specifically looked at CD25 (IL2Rα) and pSTAT5. In 13 independent patients with melanoma (representative of 52 independent fragments; 4 fragments per patient sample), TIL were collected after 3 weeks of expansion from tumor fragments and stained for pSTAT5. We measured a higher frequency of

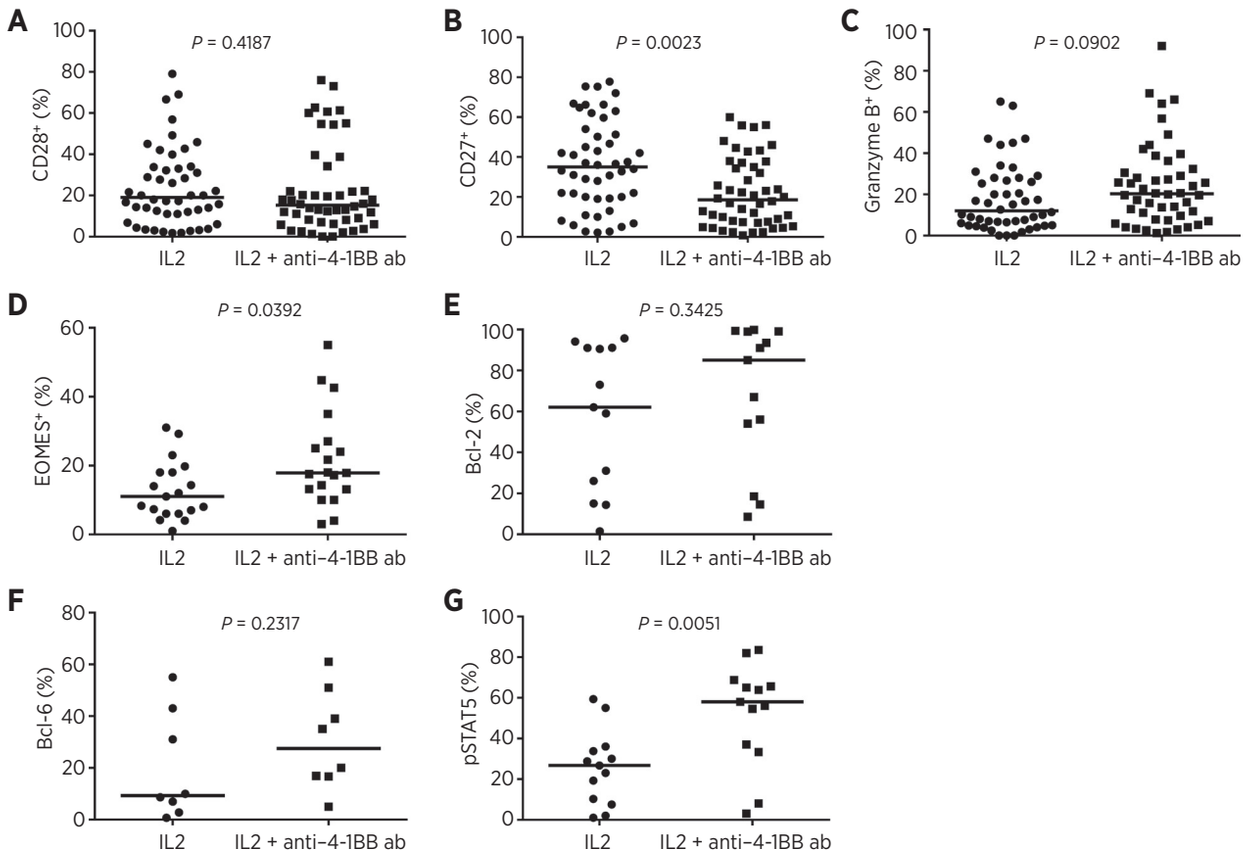

Figure 4.
Phenotypic characterization of effector-memory markers in TIL isolated from tumor fragment cultures treated with or without anti–4-1BB. Fragments from melanoma tumors were set up in a 24-well plate with or without anti–4-1BB antibody. Three weeks after the initial set-up, the TIL were harvested and flow cytometry was done to analyze effector-memory markers and cytolytic granule markers. When we measured the expression of CD28 in the $CD3^+CD8^+$ subset in 48 independent TIL lines, we found no statistical significance in the expression of CD28 in the $CD3^+CD8^+$ subset in the different conditions (A). When we measured the expression of CD27 in the $CD3^+CD8^+$ population in 48 independent TIL lines, we found that the CD27 expression in the $CD3^+CD8^+$ subset was downregulated in the TIL expanded with the anti–4-1BB antibody as compared with the TIL grown with IL2 alone (B). When we measured the expression of the cytolytic granule marker Granzyme B in the $CD3^+CD8^+$ subset in 48 independent TIL lines, we found the expression of Granzyme B was increased in the TIL expanded with IL2 and anti–4-1BB antibody as compared with TIL expanded with IL2 alone (C). When we measured the expression of Eomes using flow cytometry in 19 independent TIL, we found the TIL expanded with the anti–4-1BB antibody exhibited increased Eomes expression in the $CD3^+CD8^+$ subset (D). When we determined the percentage of the antiapoptotic molecule, bcl-2 in 13 independent TIL, we found that the TIL expanded with the anti–4-1BB antibody exhibited an increased bcl-2 percentage (E). We also determined the expression of bcl-6 (F) in eight independent TIL lines and pSTAT5 in 13 independent TIL in the $CD3^+$ $CD8^+$ subset (G). We observed that bcl-6 and pSTAT5 percentage was increased when the TIL were expanded with IL2 plus anti–4-1BB antibody for 3 weeks (F and G). Each TIL sample contained 4 pooled fragments. An unpaired, nonparametric, Mann–Whitney test was conducted to determine the statistical values for all the figures.

tyrosine-phosphorylated STAT5 (pSTAT5) expression in $CD8^+$ TIL in the 4-1BB treatment group (Fig. 4G).

Increased antitumor reactivity in TIL expanded with anti–4-1BB antibody

The induced expression of 4-1BB known to occur on antigen-activated $CD8^+$ T cells together with our detection of a significant frequency of 4-1BB$^+$ $CD8^+$ TIL within tumors (Supplementary Fig. S1) suggests that addition of agonist anti–4-1BB may also increase the tumor reactivity of the TIL grown out from the treated fragments. We analyzed the antitumor reactivity of bulk TIL cultures grown out from tumor fragments after 3 weeks against autologous or HLA-A–matched allogeneic tumor lines by measuring the extent tumor-specific IFNγ release after a 24-hour tumor cell-TIL coculture assay. We found that provision of 4-1BB costimulation to the tumor fragments yielded TIL with markedly higher antitumor reactivity after 3 weeks of culture (Fig. 5A; data from three independent experiments and melanoma patient samples shown). We performed intracellular cytokine staining (ICS) assays for IFNγ production on a single cell level from HLA-$A2^+$ patients after TIL were cultured for 3 weeks from fragments. We used HLA-$A2^+$ melanoma tumor targets and found a significant increase in the frequency in IFN-γ-positive $CD8^+$ T cells in TIL isolated from fragment cultures treated with anti-4-1BB antibody (Fig. 5B; data from 11 independent melanoma patient TIL are shown, representative of 4 fragments pooled per patient sample; 44 fragments total). We measured the frequency of CD107a release (a measure for antigen-specific T-cell degranulation) using flow cytometry. The TIL expanded with IL2 and anti–4-1BB versus IL2 alone also exhibited an increase in $CD107a^+$

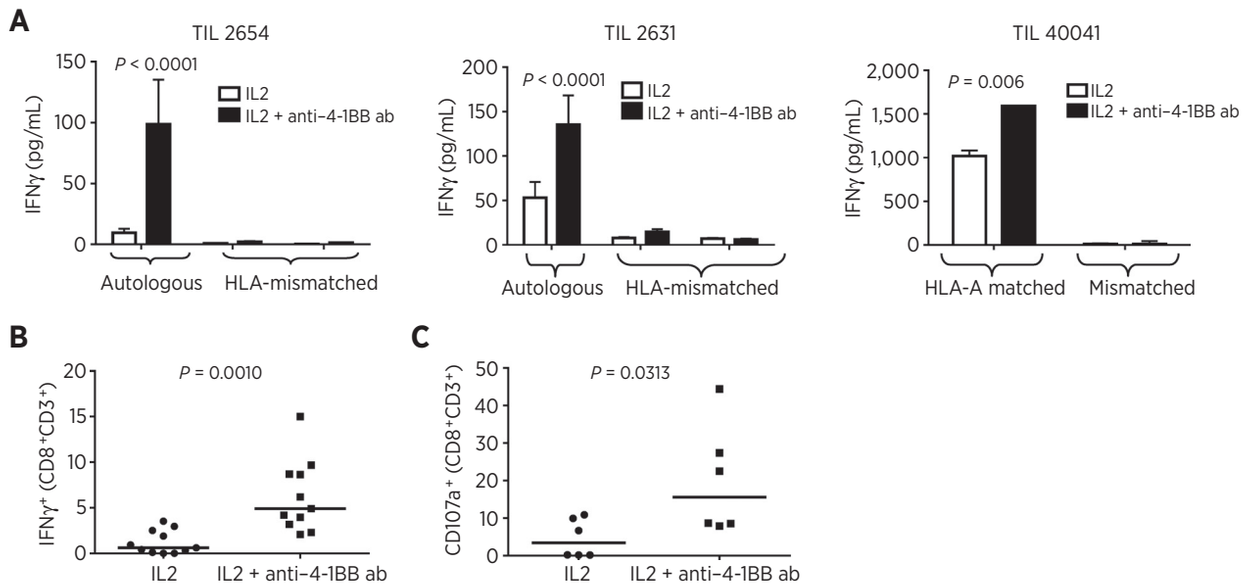

Figure 5.
Increased tumor specificity of TIL from tumor fragment cultures treated with IL2 and anti–4-1BB. After the fragments were set up with IL2 alone or IL2 and anti–4-1BB antibody and expanded over a 3-week period (pre-REP), the TIL were harvested and set up at a 1:1 ratio with autologous (A, left and middle) or HLA-A matched (A, right) melanoma tumor cells. Supernatants were collected after 24 hours and IFNγ secretion was measured using ELISA. We demonstrated in three independent TIL samples (4 pooled fragments per TIL sample) that IFNγ secretion was increased in the pre-REP TIL expanded with IL2 and anti–4-1BB compared with TIL expanded with IL2 alone (A). After 3 weeks in culture with or without the anti–4-1BB antibody, the TIL were set up at a 1:1 ratio with HLA-A matched tumor lines. Using flow cytometry, we measured the amount of IFNγ$^+$ cells in the CD3$^+$CD8$^+$ subset (B). In 11 independent TIL patient samples (44 representative fragments; 4 pooled fragments per TIL sample), the TIL expanded with IL2 and anti–4-1BB antibody exhibited an increase in IFNγ$^+$ cells in the CD3$^+$CD8$^+$ subset (B). We also used flow cytometry to determine the amount of degranulation (CD107a) in the CD3$^+$CD8$^+$ subset after setting up a 1:1 ratio with TIL and HLA-A–matched melanoma tumor lines (C). We found that in 6 independent TIL patient samples (24 representative fragments; 4 pooled fragments per TIL sample), the TIL grown with IL2 and the anti–4-1BB antibody exhibited an increase in CD107a$^+$ cells in the CD3$^+$CD8$^+$ subset (C). An unpaired, nonparametric, Mann–Whitney test was conducted to determine the statistical values for all the figures.

frequency after coincubation with HLA-A–matched tumor targets in the CD3$^+$CD8$^+$ subset (Fig. 5C; data from 6 patients with melanoma, representative of 24 melanoma patient fragments, each patient sample containing 4 pooled fragments).

Anti–4-1BB addition modulates resident DC within melanoma tumor fragments

Our observation that agonist anti–4-1BB antibodies activate CD8$^+$ T cells in the tumor microenvironment within the tumor fragments suggested that other tumor-resident leukocyte populations may be affected by 4-1BB costimulation given in this context. We performed flow cytometry analysis of cell suspensions obtained from tumor fragments that were in culture for 7 days to determine what other viable (surviving) resident cells were present and expressing 4-1BB that may be targeted by the added antibody. We stained for DC, T cells, NK cells, and B cells. We found very few NK cells (CD3$^-$CD56$^+$) and B cells (CD3$^-$CD20$^+$) in the tumor fragments and these had no detectable 4-1BB expression (data not shown). CD8$^+$ and CD4$^+$ T cells were detected, with only the CD8$^+$ cells expressing detectable 4-1BB. In the DC population (CD3$^-$CD11c$^+$ HLA class II$^+$), we found a considerable frequency of viable 4-1BB$^+$ DC persisting in these fragments. Addition of the agonistic anti–4-1BB during the tumor fragment culture led to activation of DC, manifested by a marked increase in CD86 and HLA class II expression in the gated live CD3$^-$CD11c$^+$ subset in one representative melanoma patient sample (4 fragments pooled; Fig. 6A). We measured the expression of markers on DC isolated from tumor fragment cultures from resected melanomas from 8 independent melanoma patient samples (representative of 32 fragments, 4 fragments pooled per patient sample; A), 7 independent melanoma patient samples (representative of 28 fragments, 4 fragments pooled per patient sample), and 6 independent melanoma patient samples (representative of 24 fragments, 4 fragments pooled per patient sample). We found an increase in HLA (MHC) class II (Fig. 6B), CD86 (Fig. 6C), and CD80 (Fig. 6D) on DC isolated from fragments on day 7 after addition of anti–4-1BB.

The observed activation of tumor fragment-resident DC with anti–4-1BB suggested that an ongoing presentation of antigen occurs in early tumor fragment cultures that may help drive CD8$^+$ TIL expansion. To test this, we added purified blocking anti–HLA-ABC antibody (W6/32) to the tumor fragment cultures on day 0 and harvested the TIL that had grown out of the fragments after 3 weeks. As shown in Fig. 6E, in 2 representative melanoma TIL patient samples, we found that addition of anti–HLA-ABC antibody reversed the effect of anti–4-1BB on increasing the outgrowth of CD8$^+$ T cells, as measured by the decreased CD8$^+$ T-cell numbers.

Discussion

ACT using TIL has shown considerable efficacy in mediating tumor regression (1–3). Current response rates using a nonmyeloablative chemotherapy preconditioning regimen of close to 40% to 50% have been observed at multiple centers

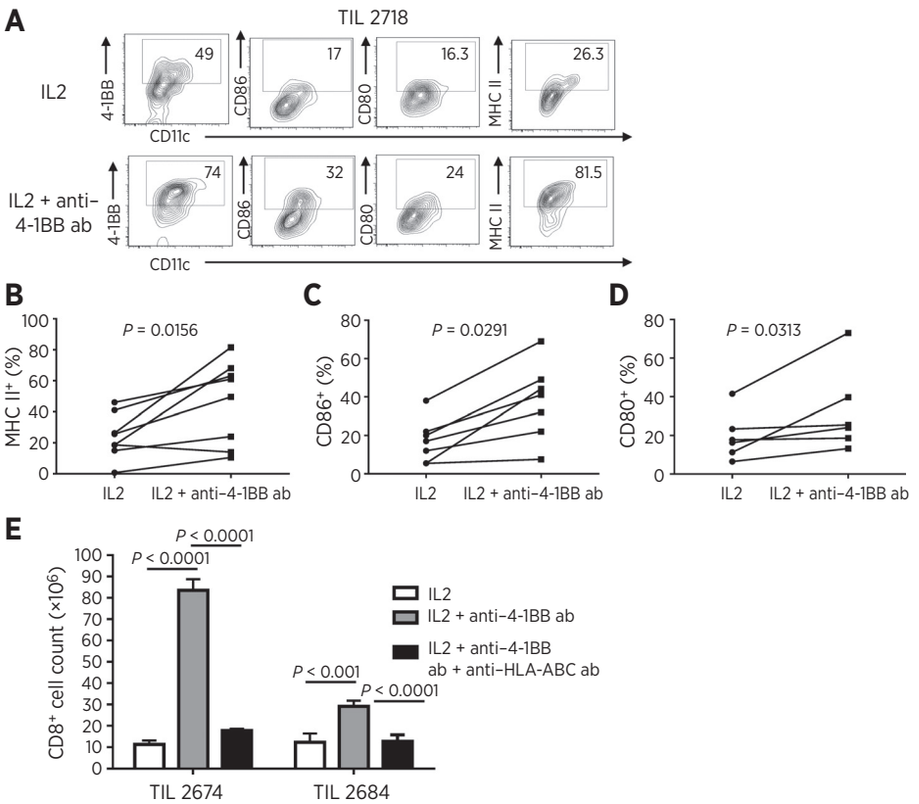

Figure 6.
Addition of anti–4-1BB antibody to the melanoma fragments modulates resident DC phenotype. The melanoma tumors were cut into multiple fragments and placed in culture with IL2 alone or IL2 and anti–4-1BB antibody for 1 week. After 1 week in culture, the fragments were mechanically disaggregated, filtered, and stained for DC and their activation markers. In one representative TIL sample (4 pooled fragments), we found that the resident DCs within the melanoma fragment expressed 4-1BB (A) and the addition of IL2 and the anti–4-1BB antibody increased CD86, CD80 and MHC II expression (A). When we observed the live, $CD3^-CD11c^+$ cells, we found that the DC had a more activated phenotype with increased MHC-II (B), CD86 (C), and CD80 (D) when anti–4-1BB antibody was added compared with the cultures grown in IL2 alone. This was demonstrated in eight independent TIL samples (32 representative fragments; 4 pooled fragments per TIL sample) for MHC II (B), seven independent TIL samples (28 representative fragments) for CD86 expression (C), and six independent TIL samples (24 representative fragments) for CD80 expression (D). The melanoma fragments were cut and set up with or without the addition of an anti–MHC-I (anti–HLA-ABC) antibody. After 3 hours, IL2 ± anti–4-1BB antibody was added to the cultures and the cultures were expanded over a 3-week period. After 3 weeks, the cells were stained using flow cytometry and viable cell counts were conducted. We found that when we blocked MHC-I, there was a decrease in the expansion of $CD8^+$ TIL, compared with the TIL expanded with IL2 and anti–4-1BB antibody (E) as demonstrated in two independent TIL lines (8 representative fragments). An unpaired, nonparametric, Mann–Whitney test was used to determine the statistical values for figures B–E.

(1, 3, 6, 24). There is growing interest in making the TIL expansion process faster and more practical. $CD8^+$ T cells have emerged to be a key subset in mediating clinical response (3). We recently demonstrated that patients with melanoma who responded to ACT using TIL had a significantly higher percentage of $CD8^+$ $BTLA^+$ cells (3). We have measured whether the addition of IL2 and anti–4-1BB antibody added during the rapid expansion (15) augmented the levels of $CD8^+BTLA^+$ in the post-REP TIL, but did not detect a significant difference between the IL2 alone condition versus IL2 and anti–4-1BB condition. However, we have not measured whether the addition of IL2 anti–4-1BB antibody added at the initial stages of melanoma TIL isolation and expansion augments the $CD8^+BTLA^+$ population.

A number of biologic issues arise in making the TIL initial expansion process not only faster to reduce the wait times for patients, but also enrich tumor-reactive T cells, especially $CD8^+$ that maintain effector-memory phenotypic properties for persistence after infusion. Although the culture of tumor fragments cut from resected metastatic lesions has been used as the most common approach for initial melanoma TIL expansion, there has been no attention paid to whether the microenvironment within these fragment cultures can be modulated to affect TIL expansion. Little is known about the dynamics of the early TIL expansion from tumor fragments and what processes take place within these small tumor pieces that can affect the yield and phenotype of the TIL. Given the emerging importance of tumor-reactive $CD8^+$ TIL in ACT and the identification of early-intermediate T-cell activation markers on these cells marking tumor-specific TIL subsets, we surmised that modulation of T-cell costimulatory pathways on these activated TIL, such as 4-1BB, may have profound effects on the yield of TIL and their phenotypic and functional properties that may be beneficial for ACT approaches. 4-1BB is a marker of tumor-reactive $CD8^+$ T cells (13) that is usually upregulated 24 to 48 hours after antigen exposure (15, 16, 25–27).

We investigated whether the provision of 4-1BB costimulation using agonist anti–4-1BB antibody at the initiation of TIL

expansion can modulate the tumor microenvironment within early tumor fragment cultures. The addition of anti–4-1BB antibody increased the rate of TIL expansion from tumor fragments, and increased the yield of tumor-specific $CD8^+$ T cells. Our data show that blockade of HLA class I during early tumor fragment cultures prevents the increased outgrowth of $CD8^+$ T cells induced by anti–4-1BB. This suggests that ongoing antigen presentation by cells within the tumor fragments stimulates resident $CD8^+$ T cells resulting in the upregulation of 4-1BB that is further costimulated with the agonist antibody. This was supported by the detection of NFκB activation and increased Ki67 expression in $CD8^+$ TIL isolated from the tumor fragments and from the surrounding culture environment after 4-1BB costimulation was provided. The 4-1BB antibody provides a potent signal enhancing $CD8^+$ costimulation leading to increased cell division in these early cultures. 4-1BB ligation, or other modulatory antibodies targeting OX40 for example, may inhibit Tregs and further facilitate the expansion of $CD8^+$ T cells in these early TIL cultures as well as alter the phenotype of $CD4^+$ TIL, but this will need to be tested in future studies.

To dissect the effects of anti–4-1BB in the early tumor fragment cultures, we examined its effects on other leukocyte subsets. We focused on tumor fragment-resident DC, as these cells are critical antigen-presenting cells in the tumor microenvironment for the activation of resident $CD8^+$ T cells. We found a subpopulation of DC expressed 4-1BB and addition of the agonist anti–4-1BB increased the expression of DC maturation markers such as MHC class II, CD86, and CD80. Increased expression of these DC maturation markers was associated with the induction of NFκB activation in the DC induced by 4-1BB costimulation. Data from *in vivo* tumor model systems have also shown that DC are critical in driving localized T-cell activation and division in the tumor microenvironment (28–30).

Activating the 4-1BB costimulatory pathway in leukocytes within tumor fragments at the earliest stages of TIL expansion from metastatic melanomas can profoundly affect the outgrowth of tumor-reactive TIL associated with the modulation of tumor fragment-resident DC. This approach can be applied clinically to rapidly expand TIL enriched for tumor specificity due to the availability of clinical-grade human or humanized anti–4-1BB antibodies. Our work sets the stage for testing the effects of other immunomodulatory agents to manipulate the tumor microenvironment. This approach can be applied to improve the output of TIL from solid tumors that have proven more difficult to expand tumor-reactive $CD8^+$ T cells maintaining effector and memory properties. The advantage of our approach, is that it can feasibly also be used with small tumors or biopsies to enhance the outgrowth of tumor-specific $CD8^+$ TIL without excessive upfront manipulation beyond cutting up the tumor into fragments for culture. This may be more amenable for clinical-grade TIL production and out-scaling TIL therapy.

Disclosure of Potential Conflicts of Interest

J. Weber is a consultant/advisory board member for Bristol-Myers Squibb and Merck. L. Radvanyi is an employee of Lion Biotechnologies, and is a consultant/advisory board member for Merck and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.A. Chacon, A.A. Sarnaik, J.Q. Chen, C. Creasy, C. Kale, J. Robinson, J. Weber, S. Pilon-Thomas, L. Radvanyi

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.A. Chacon, A.A. Sarnaik, C. Kale, L. Radvanyi

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