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ORIGINAL RESEARCH

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Analysis of the tumor microenvironment and anti-tumor efficacy of subcutaneous vs systemic delivery of the bifunctional agent bintrafusp alfa

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ABSTRACT

Most monoclonal antibodies (MAbs), including immune checkpoint inhibitor MAbs, are delivered intravenously (i.v.) to patients. Recent clinical studies have demonstrated that some anti-PD1 MAbs may also be delivered subcutaneously (s.c.), with clinical outcomes similar of those obtained with i.v.-delivered agents. Bintrafusp alfa, a first-in-class bifunctional fusion protein composed of the extracellular domain of the human transforming growth factor β receptor II (TGF-βRII or TGF-β "trap") fused to the heavy chain of an IgG1 antibody blocking programmed death ligand 1 (anti-PDL1), was designed to target two key immunosuppressive pathways in the tumor microenvironment (TME). Bintrafusp alfa is currently being administered i.v. in clinical studies. The studies reported here demonstrate that systemic or s.c. delivery of bintrafusp alfa, each administered at five different doses, induces similar anti-tumor effects in breast and colorectal carcinoma models. An interrogation of the TME for $CD8^+$ and $CD4^+$ T cells, regulatory T cells (Tregs), monocytic myeloid-derived suppressor cells (M-MDSCs) and granulocytic (G) MDSCs showed similar levels and phenotype of each cell subset when bintrafusp alfa was given systemically or s.c. Subcutaneous administration of bintrafusp alfa also sequestered TGFβ in the periphery at similar levels seen with systemic delivery. To our knowledge, this is the most comprehensive preclinical evaluation of any checkpoint inhibitor MAb given s.c. vs systemically, and the first to demonstrate this phenomenon using a bifunctional agent. These studies provide preclinical rationale to explore s.c. approaches for bintrafusp alfa in the clinic.

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Introduction

Antibodies targeting immune checkpoints (IC), including programmed cell death 1 (PD-1) receptor and its ligand PD-L1 have achieved unprecedented clinical success in subsets of cancer patients.[1,2] PD-1 is expressed on activated natural killer (NK) and T cells.[3] PD-1 interaction with its ligands PD-L1 and PD-L2, expressed on tumor cells and multiple immune cell subsets, inhibits proliferation, maturation, and effector functions on both T lymphocytes and NK cells.[2,4,5] PD-L1 overexpression is present in a wide spectrum of malignancies, correlating with poor prognosis.[6] In recent years, multiple monoclonal antibodies (MAbs) targeting the PD-1/PD-L1 axis have received regulatory approval, including nivolumab, pembrolizumab, atezolizumab, avelumab, and durvalumab. These agents were approved for parental administration; however, the subcutaneous (s.c.) route is currently being explored in several clinical studies including the anti-PD-1 PF-06801591, and envafolimab (KN035), a PD-L1–targeting nanobody.[7–9] In a phase 1 open-label, multicenter, dose-escalation trial (NCT02573259), 40 patients with locally advanced or metastatic solid tumors received PF-06801591 parentally (0.5, 1, 3, or 10 mg/kg q3w) or subcutaneously (300 mg q4w).[9] Comparable safety profile and anti-tumor activity in a variety of tumor types were observed with both s.c. and intravenous (i.v.) delivery. Collectively, these studies suggest that s.c. delivery is a more convenient and equally effective alternative to conventional parental administration of checkpoint inhibitor MAbs.

Bintrafusp alfa (previously designated M7824) is a first-in-class bifunctional fusion protein with two extracellular transforming growth factor ß receptor II (TGF-βRII) domains fused to the C-terminus of a human MAb targeting the IC PD-L1.[10,11] This TGFβ Trap/anti-PDL1 agent is designed to act both as a checkpoint inhibitor and to sequester "trap" TGFβ in the tumor microenvironment (TME) via anti-PDL1 delivery. Preclinical studies demonstrated its bifunctional targeting and immune-mediated mechanisms by which bintrafusp alfa promotes anti-tumor efficacy.[10,12–15] The first-in-human trial of bintrafusp alfa, administered i.v. to patients with advanced solid malignancies, indicated a manageable safety profile and encouraging signs of clinical efficacy across all dose levels (1, 3, 10, or 20 mg/kg), with objective and durable responses.[16,17] Several clinical studies examining safety and efficacy of bintrafusp alfa as monotherapy and in combination with other agents are currently ongoing for a wide range of tumors, including human papillomavirus (HPV)-

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associated malignancies, and carcinomas of the breast, colon, lung, and prostate, among others. Of note, bintrafusp alfa is being administered i.v. in all ongoing clinical trials.

The i.v. administration of bintrafusp alfa as well as any other MAb is labor intensive in terms of pharmacy preparation, day hospital installation, and time and effort of healthcare professionals, all of which increase the cost of health care. More importantly, it is time-consuming and inconvenient for the patient, and prone to have clinical complications when compared to an s.c. injection. Since bintrafusp alfa is a first-in-class bifunctional agent, a comparison of systemic vs. s.c. administration of bintrafusp alfa was carried out in two distinct preclinical models to define whether any differences exist in terms of resultant anti-tumor efficacy, effects on soluble factors such as TGFβ in the periphery, and mechanistically via interrogation of multiple immune subsets and their phenotype in the TME.

Materials and methods

Tumor cell line

Murine breast (EMT6) carcinoma cells were obtained from American Type Culture Collection and maintained according to the provider's recommendations. Murine colon carcinoma MC38 cells are as described.[18] Cells were used at low passage numbers (<5), and determined *mycoplasma* free (MycoAlert Mycoplasma Detection Kit, Lonza).

Reagent

Bintrafusp alfa (also known as M7824), a bifunctional fusion protein composed of two extracellular domains of TGF-βRII fused with a human IgG1 MAb targeting PD-L1, was kindly provided by EMD Serono under a Cooperative Research and Development Agreement with the National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA.

Animals

Six- to ten-week-old female Balb/c or C57BL/6 were obtained from the NCI Frederick Cancer Research Facility (Frederick, MD). Mice were housed in microisolator cages under pathogen-free conditions, in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All studies were approved by the NIH Intramural Animal Care and Use Committee (IACUC).

Murine tumor studies

On day 0, EMT6 (2.5×10^5) were implanted orthotopically into the mammary fat pad of Balb/c female mice. Alternatively, MC38 (3×10^5) were implanted subcutaneously in the right flank of C57BL/6 female mice. When tumor volume reached 50–100 mm^3 (days 7–9), mice were randomized to receive PBS (100μl, i.p.) or bintrafusp alfa (1, 2, 5, 10, or 20 mg/kg) by s.c. or intraperitoneal (i.p.) injection. Unless otherwise stated, s.c. administration was performed at a distant site (upper back) relative to the tumor. Dosing was repeated 4 days later. Survival was monitored, tumor size was measured twice weekly and tumor volume calculated as (length^2 × width)/2. On select studies, immune correlates were examined *ex vivo* 2 days after the second dose of bintrafusp alfa.

Ex-vivo analysis

Plasma collection and cytokine analysis. Peripheral blood was collected and plasma processed as previously described.[12] Plasma TGFβ1 was quantified using the Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit and by Sample Activation Kit 1 (R&D Systems) according to the manufacturer's instructions, with a limit of detection of 35.187 pg/ml. Serum cytokines were quantified using the murine V-Plex Proinflammatory Panel 1 kit and MESO QuickPlex SQ 120 (Meso Scale Diagnostics), according to the manufacturer's instructions. The assay detection limit (pg/ml) is 0.04 (IFNγ), 0.13 (TNFα), 0.06 (IL-5), 0.61 (IL-6), 0.94 (IL-10), and 0.24 (KC/GRO).

Detection of bintrafusp alfa in the TME. Detection of bintrafusp alfa bound to tumor immune and nonimmune cells was performed by flow cytometry using an anti-human IgG.

Flow cytometric analysis. Tumor single-cell suspensions were prepared using standard procedures as previously described.[12] Cell counts were performed using 123 count eBeads (eBioscience). Staining of immune cells for flow cytometry was performed using the Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's instructions. Antibodies used are listed in Supplementary Table 1 with matched isotypes obtained from the listed manufacturers. Data from >1 $\times 10^5$ cells were acquired on a BD FACSVerse or LSRII Fortessa flow cytometer (BD Biosciences) and analyzed with FlowJo Analysis Software (Treestar). Gating strategies for the identification of cell populations are in Supplementary Table 2. All frequencies of phenotypic proteins were generated by subtracting the frequency of respective isotype, set between 1–5%.

Statistics

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software). Comparisons of data presented in bar graphs was performed by one-way ANOVA with Tukey's multiple comparisons. Significant treatment effects on tumor growth were determined by two-way ANOVA. Survival was analyzed using Log-rank (Mantel-Cox) test. Statistical

Table 1. EMT6 tumor cure rate and median overall survival elicited by bintrafusp alfa (20 or 10 mg/kg) administered via i.p. or s.c. injection.

	20 mg/kg				10 mg/kg			
	Cures (%)		mOS (d)		Cures (%)		mOS (d)	
	IP	SC	IP	SC	IP	SC	IP	SC
PBS	0/8		31		0/8		31	
Bintrafusp alfa	4/8 (50%)	4/9 (44%)	51.5	52	3/8 (38%)	4/9 (44%)	39	43

IP, intraperitoneal injection; SC, subcutaneous injection; mOS (d), median overall survival in days.

Table 2. MC38 tumor cure rate and median overall survival elicited by bintrafusp alfa (20 mg/kg) administered by s.c. injection at a distant site (SC) or adjacent (SC local) to the tumor.

	Cures (%)			mOS (d)		
	IP	SC	SC local	IP	SC	SC local
PBS	0/11			31		
Bintrafusp alfa	1/11 (9.1%)	2/10 (20%)	4/11 (36.4%)	35	40.5	35

IP, intraperitoneal injection; SC, subcutaneous injection; mOS (d), median overall survival in days.

significance was set at $p < 0$ .05. *$p < .05$, **$p < .01$, ***$p < .001$, ****p < .0001.

Results

Subcutaneous administration of bintrafusp alfa induces significant anti-tumor efficacy

In preclinical models of solid tumors, bintrafusp alfa administered i.v. intraperitoneally promotes significant tumor control as a monotherapy.[10,12] To evaluate the effect of subcutaneous administration on anti-tumor efficacy elicited by bintrafusp alfa, Balb/C female mice were orthotopically implanted with EMT6 murine breast cancer cells on day 0. When tumors reached a volume of 50–100 mm^3 (day 9), mice were randomized and treated with bintrafusp alfa (10 or 20 mg/kg) administered s.c. or i.p., or received PBS. A second dose was given on day 13 (Figure 1a). Bintrafusp alfa administered at 20 mg/kg induced significant reduction in tumor growth relative to PBS-treated controls, regardless of route of administration (Figure 1b). Subcutaneous administration at 20 mg/kg eradicated 44% (4/9) of tumors (Table 1), significantly increasing median overall survival (mOS) by 67.7% relative to PBS controls (Figure 1b, Table 1). Similarly, i.p. administration with the same dose cured 50% of mice (4/8), increasing mOS by 66.1% versus controls (Figure 1b, Table 1).

Next, we examined the effect of a lower dose administered by either route. As shown in Figure 1c, significant antitumor efficacy was also observed at 10 mg/kg, with both routes of administration resulting in tumor control of similar magnitude. Treatment with bintrafusp alfa by systemic or s.c. routes cured 38% or 44% of mice, resulting in similar mOS increases relative to PBS-treated mice, respectively (Figure 1c and Table 1). These data suggest that s.c. administration of bintrafusp alfa results in similar tumor control as systemic administration.

Next, we examined the antitumor activity of bintrafusp alfa administered by either route in a second tumor model. C57BL/6 female mice were implanted with MC38 murine colorectal cancer cells on day 0. When tumors reached a volume of 50–100 mm^3 (day 10), mice were randomized and treated with PBS, or bintrafusp alfa (20 mg/kg) administered i.p., or by s.c. injection. A second dose was given on day 14 (Figure 1d). Bintrafusp alfa induced significant tumor control relative to PBS-treated controls, regardless of route of administration (Figure 1e; Table 2). This also translated into significant survival benefit irrespective of route of administration (Figure 1e). Peritumoral (local) administration of bintrafusp alfa elicited comparable results (Table 2). These data suggest that s.c. administration of bintrafusp alfa elicits non-inferior antitumor efficacy relative to systemic administration.

To determine whether the similarity in anti-tumor efficacy would be maintained at lower doses of bintrafusp alfa, mice harboring orthotopic EMT6 tumors were treated on days 7 and 11 with 5, 2, or 1 mg/kg bintrafusp alfa given i.p. or s.c., or PBS (Figure 2a). As shown in Figure 2b, 5 mg/kg bintrafusp alfa significantly decreased tumor growth when administered i.p. or s.c., with no significant difference observed between both routes of administration. Similar results were observed with a dose of 2 mg/kg, with i.p. or s.c. administration eliciting comparable tumor control. Bintrafusp alfa administered at a dose of 1 mg/kg did not promote significant antitumor effects, regardless of route of administration. Noteworthy, no signs of toxicity, including skin reactions probed by the presence of redness and/or swelling, were observed in these studies.

SC and systemic administration of bintrafusp alfa are equally effective in reducing plasma TGFβ1

We next examined if either route of administration of bintrafusp alfa would result in a similar ability to sequester peripheral TGFβ. To this end, we examined plasma TGFβ1 protein level in EMT6 and MC38 tumor-bearing mice 2 days after the second dose (20 or 10 mg/kg) of bintrafusp alfa. As shown in Figure 3a, administration of 20 mg/kg to EMT6 tumor-bearing mice resulted in a significant and comparable reduction of plasma TGFβ1 with both routes of administration. Quantification of other plasma cytokines in this model indicated no significant modulation of IFNγ upon administration by either route, albeit IFNγ levels trended higher upon s.c. injection. We observed significant elevation of IL-5 after i.p. administration of bintrafusp alfa, with values trending toward significance (p = .0583) upon s.c. injection. Both routes of administration resulted in unremarkable alteration in TNFα, IL-6, IL-10, and KC/GRO (Figure 3a) in EMT6 tumor-bearing mice. Analysis of TGFβ1 and other cytokines 2 days after i.p. or s.c. administration of bintrafusp alfa at 10 mg/kg also showed no significant alteration in any cytokine level (Figure 3b), with both routes of administration eliciting similar results. In the MC38 model, analysis of plasma TGFβ1 protein levels 2 days after the second dose (20 mg/kg) of bintrafusp alfa resulted in a significant reduction of TGFβ1 irrespective of route of administration. No significant alterations were observed in protein levels of any of the additional cytokines examined with i.p. or s.c. administration (Figure 3c).

These data suggest that despite potential differences in pharmacokinetics between both routes of administration, the ability of bintrafusp alfa to sequester peripheral TGFβ1 remained similar following either route of agent administration.

Bintrafusp alfa localizes to the tumor microenvironment after s.c. administration

We next examined the presence of bintrafusp alfa on the surface of immune and nonimmune cell populations in the TME. On days 7 and 11 after tumor implant, EMT6 tumor-bearing

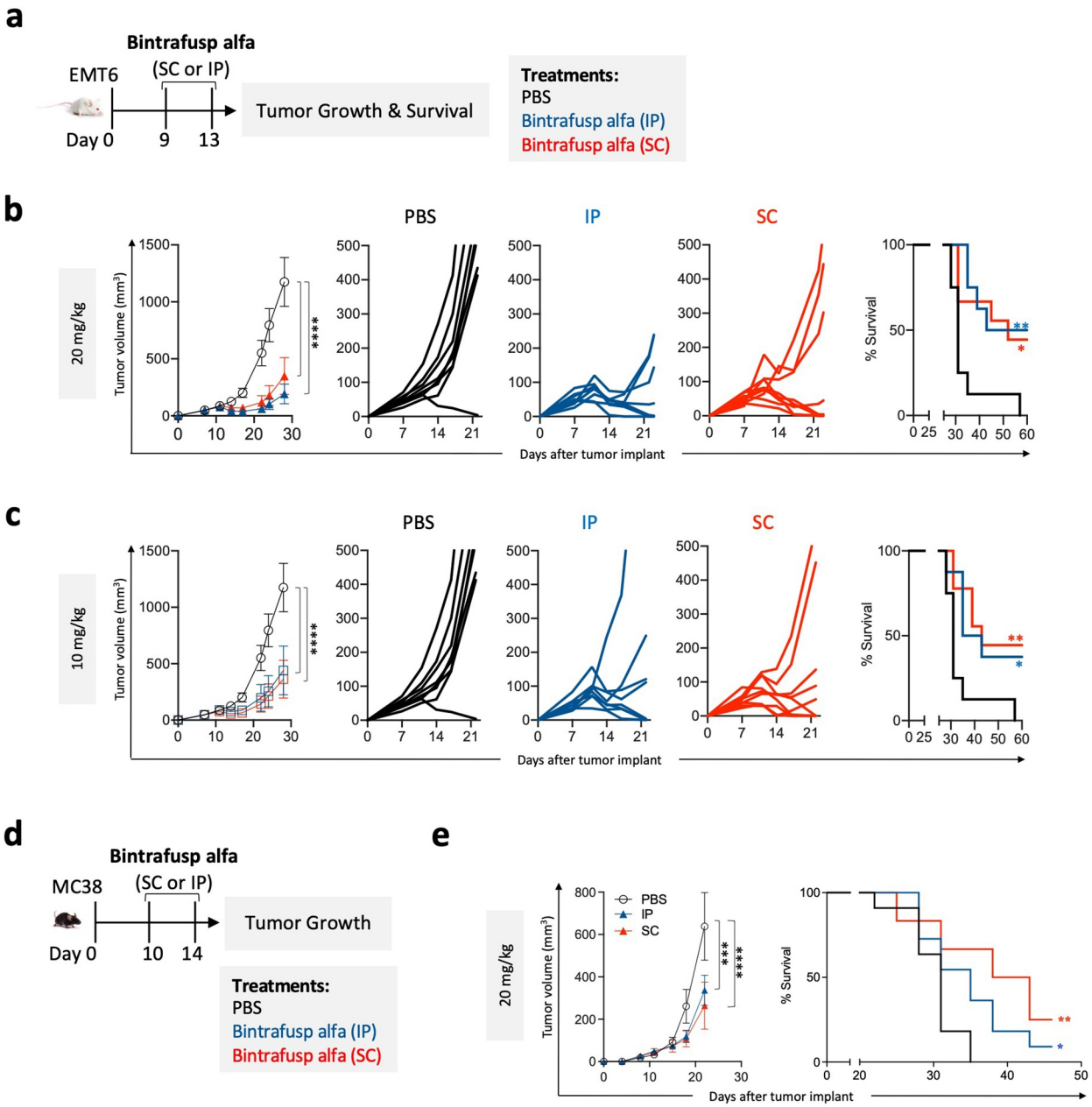

Figure 1. Effect of bintrafusp alfa route of administration on tumor growth. (a-c) EMT6 murine breast carcinoma cells (2.5×10^5) were implanted in the mammary fat pad of Balb/C female mice on day 0. When tumor volume reached 50–100 mm^3 (day 9), mice were randomized (n = 8–9/group) and treated on days 9 and 13 with PBS (100µl, i.p.), or with two different doses (20 mg/kg or 10 mg/kg) of bintrafusp alfa via i.p. or s.c. injection, as depicted in the schematic (a). Tumors were measured twice weekly using digital calipers, and tumor growth and survival were monitored. Tumor mean (± SEM) growth curves, individual tumor growth curves, and survival of mice treated with 20 mg/kg (b), or 10 mg/kg (c). (d-e) MC38 murine colorectal carcinoma cells (3×10^5) were implanted in the right flank of C57BL/6 female mice on day 0. When tumor volume reached 50–100 mm^3 (day 10), mice were randomized (n = 10–11/group) and treated on days 10 and 14 with PBS (100µl, i.p.) or bintrafusp alfa (20 mg/kg) via i.p. or s.c. injection, as depicted in the schematic (d). Tumors were measured twice weekly using digital calipers, and tumor growth and survival were monitored. Graphs depict tumor mean (± SEM) growth curves and survival of mice (e). Mantel Cox used for survival comparisons, and two-way ANOVA for tumor growth. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

mice received PBS, or bintrafusp alfa (10 or 20 mg/kg) given i.p. or s.c. Two days after the last dose, the presence of bintrafusp alfa in the TME was examined by flow cytometry using a fluorescently labeled anti-human IgG antibody (Figure 4a).

As shown in Figure 4b (*upper panel*), administration of 20 mg/kg bintrafusp alfa resulted in significant and comparable binding magnitude of the bifunctional molecule to the surface of nonimmune ($CD45^{neg}$) cells in the TME when administered by either route. However, at the lower dose of 10 mg/kg, s.c. administration resulted in a significantly higher level of binding to nonimmune cells relative to i.p. dosing (Figure 4b, *lower panel*). Examining immune subsets in the TME after a dose of 20 mg/kg i.p. revealed that bintrafusp alfa was bound in significant levels to $CD8^+$ and $CD4^+$ tumor-infiltrating lymphocytes (TILs), regulatory T cells (Tregs), and to a higher degree monocytic myeloid-derived suppressor cells (M-MDSCs) and granulocytic (G) MDSCs (Figure 4c, *upper panels*). Similar results at this dose level were observed with s.c. dosing, albeit a non-significant reduction in bintrafusp alfa binding to $CD4^+$ and Treg TILs was observed. At 10 mg/kg

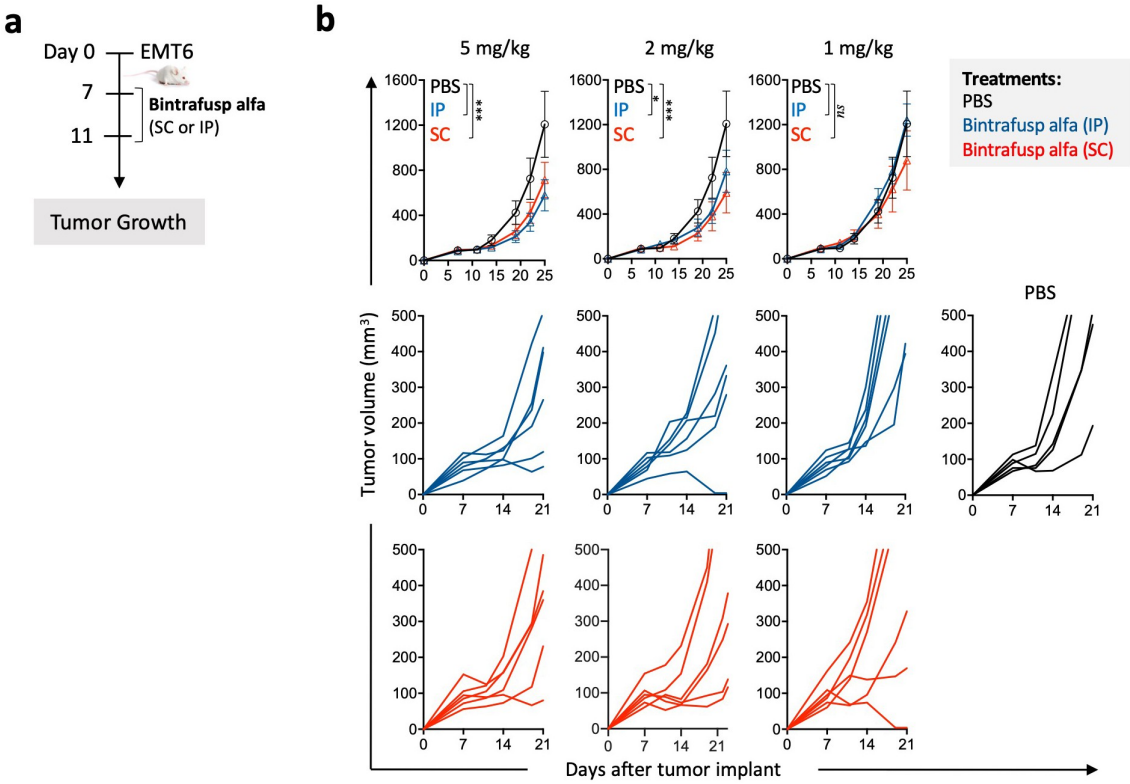

Figure 2. Dose de-escalation of bintrafusp alfa administered by i.p. or s.c. routes of administration. EMT6 murine breast carcinoma cells (2.5×10^5) were implanted in the mammary fat pad of Balb/C female mice on day 0. When tumor volume reached 50–100 mm^3 (day 7), mice were randomized (n = 5–6/group) and treated on days 7 and 11 with PBS (100µl, i.p.), or 5, 2, or 1 mg/kg of bintrafusp alfa via i.p. or s.c. injection, as depicted in the schematic (a). Tumors were measured twice weekly using digital calipers, and tumor growth was monitored. **b**, Tumor mean (± SEM) growth curves, and individual tumor growth curves of mice treated at the indicated doses via i.p. (blue lines) or s.c. (red lines) routes. Two-way ANOVA for tumor growth. *$p < .05$, ***$p < .001$; *ns*, not significant.

(Figure 4c, *lower panels*), s.c. administration elicited a significant presence of bintrafusp alfa bound to $CD8^+$ and $CD4^+$ TILs, which was not observed upon i.p. administration at this dose level. No appreciable binding to Tregs was observed with either route of administration at 10 mg/kg. However, at this dose level, both routes of administration resulted in similar and significant bintrafusp binding to both MDSC subsets, albeit a non-significant difference favoring s.c. administration. These data indicate that s.c. administration is clearly non-inferior to systemic i.p. administration in promoting the presence of bintrafusp alfa binding to immune and nonimmune cells in the TME.

Next, we examined the presence of bintrafusp alfa in the TME of MC38 tumors 2 days after the second dose (20 mg/kg) given i.p. or s.c. (Figure 4d). As shown in Figure 4e, bintrafusp alfa displayed comparable binding magnitude to the surface of nonimmune ($CD45^{neg}$) cells in the TME when administered by i.p. or s.c. routes. Bintrafusp alfa (i.p.) was bound in significant levels to $CD8^+$ and $CD4^+$ TILs, M-MDSCs and G-MDSCs, and to a lesser extent to Tregs (Figure 4f). Subcutaneous administration elicited similar results, in addition to significant binding to Tregs. Collectively, these data indicate that s.c. dosing is non-inferior to i.p. administration in promoting the presence of bintrafusp alfa binding to immune and nonimmune cells in the TME.

Subcutaneous administration of bintrafusp alfa results in PD-L1 blockade in the tumor microenvironment similar to that obtained via systemic administration

To investigate the impact of route of administration on the ability of bintrafusp to target PD-L1 in the TME, EMT6 tumors excised 2 days after the last dose of 10 or 20 mg/kg were examined by flow cytometry for the detection of PD-L1 on the surface of immune and nonimmune cells (Figure 5a). Administration of 20 mg/kg resulted in a >4.5-fold decrease in detected PD-L1 on nonimmune/tumor cells on a per cell basis, with no significant difference observed between i.p. and s.c. administration (Figure 5b, *upper panel*). No significant PD-L1 blockade in $CD45^{neg}$ cells was observed with either route of administration at the lower dose (Figure 5b, *lower panel*). Administration of bintrafusp alfa at 20 mg/kg resulted in significant PD-L1 blockade on $CD8^+$, $CD4^+$, and Treg TILs, and both M- and G-MDSCs, with i.p. and s.c. administration attaining similar magnitude of effects (Figure 5c, *upper panels*). Despite a lesser magnitude of PD-L1 blockade observed at 10 mg/kg, both routes of administration attained similar results, both eliciting a significant reduction of detectable PD-L1 on the surface of $CD4^+$ and Treg TILs (Figure 5c, *lower panels*).

Next, we examined PD-L1 levels in immune and nonimmune cells in the TME of MC38 tumor-bearing mice 2 days after the last administration of bintrafusp alfa (20 mg/kg) (Figure 5d). In findings similar to those in EMT6 tumors, administration of bintrafusp alfa resulted in significant and comparable blockade of PD-L1 in both nonimmune $CD45^{neg}$ cells (Figure 5e) as well as in all immune subsets examined (Figure 5f) with either route of administration. Collectively,

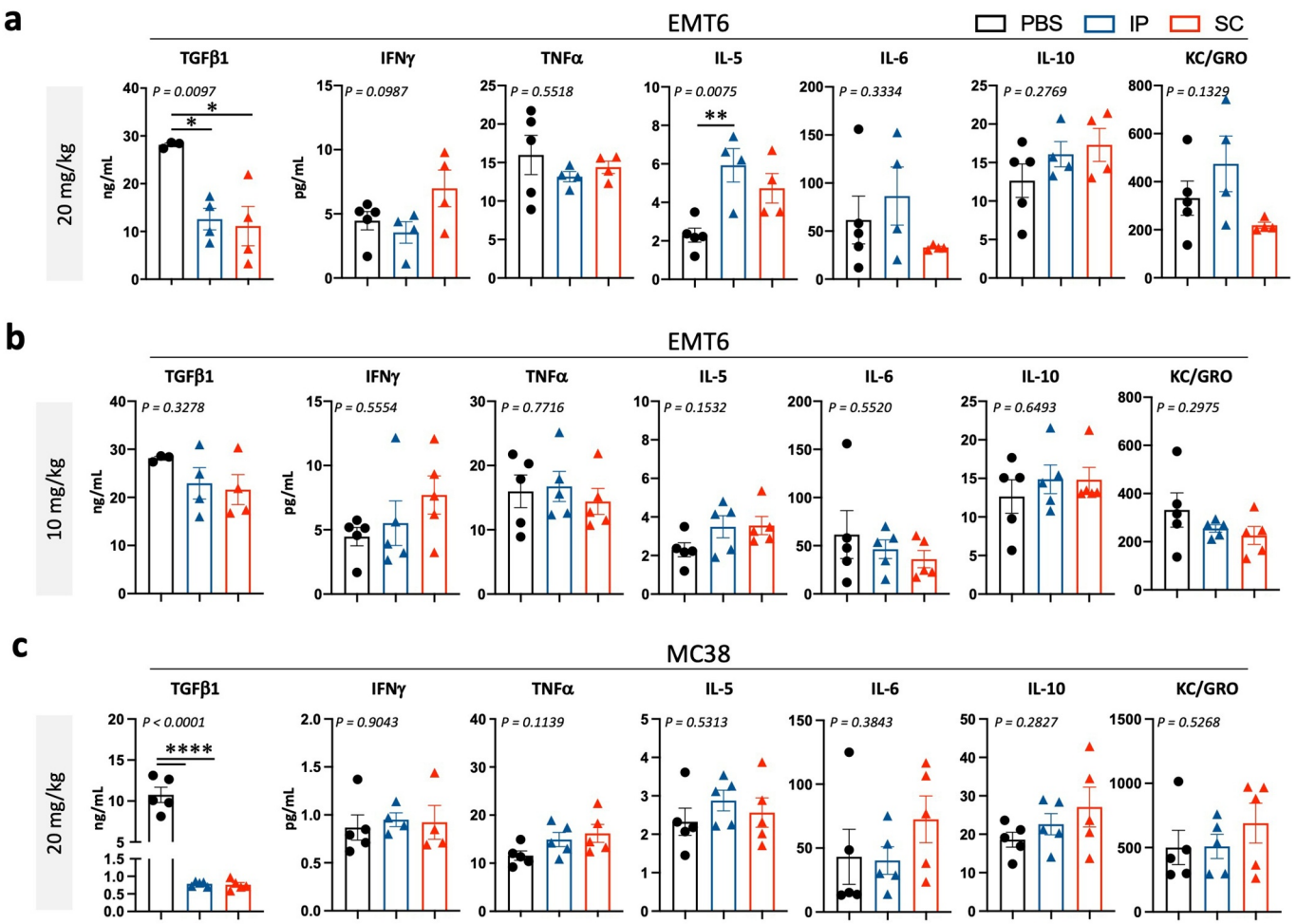

Figure 3. Effect of bintrafusp alfa administered s.c. or i.p. on plasma TGFβ and other cytokines. EMT6 tumors were implanted and treated as in Figure 1a. Two days after the last treatment with PBS, or 20 mg/kg (a) or 10 mg/kg (b) of bintrafusp alfa administered i.p. or s.c., plasma levels of TGFβ, IFNγ, TNFα, IL-5, IL-6, IL-10 and KC/GRO were analyzed in individual mice. **c**, MC38 tumors were implanted and treated as in Figure 1d. Two days after the last treatment with PBS, or bintrafusp alfa (20 mg/kg) administered i.p. or via s.c. injection, protein plasma levels of designated cytokines were quantified. Data shown as mean ± SEM. One-way ANOVA with Tukey's comparison, *$p < .05$, **$p < .01$, ****$p < .0001$.

these findings demonstrate that s.c. administration of bintrafusp alfa results in similar tumor targeting, PD-L1 blockade, and consequent antitumor efficacy compared to systemic administration.

Bintrafusp alfa promotes tumor infiltration of cytotoxic CD8$^+$ T cells while reducing immunosuppressive cells irrespective of route of administration

Next, we examined the impact of route of administration on the phenotype of immune cells infiltrating EMT6 and MC38 tumors 2 days after the last bintrafusp alfa dose. In prior preclinical solid tumor models, the mechanism of action of bintrafusp alfa has been shown to be mostly mediated by CD8$^+$ T cell cytotoxicity.[12,14] As shown in Figure 6, whereas neither route affected the infiltration of CD8$^+$ T cells in EMT6 tumors upon administration of 20 mg/kg (*upper panels*) or 10 mg/kg (*middle panels*), both i.p. and s.c. administration induced a significant and comparable increase in the population of CD8$^+$ TILs containing granzyme B with either dose. However, s.c. administration resulted in higher levels of granzyme B per CD8$^+$ T cell (geometric mean fluorescence intensity; gMFI) in the EMT6 TME relative to i.p. dosing, with significant difference observed at the lower dose. Bintrafusp alfa did not significantly alter the infiltration of CD4$^+$ TILs in EMT6 tumors, regardless of dose and route of administration. We observed that both i.p. and s.c. administration significantly decreased the number of Tregs in the EMT6 TME to a similar extent, resulting in similar effects on CD8/Treg ratio. Additionally, both routes of administration resulted in a similar and significant decrease in infiltration of M-MDSCs. Analysis of immune cells in MC38 tumors upon i.p. or s.c. dosing of bintrafusp alfa (20 mg/kg) elicited comparable results (Figure 6, *lower panels*). No significant effects were observed in tumor infiltration of CD4$^+$ TILs, Tregs, or M-MDSCs. Similar to observations in the EMT6 model, both routes of administration resulted in a non-significant trending elevation of CD8/Treg ratio. Neither route of administration impacted the infiltration of CD8$^+$ TILs. However, both routes of administration equally induced a significant increase in the population of CD8$^+$ TILs containing cytolytic granzyme B. Overall, these data indicate that the immune modulatory effects and antitumor efficacy of bintrafusp alfa are not significantly different between i.p. and s.c. administration.

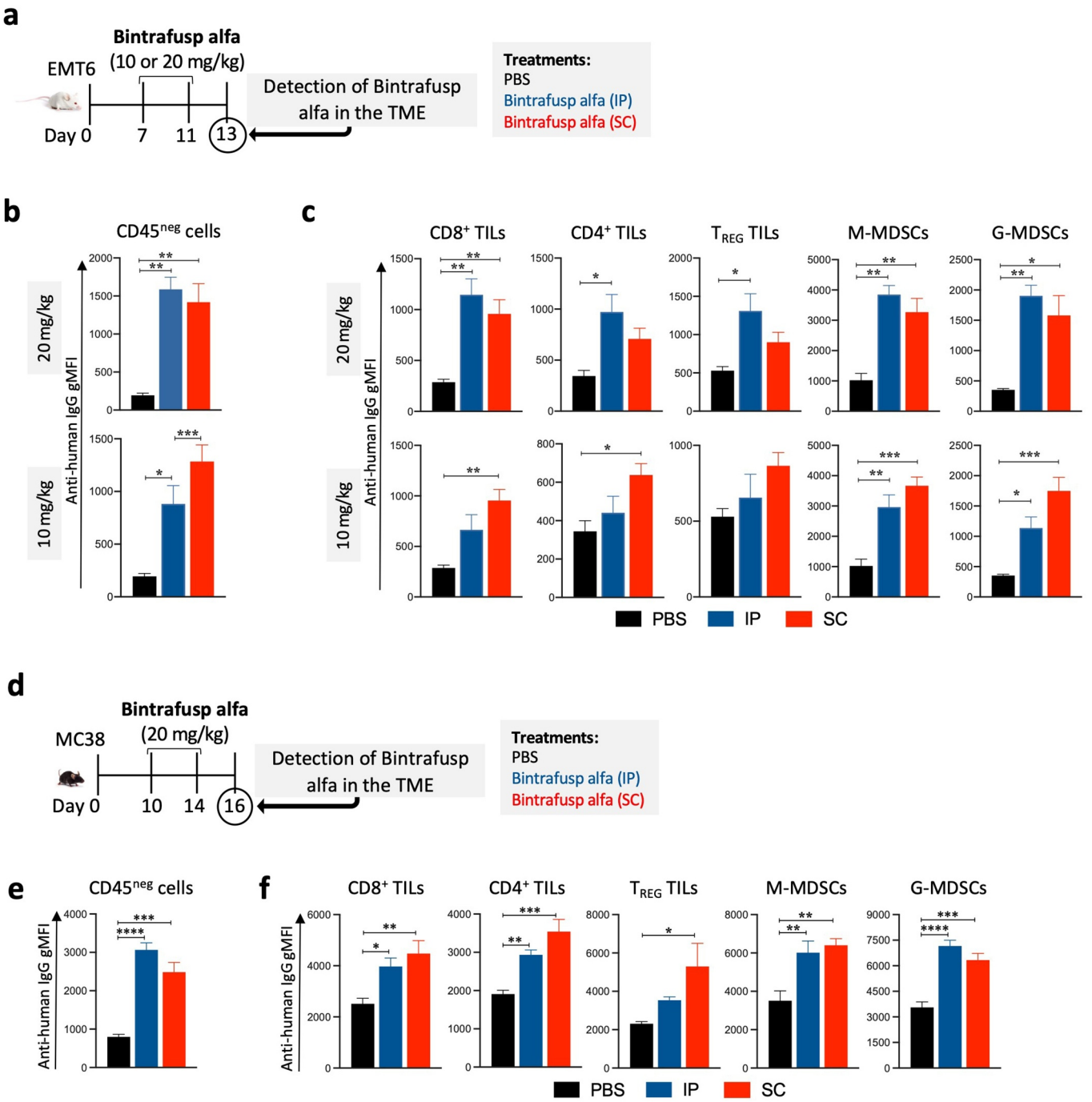

Figure 4. Effect of route of administration on bintrafusp alfa localization in the tumor microenvironment (TME). EMT6 tumors were implanted as in Figure 1a and treated as depicted in the schematic (a). Two days after the last treatment with PBS or bintrafusp alfa (20 or 10 mg/kg), tumor (n = 5/group) single-cell suspensions were analyzed by flow cytometry for the presence of bintrafusp alfa on the surface of (b) $CD45^{neg}$ tumor/stromal cells, or (c) $CD8^+$ tumor-infiltrating lymphocytes (TILs), $CD4^+$ TILs, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) of monocytic (M-MDSCs) or granulocytic (G-MDSCs) lineage. MC38 tumors (n = 5/group) implanted as in Figure 1d and treated as depicted in the schematic (d) were analyzed by flow cytometry two days after the last treatment with PBS or bintrafusp alfa (20 mg/kg) for the presence of bintrafusp alfa on the surface of (e) $CD45^{neg}$ tumor/stromal cells, or (f) designated tumor-infiltrated immune cells. Bintrafusp alfa was detected using an anti-human antibody. Data is shown as mean ± SEM. One-way ANOVA with Tukey's comparison, $*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$. gMFI, geometric mean fluorescence intensity.

Discussion

In addition to immune checkpoint inhibitor MAbs, the emergence of MAbs targeting tumor-associated antigens has dramatically altered the oncology field in recent decades by significantly improving clinical responses for patients with certain malignancies.[19] This led to regulatory approval of multiple agents targeting diverse antigens overexpressed in multiple malignancies, including trastuzumab for human epidermal growth factor receptor 2 (HER2)-positive breast cancer, and rituximab for multiple CD20-expressing hematological cancers, among others.[20,21] Whereas MAbs have been traditionally developed and approved to be administered i.v., various randomized clinical studies with

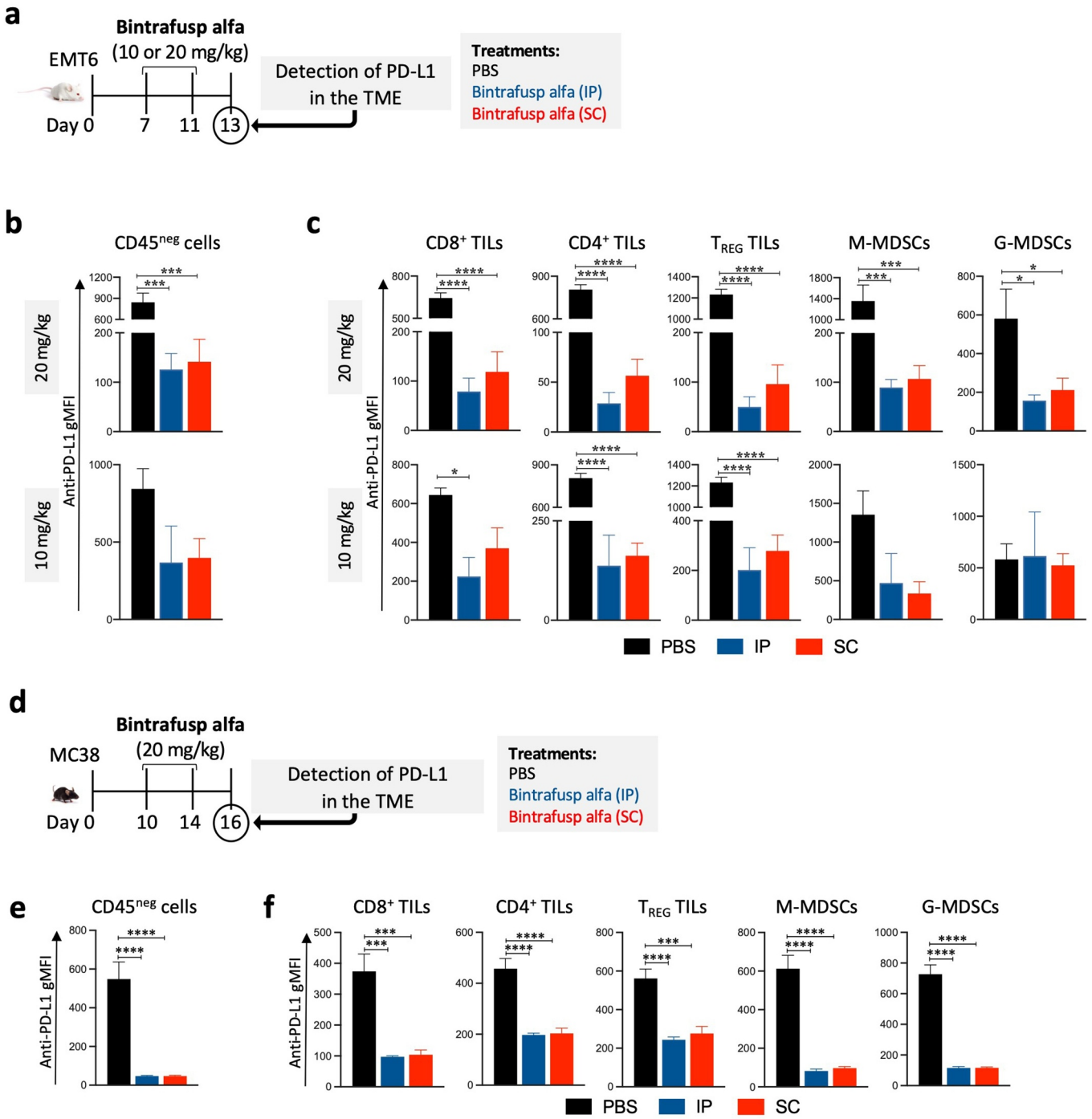

Figure 5. Effect of route of administration on PD-L1 detection in the tumor microenvironment (TME). EMT6 tumors were implanted as in Figure 1a and treated as depicted in the schematic (a). Two days after the last treatment with PBS or bintrafusp alfa (20 or 10 mg/kg), tumor (n = 5/group) single-cell suspensions were analyzed by flow cytometry for the presence of PD-L1 on the surface of (b) $CD45^{neg}$ tumor/stromal cells, or (c) $CD8^+$ and $CD4^+$ TILs, Tregs, and myeloid-derived suppressor cells (MDSCs) of monocytic (M-MDSCs) or granulocytic (G-MDSCs) lineage. MC38 tumors (n = 5/group) implanted as in Figure 1d and treated as depicted in the schematic (d) were analyzed by flow cytometry 2 days after the last treatment with PBS or bintrafusp alfa (20 mg/kg) for the presence of PD-L1 on the surface of (e) $CD45^{neg}$ tumor/stromal cells, or (f) designated tumor-infiltrated immune cells. Data shown as mean ± SEM. One-way ANOVA with Tukey's comparison, $*p < .05$, $***p < .001$, $****p < .0001$.

trastuzumab have identified s.c. administration as a viable alternative.[22–24] Based on these studies, subcutaneous trastuzumab (Herceptin Hylecta®) is now approved by the Food and Drug Administration (FDA) and the European Medicines Agency as an alternative to standard parental infusion. Clinical studies with rituximab have also compared s.c. to conventional i.v. administration, demonstrating non-inferior pharmacokinetics, and a similar safety and efficacy profile as with rituximab i.v.[25–27] Based on these clinical findings, rituximab s.c. (Rituxan Hycela®) is now FDA approved for multiple indications.

To the best of our knowledge, there are no reports examining the efficacy of s.c. administration of any bifunctional agent. Previous reports have demonstrated significant antitumor

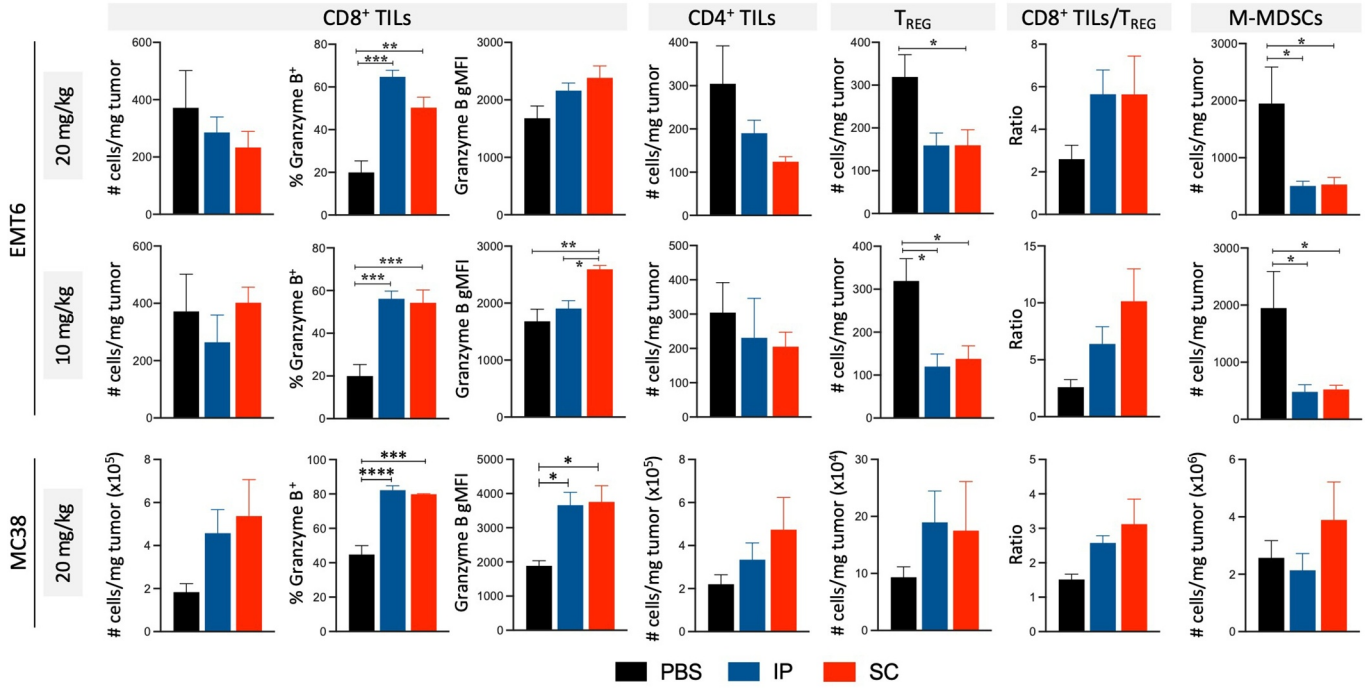

Figure 6. Immune effects in the tumor microenvironment elicited by bintrafusp alfa administered s.c. or i.p. EMT6 and MC38 tumors were implanted and treated as in Figure 4. Two days after the last treatment with PBS or bintrafusp alfa (20 or 10 mg/kg) administered i.p. or via s.c. injection, tumor (n = 4–5/group) single-cell suspensions were analyzed by flow cytometry for the presence of $CD8^+$ tumor-infiltrating lymphocytes (TILs), Granzyme B on $CD8^+$ TILs, $CD4^+$ TILs, regulatory T cells (Tregs), and myeloid-derived suppressor cells (MDSCs) of monocytic lineage (M-MDSCs). Data shown as mean ± SEM. One-way ANOVA with Tukey's comparison, $^*p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

efficacy of bintrafusp alfa when given systemically in multiple murine models of solid cancers, including EMT6 breast and MC38 colorectal tumors.[10,12] Here, we report our preclinical findings comparing the efficacy and immune correlates of s.c. vs. systemic i.p. administration of bintrafusp alfa at multiple dose levels in the EMT6 breast tumor model, and in the MC38 colorectal tumor model. Our findings in the EMT6 model indicate no significant difference between both routes of administration in attaining antitumor efficacy, cure rate, or survival elicited by a range of doses. Similar findings were observed in MC38 tumor-bearing mice. Importantly, no signs of toxicity, including injection-site reactions, were observed with either route of administration in these studies.

We have previously reported[12] that upon i.p. administration of 20 mg/kg, bintrafusp alfa was bound to a significant proportion (~40%) of nonimmune $CD45^{neg}$ cells in the TME, resulting in significant reduction in PD-L1 detection on the surface of those cells. This reduction was not observed upon administration of a mutant bintrafusp alfa devoid of PD-L1 binding ability. These findings were consistent with results from in vitro studies demonstrating bintrafusp alfa–mediated concentration-dependent decrease in PD-L1 detection on the surface of murine tumor cells at 4°C, which was not observed with mutant bintrafusp alfa.[12] This strongly suggests that the reduced detection of PD-L1 elicited by bintrafusp alfa is a result of agent binding to PD-L1 as opposed to PD-L1 downregulation or agent binding to TGFβ on the cell surface. Here, we extended these findings to examine the magnitude of bintrafusp alfa binding on a per cell basis on both immune and nonimmune cells present in the EMT6 and MC38 TMEs upon administration of 10 mg/kg and/or 20 mg/kg. These data indicate that bintrafusp alfa localizes to the TME when administered i.p. or s.c., with both routes resulting in similar levels of the molecule detected per cell in nonimmune cells with the highest dose. Interestingly, at the lower dose level, we observed a significantly higher localization of the agent on nonimmune cells when administered subcutaneously.

Analysis of the EMT6 TME immunome revealed extensive binding of bintrafusp alfa to both regulatory (MDSCs, Tregs) and effector (CD4, CD8) immune cell subsets, with highest levels observed in M-MDSCs, which displayed the highest level of PD-L1 expression. Significant binding was also observed in G-MDSCs and Tregs, with $CD4^+$ and $CD8^+$ T cells having lower levels of bound bintrafusp alfa. Notably, at the highest dose both systemic and subcutaneous administration resulted in similar extent and profile of binding, with the lowest dose (10 mg/kg) favoring s.c. administration. Despite these differences, however, both routes of administration resulted in a similar magnitude of PD-L1 blockade in both immune and nonimmune cells in the TME, as well as comparable ability of bintrafusp alfa to reduce plasma TGFβ1 levels. Similar results were observed in MC38 tumor-bearing mice.

These findings are consistent with data from several clinical studies comparing subcutaneous and intravenous administration of MAbs other than checkpoint inhibitors in cancer patients.[22,25,26,28–31] This paradigm shift in the delivery platform is supported by clinical studies analyzing other important aspects of healthcare delivery, such as patient preference and

healthcare-associated costs. Multiple studies with tumor-targeted MAbs have indicated a clear and consistent patient preference for s.c. administration relative to i.v. administration.[23–25,27,32–34] In one example, an international clinical study of 488 patients with early breast cancer randomized to receive s.c. trastuzumab followed by i.v., or vice-versa (PrefHer study, NCT0141166), revealed consistent patient preference for s.c. (88.9%) over i.v. (9.6%) administration, with safety consistent with previous reports.[23,24,33,34] Similar patient preference was observed in a randomized trial with 113 patients in the metastatic setting (MetaspHer study, NCT01810393).[25] Patient preference for either route of rituximab administration was also assessed on a randomized study (PrefMab, NCT01724021) with 743 previously untreated patients with $CD20^+$ diffuse large B-cell or follicular lymphoma. Safety was similar between both routes of administration, with 81% of patients preferring s.c. rituximab.[27]

Treatment regimens with parental administration of MAbs require more frequent and longer patient visits to the clinic.[32] Quantification of healthcare professional (HCP) and patient chair times in the SABRINA trial in eight countries revealed that s.c. administration resulted in significant reductions in HCP time (32%) and mean chair time (74%), potentially translating into higher efficiency of day oncology units and reduced healthcare costs.[32] Thus, by reducing patient treatment time and frequency, s.c. administration may increase the efficiency of the day clinic units and reduce costs associated with treatment and loss of patient productivity, while increasing patient comfort and satisfaction.[35–37]

The studies reported here provide the rationale to evaluate the delivery of bintrafusp alfa, a bifunctional agent that both targets PD-L1 and sequesters TGFβ, systemically vs s.c. injection in clinical studies to determine whether the s.c. route of administration, with its advantages described above, will attain similar clinical efficacy.

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