Research Article

Cancer Immunology Research

Requirement for Innate Immunity and CD90$^+$ NK1.1$^-$ Lymphocytes to Treat Established Melanoma with Chemo-Immunotherapy

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Abstract

We sought to define cellular immune mechanisms of synergy between tumor-antigen–targeted monoclonal antibodies and chemotherapy. Established B16 melanoma in mice was treated with cytotoxic doses of cyclophosphamide in combination with an antibody targeting tyrosinase-related protein 1 (αTRP1), a native melanoma differentiation antigen. We find that Fcγ receptors are required for efficacy, showing that antitumor activity of combination therapy is immune mediated. Rag1$^{-/-}$ mice deficient in adaptive immunity are able to clear tumors, and thus innate immunity is sufficient for efficacy. Furthermore, previously treated wild-type mice are not significantly protected against tumor reinduction, as compared with mice inoculated with irradiated B16 alone, consistent with a primarily innate immune mechanism of action of chemo-immunotherapy. In contrast, mice deficient in both classical natural killer (NK) lymphocytes and nonclassical innate lymphocytes (ILC) due to deletion of the IL2 receptor common gamma chain IL2γc$^{-/-}$) are refractory to chemo-immunotherapy. Classical NK lymphocytes are not critical for treatment, as depletion of NK1.1$^+$ cells does not impair antitumor effect. Depletion of CD90$^+$NK1.1$^-$ lymphocytes, however, both diminishes therapeutic benefit and decreases accumulation of macrophages within the tumor. Tumor clearance during combination chemo-immunotherapy with monoclonal antibodies against native antigen is mediated by the innate immune system. We highlight a novel potential role for CD90$^+$NK1.1$^-$ ILCs in chemo-immunotherapy. *Cancer Immunol Res; 3(3); 296–304. ©2015 AACR.*

Introduction

Immunotherapy has yielded exciting results in clinical cancer care. Ipilimumab (Yervoy; Bristol-Myers Squibb), an anti-cytotoxic T-lymphocyte antigen 4 (CTLA-4) antibody, was FDA approved in 2011 for the treatment of metastatic melanoma; pembrolizumab, an anti-programmed cell death 1 (PD-1) antibody (pembrolizumab, Keytruda; Merck), was approved for melanoma in 2014 with a reported response rate of 38% (1, 2). The response rate to ipilimumab doubles when it is combined with dacarbazine, and multiple studies combining pembrolizumab with chemotherapy are ongoing (3–5). However, the role of chemotherapy in combination with immunotherapy is yet to be established. It is not known how chemotherapy may affect overall survival in patients treated with chemo-immunotherapy, particularly as the reported survival at 4 years after treatment with the combination did not appear to differ significantly from survival rates with ipilimumab alone (6).

Similarly, chemotherapy is known to enhance the response rates of tumor-antigen–targeted monoclonal antibodies (mAb), and the combination regimens have documented efficacy against solid tumors of the breast, head and neck, and colon. Tumor-antigen–targeted mAbs used as a single agent have limited clinical response rates of 8% to 10%, and when used in combination with radiotherapy or chemotherapy, the response rates increase up to 50% (7). Thus, chemotherapy significantly improves the clinical benefit of tumor-antigen–targeted mAbs. The antitumor activity of combination therapy using tumor-antigen–targeted mAb is complex, and effector mechanisms via both innate and adaptive immunity have been proposed. Furthermore, results from recent studies have suggested that there are commonalities in therapy-induced immunologic mechanisms of response between solid tumor types (8). An understanding of the mechanisms whereby chemotherapy improves the efficacy of tumor-antigen–targeted

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mAbs would inform the design of future combination trials using tumor-antigen–targeted mAbs as well as other immunotherapies.

Tumor-antigen–targeted mAbs can eliminate cancer cells in patients through both immune-mediated and non–immune-mediated mechanisms (9). Some antibodies, such as trastuzumab (Herceptin; Genentech), target an oncogenic protein HER2/neu, and are hypothesized to disrupt oncogenic signaling pathways (10). Other antibody targets, such as CD20 (Rituxan; Genentech), have no established role in carcinogenesis, but ligation of these molecules is nevertheless efficacious in the treatment of lymphoma, most likely via binding of effector cells to the Fc domain of the tumor-bound antibody (11). Established immune mechanisms for antitumor mAbs include complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and induction of adaptive immunity. CDC occurs when complement binds and lyses tumor cells ligated by antibody. In contrast, ADCC occurs when activating Fc receptors, expressed on the surface of innate immune cells, bind to the Fc domain of antibodies and activate killing mechanisms. Fc receptor genotype has been shown to affect clinical response to many antitumor antibodies, validating the importance of this mechanism in patients (12, 13). Finally, adaptive immunity has been proposed as a contributor to antitumor efficacy, and preclinical studies have shown that tumors coated with antibody can be phagocytosed by antigen-presenting cells, improving the generation of T-cell responses against the tumor and yielding a vaccine effect (14).

To define the mechanisms of synergy between chemotherapy and mAbs, we treated established melanoma with a combination of an IgG2a murine antibody to tyrosinase-related protein 1 (αTRP1) and cyclophosphamide. TRP1 is a native self-differentiation antigen against which normal tolerance is well established, and it is expressed by melanomas and melanocytes. A humanized analogue to TRP1 has been tested in clinical trials (15). Previous studies have shown that αTRP1 protects mice from B16 melanoma when administered synchronously but αTRP1 is not protective when administered after tumor engraftment (16). It is important to note that although TRP1 is an intracellular melanosome antigen, it is expressed on the tumor-cell surface *in vivo* (17, 18), and efficacy of αTRP1 monotherapy for nonestablished tumors is dependent on Fc receptors (19). However, the specific cellular effector mechanism is unknown with roles proposed in the literature for natural killer (NK) cells, macrophages, and CD4$^+$ T cells (19–21).

When established melanoma is treated with αTRP1 and cyclophosphamide, we find that the innate immune system is fully competent to mediate tumor clearance in the absence of adaptive immunity, but that Fc receptors are required. Furthermore, we see no significant evidence that antibody therapy produces long-term protection against reinoculation with B16. We find a requirement for innate lymphocytes (ILC), as treatment is not effective in animals with a deficiency in the gamma chain of the IL2 receptor (*IL2γc*$^{-/-}$; refs. 22–24). Among the ILC populations, we find that CD90$^+$NK1.1$^-$ ILCs play a key role in tumor clearance. CD90$^+$NK1.1$^-$ cells found within the tumor do not express Fc receptors, but elimination of this population both limits therapeutic effect and diminishes tumor infiltration by macrophages, cells that have been proposed to play a role in the activity of anti-TRP1 (19, 25, 26). ILCs lack rearranged antigen receptors and function in lymphoid organogenesis, tissue remodeling, antimicrobial immunity, and inflammation (27). This work suggests a potential role for innate lymphoid cells in the efficacy of chemo-immunotherapy using tumor-antigen–targeted mAbs.

Materials and Methods

Mice

C57BL/6 mice (6–8 weeks) were obtained from NCI, B6.129P2-Fcer1gtm1Rav N12 (*FcγR*$^{-/-}$) and B6.129 mice from Taconic, and *Rag1*$^{-/-}$ B6.129S4-IL2rgtm1Wjl/J (*IL2γc*$^{-/-}$) mice from The Jackson Laboratory. All mice were female and weighed 16 to 22 g. All experiments were performed in compliance with the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee approved protocol (LA09-00405).

Cell lines and antibodies

The B16 F10 melanoma cell line was obtained courtesy of Drs. Alan Houghton and Jedd Wolchok (Memorial Sloan Kettering Cancer Center, New York, NY) in 2010. Hybridoma cell lines producing αTRP1 (TA99), isotype control C44 IgG2A anti-colchicine (C44), anti-NK1.1 (PK136), and anti-CD8 (2.43) were purchased from the ATCC and grown in hybridoma serum-free media (Invitrogen) in 2010. The cell lines were not tested or authenticated.

Production of antibodies

Hybridoma cell lines were grown in SFM hybridoma medium (Life Technologies), and then transferred into the small chamber of CELLine bioreactor flask (Integra Biosciences AG). After 2 weeks, the supernatant was removed and filtered with a 0.22-μm filter (Fisher Scientific) and antibody was purified using PD-10 desalting columns according to the manufacturer's protocol (GE Healthcare). Each dose of TA99 was titrated for antitumor activity in mice synchronously injected with B16 tumor cells before use in experiments.

Tumor inoculation and monitoring of tumor growth

Mice were inoculated s.c. on the right flank with 75,000 B16 cells. Tumor growth was monitored via biweekly measurement of perpendicular diameters and height using a caliper. Animals that were moribund or had tumors greater than 300 mm^3 were scored as dead in accordance with our IACUC protocol.

Cyclophosphamide and mAb treatment

Mice were injected i.p. with cyclophosphamide (Baxter) at 225 to 250 mg/kg on day 4 to 7 after tumor inoculation. One day after cyclophosphamide was administered, mice were injected i.p. with 500 μg of αTRP1 followed by two injections per week of the same dose for a total of seven doses.

In vivo antibody depletions

Mice were injected i.p. on day −1 with 500 μg αNK1.1 (PK136; ATCC), or with 250 μg αCD90 (30H12; Bio X cell), or with 500 μg αCD8; injections were repeated weekly for the duration of the experiment.

Flow cytometry

For analysis of tumor-infiltrating leukocytes, mice bearing tumors at least 300 mm^3 (newly scored dead on survival curve) were sacrificed by CO_2 inhalation and tumors were dissected. The tumor mass was homogenized, digested with 0.4 mg/mL

collagenase (Sigma-Aldrich), incubated at 37°C for 1 hour, and strained through 100-μm nylon filters (Fisher Scientific). Cells were incubated with antibodies for 25 minutes at 4°C, acquired with a BD LSRII Flow Cytometer or a BD Fortessa Flow Cytometer, and analyzed with FlowJo software (TreeStar). Fluorochrome-conjugated antibodies specific for mouse CD45 (clone 30-F11), CD3e (clone 500A2), B220 (clone RA3-6B2), NK1.1 (clone PK136), NKp46 (clone 29A1.4) or CD11b (clone M1/70), CD103 (clone CD103), F480 (clone BM8), CD11c (clone N418), GR1 (clone RB6-8C5), CD64 (clone X34-5/7.1), CD16/32 (clone 93), mouse IgG PE control (clone M1-14D12), and IA/IE (clone M5/114.15.2) were obtained from eBioscience Inc. and CD90 (clone 53-2.1) from BD Biosciences. For stains, including CD64 antibody, cells were incubated in a 50 μL volume with 5 μg IgG (BioXCell), 1 μg anti-CD16/32 (clone 2.42G2; BD Biosciences), and 5 μg anti-FcRIV (Clone 9E9; The Rockefeller University, New York, NY) alone and in combination for 15 minutes before addition of antibody.

Statistical analysis

Statistical analysis was performed using Prism Software (GraphPad Software, Inc.). Survival analysis was performed using the log-rank (Mantel–Cox) test with Bonferroni correction. Significance was defined at a P value of <0.05, using a nonparametric Mann–Whitney t test. Bonferroni correction was used for multigroup comparisons.

Results

Synergistic antitumor benefit is achieved through treatment of established melanoma with cytotoxic dose of cyclophosphamide combined with anti-TRP1 mAb

To understand the mechanism of combination therapy, including antitumor antibody and chemotherapy, we established a murine treatment model using B16 melanoma (Fig. 1A). Mice received cyclophosphamide at cytotoxic doses (250 mg/kg), resulting in tumor growth arrest. Similar to what is observed

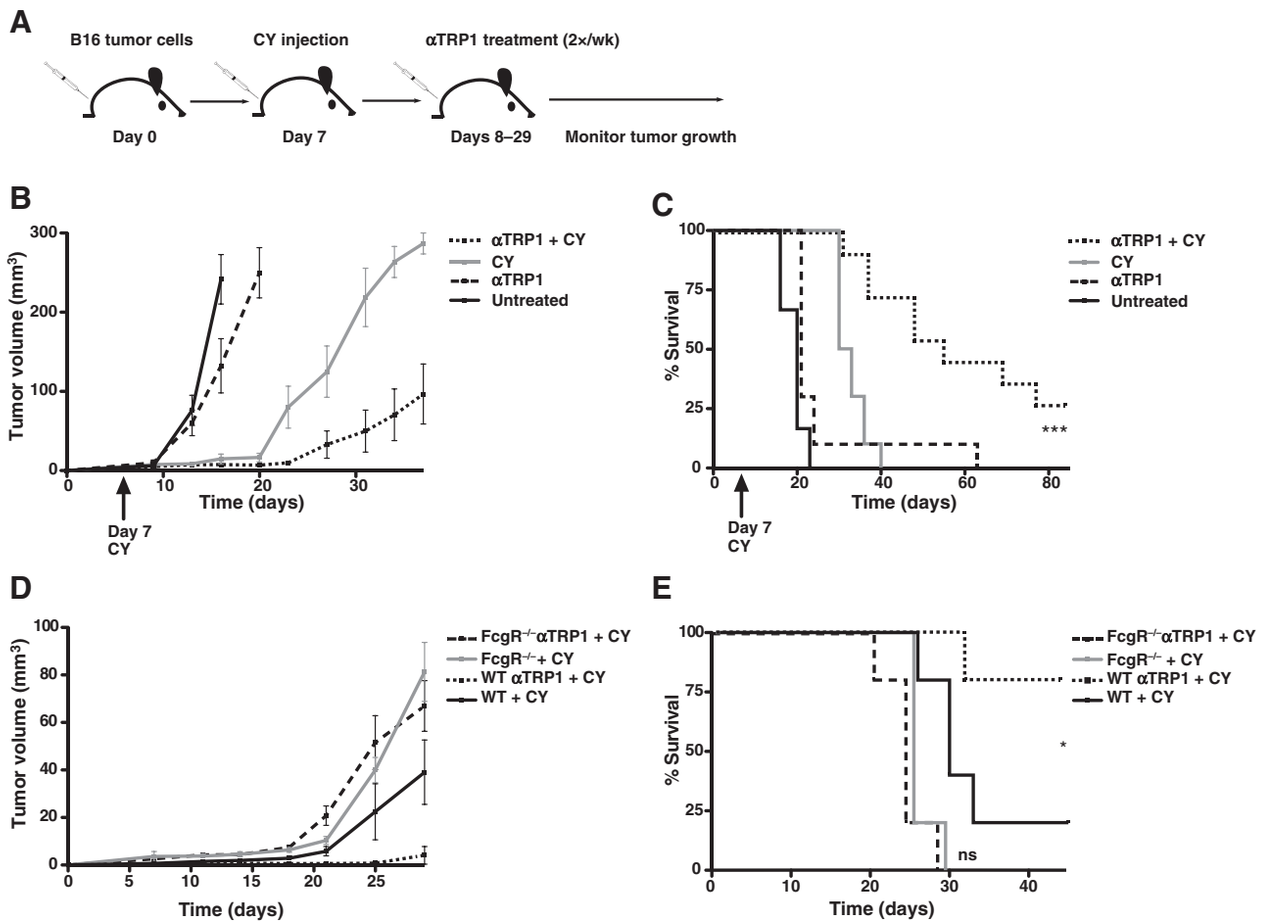

Figure 1.
αTRP1 combined with cyclophosphamide (CY) has efficacy in established melanoma. A, C57BL/6 mice (n = 9–11/group) were inoculated with B16 on day 0 and treated with 250 mg/kg of cyclophosphamide on day 7 followed by 0.5 mg of αTRP1 or isotype control (C44) biweekly for 3 weeks. Tumor volumes were assessed biweekly. B, mean tumor size is shown for each group. Note that curves are cut off when mice started to die in each group and can distort the growth curves. C, survival was statistically increased in the combination therapy group compared with that of cyclophosphamide alone (P = 0.0002), αTRP1 alone (P = 0.0002), and untreated (P < 0.0001). C and D, the same protocol was applied to mice on the B6.129 background with or without deletion of the Fc receptor common γ chain (FcγR$^{-/-}$). Survival was statistically increased in the B6.129 mice compared with FcγR$^{-/-}$ receiving combination treatment (P = 0.002). *, P < 0.05; ***, P < 0.0001.

clinically in patients with cancer, this growth arrest was temporary, and all animals receiving only cyclophosphamide subsequently grew large tumors (Fig. 1B and 1C). Meanwhile, αTRP1 mAb alone had minimal impact on the growth of established tumors. The addition of αTRP1 to cyclophosphamide treatment significantly improved survival, with some animals displaying long-term responses (Fig. 1B and C). Pigmented scars were observed at the site of tumor inoculation in long-term responders, showing appropriate tumor engraftment. Cyclophosphamide has also been reported to have favorable immunologic effects at lower doses through the selective depletion of regulatory T cells (28). Although low-dose cyclophosphamide (150 mg/kg) synergized with αTRP1, it did not produce long-term responders, the clinically desired outcome, even when treatment was administered before tumors were visible (day 4; Supplementary Fig. S1). Thus, a treatment model using cytotoxic doses of cyclophosphamide was used for all subsequent studies as it achieves the clinically relevant outcome of long-term survival benefit in established tumors.

Efficacy of combined therapy with αTRP1 and cyclophosphamide is Fc receptor-dependent

Tumor-antigen–targeted antibodies induce tumor regression through both immune and nonimmune mechanisms. Antibodies can mediate tumor-cell elimination in a mechanism dependent on Fc receptors expressed on the surface of immune cells (29). Thus, we investigated whether the mechanism of action of combination therapy was dependent on ligation of Fc receptors by the antibody. Prior work with αTRP1 has shown that it prevents tumor engraftment as a single agent in prophylactic models (administered synchronously with tumor) in a manner dependent on activating Fc receptors (19). This is consistent with the proposed mechanisms of action of multiple antibodies in clinical use, including αher2neu and αCD20, antibodies known to act via Fc receptors (30). We tested our combination regimen in mice deficient in the common γ chain required for expression of activating Fc receptors (Fig. 1D and E; ref. 31). Treatment was not effective in Fc receptor–deficient animals, confirming that chemotherapy does not alter the requirement for Fc receptors and that the immune system plays a necessary role in the efficacy of our combination treatment.

The innate immune system is competent to induce tumor clearance during therapy with αTRP1 + cyclophosphamide

By binding to Fc receptors, antibodies can eliminate tumor cells through ADCC, whereby antibody-bound tumor cells are ligated by Fc receptors on NK cells and macrophages. ADCC is mediated by innate immune cells and does not require antigen-specific T-cell or B-cell responses. However, a role for adaptive immunity in the efficacy of mAbs has been proposed for αTRP1 as well as αher2neu and αCD20. This is because antibodies can modulate antigen presentation through ligation of Fc receptors on dendritic cells and other antigen-presenting cells (APC), which can then interact with and activate T cells, potentially converting passive immunization into active immunization (32).

To test whether the adaptive immune system is required for efficacy of therapy with αTRP1 and cyclophosphamide, we treated *Rag1*$^{-/-}$ mice lacking all adaptive lymphocyte populations, including B and T cells, with the combined therapy (33). Therapeutic efficacy was completely preserved in *Rag1*$^{-/-}$ mice (Fig. 2A), establishing that cell populations in the innate immune

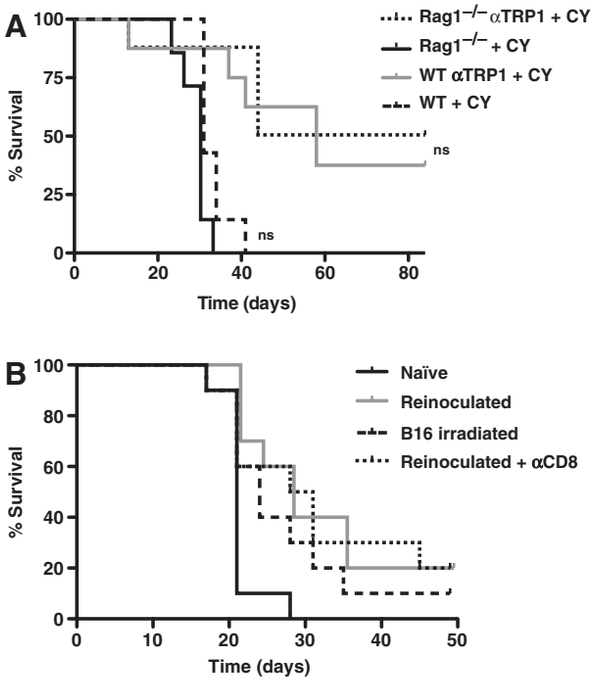

Figure 2.
Efficacy of αTRP1 combined with cyclophosphamide (CY) is dependent on the innate immune system. A, wild-type and *Rag1*$^{-/-}$ mice on B6 background (n = 7–8/group) were treated as described in A. Therapy with cyclophosphamide and αTRP1 prolonged survival in both wild-type and *Rag1*$^{-/-}$ mice (P = 0.0040). B, mice previously treated with cyclophosphamide and αTRP1, or depleted of CD8$^+$ T cells, were reinoculated with the same B16 clone 60 days following initial tumor inoculation. Control animals had received irradiated B16 60 days preceding tumor challenge and had not grown any visible tumors. Mice treated with combination immunotherapy did not have a significantly improved survival compared with mice previously exposed to irradiated B16 (P > 0.05), although they both had improved survival compared with completely naïve animals (P = 0.0169 and P = 0.0057, respectively).

compartment are competent to mediate effective chemo-immunotherapy. Thus, therapeutic efficacy of chemotherapy combined with mAb is mediated by the innate immune compartment with requirement for Fc receptors.

Therapy with αTRP1 + cyclophosphamide does not induce long-term protection against tumor reinoculation

On the basis of results from the above experiments, we concluded that efficacy of our combination regimen is mediated by the innate immune system, most likely via ADCC. However, considerable evidence in the literature showed that mAbs can improve antigen presentation (34, 35). We reasoned that adaptive immunity may be induced during treatment even if it is not essential for initial tumor clearance. Thus, we sought to test whether our combination regimen produces a vaccine-like effect. The value of successful immunotherapy is achieving long-term protection. To test whether chemo-immunotherapy induces long-term protection, we reinoculated surviving mice who did not exhibit tumor progression after treatment with cyclophosphamide and αTRP1 60 days after the previous challenge (Fig. 2B). Survival was significantly improved in the treated mice compared with naïve controls, yet not improved relative to

untreated mice inoculated 60 days earlier with irradiated B16 cells. Thus, although some long-term protection was induced, it is attributable to exposure to the B16 cells and was not specific to immunotherapy with αTRP1 combined with cyclophosphamide. Furthermore, depletion of $CD8^+$ T cells, hypothesized to be activated through antibody-mediated cross-priming, did not affect the long-term protection that was achieved (Fig. 2B). Thus, although prior exposure to B16 tumor cells confers a slight degree of protection, the addition of the antibody and cyclophosphamide does not further enhance long-term adaptive antitumor immunity. These results confirm an innate immune mechanism of action for this combination treatment.

$NK1.1^-CD90^+$ cells, but not classical $NK1.1^+$ cells, mediate antitumor efficacy during treatment with chemo-immunotherapy

As T and B cells are not required for efficacy of our treatment, and a protective T-cell response is not induced, we next tested whether lymphocytic populations, as opposed to myeloid cells, are critical for the efficacy of our combination regimen. We inoculated mice lacking the common gamma chain of the IL2 receptor ($IL2\gamma c^{-/-}$), which have a global defect in lymphocyte development (22). Therapeutic efficacy was abrogated in $IL2\gamma c^{-/-}$ mice (Fig. 3A), showing that lymphoid populations are required for tumor eradication.

Two populations of lymphocytes found in $Rag1^{-/-}$ mice, but lacking in $IL2\gamma c^{-/-}$ mice, are NK cells and nonclassical ILCs (23, 24, 36). NK cells express activating Fc receptors and can kill tumor cells via ADCC (16, 17). However, in line with prior work by the Houghton and Ravetch laboratories when using the beige/SCID model (19), depletion of NK1.1 cells within the tumor did not affect the activity of combination therapy (Fig.s 3B and Supplementary Fig. S2). Phenotyping of lymphocytes within the tumors from $Rag1^{-/-}$ mice revealed an additional population of ILCs present within the tumor that are negative for NK1.1 but express the common lymphocyte marker and adhesion molecule CD90 (Fig. 4A). $IL2\gamma c^{-/-}$ mice are deficient in NK cells but also in other ILCs, including lymphoid tissue inducer (LTi) cells because of defective signaling through the IL7 receptor (37). To define the role of ILC populations in chemo-immunotherapy, we depleted $Rag1^{-/-}$ mice of $CD90^+$ cells and then treated the mice with either cyclophosphamide alone or cyclophosphamide in combination with αTRP1. CD90 depletion decreased infiltration of $CD90^+NK1.1^-$ cells into the tumor and resulted in diminished efficacy of treatment (Fig. 4B and Supplementary Fig. S2). Therefore, ILCs expressing CD90, but not NK cells, are required for antitumor response induced by this combined regimen.

$NK1.1^-$ $CD90^+$ cells express RORγt and lack Fcγ receptors

Innate lymphoid cells are dependent on the transcription factor Id2 and can be broadly classified into subgroups depending on their function and marker expression. LTi cells are characterized by their dependence on retinoic acid receptor-related orphan receptor gamma t (RORγt; ref. 24). To better identify our cell population of interest, we analyzed the phenotype of $NK1.1^-$ $CD90^+$ cells. They were positive for the expression of RORγt and negative for NKp46 (Fig. 4C). This is consistent with an ILC3/LTi phenotype (38). To determine whether the $NK1.1^-$ $CD90^+$ cells might mediate ADCC, we analyzed the population for expression of Fcγ receptors. Our results show that these cells lack the expression of both FcγRI and FcγRII/III (Fig. 4C). Anti-TRP1 mAb treatment efficacy is known to be dependent on FcγRI and FcγRIV in melanoma (19, 39, 40), and our data highlight that activating Fc receptors are required for therapeutic efficacy of αTRP1 and cyclophosphamide. Thus, $NK1.1^-$ $CD90^+$ cells are not likely the direct effectors of our combination therapy and must instead function as mediators in conjunction with other cells that express activating FcRs.

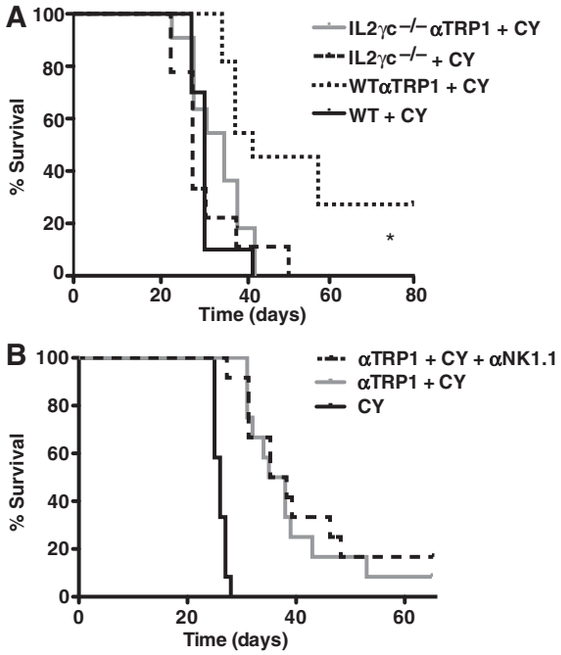

Figure 3.
Depletion of $NK1.1^+$ innate lymphoid cells did not alter efficacy. A, mice deficient in the $IL2\gamma c^{-/-}$ and wild-type controls (n = 9–11/group) were treated with cyclophosphamide (CY) and αTRP1 or isotype control as in schema shown in Fig. 1A. Survival was enhanced in the wild-type ($P = 0.0002$) animals but not in $IL2\gamma c^{-/-}$ mice ($P = 0.2380$). B, mice (n = 10–11) were treated with cyclophosphamide plus αTRP1 or isotype control and administered i.p. α-NK1.1, or given isotype control. Survival in mice depleted of $NK1.1^+$ cells and receiving combination therapy was statistically significant when compared with that in isotype control mice ($P < 0.0001$) but not compared with control animals receiving combination therapy. *, $P > 0.05$.

$F4/80^+$ macrophage cell infiltration is induced by cyclophosphamide and αTRP1 combination therapy and abrogated in CD90-depleted mice

ILCs are known to regulate myeloid-cell populations and have been implicated in mediating antitumor efficacy via recruitment of other leukocytes into the tumor (37, 41). Multiple prior studies have proposed a role for macrophages in the antitumor mechanism of anti-TRP1 (19, 40, 42). To test the hypothesis that ILCs facilitate entry of macrophages, we examined the quantity and composition of myeloid cells infiltrating the tumor. These populations do not express CD90, and we confirmed this within the tumor setting (Supplementary Fig. S4). Depletion of $CD90^+$ lymphocytes resulted in a net reduction of macrophages and granulocyte receptor-1 antigen (Gr1) low myeloid cells, but not of Gr1 intermediate or Gr1 high myeloid cells (Fig. 5A–C). These data show that $CD90^+$ cells alter the tumor microenvironment by

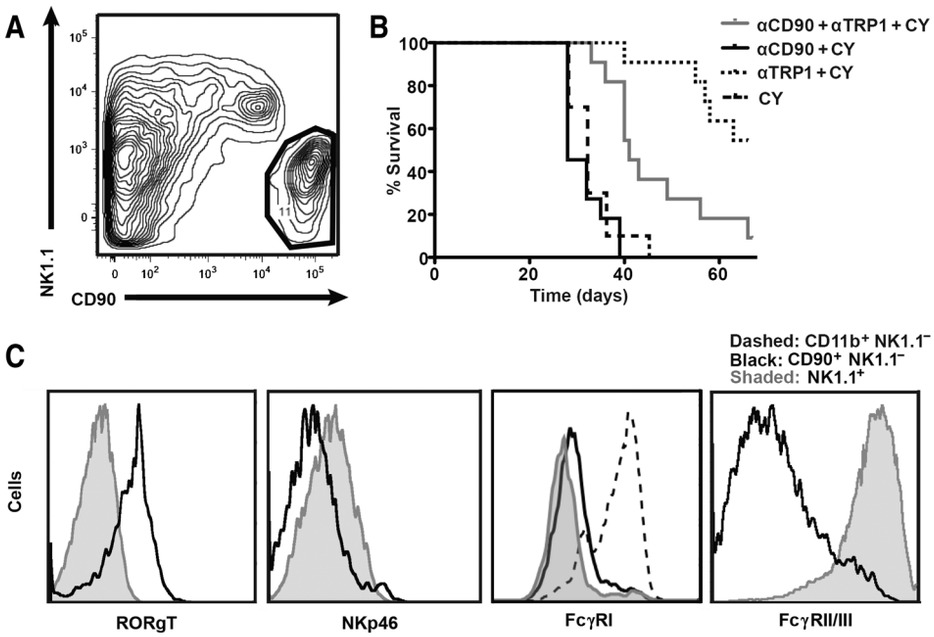

Figure 4.
Therapeutic efficacy of αTRP1 combined with cyclophosphamide (CY) is dependent on signaling through the IL2 receptor common γ chain and mediated by CD90$^+$ NK1.1$^-$ innate lymphoid cells. A, tumors from *Rag1*$^{-/-}$ mice were homogenized, digested with collagenase, and stained with antibodies as described in Materials and Methods. Results shown (11% CD90$^+$ NK1.1$^-$ cells) are gated on CD3$^-$B220$^-$ lymphocytes and are representative of 5 mice. B, mice ($n = 10$–11) were treated with cyclophosphamide plus αTRP1 or isotype control and administered i.p. α-CD90, or given isotype control. Survival in mice depleted of CD90$^+$ cells and receiving combination therapy was statistically significant when compared with that in isotype control mice ($P = 0.0037$). C, phenotyping of CD90$^+$ NK1.1$^-$ population in tumors of wild-type mice shows positive expression of RORγt and negative expression of NKp46, FcγRI, and FcγRII/III compared with CD90$^-$NK1.1$^+$ and CD11b$^+$ NK1.1$^-$ (for FcγRI only) population (representative of 5 tumors).

modulating the numbers of macrophages and myeloid-derived suppressor cells entering into the tumor bed.

Discussion

Chemo-immunotherapy regimens using antitumor mAbs are widely used in clinical practice, inducing high response rates in patients with advanced solid tumors. However, the mechanisms of antitumor activity of chemo-immunotherapy in the clinic are not clearly delineated. In our study, we use an antibody targeting TRP1, a syngeneic antigen expressed on murine melanoma and melanocyte tissues, in combination with cyclophosphamide to treat established melanoma. TRP1 has been shown to be both an intracellular and a surface antigen on B16 tumor cells *in vivo*, making it a good target for antibody binding. This melanoma model is suitable to mimic the clinical setting in immunotherapy for solid tumors because the B16 cell line is not genetically manipulated and TRP1 antigen is endogenous to the mouse. Thus, there is low potential for artifact due to cross-species differences in protein sequence that can lead to adaptive immunity as may be observed in other models.

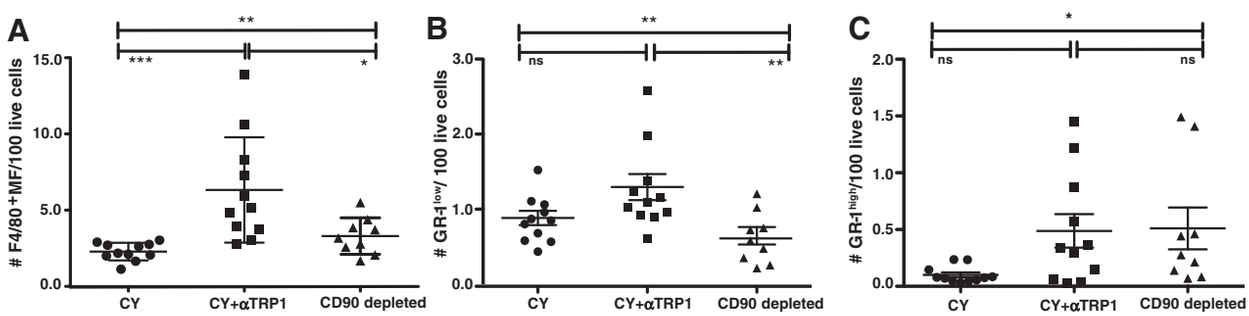

Figure 5.
Effect of CD90 depletion on infiltration of myeloid cells. A, mice ($n = 10$–11) were treated with cyclophosphamide (CY) plus αTRP1 or with isotype control and administered either αCD90 i.p. or given isotype control. Each data point represents one animal. B, Gr-1^{low} CD11b$^+$ immune suppressor cells had differences in CD90-depleted mice compared with CD90$^+$ mice ($P = 0.0076$), while (C) Gr-1^{high} CD11b$^+$ immune suppressor cells showed no significant difference in the two groups ($P > 0.05$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$; ns, not statistically significant.

We find that the antitumor activity of the combined αTRP1 mAb and cyclophosphamide regimen depends on Fc receptors, which are known to recognize mAbs bound to tumor antigen leading to ADCC and/or CDC (19). This is consistent with several prior studies published by the Ravetch and Houghton laboratories using anti-TRP1 as a single agent (19, 25, 43, 44). To distinguish between innate and adaptive immunity, we tested therapeutic efficacy in $Rag1^{-/-}$ mice, which lack all adaptive immune responses. Strikingly, therapy was equally efficacious in both $Rag1^{-/-}$ and control mice, highlighting the importance of innate immunity in the antitumor activity of this chemo-immunotherapeutic regimen. We show that efficacy is abrogated in $IL2\gamma c^{-/-}$ mice lacking ILCs, and that depletion of $CD90^+$ cells, but not $NK1.1^+$ cells, impairs the efficacy of the combined αTRP1 mAb and cyclophosphamide regimen. Phenotyping the ILCs within the tumor revealed an absence of expression of Fc receptors. Thus, these cells most likely not direct effector cells in tumor suppression. However, depletion of $CD90^+$ cells also impaired macrophage penetration into the tumor, consistent with a role for macrophages in tumor clearance. Our data add to prior literature by confirming a predominately innate mechanism of action of the anti-TRP1 antibody and a previously proposed role for macrophages, while identifying a potential new role for ILCs in promoting tumor regression.

Tumor eradication by anti-TRP1 combined with chemotherapy is mediated by the innate immune system. Our data complement work published by Park and colleagues (45) regarding the potential of chemotherapy to ablate the adaptive immune response induced by mAb therapy against her2neu. Thus, although there is significant evidence that chemotherapy at lower doses may be immune-stimulatory, higher doses required to induce significant tumor killing may adversely affect adaptive immune responses (26). In this regard, our work differs in its conclusions from recent studies proposing a vaccine effect induced by mAbs (11, 45). The fact that we do not observe significant long-term protection upon tumor rechallenge may be due to both the presence of the high-dose chemotherapy and the nature of our model where neither the antigen nor the tumor has been genetically manipulated to favor an adaptive immune response.

In this work, we do not wish to imply that T-cell responses cannot be induced by antibody. T-cell responses have been observed in breast cancer patients treated with anti-her2neu (46), and intriguingly some activation of adoptively transferred anti-melanoma PMEL T cells did occur in the context of therapy as shown in Supplementary Fig. S5. However, clinical experience with vaccine studies has demonstrated that heightened stimulation of peripheral T cells often does not translate into impactful antitumor immunity because of the immunosuppressive nature of the tumor microenvironment (47–49). Thus, although minimal protective immunity was observed in long-term responders to initial treatment with αTRP1 + cyclophosphamide, this immunity was not enhanced by inoculation with irradiated B16. This result suggests that the mild immunogenicity came from the B16 tumor cells and not from the combined therapy and that, in the context of high-dose cytotoxic chemotherapy, further immune stimulation would be required to produce clinically relevant long-term immunity. These results highlight the fact that long-term remission with strong protective immunity is rarely induced in solid tumors through combined mAb and chemotherapy.

Within the context of the innate immune system, our data highlight a potential role for $NK1.1^-$ ILCs in the efficacy of combination therapy with cyclophosphamide and αTRP1. Although treatment was effective in $Rag1^{-/-}$ mice, $IL2\gamma c^{-/-}$ animals did not benefit. Notably, the common gamma chain of the IL2 receptor is required for the growth and development of both NK cells and ILCs (50). Furthermore, depletion of $CD90^+$ cells, but not NK1.1 cells, diminished the benefits of treatment. This is supported by prior work showing that NK cells are not required for activity of anti-TRP1 as a single agent (16, 17, 19). We identified a population of cells in the tumor infiltrate consistent with LTi cells, which were recently classified by Spits and colleagues (24, 51) as group 3 ILCs and phenotyped as $CD90^+$ $ROR\gamma t^+$ $NKp46^-$. LTi cells have been implicated in secondary lymphoid organogenesis and secrete IL17A and IL22 upon stimulation, suggesting an effector role in immune function (52). They have been shown to mediate cross-talk of myeloid cells via secretion of GM-CSF and may induce immune tolerance by recruiting myeloid suppressor cells (41). Furthermore, we find that $NK1.1^-$ ILCs within the tumor do not express FcRs; therefore, it is unlikely that they are the sole or primary mediators of antitumor activity for the combined regimen of αTRP1 and cyclophosphamide.

It is intriguing that depletion of $CD90^+$ cells decreases infiltration of macrophages into the tumor. The function of ILCs in tumorigenesis is poorly understood. Recent results from Eisenring and colleagues (37) implicated $NKp46^+$ LTi cells as mediators of IL12-initiated tumor rejection in a B16 melanoma model. $NKp46^+$ LTi cells induced upregulation of adhesion molecules vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM) in the tumor vasculature allowing for increased leukocyte infiltration. In contrast, Shields and colleagues (20) showed that ILCs, including LTi cells, recruited to the tumor site via tumor-produced chemokine CCL21 enhanced tumor growth by promoting infiltration of regulatory T cells and other immunosuppressive populations. Thus, ILCs may modulate the immune microenvironment and recruit either suppressive or effector populations, depending on the presence of additional immunostimulatory molecules. Chemotherapy and/or antitumor antibody may affect the effect of ILCs by altering the phenotypes of myeloid cells within the tumor. Low-dose cyclophosphamide has recently been implicated in inducing the transient release of cytokines such as CCL4, IL8, VEGF, and TNFα from tumor cells, sensitizing the tumor microenvironment to macrophage-mediated phagocytosis when combined with antibody treatment (53). Thus, LTis may play different roles depending on the local microenvironment, and may play a beneficial role when leukocyte infiltration is required for therapeutic benefit.

In the context of αTRP1, macrophages likely play a proactive role in tumor clearance by binding tumor cells opsonized by the antibody. This hypothesis is consistent with prior work by several groups showing a role for macrophages in tumor clearance mediated by anti-TRP1 (19, 40, 42), and that CD11b is required (54). A recent study by Gul and colleagues (42) identified a role for Kupffer cells in the clearance of B16 cells inoculated into the mouse liver. Our model suggests that macrophages may play a similar role in chemotherapy and αTRP1 treatment of established tumors. Although we observed a correlation between the presence of $CD90^+$ ILCs and increased infiltration of macrophages, it is unknown whether

the effect was causative or if there are additional regulating factors involved. Further investigation is necessary to understand this relationship between ILCs and myeloid cells in tumorigenesis. Although our results show potential roles for group 3 ILCs in the efficacy of chemo-immunotherapy in mice, the role of the human ILC3 counterpart may differ. Human ILC3s have been implicated in the development of colorectal carcinoma, wound repair, and the pathology in psoriasis (55). Their role in melanoma is unknown. Furthermore, our findings suggest a different mechanism of function from that seen in ipilimumab and anti–PD-1 immunotherapy in which adaptive immunity plays a larger role (56).

Our data identify CD90$^+$NK1.1$^-$ ILCs as important mediators in the efficacy of chemo-immunotherapy and highlight their potential role as facilitators for macrophages entering the tumor. These results support the importance of the innate immune system in mediating effects of chemo-immunotherapy. Although, anti–PD-1 and anti–CTLA-4 have shown that activation of adaptive lymphocytes has tremendous potential in cancer care, some tumors remain refractory to checkpoint blockade and agents targeting other immune cells besides T cells may be required. Our results support the importance of the innate immune system in mediating effects of chemo-immunotherapy and thus the disease course. The role of NK1.1$^-$ innate lymphoid cells in the treatment of solid tumors using chemotherapy and tumor-antigen–targeted mAbs can be a potential source for therapeutic intervention and merits further study.

Disclosure of Potential Conflicts of Interest

A.G. Sikora reports receiving a commercial research grant from Advaxis Pharmaceuticals and is a consultant/advisory board member for Leerink Partners LLC. No potential conflicts of interest were disclosed by the other authors.

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