In vivo imaging reveals a tumor-associated macrophage–mediated resistance pathway in anti–PD-1 therapy

Sean P. Arlauckas, Christopher S. Garris, Rainer H. Kohler, Maya Kitaoka, Michael F. Cuccarese, Katherine S. Yang, Miles A. Miller, Jonathan C. Carlson, Gordon J. Freeman, Robert M. Anthony, Ralph Weissleder, Mikael J. Pittet

Monoclonal antibodies (mAbs) targeting the immune checkpoint anti–programmed cell death protein 1 (aPD-1) have demonstrated impressive benefits for the treatment of some cancers; however, these drugs are not always effective, and we still have a limited understanding of the mechanisms that contribute to their efficacy or lack thereof. We used in vivo imaging to uncover the fate and activity of aPD-1 mAbs in real time and at subcellular resolution in mice. We show that aPD-1 mAbs effectively bind PD-1–tumor-infiltrating CD8+ T cells at early time points after administration. However, this engagement is transient, and aPD-1 mAbs are captured within minutes from the T cell surface by PD-1–tumor-associated macrophages. We further show that macrophage accrual of aPD-1 mAbs depends both on the drug’s Fc domain glycan and on Fcγ receptors (FcγRs) expressed by host myeloid cells and extend these findings to the human setting. Finally, we demonstrate that in vivo blockade of FcγRs before aPD-1 mAb administration substantially prolongs aPD-1 mAb binding to tumor-infiltrating CD8+ T cells and enhances immunotherapy-induced tumor regression in mice. These investigations yield insight into aPD-1 target engagement in vivo and identify specific Fc/FcγR interactions that can be modulated to improve checkpoint blockade therapy.

INTRODUCTION

Immune checkpoint blockade is a recent development in cancer therapy that has shown remarkable results in certain cancers and patient groups (1–3). Currently approved immune checkpoint blockers are monoclonal antibodies (mAbs) that target the programmed cell death protein 1 (PD-1) or cytotoxic T lymphocyte–associated protein 4 pathways, and agents targeting other pathways are in clinical development (including OX40, Tim-3, and LAG-3) (4). Checkpoint inhibitors are used to reactivate exhausted tumor-specific T cells and reinstate cancer immunosurveillance (5, 6). Some cancer tissues limit antitumor immunity by up-regulating immunosuppressive factors such as PD-1 ligand (PD-L1) that binds to PD-1 on tumor-specific CD8+ T cells (7). Drugs targeting the PD-1/PD-L1 immune checkpoint axis can block immunosuppressive signals and enable T cell–mediated elimination of cancer cells (8). However, immune checkpoint blockade is not always effective, and we lack a complete understanding of the mechanisms that contribute to efficacy and resistance (9).

At present, experimental and clinical evidence suggests that a pre-existing tumor infiltrate of CD8+ T cells is one of the most favorable prognostic indicators of checkpoint inhibitor response (10). Also, patients with the highest degree of neoantigen burden (high mutational load) in their cancers may have increased tumor infiltration by T cells and more robust responses to checkpoint blockade (11, 12). Histology and sequencing methodologies have been used to define metrics of cytotoxic T cell infiltration in tumors (13), with a focus on the identification of tumor neoantigens and the resultant antigen-specific T cell expansion after immunotherapy (11). These studies have provided insight into the mechanism of anti–PD-1 (aPD-1)–induced antitumor T cell activation and spurred efforts focused on identifying new strategies that foster T cell recruitment to tumors (14–16).

Much less is known about checkpoint inhibitors in vivo pharmacokinetics and interactions with host components in the tumor bed. Studying these parameters is likely essential to identifying resistance mechanisms and developing improved therapeutic options. Here, we used intravital imaging to follow fluorescently labeled aPD-1 mAbs in real time and at subcellular resolution. Because tumor microenvironments are home to diverse host cell types, and immune checkpoint blockers are unlikely to solely act on T cells, we focused on aPD-1 mAb interactions with various host components by simultaneously assessing aPD-1 mAbs, tumor cells, CD8+ T cells, and myeloid cells/macrophages. We investigated tumor-infiltrating CD8+ T cells because they express PD-1 and are the expected targets of aPD-1 mAbs. We also investigated myeloid cells because they are frequently found in the stroma of growing tumors (17), and emerging evidence indicates that they can affect virtually all therapeutic modalities, including immunotherapy (18). Our results not only confirm existing knowledge on PD-1 inhibition but also uncover findings with therapeutic implications to further improve immunotherapy.

RESULTS

Global aPD-1 mAb biodistribution

We initially sought to track the temporal distribution of aPD-1 mAbs in vivo at the organ level. We thus covalently labeled aPD-1 mAb (clone 29F.1A12) with an Alexa Fluor 647 dye (AF647–aPD-1) using N-hydroxysuccinimide (NHS) chemistry. For maximal brightness without dye quenching, we optimized the labeling conditions to achieve about four fluorochrome molecules per antibody. For in vitro studies, the EL4 mouse lymphoma cell line was used as a T cell model because of its stable PD-1 expression and its broad adaptation to in vitro culture
(19). Using this cell line, we confirmed that fluorescent labeling of aPD-1 did not interfere with the drug’s binding specificity (Fig. 1A). In addition, AF647–aPD-1 retained therapeutic activity in the ovalbumin-expressing MC38 tumor model, which is responsive to single-agent aPD-1 therapy (Fig. 1B) (20). Collectively, these data indicate that AF647–aPD-1 retains PD-1 tropism and antitumor activities.

We next examined the in vivo biodistribution of AF647–aPD-1 in the wild-type MC38 tumor model, which also responds to aPD-1 treatment, but less efficiently than the ovalbumin-expressing MC38 counterpart in which aPD-1 treatment results in uniform tumor rejection. Mouse cohorts were sacrificed at times ranging from 0.5 to 72 hours after treatment, and organs were removed for fluorescence measurements (Fig. 1C). AF647–aPD-1 signal was primarily retained within tumors over time (Fig. 1D and fig. S1). We observed a large spike of AF647–aPD-1 in the liver, lungs, kidney, and spleen at 0.5 hours after injection, followed by a subsequent decrease over time that coincided with increases in AF647–aPD-1 signal in the tumor (Fig. 1D). Collectively, these data indicate that the drug started to accumulate in tumors within minutes after injection but that maximal aPD-1 accumulation in the tumor was achieved after 24 hours.

Cellular kinetics and dynamics of aPD-1

After observing that aPD-1 mAbs collect within the tumor microenvironment shortly after administration, we further aimed to study whether the drugs bind their intended target T cells at the tumor site. To this end, we used intravital microscopy in dorsal skinfold chambers, which enabled us to examine the distribution and tropism of AF647–aPD-1 at subcellular resolution within the tumor stroma and longitudinally after drug administration (Fig. 2A). The experimental system allowed simultaneous tracking of four components: aPD-1 mAbs (labeled with AF647), MC38 tumor cells (labeled with H2B-mApple), T cells [labeled with green fluorescent protein (GFP) or yellow fluorescent protein (YFP)], and tumor-associated macrophages (TAMs) (labeled with Pacific Blue–dextran nanoparticles). We used two different reporter mouse models to visualize T cells: (i) DPE-GFP mice (21) in which all GFP-expressing cells are CD90+ (fig. S2A) and (ii) interferon-γ (IFN-γ) reporter (GREAT) mice (22), which were useful because tumor-infiltrating YFP+ cells in these mice were almost exclusively CD8+ T cells (fig. S2B). The fluorescent Pacific Blue–dextran nanoparticle has been validated for intratumoral macrophage identification (23), and we confirmed its specificity for macrophages (F4/80+ cells) in the tumor stroma (fig. S2C). Finally, we verified that single-agent aPD-1 treatment was able to suppress MC38–H2B-mApple tumor growth in the window chamber system (Fig. 2B).

Upon administration of AF647–aPD-1, we found that the antibody rapidly perfused tumor vessels and gradually disseminated out of the vasculature and into the tumor interstitium (Fig. 2C and movie S1). AF647–aPD-1 was observed on GFP-labeled T cells as early as 5 min after injection, and these were the first cells in the tumor microenvironment to be detectably labeled by the drug. AF647–aPD-1 binding to tumor-infiltrating T cells was initially pericellular but, within minutes, formed puncta on the cell surface (fig. S3). These rearrangements occurred without apparent decreased T cell motility (fig. S4, A to C). Later time points revealed that TAMs, which were stationary in the tumor microenvironment (fig. S4D), had collected most of the AF647–aPD-1; T cells were not associated with AF647–aPD-1 at these time points (Fig. 2, D and E). T cells were present in the tumor microenvironment at all times examined, removing the possibility that the drug biodistribution was an artifact of T cell loss after therapy. Also, tracing AF647–aPD-1 across all time points failed to show binding to tumor cells, precluding the possibility that aPD-1 mAbs had direct effects on cancer cells (fig. S5).

Quantification of the tumor microenvironment images taken at 15 min or 24 hours after drug administration showed a pattern of

**Fig. 1. aPD-1 mAb labeling facilitates tracking of tissue biodistribution.** (A) The rat anti-mouse PD-1 29F.1A12 clone, conjugated to AF647 via NHS ester linkage, efficiently binds PD-1+ T cells (here, EL4 cells) as detected by flow cytometry (gray histogram). Isotype control staining is shown in white. (B) MC38 tumors were equally responsive to single-dose AF647–aPD-1 and unlabeled aPD-1, whereas tumor sizes increased 72 hours after control immunoglobulin G2a (IgG2a) treatment. (C) Fluorescence reflectance imaging of three tumors compared to tumor-draining (tdLN) and nondraining (ndLN) lymph nodes 24 hours after AF647–aPD-1 treatment (AF647: λex = 620 to 650 nm, λem = 680 to 710 nm). Scale bar, 5 mm. (D) Quantified AF647–aPD-1 in each tissue demonstrating tumor accumulation. Values represent SEM and n = 3 unless otherwise noted. ***P < 0.001, unpaired, two-tailed t test.
AF647 signal loss on T cells over time and a concomitant AF647 signal increase on macrophages (Fig. 3A). Although DPE-GFP labels CD90+ lymphocytes (fig. S2A), the GREAT mouse model allowed us to focus specifically on IFN-γ–expressing cells (movie S2), which are almost exclusively CD8+ T cells in the tumor microenvironment (fig. S2B). Intravital imaging of these cells confirmed surface binding of AF647–aPD-1 within minutes after drug administration (fig. S3). Longitudinal studies further showed that T cells eventually lose AF647–aPD-1 mAbs, which are physically transferred to, and retained by, neighboring macrophages (Fig. 3B). Consistent with our observations in DPE-GFP mice, we found in IFN-γ reporter mice that aPD-1 transfer from T cells to macrophages limited the overall duration of drug binding to their intended target cells (Fig. 3C).

To address whether checkpoint blockade agent uptake by macrophages could be independently validated using a bulk tissue measurement, we performed flow cytometry of tissues excised from tumor-bearing animals at 0.5 and 24 hours after AF647–aPD-1 administration (Fig. 3D). We evaluated several relevant cell populations for aPD-1 binding and confirmed our intravital microscopy observations: aPD-1 mAbs were bound mostly to CD8+ T cells at 0.5 hours but to macrophages at 24 hours (Fig. 3, D and E). Tumor macrophages were positive for rat IgG2a (fig. S6), confirming that the aPD-1 mAb, and not just the fluorophore, was transferred in vivo. Other cell types investigated, including CD4+ T cells, CD45+ CD11b− F4/80+ cells (which can include granulocytes and monocytes), and dendritic cells, did not display significant binding of aPD-1 at any time point tested (Fig. 3, D and E).

Analysis of B16 melanoma and KP1.9 lung adenocarcinoma tumor models also demonstrated that most AF647–aPD-1 mAbs accumulate within TAMs at 24 hours, similar to the findings obtained with MC38 colon adenocarcinoma (fig. S7A). Most of the intratumoral macrophages were bound by AF647–aPD-1 in all three tumor models (fig. S7B). Linear regression analysis of combined data further indicated that aPD-1 mAb
uptake by macrophages is independent of macrophage number and can also occur when TAM numbers are relatively low (fig. S7C).

aPD-1 mAb transfer mechanism

To explore the removal of aPD-1 mAbs on T cells by macrophages, we first asked whether the latter may also express PD-1 on their surface. However, ex vivo flow cytometry analysis indicated that TAMs, in contrast to tumor-infiltrating CD8+ T cells, were PD-1− (Fig. 4A). PD-1 was also absent in natural killer and B cells in these tumors (fig. S8). We then designed an in vitro coculture system combining bone marrow–derived macrophages and T cells that constitutively express PD-1 to create a controlled system to study the mechanism of drug collection by macrophages (Fig. 4B). T cells were preincubated with AF647–aPD-1 to emulate the antibody initially bound to T cells we observed in vivo and then cocultured with macrophages. Using this experimental setting, AF647–aPD-1 mAbs effectively relocated from T cells to macrophages within several minutes, as detected by the formation of drug puncta within macrophages (Fig. 4, B and C, and movie S3). The transfer could not be attributed solely to phagocytosis of cell debris because it occurred even in the presence of the phagocytosis inhibitor dynasore (Fig. 4B).

Because tumor macrophages did not capture AF647–aPD-1 in substantial quantities early after administration, did not express PD-1 on their cell surface, and withdrew drug bound to the T cell surface, we reasoned that AF647–aPD-1 mAbs must accumulate in macrophages through a non–antigen-specific mechanism. The aPD-1 clone 29F.1A12 is a rat IgG2a isotype that is used to mimic the biological properties of human IgG4. Both rat IgG2a and human IgG4 have been demonstrated to bind inhibitory FcγRs (mouse FcγRIIb and human FcγRIIB, respectively) (24, 25). Adding FcγRIIb/III blocking antibodies to the in vitro coculture system diminished AF647–aPD-1 transfer from T cells to macrophages (Fig. 4, B and C). The effect was specific because blocking FcγRIIb/III failed to inhibit AF647–aPD-1 transfer (Fig. 4, B and C).

To substantiate these findings, we developed a flow cytometry–based antibody transfer assay: Macrophages were incubated with T cells previously labeled with AF647–aPD-1 mAbs for 30 min and analyzed by flow cytometry. AF647–aPD-1 signal was detected on macrophages in this experimental setting (Fig. 4D); however, aPD-1 transfer could be
neutralized by adding a blocking antibody to FcγRIIb/III to the coculture system (Fig. 4, D and E). AF647–aPD-1 mAbs added directly to the culture medium in the absence of T cells were not efficiently taken up by macrophages, further suggesting that T cells are the major source of aPD-1 for macrophages (Fig. 4, D and E). We also tested whether aPD-1 loss on the T cell surface might be due to receptor internalization independent of macrophage uptake. T cells were exposed to AF647–aPD-1 for 1 hour at 37°C, treated with an acid solution to remove cell surface antibodies, and analyzed by flow cytometry to assess remaining (internalized) fluorescent signals (fig. S10A) (26). Acid stripping strongly reduced AF647–aPD-1 detection, indicating that antibody internalization is likely not the primary contributor to aPD-1 loss on T cells (fig. S10B).

Macrophages cocultured with AF647–aPD-1–coated T cells were positive not only for AF647 but also for rat IgG2a, as assessed by enzyme-linked immunosorbent assay (fig. S11A). The eventual decline in macrophage rat IgG2a was not accompanied by the release of IgG2a into the supernatant, suggesting that acquired antibody is eventually degraded by the macrophage (fig. S11B). Collectively, these data indicate that aPD-1 mAb removal from the T cell surface receptor by macrophages is a pharmacologic end point elicited by FcγR interactions with T cell–bound antibody complexes.

To assess whether the PD-1 receptor is transferred during aPD-1 removal, PD-1+ T cells were exposed to unlabeled aPD-1, cocultured with macrophages (to enable aPD-1 capture), and reprobed for cell surface PD-1 expression with a fluorescent aPD-1. Instead, T cells from which aPD-1 had been captured were efficiently reprobed, indicating that aPD-1 removal frees up PD-1 molecules, which become available to fluorescent aPD-1 mAb (fig. S12A). Control experiments confirmed that the increased reprobing signal was not contributed by new PD-1 molecules on the surface of T cells (fig. S12B). Together, these data indicate that PD-1 remains on the T cell membrane after aPD-1 capture.

Because Fc/FcγRI binding interactions of many IgG subclasses depend upon mAb Fc glycosylation (27), we profiled the glycan structures from the murine aPD-1 mAb (29F.1A12) and further extended our analysis to the human aPD-1 mAb nivolumab. Both antibodies were treated with peptide N-glycosidase F (PNGase F), a glycosidase that cleaves N-linked glycan, to remove glycan from each antibody, and the digested...
products were analyzed by high-performance liquid chromatography (HPLC) (Fig. 5A). We found that murine and human aPD-1 mAbs share the same predominant glycoform that lacks terminal galactose residues (G0F) and is fucosylated on the penultimate N-acetylglucosamine (fig. S13). Both mouse and human aPD-1 contained substantial fractions of terminally galactosylated glycoforms, indicating a high degree of Fc glycan heterogeneity in these aPD-1 mAb preparations.

The similarity in glycan pattern between mouse and human aPD-1 mAbs led us to hypothesize that FcγR-mediated antibody transfer is relevant to human aPD-1 interactions. We fluorescently labeled the anti-human PD-1 mAb nivolumab with AF647 and adapted the in vitro coculture system (Fig. 4B) to primary human cells. We differentiated human macrophages from blood monocytes using macrophage colony-stimulating factor, and PD-1+ expressing CD8+ T cells were generated by isolating primary human CD8+ T cells and stimulating them with plate-bound aCD3 mAbs for 3 days. Coculture of AF647-nivolumab–labeled human CD8+ T cells with macrophages resulted in mAb transfer from CD8+ T cells to macrophages (movie S4), and the transfer was inhibited by blocking FcγRs using aFcγRIIB/III (Fig. 5B). There was no evidence of CD8+ T cell membrane components in the macrophages, consistent with the antibody alone being removed from the surface PD-1 receptor (fig. S14). Furthermore, blocking FcγRs decreased AF647-nivolumab puncta inside of macrophages, implying that FcγRs also regulate nivolumab uptake in human cells (Fig. 5C).

In an attempt to minimize aPD-1/FcγR interactions, we used PNGase F to remove the glycan from murine aPD-1 mAbs and confirmed cleavage of glycan by lens culinaris agglutinin lectin blot (fig. S15A). The PNGase F–treated aPD-1 was labeled with AF647, and flow cytometry confirmed that the presence of glycan was not required for aPD-1 tropism because PD-1+ T cells were still efficiently labeled with PNGase F–treated AF647–aPD-1 (fig. S15B). However, live cell imaging demonstrated that glycan removal diminished antibody transfer from T cells to macrophages (Fig. 6A), and these results were confirmed by flow cytometry (Fig. 6, B and C).

We then aimed to uncover the in vivo activity of aPD-1 mAbs when aPD-1/FcγR interactions were therapeutically inhibited. To this end, we tracked aPD-1, CD8+ T cells, macrophages, and tumor cells in mice in which we inhibited FcγRs by infusing FcγRIIb/III blocking antibodies (2.4G2 clone) before delivering AF647–aPD-1 mAbs (Fig. 6D). We found that administration of the FcγR blocking agent substantially prolonged the occupancy time of AF647–aPD-1 mAbs on CD8+ T cells in the tumor bed (Fig. 6E). Furthermore, whereas the response of MC38 tumors to aPD-1 therapy typically varies among animals (fig. S16), blocking FcγR interactions completely eliminated the fraction of nonresponders observed, with complete tumor rejection in all mice that received the combination treatment (Fig. 6F). These data provide evidence that mAb/FcγR interactions abbreviate aPD-1 mAb occupancy time on tumor-infiltrating CD8+ T cells and limit therapy response; conversely, aPD-1 mAb therapy can be improved by blocking FcγR interactions (fig. S17).

**DISCUSSION**

Many cancer patients do not respond to immune checkpoint blockade therapy, and we lack a complete understanding of the mechanisms that...
contribute to treatment efficacy and resistance. Here, by using time-lapse intravital microscopy, we uncovered in real time how the immune checkpoint blocker aPD-1 mAb distributes in tumors and physically interacts with tumor microenvironment components. This approach enabled us to detect aPD-1 mAb association with cytotoxic T cells infiltrating tumors in vivo. Furthermore, by following the drug’s pharmacokinetics over time, we found the drug to be rapidly removed from PD-1+ CD8+ T cells and transferred to neighboring PD-1+ TAMs. The transfer of aPD-1 mAbs from T cells to macrophages was unexpected because macrophages do not directly take up aPD-1 mAbs in culture. We further identified that aPD-1 uptake by macrophages depends both on the Fc domain of the antibody and on FcγRs expressed by macrophages. Interactions between the drugs and macrophages are likely important because blocking Fc/FcγR binding inhibited aPD-1 transfer from CD8+ T cells to macrophages in vivo and enhanced aPD-1 therapeutic efficacy.

Although clinical aPD-1 has an extensive circulation half-life (~26 days), our observations suggest that the time of target engagement in the local tumor environment may be much shorter. This engagement time is reduced at least in part by FcγRIIb/III, which mediate aPD-1 mAb uptake from T cells to macrophages. Accordingly, previous work in mice has shown that IgG2a isoforms preferentially bind FcγRIIb/III and that aPD-1 therapy with an IgG2a mAb is more effective in FcγRIIb knockout animals (25). Our studies further suggest that FcγR-mediated aPD-1 removal does not involve transfer of cell membrane components or trogocytosis, which has been described for other mAbs including rituximab (27, 28). However, we found that aPD-1 uptake by macrophages is favored when the mAb is bound to PD-1 on T cells, which aligns with previous findings that FcγRs bind IgGs more efficiently when they form immune complexes (29, 30). We also found that PD-1 remains on the T cell surface after aPD-1 removal, but we did not observe these PD-1 molecules to bind new aPD-1. Overall, aPD-1 transfer from T cells to macrophages appears to be faster than aPD-1 uptake by T cells in the tumor stoma.

The human aPD-1 drugs nivolumab and pembrolizumab were designed as human IgG4 antibody isotypes that are not known to fix complement or trigger antibody-dependent cell-mediated cytotoxicity (ADCC) (31). However, IgG4 can bind FcγRI and FcγRIIb, and these interactions can have profound clinical consequences (32, 33). Awareness of the FcγR binding profile of an mAb offers an opportunity to improve upon existing monoclonal therapies, exemplified by obinutuzumab, a defucosylated IgG1 biosimilar of rituximab designed to bind FcγRIIA and enhance ADCC against CD20+ cells in chronic lymphocytic leukemia (34). Human germline variants of FcγRs that display altered Fc binding tropism have been identified and are an important focus in the effort to understand responses to mAb therapies that rely on FcγRs for therapeutic
function, like cetuximab (35), rituximab (36), trastuzumab (37), and other mAb therapies (38).

We suggest that nivolumab and other IgG4-based mAbs are not exceptions to the rules of FcγR binding, and therefore, Fc interactions should be considered in pharmacologic models. This is particularly important because there is growing interest in immune checkpoint molecules as diagnostic tools to identify PD-1/PD-L1+ tumors. For example, previous efforts to image PD-1 expression using positron emission tomography (PET) radioligands have focused on gross tissue distribution and lack the resolution to identify cellular tropisms in vivo. Natarajan et al. used hamster anti-mouse PD-1 (39), but it is not fully understood how hamster mAbs interact with mouse FcγRs in this context. A secondary aPD-1 PET imaging study reported the use of the RMP1-14 rat IgG2a anti-mouse PD-1 clone to cross-correlate with ex vivo PD-1 staining (40), but drug withdrawal by macrophages could complicate the relationship between PET signal and PD-1 expression. Future preclinical diagnostic efforts to image PD-1 expression should consider imaging agents that avoid FcγR interactions; however, antibodies meant to mimic nivolumab and pembrolizumab should accurately represent the Fc status of the human IgG4 antibodies.

The findings from the present study also have potential implications for improving current treatment options. From our understanding of Fc interactions with aPD-1, Fc-engineered IgG variants that abrogate FcγR binding and mAb effector functions (41) or combinations with therapies that inhibit FcγR binding in vivo may enhance the effects of treatment. In addition, therapies designed to target tumor macrophages (18), when combined with aPD-1, may provide additional benefit by increasing immune checkpoint blockade drug delivery to CD8+ T cells, thereby enhancing activity of immunotherapy. Clinical trials combining macrophage targeting therapeutics and immune checkpoint blockers are underway (42) and should answer this question. In support of the notion that myeloid cells interface with aPD-1 immunotherapy, recently identified correlates of aPD-1 response in tumors suggest that alterations in macrophage gene signatures are associated with nonresponsiveness to aPD-1 (43).

Comparisons between mouse and human Fc/Fc receptor interactions are complex because both antibody Fc isotypes and Fc receptor biological functions are divergent between species. Our initial findings in mice, which established the basic principle of Fc receptor–mediated antibody transfer, were recapitulated using primary human cells and nivolumab. However, it will be important to understand the impact of such interactions in patients. In addition, one should clarify whether all macrophages, or only a subset thereof, contribute to mAb uptake in tumors. Our understanding of the complexity of macrophages and other myeloid cells remains limited (18), and it is possible that those with discrete phenotypes, for example, with distinct proportions of activating versus inhibitory Fc receptors, play different roles in response to immune checkpoint blockade therapy (44). Uncovering the mechanisms that control mAb drug interactions with host cells is an ongoing challenge, although the findings could have far-reaching consequences for diagnostic and therapeutic approaches.

**Animal models**

Animal research was performed in accordance with the Institutional Animal Care and Use Committees at Massachusetts General Hospital (MGH). DPE-GFP mice (21) were provided by U. von Andrian (Department of Microbiology and Immunobiology, Harvard Medical School). IFN-γ reporter with endogenous polyadenylated tail (GREAT) mice (22) were provided by A. Luster (Division of Rheumatology, Allergy and Immunology, MGH). Tumor growth was monitored by caliper measurement, and the area (A) of these predominantly two-dimensional tumors was calculated using the formula A = length × width. Tumor implantation was performed by intradermal injection of tumor cells (2 × 10^6 MC38/MC38-H2B-mApple, 5 × 10^5 B16-F1–ovalbumin, and 5 × 10^5 KP1.9). MC38 cells were gifted by M. Smyth (QIMR Berghofer, Brisbane, Australia), B16 cells were from American Type Culture Collection, and KP1.9 cells were a gift from A. Zippelius (University Hospital Basel, Switzerland). Experiments were generally started when tumors became vascularized, which was after 8 days. For aPD-1 and AF647–aPD-1 treatments, mice were given 200 μg (intraperitoneally) of the 29F.1A12 aPD-1 clone. For in vivo Fc blocking experiments, mice were infused intraperitoneally with 200 μg of monoclonal antibody specific to mouse FcγRII and FcγRIII (clone 2.4G2, Bioxcell) daily for 5 days. Control mice received 200 μg of rat IgG2a isotype control (clone 2A3, Bioxcell).

**In vivo microscopy**

Intravital microscopy was performed in dorsal skinfold window chambers installed on DPE-GFP or GREAT mice inoculated with MC38–H2B-mApple tumors. Mouse macrophages and/or vasculature were labeled with Pacific Blue–ferumoxytol and Pacific Blue–dextran, respectively. AF647–aPD-1 (200 μg) was delivered intravenously, and its tumor distribution was observed using an Olympus Fluoview FV1000MPE confocal imaging system (Olympus America), as described previously (45). Pacific Blue, GFP/YFP, mApple, and AF647 were imaged sequentially using 405-, 473-, 559-, and 635-nm lasers and BA430–455, BA490–540, BA575–620, and BA575–675 emission filters with DM473, SDM560, and SDM640 beam splitters, all sourced from Olympus America. Time-lapse images were acquired continually over the first hour after AF647–aPD-1 injection, after which the mice were allowed to recover before subsequent imaging.

**Statistical analysis**

Data points were compiled in Microsoft Excel, and statistical analyses were performed using GraphPad Prism 6. α levels of 0.05 were used to
define statistical significance, and error bars represent SEM unless otherwise noted.

SUPPLEMENTARY MATERIALS
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Materials and Methods
Fig. S1. AF647–apD1 mAb quantification in various tissues at 24 hours after injection.
Fig. S2. Specificity of T cell reporter mice and dextran nanoparticles.
Fig. S3. Representative intravital microscopy time course of AF647–apD1 from an IFN-γ–YFP mouse.
Fig. S4. T cell and macrophage motility before and after apD1 treatment.
Fig. S5. Assessment of apD1 binding to tumor cells.
Fig. S6. apD1 transfer to tumor macrophages in vivo.
Fig. S7. Distribution of AF647–apD1 across tumor models.
Fig. S8. PD1 expression by T cell in the MC38 tumor microenvironment.
Fig. S9. apD1–mAb transfer from T cells to macrophages in vitro.
Fig. S10. Assessment of apD1 internalization after binding to PD-1.
Fig. S11. apD1 degradation by macrophages.
Fig. S12. PD-1 localization following apD1 internalization after binding to PD-1.
Fig. S13. Comparative analysis of mAb glycosylation patterns between mouse and human PD-1 antibodies.
Fig. S14. Assessment of cell membrane component exchange during apD1 transfer.
Fig. S15. Confirmation of deglycosylation and antigen binding affinity for rat anti-mouse PD-1.
Fig. S16. Impact of Fc blockade on apD1–mAb transfer from T cells to macrophages in vitro.
Fig. S17. Proposed resistance mechanism and potential improvement of apD1–mAb therapy.
Movie S1. Intravital microscopy imaging of AF647–apD1 injection in a D佩-GEF mouse bearing an MC38–H2B–mApple tumor in a dorsal skinfold chamber.
Movie S3. Live imaging of mouse T cell apD1 transfer to macrophages.
Movie S4. Live imaging of human T cell apD1 transfer to macrophages.

REFERENCES AND NOTES
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In vivo imaging reveals a tumor-associated macrophage–mediated resistance pathway in anti–PD-1 therapy

Sean P. Arlauckas, Christopher S. Garris, Rainer H. Kohler, Maya Kitaoka, Michael F. Cuccarese, Katherine S. Yang, Miles A. Miller, Jonathan C. Carlson, Gordon J. Freeman, Robert M. Anthony, Ralph Weissleder and Mikael J. Pittet

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Tug-of-war with anti–PD-1

Antibodies against immune checkpoints such as programmed death–1 (PD-1) are gaining increasing prominence in cancer treatment, but even these promising therapeutics do not always work. To be effective in preventing T cells from becoming exhausted, anti–PD-1 antibodies must be able to remain bound to the T cells. Unfortunately, this does not always happen, as Arlauckas et al. discovered. Although anti–PD-1 antibodies initially bound to T cells as intended, the authors found that tumor-associated macrophages quickly removed these antibodies from T cells, thus inactivating them. The researchers also identified a potential way to overcome this problem, showing that inhibition of Fc \( \gamma \) receptors prevented removal of anti–PD-1 and prolonged its effects in vivo.