Intratumourally injected alum-tethered cytokines elicit potent and safer local and systemic anticancer immunity

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Anti-tumour inflammatory cytokines are highly toxic when administered systemically. Here, in multiple syngeneic mouse models, we show that the intratumoural injection of recombinantly expressed cytokines bound tightly to the common vaccine adjuvant aluminium hydroxide (alum) (via ligand exchange between hydroxyls on the surface of alum and phosphoserine residues tagged to the cytokine by an alum-binding peptide) leads to weeks-long retention of the cytokines in the tumours, with minimal side effects. Specifically, a single dose of alum-tethered interleukin-12 induced substantial interferon-γ-mediated T-cell and natural-killer-cell activities in murine melanoma tumours, increased tumour antigen accumulation in draining lymph nodes and elicited robust tumour-specific T-cell priming. Moreover, intratumoural injection of alum-anchored cytokines enhanced responses to checkpoint blockade, promoting cures in distinct poorly immunogenic syngeneic tumour models and eliciting control over metastases and distant untreated lesions. Intratumoural treatment with alum-anchored cytokines represents a safer and tumour-agnostic strategy to improving local and systemic anticancer immunity.

Immune checkpoint blockade therapy has improved progression-free survival in patients suffering from cancer over previous treatment modalities1–4. However, immune checkpoint blockade typically elicits durable responses in a minority of patients, in part because of the highly immunosuppressive tumour microenvironment (TME)5,6. Although rational combinations with inflammatory cytokines or immune agonists can relieve some immunosuppression7,8, systemic dosing of these proteins is impeded by severe immune-related adverse events. Early phase 1 clinical trials involving promising cytokines such as interleukin-2 (IL-2) and interleukin-12 (IL-12) resulted in sub-optimal anti-tumour efficacy with high treatment-related morbidity and even mortality, partially due to limited drug exposure within the tumour and over-stimulation of lymphocytes in healthy tissue9–14. Thus, there is great promise for strategies that could localize cytokine effects to the TME.

One approach to focus the activity of immunostimulatory agents in tumours while lowering systemic toxicity is to administer these drugs intratumourally. With advances in interventional radiology, endoscopy and laparoscopic surgery procedures, most lesions in the human body are now accessible for intratumoural (i.t.) dosing15. Moreover, a locally stimulated immune response in one lesion can elicit systemic anti-tumour immunity to promote control over untreated lesions in patients, especially in combination with systemic checkpoint blockade therapy16–20. However, i.t. injection of therapeutics does not ensure persistence in the TME, since free drugs are quickly cleared via lymphatics and/or the tumour vasculature, rapidly leading to toxic accumulation in the circulation21,22. For instance, while there is extensive interest in the local delivery of IL-1222–24, these approaches are typically accompanied by rapid leakage of IL-12 into the circulation, which in turn triggers systemic interferon-γ (IFN-γ) production (a biomarker for IL-12-related immune-related adverse events)25–27. We have previously reported a strategy of fusing cytokines to collagen-binding proteins to enhance TME retention following i.t. administration, which reduced toxicities of these potent agents while enhancing therapeutic efficacy28. This strategy extends drug persistence over a period of a few days, but is dose-limited by the quantity of collagen available in the TME, which varies from patient to patient and tumour to tumour. Further, drug is spatiotemporally governed by the distribution and turnover of collagen in the tumour.

In this Article, we demonstrate an approach for the i.t. delivery of engineered cytokines using the US Food and Drug Administration (FDA)-approved vaccine adjuvant aluminium hydroxide (alum). Alum has nearly 100 years of history of safe use in humans and is administered annually to millions of people in over 20 vaccine formulations. Aluminium hydroxide adjuvants are composed of micrometre-scale aggregates of nanometre-scale rod-shaped nanocrystals; these alum aggregates form a physical depot at injection sites in tissue that is persistent over a period of weeks29. Phosphorylated proteins bind tightly to alum through a ligand exchange reaction with surface hydroxyls, enabling retention of bound molecules in the presence of interstitial fluid in vivo30–32. To exploit this chemistry, we...
A single kinase, Fam20C, is responsible for phosphorylation of Targeted phosphorylation via an in-cell approach is robust. Results blockade therapy. genic preclinical models when combined with systemic checkpointrobust systemic anti-tumour responses in multiple poorly immuno-
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and eliminated systemic toxicities seen upon i.t. injection of the free
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in tumours for more than a week. Applied to the cytokine IL-12,
reduce a series of ABP-labelled cytokines, which rapidly adsorbed
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developed an approach for in-cell site-specific protein phosphoryla-
tion to synthesize bioactive proteins fused with a phosphorylated
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duce a series of ABP-labelled cytokines, which rapidly adsorbed
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robust systemic anti-tumour responses in multiple poorly immuno-
genic preclinical models when combined with systemic checkpoint
blockade therapy.

Results
Targeted phosphorylation via an in-cell approach is robust.
A single kinase, Fam20C, is responsible for phosphorylation of
the majority of the mammalian secreted phosphoproteome. We
hypothesized that co-expression of Fam20C together with therapeu-
tic proteins fused to a short peptide containing consensus motifs
for the kinase (alum binding peptide or ABP) would lead to the spe-
cific phosphorylation of the ABP (Fig. 1a). Fam20C recognizes and
phosphorylates serines contained within a well-defined consensus
motif (S-x-E) in mammalian cells, and can be engineered for
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The data from the co-induced experiments with n = 3 technical replicates per group and presented as mean ± s.d. P values were determined by ordinary one-way ANOVA followed by Tukey’s multiple comparison test (d,e) or two-way ANOVA with Šidak’s multiple comparisons test (b) using GraphPad PRISM, and exact P values are indicated (NS, not significant, P > 0.05).

Fig. 1 | Co-expression of cytokines and Fam20C enables in-cell site-specific serine phosphorylation of interleukins. a, Manufacturing workflow for ABP-fusion proteins with IL-12 fused to ABP10 as an example. b, Phosphorylation as measured by malachite green assay for IL-12 and IL-12–ABP either expressed alone (np) or co-expressed with Fam20C-KDEL (p). c, Indicated proteins were run on an SDS–PAGE gel stained with Coomassie Blue (Simple Blue, orange) or transferred to a membrane and stained with an anti-pSer antibody followed by an IR800 secondary (purple). Shown are bands for ~65 kDa purified protein. The blot was analysed by Fiji (ImageJ). The unedited blot image is available as source data. d, Phosphorylation was measured as in b for indicated proteins. e, Fluorophore-conjugated IL-12 fusion proteins (10 μg ml−1) were mixed with Alhydrogel (100 μg ml−1) for 30 min in TBS, then incubated in 10% mouse serum in PBS for 1 h, followed by fluorescence spectroscopy to measure protein remaining bound to alum. f, IL-12 proteins at indicated concentrations (maximum alum concentration was 6 ng ml−1) were incubated with murine splenocytes for two days. Shown are the IFN-γ concentrations measured in culture supernatants by ELISA. ABP refers specifically to ABP10. Data are representative of at least two independent experiments with n = 3 technical replicates per group and presented as mean ± s.d. P values were determined by ordinary one-way ANOVA followed by Tukey’s multiple comparison test (d,e) or two-way ANOVA with Šidak’s multiple comparisons test (b) using GraphPad PRISM, and exact P values are indicated (NS, not significant, P > 0.05).
co-expressed with the kinase (IL-12–ABP-p) but not when expressed without kinase (IL-12–ABP-np), and IL-12 lacking the ABP was not phosphorylated regardless of the presence of the kinase (Fig. 1b,c).

Phosphorylation was dependent on the presence of the target serines in the S-x-E motifs and was sensitive to the spacing between these motifs as well as the sequence of flanking residues (Supplementary Information).
Fig. 3 | A single dose of alum-anchored i.t. IL-12 or IL-2 elicits long-term tumour regression in multiple syngeneic cancer models. a–d. Overall survival over time for mice bearing flank Ag104A (a), B16F10 (b,c) or MC38 (d) tumours after a single i.t. dose administered on day 7 (a,d) or day 6 (b,c) after inoculation. Groups for a were untreated (n = 10), IL-12-ABP-p (20 μg) i.t. (n = 13), and IL-12-ABP-p (20 μg)/alu m (100 μg) i.t. (n = 13). Treatments for b were saline i.t. + saline i.p. (n = 10), saline i.t. + TA99 i.p. (n = 15), MSA–IL-2–ABP-p (36 μg) i.t. + TA99 i.p. (n = 8), MSA–IL-2 (34 μg)/alum (90 μg) i.t. + TA99 i.p. (n = 15), MSA–IL-2–ABP-p (36 μg)/alum (90 μg) i.t. + TA99 i.p. (n = 15), MSA–IL-2–ABP-p (36 μg)/alum (90 μg) i.t. + TA99 i.p. (n = 15), MSA–IL-2–ABP-p (36 μg)/alum (90 μg) i.t. + TA99 i.p. (n = 15), and lumican–MSA–IL-2 (52 μg) i.t. + TA99 i.p. (n = 10). TA99 was dosed at 200 μg per injection. Treatments for c were untreated (n = 10), saline i.t. + anti-PD1 i.p. (n = 10), alum (100 μg) i.t. + anti-PD1 i.p. (n = 9), IL-12-ABP-p (20 μg) i.t. (n = 15), and IL-12-ABP-p (20 μg)/alum (100 μg) i.t. (n = 28). Treatments for d were untreated (n = 5), untreated i.t. + anti-PD1 i.p. (n = 5), IL-12-ABP-p (20 μg) i.t. (n = 10), and IL-12-ABP-p (20 μg)/alum (100 μg) i.t. (n = 20). Anti-PD1 was dosed at 200 μg per injection. e,f. Mice with B16F10 tumours were treated on day 6 i.t. with saline (n = 5), alum (100 μg, n = 5), IL-12-ABP-p (20 μg) + MSA–IL-2–ABP-p (36 μg, n = 10), or IL-12-ABP-p + MSA–IL-2–ABP-p + alum (n = 10); all groups received anti-PD1 i.p. (black arrows). Shown is the overall survival (e) and percent change in body weight (mean ± s.d., f) over time after treatment. Anti-PD1 was administered on days 6, 9, 12, 15 and 18 for b,f and days 7 and 13 for c. Red and black arrows indicate timing of i.t. and i.p. treatments, respectively. ABP refers specifically to ABP10. P values were determined by the log-rank (Mantel–Cox) test (a,d). Gehan–Breslow–Wilcoxon test (b,e) or two-way ANOVA followed by Tukey’s multiple comparison test versus alum i.t. at indicated timepoints (f) using GraphPad PRISM.

Fig. 1i–l). When fused to other therapeutic cytokines (IL-2 fused to mouse serum albumin (MSA, to enhance expression) or a super-agonist complex of interleukin-15 (IL-15) with the IL-15α chain (IL-15αa)), we observed consistent phosphorylation of the ABP (Fig. 1d), indicating that this in-cell Fam20C-dependent phosphorylation approach is robust and modular.

Initial adsorption of IL-12 to alum in buffer was similar irrespective of the presence of the ABP tag (Supplementary Fig. 2a). However, the majority of IL-12 with a phosphorylated ABP remained bound following incubation of the cytokine-loaded alum with mouse serum, while unphosphorylated IL-12 rapidly desorbed (Fig. 1e). Tracked over time, phosphorylated IL-12 was slowly
released from alum over ~2 weeks (Supplementary Fig. 2b). IL-12 anchoring on alum increased the size of alum crystal microaggregates in saline (Supplementary Fig. 2c), but the nanoscale morphology of alum nanorods appeared unchanged (Supplementary Fig. 2d,e). Further, the bioactivity of IL-12–ABP-p was similar to native single-chain IL-12 as measured by splenocyte activation (Fig. 1f) and HEK-Blue IL-12 reporter cell activation (Supplementary Fig. 2f,g). Interestingly, IL-12–ABP-p remained functional while adsorbed to alum particles, although with a several-fold reduction in half-maximal effective concentration (EC50) (Fig. 1f) and HEK-Blue IL-12 reporter cell activation (Supplementary Fig. 2f,g). The bioactivity of alum-bound IL-12–ABP-p was essentially constant even following multiple days of incubation in serum at 37 °C, indicating high in vitro stability for alum–cytokine complexes (Supplementary Fig. 2i). We also analysed in vitro alum binding and bioactivity of IL-2–ABP and IL-15sa–ABP fusion proteins. Similar to IL-12, when tagged with the phosphorylated ABP, these cytokines also showed enhanced retention on alum following serum exposure (Supplementary Fig. 2j,k). IL-2–ABP-p exhibited a four-fold loss in activity when bound to alum similar to IL-12–ABP-p, while IL-15sa–ABP-p showed no change in bioactivity when bound to alum (Supplementary Fig. 2l,m). Thus, ABP-fusion cytokines produced by in-cell phosphorylation exhibit stable alum binding and remain functional while immobilized on alum in vitro.

Alum-bound IL-12 safely persists in vivo after treatment. We next assessed the biodistribution and pharmacokinetics of alum-bound IL-12–ABP-p in vivo. We previously reported a strategy to stably label alum particles using an AlexaFluor 488 dye conjugated to a solid phase-synthesized poly-phosphoserine (pSer4) peptide16. Alum was labelled using this approach and combined with IL-12–ABP-p labelled with AlexaFluor 568 dye. Immediately following i.t. injection in subcutaneously (s.c.) implanted B16F10 melanoma tumours, we observed that alum and phosphorylated protein were co-localized and distributed throughout the tumour bed (Fig. 2a). Measurement of IL-12 remaining in tumours 24, 72 or even 144 h later revealed a >400-fold greater retention of alum-tethered IL-12–ABP-p versus free IL-12–ABP-p (Fig. 2b). IVIS whole-animal fluorescence imaging of labelled alum-bound IL-12–ABP-p showed persistence of the cytokine at high levels in injected tumours for weeks after a single dosing, while signal from IL-12–ABP-p injected without alum was rapidly cleared (Fig. 2c,d and Supplementary Fig. 3a). Note that the high density of dye-labelled protein bound to alum leads to some fluorescence quenching at time zero17, which is alleviated over the first few days as some cytokine is released, lowering the dye density and causing an artificial increase in signal over the first few days post injection. Intratumoural injection of free IL-12–ABP-p or alum mixed with non-phosphorylated IL-12 led to high levels of the cytokine in serum a few hours after treatment, while alum/IL-12–ABP-p injection led to IL-12 levels that were not statistically different than the baseline of untreated mice (Fig. 2e and Supplementary Fig. 3b).

IL-12 induces IFN-γ secretion by lymphocytes, which has been associated with toxicity in IL-12 clinical trials18. To assess the impact of altered IL-12 pharmacokinetics on safety, we established Ag104A fibrosarcoma flank tumours in C3H-Hej mice, which are known to better model human sensitivity to IL-12 than C57Bl/6 mice19. Unanchored IL-12 rapidly dispersed in the blood as observed in C57Bl/6 mice (Supplementary Fig. 3c). Intratumoural injection of free IL-12 or alum mixed with non-phosphorylated IL-12...
led to animal weight loss, significant elevations in serum IFN-γ and alanine transaminase (ALT, indicating liver toxicity), and reduced albumin and total protein levels in blood after a single dose (Fig. 2f–h and Supplementary Fig. 3d–f). By contrast, i.t. injection of alum-anchored IL-12–ABP-p elicited significantly lower serum IFN-γ levels, prevented ALT levels from exceeding the normal clinical range, and left blood chemistry unaffected, correlating to no weight loss in treated animals (Fig. 2f–h and Supplementary Fig. 3d–f). By contrast, i.t. injection of MSA–IL-2 elicited little survival benefit over treatment with the TA99 alone, with no long-term survivors while many cytokines have unacceptable toxicity when administered systemically, checkpoint blockade antibodies and antibodies against tumour cell surface antigens are better tolerated and are approved as systemic treatments. Thus, we also investigated the anti-tumour efficacy of combining a single i.t. alum/ABP–cytokine dose with systemic administration of antibodies modelling clinically relevant treatment combinations. We first treated mice bearing established B16F10 melanoma tumours intratumourally with a single dose of MSA–IL-2 accompanied by systemic administration of an antibody against tyrosinase-related protein 1 (anti-TYRP-1 or TA99). We previously reported that TA99 combined with MSA–IL-2 fused to the collagen-binding protein lumican leads to some curative responses in this model following multiple doses28. Alum-bound phosphorylated MSA–IL-2–ABP-p combined with TA99 regressed tumours and elicited complete responses in the majority of treated animals following a single dose (Fig. 3b and Supplementary Fig. 4b–e). By contrast, injection of MSA–IL-2–ABP-p in the absence of alum or administration of alum mixed with unphosphorylated MSA–IL-2 elicited little survival benefit over treatment with the TA99 alone, with no long-term survivors (Fig. 3b and Supplementary Fig. 4b–e). Moreover, a single injection

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Anti-tumour efficacy is stronger with i.t. IL-12 retention. Dosing of alum-bound IL-12 intratumourally was not only safer but also much more effective. A single i.t. dose of IL-12–ABP-p + alum into large subcutaneous Ag104A tumours led to complete responses in 11 of 13 animals, while unanchored IL-12–ABP-p was only moderately effective and led to treatment-related mortality in 1 of 13 animals (Fig. 3a and Supplementary Fig. 4a).
of lumican–MSA–IL-2 in combination with TA99 was significantly less effective than alum-bound IL-2 (Fig. 3b and Supplementary Fig. 4d). The majority of mice that rejected their primary tumours also rejected cancer cells on rechallenge for the IL-2/alum/TA99 combination (Supplementary Fig. 4f).

We next tested i.t. IL-12 treatment in the same tumour model in combination with anti-PD1 therapy. Intratumoural alum/IL-12–ABP-p combined with systemic anti-PD1 elicited complete responses in 12 of 23 mice, while unanchored IL-12–ABP-p provided only a modest tumour growth delay and no long-term survival (Fig. 3c and Supplementary Fig. 4g). Notably, anti-PD1 played an important role in the high efficacy of the alum-anchored IL-12/checkpoint blockade combination (Supplementary Fig. 4h). In this study, we also assessed the immunogenicity of IL-12–ABP-p constructs: compared with the positive control of mice immunized with ovalbumin (OVA) mixed with alum, no detectable anti-drug antibody responses were detected by enzyme-linked immunosorbent assay (ELISA) (Supplementary Fig. 4i). In another syngeneic tumour model, the MC38 colon carcinoma, single shot alum/IL-12–ABP-p therapy elicited even stronger responses (9/10 complete responses) in combination with systemic anti-PD1, while IL-12–ABP-p in the absence of alum was substantially less potent (Fig. 3d and Supplementary Fig. 4j).

The ability of alum anchoring to block cytokine dissemination into the blood prompted us to next test combinations of alum-bound cytokines. Treatment of B16F10 tumours with a single i.t. dose of alum-tethered MSA–IL-2–ABP-p and IL-12–ABP-p accompanied by systemic anti-PD1 led to tumour eradication in nine of ten mice with no weight loss (Fig. 3e,f). By contrast, i.t. administration of this cytokine combination in the absence of alum led to pronounced weight loss and much poorer efficacy (Fig. 3e,f). In summary, by anchoring cytokines to alum via phosphorylation, both the safety and anti-tumour efficacy are enhanced in diverse tumour models, and clinically relevant combination treatments achieve high levels of therapeutic efficacy following a single i.t. injection.

Alum–cytokine treatment promotes control over distal lesions.

The success of any i.t. therapy in the clinic will depend on its ability to promote systemic anti-tumour responses to control distal, untreated lesions and micro-metastases6,36. Therefore, we tested the ability of single-shot alum–IL-12 therapy to promote abscopal responses using two distinct models. First, we established Ag104A tumours on opposite flanks of mice and treated only one of the tumours with a single dose of alum with anchored IL-12–ABP-p (Fig. 4a). Even though IL-12 leakage into the blood was eliminated by alum binding, single-dose IL-12–ABP-p/alum exhibited greater efficacy than IL-12 alone, and elicited a systemic response that eliminated established distal untreated tumours in the absence of systemic checkpoint blockade therapy (Fig. 4b–f). Alum-anchored IL-12–ABP-p also significantly outperformed free IL-12–ABP-p in...
mice with bilateral B16F10 tumours in combination with systemic anti-PD-1.

A second important clinical treatment scenario is neoadjuvant therapy. To assess the potential of alum-bound cytokines to be effective in this setting, we orthotopically implanted spontaneously metastatic 4T1 breast cancer cells in the mammary fat pad of BALB/c mice, and treated established tumours with a single dose of alum, IL-12–ABP-p or IL-12–ABP-p + alum, in combination with systemic anti-PD1. Nine days after treatment, we surgically resected the primary tumours and monitored the mice for survival (Fig. 5a). Alum-bound IL-12–ABP-p notably slowed primary tumour progression relative to treatment with alum alone or free IL-12–ABP-p (Fig. 5b). In this model, animals are susceptible to relapse due to micro-metastases in the lungs. The combination of alum-bound IL-12–ABP-p and anti-PD1 led to long-term survival of ~75% of animals, in contrast to minimal benefit achieved by alum/anti-PD1
or IL-12/anti-PD1 (Fig. 5c). To evaluate whether this supplementary survival was due to improved tumour-specific T-cell responses, we analysed the peripheral blood of all survivor mice for the frequency of CD8+ T cells specific to gp70, an endogenous retroviral antigen expressed by 4T1 cells38. Impressively, a single dose of alum-anchored IL-12 promoted high gp70 tetramer+ T-cell responses over both age-matched untreated mice bearing orthotopic 4T1 tumours and the few long-term survivors treated with free IL-12–ABP-p (Fig. 5d,e). Thus, a single dose of alum-anchored IL-12 elicits systemic immunity enabling control over non-injected distal tumours in multiple tumour models.

Alum/IL-12-induced IFN-γ drives early tumour regression. IL-12 can activate cells from both the innate and adaptive immune compartments either directly or indirectly via IFN-γ4,20,41-48. To determine the cellular and molecular effectors implicated in the context of persistent i.t. IL-12, we first carried out quantification of cytokines and chemokines generated in the TME of B16F10 tumours. Three days post treatment, alum/IL-12–ABP-p upregulated a battery of inflammatory effector proteins, including IL-6, TNF-α, IL-1β, CXCL9 and CXCL10 (Fig. 6a and Supplementary Fig. 5a,b). By this timepoint, IFN-γ production was increased five-fold over treatment with alum alone or IL-12–ABP-p alone, and was sustained for at least 6 days (Fig. 6b), indicating that high amounts of intratumourally retained IL-12 are still active at this timepoint. Although alum is known to activate the NLRP3 inflammasome48, cytokine release in tumours and anti-tumour efficacy were unaffected in Nlrp3−/− mice (Supplementary Fig. 5c–g).

Successful checkpoint blockade therapy in mice relies on strong collaboration between T-cell-produced IFN-γ and dendritic cell (DC)-produced IL-12. Here, treatment in the presence of an IFN-γ-neutralizing antibody entirely eliminated the efficacy of alum-anchored IL-12 with immediate loss of tumour control (Fig. 6c and Supplementary Fig. 6a). Thus, we asked what key cellular effectors drive IFN-γ expression in response to IL-12 (Supplementary Fig. 7). While IFN-γ production was upregulated in multiple cell types by alum-anchored IL-12, CD8+ T cells and natural killer T (NKT) cells were upregulated by the NLRP3 inflammasome, cytokine release in tumours and anti-tumour efficacy were unaffected in Nlrp3−/− mice (Supplementary Fig. 5c–g).

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expanded a greater dLN-infiltrating MHC-II+ and CD86+Zsgreen+ monocyte population in dLNs, which peaked 3 days post treatment (Fig. 8f,g and Supplementary Fig. 11f,g). IL-12–ABP-p + alum also promoted CD11c expression in dLN monocytes, suggesting their differentiation toward monocyte-derived DCs (Fig. 8h). Other DC and macrophage populations in the dLNs, particularly migratory CD11b+ DCs and medullary chord macrophages, also had higher levels of CD86 and Zsgreen uptake over time after treatment with alum-bound IL-12–ABP-p (Supplementary Fig. 11h–j). Consistent with these shifts in antigen uptake in antigen presenting cells (APCs), an IFN-γ enzyme-linked immune absorbent spot (ELISPOT), performed on irradiated B16F10 cells cultured with splenocytes collected 10 days after i.t. treatment of B16F10 tumours revealed substantially higher tumour-specific responses in mice treated with alum/IL-12–ABP-p than those treated with IL-12–ABP-p alone (Fig. 8i). In summary, by tethering IL-12 to alum and improving its
i.t. persistence following a single dose, several antigen-presenting myeloid cell types remain highly activated while presenting antigen in tumour dLNs and enhancing tumour-specific T-cell priming.

Discussion
Clinical translation of cytokine-based cancer immunotherapy has been challenging. So far, only IFNα and IL-2 have been approved by the US FDA for use in certain indications, and even those have fallen out of favour because of toxicity concerns63. IL-12 potently drives anti-tumour immunity in mouse models but has been found to have an unmanageably narrow therapeutic window in humans64. Here we report a modular approach for the local delivery of high-dose cytokine therapy with negligible toxicity. A single i.t. dose of alum-anchored cytokines enabled in situ vaccination, promoting durable local and systemic anti-tumour responses in several mouse cancer models.

Although other biomaterials such as injectable hydrogels65–67, polymeric microspheres68, Montanide ISA-51, mesoporous silica nanoparticles69, microneedle patches70,71 and other materials72,73,74 have been explored for the local and sustained release of cytokines and chemokines in tumours, the FDA approval for use of these approaches in cancer remains a constant hurdle due to regulation, manufacturing and scalability challenges75. Further, precise engineering of the release rate of drugs to match their consumption rate in tumours has been extremely difficult, with clear evidence of drug leakage into the systemic circulation for most of these platforms66,67. For IL-12 in particular, there is considerable interest in the i.t. delivery of the IL-12 gene either via plasmid electroporation10 or several viral and non-viral vectors76–78. However, this strategy also often results in the swift accumulation of IL-12 and IFN-γ in the blood10. By anchoring IL-12 to alum using phosphorylated peptide tags, we are able to dramatically limit such systemic dissemination, while maintaining high efficacy.

The number of clinical trials involving i.t. immunotherapy has increased swiftly over the past decade, and the oncolytic viral therapy talimogene laherparepvec (TVEC) became the first FDA-approved local immunotherapy in 2015. With innovations in interventional radiology and surgical techniques, essentially any lesion has become accessible for i.t. injections, making human i.t. immunotherapy a viable route for the administration of cancer drugs79. However, for lesions requiring surgery, treatments that can elicit prolonged immunostimulation following infrequent injections will be desirable, in combination with systemic dosing of approved therapies such as checkpoint blockade or tumour-targeting antibodies. In the single i.t. dose context, we show that alum-anchored IL-2 and IL-12 can greatly improve outcomes in both immunologically ‘hot’ (MC38) and ‘cold’ (B16F10) tumours in combination with checkpoint blockade or anti-tumour antibodies. We also see improved survival over collagen-anchored IL-2 in the single-dose setting. Strikingly, a single local dose of IL-12 also elicited systemic responses, delaying the growth of established untreated distal tumours in two different solid tumour models and greatly decreased lung metastases after neoadjuvant treatment of orthotopic 4T1 tumours, a model for triple-negative breast cancer.

Whereas enhancing i.t. residence of IL-12 led to the expected stimulation of T cells and NK cells to produce IFN-γ, we also observed notable activation of APC populations in dLNs, and increased tumour antigen accumulation in these cells. This effect could result from direct IL-12 signalling80,81, IFN-γ-mediated reprogramming of DCs and monocytes82,83 or an indirect outcome of tumour antigen and damage-associated molecular pattern release following cancer cell killing triggered by IFN-γ and granzyme B-producing T and NK cells. MHC-I upregulation by tumour cells after IFN-γ release is expected to promote cytolytic T-cell killing. Antibody-mediated neutralization experiments indicated that, while early tumour control may be entirely dependent on IFN-γ-driven immune responses, long-term tumour regression required both innate (cDC1, CSF1R+ monocytes and macrophages) and adaptive (CD8+ T cells) immune cells. The observed reprogramming of i.t. monocytes might also contribute to increased tumour-cell killing. Thus, by tethering IL-12 to alum for i.t. treatment, we not only restrict toxicity but also potentiate IL-12’s pleiotropic therapeutic mechanisms of action.

Alum mixed with IL-12 was previously used to promote Th1 responses to sub-unit vaccines84,85. We used alum for the multi-day persistent delivery of cytokines to tumours. In the vaccine context, alum has been reported to improve humoral immunity by enabling sustained retention of antigen at injection sites, improving uptake by antigen-presenting cells, promoting necrosis and release of damage-associated molecular patterns at the injection site and signal through the NLRP3 inflammasome to elicit type-2 helper-T-cell-based antibody responses86. We found, however, that the NLRP3 inflammasome was not necessary for either inflammatory cytokine release or overall therapeutic efficacy for single-dose alum–IL-12 therapy. Some release of IL-6 and TNF-α does occur with i.t. treatments of just alum 1 day after treatment. This could be attributed to alum promoting necrosis in the tumour. However, this inflammation was only transient since i.t. injections of alum alone never led to substantial tumour control in any of the models we tested. Site-specific anchoring of proteins to solid surfaces can enhance stability and protect from endogenous, extracellular processes. So, alum may also delay cytokine degradation in vivo and enhance the signalling period for cytokines.

In this study, we report the retention of alum-bound cytokines at injection sites for weeks after administration. Further, therapeutic efficacy appears to rely on strong activation driven by early IL-12 retention with enhanced IFN-γ secretion in the tumour lasting at least a week after treatment. Although delayed-type hypersensitivity to alum is rare and clinically manageable in humans87, we did not observe anti-ABP or anti-cytokine antibodies in treated animals, chronic inflammation due to long-term cytokine release at the sites of cured tumours could promote autoimmune reactions and granulomas88. Thus, it will be of interest in future work to define an optimal window for cytokine signalling and evaluate the long-term degradation, accessibility and bioactivity of the alum–cytokine depot. If there is active, accessible IL-12 months after treatment, alterations in the ABP designs such as the addition of a protease cleavage tag or a pH-responsive linker89 could be made to ensure the removal of the cytokine after primary tumour cure.

The intracellular Fam20C phosphorylation method was developed with manufacturability and generalizability in mind90. For bio-manufacturing generality and to take advantage of highly developed good manufacturing practice (GMP) cell expression platforms, we developed an in-cell phosphorylation strategy such that proteins are modified during secretion from mammalian cells. Stable cell lines expressing Fam20C could be next used for the transient transfection of any ABP-fusion protein of interest. Further, even proteins with internal S-x-E sites (IL-12p40 has two S-T-E sites) were only phosphorylated with the ABP present, indicating that the clustering of the motifs on one unstructured small peptide dominates phosphorylation. With some optimization in ABP design, linker sequence between ABP and target protein, and position of ABP within the protein, we have been able to apply this approach to antibodies, antibody–cytokine fusion proteins, other cytokines such as interferons, and other interleukins.

Finally, effective temporal programming may be critical to harnessing the true power of combination immunotherapy91. The customizability of the ABP approach may enable one to precisely control and tune release of proteins from alum in vivo and, eventually, programme timing such that a single shot of alum-anchored proteins would lead to release of distinct therapies at different times after treatment.
Methods

Cell lines and animals. Cell lines B16F10 (ATCC), HEK-Blue IL-12 (InvivoGen), HEK293-F (Gibco), 4T1 (ATCC) and CTL-2 (ATCC) cells were cultured following vendor instructions. 4T1-GFP-Luc (4T1-Luc) cells were generated by transfection of the 4T1 cell line with pGFPv6-1 (Clontech, System Biosciences), and B16F10–Tryp knock-out (KO) cells were generated as previously described by Mohiyuddin et al. Ag04A, MC38 and B16F10–Zagreen cells were a gift from H. Schreiber (University of Chicago, I. Schlom (National Cancer Institute) and R. Hynes (Massachusetts Institute of Technology (MIT)), respectively. 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (ATCC) supplemented with 10% foetal bovine serum (FBS), 100 units ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin, while Ag04A, MC38 and B16F10–Zagreen were all cultured in complete Dulbecco’s Modified Eagle’s Medium supplemented with 10% FBS, 100 units ml$^{-1}$ penicillin and 100 μg ml$^{-1}$ streptomycin. All cell lines were maintained at 37 °C and 5% CO$_2$, and all tested negative for mycoplasma.

Cloning, protein purification and phosphorylation analysis. Genes for single-stranded DNA primers (Integrated DNA technologies) that were then exchanged into Tris-buffered saline (TBS, 1×$20$ mM Tris–HCl, 150 mM NaCl, pH 7.5) were further purified using custom anion exchange chromatography salt gradients from cell culture supernatants were then purified using HisPur Ni-NTA metal affinity resin (Thermo Scientific), and data were normalized to maximum radiance throughout the experiment per protein.

In vitro alumin-binding and bioactivity assays. All alum used in the study was Alhydrogel purchased from Invivogen. Alumin-binding assays were performed as described previously. Briefly, proteins were conjugated to Alexa Fluor 647 (AF647) via NHS labelling (Invitrogen), mixed with alum with a mass ratio of 10:1 alumin:protein unless otherwise noted in TBS and rotated at room temperature (r.t.) for 20 min to enable adsorption. Subsequently, the samples were centrifuged at 10,000 g for 10 min to pellet alum, and the supernatant was aliquoted and replaced with 10% mouse serum-containing PBS. The tubes were then moved to a rotator at 37 °C for indicated amounts, the samples treated, and the supernatant was replaced with free 10% mouse serum-containing PBS. All removed supernatants were analysed for fluorescence using a Tecxan Infinite M200 absorbance/fluorescence plate reader, and the results were normalized to samples that had no alum.

All bioactivity assays were performed in U-bottom plates to maximize alum–protein–cell interactions. For IL-12 bioactivity, two in vitro assays were used: HEK-Blue IL-12 reporter assay and ex vivo spleenocyte stimulation assay. The HEK-Blue assay (Invivogen) was run according to manufacturer’s instructions with 5:1 alum:IL-12 mass ratio. For serum stability assay, alum/IL-12 mixtures were incubated in TBS supplemented with 20% FBS for the indicated time before adding to cells. For the spleenocyte assay, spleens were collected from C57BL/6 mice and processed into single-cell suspensions. Red blood cells were lysed with ACK lysis buffer (Gibco) and spleenocytes plated in a 96-well plate at 500,000 cells per well. Then, IL-12, IL-12–ABP-p, IL-12–ABP-p + alum or just alum were added at indicated concentrations and incubated with spleenocytes for 48 h at 37 °C. Supernatants were diluted 3x and analysed for IL-12 via ELISA (Mouse IFNγ DuoSet ELISA, R&D Systems). Culture medium was RPMI supplemented with 10% FBS, 1% penicillin–streptomycin, 1× non-essential amino acids (Invitrogen), 1× GlutaMAX (Invitrogen) and 10 ng ml$^{-1}$ mouse IL-2. For IL-2 and IL-15 bioactivity, 10,000 CTL-2 cells were plated in incomplete medium (no T-STIM w/ ConA) with indicated concentrations of recombinant MSA–IL-2 or IL-15 proteins with or without alum and incubated for 48 h at 37 °C. Mass ratio used was 2.5:1 and 5:1 alum:protein for MSA–IL-2 and IL-15, respectively. Cell viability was determined via the ATP-detecting CellTiter-Glo 2.0 assay (Promega) as per the manufacturer’s instructions. Luminescence was measured using the Tecxan plate reader with 0.25 s integration time.

Morphology and size characterization of alum/cytokine particles. Transmission electron microscopy was used to analyse morphology of particles. B16F10 and MC38 cultures were plated on 0.2 μm polycarbonate filters. For the splenocyte assay, spleens were collected from C57BL/6 mice and processed into single-cell suspensions. Red blood cells were lysed with ACK lysis buffer (Gibco) and spleenocytes plated in a 96-well plate at 500,000 cells per well. Then, IL-12, IL-12–ABP-p, IL-12–ABP-p + alum or just alum were added at indicated concentrations and incubated with spleenocytes for 48 h at 37 °C. Supernatants were diluted 3x and analysed for IL-12 via ELISA (Mouse IFNγ DuoSet ELISA, R&D Systems). Culture medium was RPMI supplemented with 10% FBS, 1% penicillin–streptomycin, 1× non-essential amino acids (Invitrogen), 1× GlutaMAX (Invitrogen), 1× penicillin/ streptomycin (Invitrogen) and 10 ng ml$^{-1}$ mouse IL-2. For IL-2 and IL-15 bioactivity, 10,000 CTL-2 cells were plated in incomplete medium (no T-STIM w/ ConA) with indicated concentrations of recombinant MSA–IL-2 or IL-15 proteins with or without alum and incubated for 48 h at 37 °C. Mass ratio used was 2.5:1 and 5:1 alum:protein for MSA–IL-2 and IL-15, respectively. Cell viability was determined via the ATP-detecting CellTiter-Glo 2.0 assay (Promega) as per the manufacturer’s instructions. Luminescence was measured using the Tecxan plate reader with 0.25 s integration time.

Histopathology analysis. C3H/HeJ mice (n = 3) were left untreated or treated s.c. with 20 μg IL-12–ABP-p alone or mixed with 100 μg alum. Three days after treatment, lungs and livers of the mice were collected and fixed in 10% formalin. The organs were embedded in paraffin, and blocks were cut into 4 μm sections followed by haematoxylin and eosin staining. The slides were imaged using the Aperio Digital Slide Scanning System (Leica). The slides were then blindly assessed for tissue damage, by the pathologist Dr. R. Bronson.

IVIS. Albino B6 mice were inoculated with one million agminated B16F10–Trp2 KO cells s.c. on the right flank. After the tumours became palpable (day 6), tumours were intratumourally treated with 30 μl of AF647-labelled proteins mixed with alum. For IL-12, 20 μg (~316 pmol) of protein (IL-12, IL-12–ABP , IL-12–ABP-p) was mixed with 100 μg (~18 μmol) of pre-mixed alum/IL-12 or alum alone was dropped onto the mouse copper grids coated with a continuous carbon film and dried at r.t. Grids were then mounted on a JEOL single tilt holder equipped in the JEOL 2100 FEG microscope. The microscope was operated at 200 kV and with a magnification in the range of ~10,000–60,000 for assessing particle size and distribution. All images were recorded on a Gatan 2 k × 2 k Ultrascan CCD camera.

The size of alum-bound protein particles was assessed using a Horiba Partica LA-950V2 Laser Diffraction Particle Size Distribution Analyzer with a FractionCell Ultra-small Volume Solvent Resistant Cuvette.

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IFN-γ ELISPOT. C57BL/6 mice (n = 5 animals per group) bearing 25 mm² B16F10 tumours (~day 6 after 10⁶ cells were inoculated in the flank) were treated with no i.t. treatment, 100 μg alum intratumourally, 20 μg IL-12–ABP-p intratumourally or 20 μg IL-12–ABP-p + 200 μg IL-12–ABP-p + 200 μg anti-PD1 p.i. on days 6, 9, 12 and 15. On day 16 (10 days after i.t. treatment), spleens were isolated from mice, mechanically digested through 70 μm nylon cell strainers to prepare single-cell suspensions in RPMI supplemented with 10% FBS, 1% penicillin–streptomycin, 1X non-essential amino acids (Invitrogen), and 2-mercaptoethanol (Invitrogen). Red blood cells were lysed in ACK Lysis Buffer (Gibco). On the same day, B16F10 cells (treated with 500 U mL⁻¹ IFN-γ overnight) were subjected to 120 Gy radiation and trypsinized into a single-cell suspension in the same supplemented RPMI. Then, 25 000 irradiated B16F10 cells were mixed with 2.5 × 10⁶ splenocytes per sample and seeded in a 96-well ELISpot plate (BD Biosciences) that was pre-coated with IFN-γ-capture antibody (BD Biosciences). Plates were incubated in a humidified incubator for 24 h at 37 °C, then developed according to the manufacturer’s protocol. Plates were scanned using a CTL-ImmunoSpot Plate Reader, and data were analysed using CTL-ImmunoSpot Software.

Depletion studies. Depletions of immune cells were done using antibodies against CD8α (clone 2.43, BioXCell 400 μg i.p. twice weekly), Ly6g (clone 1A8, BioXCell, 400 μg i.p. twice weekly) or CSFR1 (clone AF589, BioXCell, 300 μg i.p. every other day) as previously described. Cytokine neutralization was done using intraperitoneal treatment with 200 μg antibodies against IFN-γ (clone XMG1.2), IL-1β (clone B122, BioXCell) and IL-18 (clone YIGF74-G7, BioXCell) every other day. All depletion antibodies dosing was initiated 1 day before i.t. treatment and continued until at least 1 month after treatment.

Tumour inoculation, treatment and surgery for 4T1 orthotopic model. A total of 5 × 10⁵ 4T1-Luc tumour cells were injected into the fourth mammary fat pad of BALB/c mice, and palpable tumours 5 days after inoculation were treated intratumourally with 20 μg IL-12–ABP-p, 100 μg alum or a mixture of the two. Mice were also treated with 200 μg αPD-1 i.p. on days 5, 8 and 11. Nine days after i.t. treatment (day 14), mice were anaesthetized with isoflurane and prepared for intraperitoneal injection of Anti-CD8α (clone YTS191), Ly6G (clone 1A8), CD62L (MEL-14), CD25 (PC61) and I-A/E (M5/114.15.2), CD24 (30-F1), CD11c (N418), CCR7 (4B12), CD169 (SD16.12.2), Ly6G (1A8), CD3 (17A2), NKp46 (29A1.4), Ki67 (16A8), NK1.1 (PK136), Tim3 (RMT-23), granzyme B (Q17/102), FoxP3 (MF44), CD14 (M17), IFN-γ (XMG1.2), CD167a (1D4B), CD62L (MEL-14), CD25 (PC61) and TNF-α (MP6-XT22) were obtained from Biolegend. Antibodies to CD8α (53-6.7), CD103 (I-A/E), CD107a (1D4B), CD62L (MEL-14), CD25 (PC61) and TNF-α (MP6-XT22) were obtained from Biolegend. Antibodies to CD8α (53-6.7) and CD4 (GK1.5) were purchased from BD Biosciences. All antibodies were diluted 1:100. Gp70 tetramer (T-Select H-2L* MuLV gp70 Tetramer-SPSYYHQFPE) was purchased from MBL. Tetramer staining was performed in buffer containing 50 mM disodium ATP with 1.50 mM ATP disodium. Viability was assessed using Zombie Aqua and UV (Biolegend, 11,000) for tumour and dLN samples or using DAPI for tetramer staining of blood samples. Intracellular staining for FoxP3, K67, IFN-γ, TNF-α and granzyme B was performed using the Foxp3 Transcription Factor Buffer Set (eBioscience).

B16F10 tumours and dLNs were collected 1, 3, 6 or 9 days after i.t. treatment of day 8 tumours. Both were mechanically digested through 70 μm nylon cell strainers to prepare single-cell suspensions. Blood was collected by submandibular bleeding into K²-EDTA tubes (Greiner-Bio), and red blood cells were lysed in ACK Lysis Buffer (Gibco). All samples were then resuspended in ice-cold PBS containing 1% (w/v) bovine serum albumin and 2 μm EDTA (fluorescence-activated cell sorting (FACS) buffer) with precision count beads (Eve Technologies) or analysed in-house using a 31-plex Luminex analysis or analysed in-house using a 31-plex Luminex analysis or analysed in-house using a 31-plex Luminex analysis or analysed in-house using a 31-plex Luminex analysis. Data were analysed in FlowJo.

Statistics and reproductibility. Statistics were performed using Prism (GraphPad). Statistical methods were not used to determine sample size, but sample numbers were chosen based on estimates from pilot studies and published results, such that appropriate statistical tests would yield statistically significant results. For survival studies, log-rank (Mantel–Cox) tests were used. For FACS studies involving multiple timepoints, two-way analyses of variance (ANOVA)s followed by Tukey’s multiple comparison tests were used, while one-way ANOVA used Student’s t-tests. All experiments were performed in triplicate, and the sample size for in vitro analysis was three and for in vivo analysis was as annotated in figure legends. The details of statistical analysis for figures and Supplementary figures are included in the source data files.

Data availability

The main data supporting the results in this article are available within the paper and its Supplementary Information. Any data supporting the findings of this study are also available from the corresponding authors on reasonable request. Source data are provided with this paper.

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**Author contributions**

Y.A., K.D.W. and D.J.I. conceived of the study and wrote the manuscript. Y.A. designed the experiments and analysed data. Y.A., L.E.M., J.Y.H.C., L.S., A.S., E.A.L., A.T. and J.S. performed the experiments and data analysis. K.A.R. assisted with the Horiba experiment, and K.N. performed the transmission electron microscopy imaging. K.A.R. and T.J.M. provided the AF488-pSer4 peptide, and M.D.M provided the plasmid for murine IL-15sa.

**Competing interests**

Y.A., D.J.I., K.D.W. and T.J.M. are named as inventors in patent applications filed by MIT related to the data presented in this work (US20200405950A1). K.D.W. and D.J.I. are co-founders of Ankyra Therapeutics, which has licensed rights to the MIT intellectual property mentioned above.

**Additional information**

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*Our web collection on statistics for biologists contains articles on many of the points above.*

**Software and code**

Policy information about availability of computer code.

**Data collection**

FACS data were obtained using the BD FACSDiva software. ELISA plates and plate-based fluorescent/absorbance experiments were measured using the Tecan Infinite M200 Pro absorbance/fluorescence plate reader and software. ELISPOT measurements were taken using the CTL Immunospot Analyzer and associated software. Western Blot analysis was made using a LI-COR ODyssey reader and LI-COR imaging software. Confocal images were taken on a Leica SP8 laser scanning confocal microscope and associated software. IVIS data were collected on the IVIS Spectrum In Vivo Imaging system. TEM images were collected using the JEOL 2100 FEG microscope, whereas particle size was collected with a Horiba Partica LA-950V2 Laser Diffraction Particle Size Distribution Analyzer.

**Data analysis**

FlowJo 10.7.1 was used for the analysis of FACS data. GraphPad Prism 9.1.2 was used for plotting and statistical analysis. Cluster 3.0 was used for hierarchical clustering of tumour cytokine/chemokine data. Fiji (Image J v2.1.0) was used for histology image analysis. Excel, Word and Powerpoint from Microsoft Office (version 16.49) were used to draft the manuscript. IVIS data were analysed using Living Image v4.5.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | Sample sizes were predetermined from past experiments, to obtain statistically significant data. |
| Data exclusions | No mice were excluded from the study. |
| Replication | All in vitro experiments were performed at least twice, for independent confirmation of the results. Mouse tumour studies were done with the reported biological replicates; and for n>7 they include pooled results from multiple independent experiments. All other experiments were done once for each time point with at least three (and in most cases, more than five) biological repeats. All attempts at replication were successful. |
| Randomization | Mice were randomized into treatment groups, to provide the same mean tumour size at the start of the treatment. |
| Blinding | No blinding was done for the study. |

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Materials & experimental systems

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Methods

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| --- | --- |
| [ ] | ChIP-seq |
| [ ] | Flow cytometry |
| [ ] | MRI-based neuroimaging |

Antibodies

Flow cytometry: antibodies to CD8α (53-6.7), CD103 (2E7), Ly6C (HK1.4), F4/80 (BM8), CD11b (M1/70), CD86 (GL1), MHC2 or I-A/I-E (MS/114.15.2), CD24 (30-F1), CD11c (N418), CCR7 (4B12), CD169 (3D6.112), Ly6G (1A8), CD3 (17A2), Nkp46 (29A1.4), Ki67 (16A8), NK1.1 (PK136), Tim3 (RMT3-23), Granzyme B (QA17A02), FoxP3 (MF14), CD44 (1M7), IFN-γ (XMG1.2), CD107a (1D4B), CD62L (MEL-14), CD25 (PC61) and TNFα (MP6-XT22) were obtained from Biolegend. Antibodies to CD45 (30-F11), CD8α (53-6.7) and CD4 (GK1.5) were purchased from BD Biosciences. All antibodies were diluted 1:100. Gp70 tetramer (T-Select H-2Ld MuLV gp70 Tetramer-SPSYYVHQQF-PE) was purchased from MBL. Tetramer staining was performed in buffer containing 50 nM dasatinib with 1:50 antibody dilution. Viability was assessed using Zombie Aqua and UV (Biolegend, 1:1000) for tumour and dLN samples or using DAPI for tetramer staining of blood samples. Intracellular staining for FoxP3, Ki67, IFN-γ, TNFα and Granzyme B was performed using the FoxP3 Transcription Factor Buffer Set (eBioscience).
For histology, BV421 anti-mouse CD11b antibody (clone M1/70, Biolegend) and APC anti-mouse CD8α Ly 2 (clone CT-CD8α, Cedarlane) were used with dilution 1:100. For ELISA, anti-mouse IgG-HRP antibody (Biolegend) was used with a 1:5000 dilution and for western blot, a rabbit anti-pSer antibody (Abcam, ab9332, 1:125) and anti-rabbit IR800 antibody (LI-COR, 1:10000) with indication dilution ratios. All other antibodies used for ELISA/ELISPOT purposes were from commerical kits cited in Methods.

For treatments, anti-PD1 (clone 29F.1A12, BioXCell) and TA99 (synthesized in-house) were used at 200 ug per i.p. dose. Depletions of immune cells were done using antibodies against CD8α (clone 2.43, BioCell 400 ug i.p. twice weekly), NK1.1 (clone PK136, BioXCell, 400 ug i.p. twice weekly), Ly6g (clone 1A8, BioXCell, 400 ug i.p. twice weekly) or CSF1R (clone AFS98, BioXCell, 300 ug i.p. every other day) as previously described. Cytokine neutralization was done using i.p. treatments with 200 ug of antibodies against IFN-γ (clone XMG1.2, BioXCell), IL-1b (clone PK136, BioXCell) and IL-18 (clone YIGIF74-1G7, BioXCell) every other day.

Validation
All antibodies other than TA99 were validated by the vendors: Biolegend, BD Biosciences, ThermoFisher, Abcam and BioXCell. TA99 was synthesized in-house and validated by a flow cytometry B16F10 binding assay and in a previous publication (Momin et al., Sci. Transl. Med. 11, eaaw2614 (2019)).

Eukaryotic cell lines

Policy information about cell lines
Cell line source(s)
Cell lines B16F10 (ATCC), HEK-Blue IL-12 (Invivogen), HEK293-F (Gibco), 4T1 (ATCC) and CTLL-2 (ATCC) cells were cultured following vendor instructions. 4T1-GFP-Luc (4T1-Luc) cells were generated by transfection of the 4T1 cell line with pGreenFire lentiviral vector (System Biosciences) and B16F10-Trp2 KO cells were generated as previously described (Moynihan et al.). Ag104A, MC38 and B16F10-Zsgreen cells were a gift from H. Schreiber (University of Chicago), J. Schlom (National Cancer Institute) and R. Hynes (MIT) respectively. 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media (ATCC) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin while Ag104A, MC38 and B16F10-Zsgreen were all cultures in complete Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin.

Authentication
Each cell line was maintained separately and stocked in early passages, to minimize contamination and to preserve cell identity.

Mycoplasm contamination
All cell lines that were inoculated in mice were confirmed to be absent of mycoplasma contamination by PCR.

Commonly misidentified lines
No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Female C57BL/6 (Taconic, C57BL/6NTac), B alb/C (JAX, 000651), Batf3−/− (JAX, 013755), Nlrp3−/− (JAX, 021302), albino B6 (JAX, 000058), C3H/Hej (JAX, 000659) and IFN-γ-reporter GREAT (JAX, 017581) mice at 6–10 weeks age (~20 g) were purchased and maintained in the animal facility at the Massachusetts Institute of Technology.

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve samples collected from the field.

Ethics oversight
All animal studies and procedures were carried out following federal, state and local guidelines under an animal protocol approved by the institutional animal care and use committee at MIT.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
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☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
B16F10 tumours and dLNs were harvested 1, 3, 6 or 9 days after i.t. treatment of day-8 tumours. Both were mechanically digested through 70-um nylon cell strainers to prepare single-cell suspensions. Peripheral blood was collected by submandibular bleeding into K2-EDTA tubes (Greiner-Bio), and red blood cells were lysed in ACK Lysis Buffer (Gibco). All samples were then resuspended in ice-cold PBS containing 1% (w/v) BSA and 2 mM EDTA (FACS buffer) with precision count beads (Biolegend, normalized to the weight of tissue per sample) before staining. For intracellular cytokine/granule staining
of tumour and dLN infiltrating cells, digested tissue samples were resuspended in RPMI supplemented with 10% FBS, 1% penicillin-streptomycin, 1X non-essential amino acids (Invitrogen), 1X sodium pyruvate (Invitrogen), 1X 2-mercaptoethanol (Invitrogen) and 5 ug/mL brefeldin A (Sigma-Aldrich) for 4 hours at 37 °C prior to staining.

| Instrument | Cells were analysed using BD FACS LSR Fortessa, or BD FACS Symphony A3 flow cytometers. |
|------------|------------------------------------------------------------------------------------------|
| Software   | BD FACSDiva (BD Biosciences) was used for the collection of FACS data, and FlowJo was used for analysis. The collected data were plotted with statistical analysis by GraphPad Prism. |
| Cell population abundance | No sorting was used in this study. |
| Gating strategy | For Fig 5 and Extended Data Figs. 6 and 8, representative gating is provided in Extended Data Fig. 7. IFNy, Granzyme B positive gating is shown in Fig 5g.  
For Fig. 6 and Extended Data Fig. 11, representative gating is provided in Extended Data Fig. 10. Zsgreen, CD86-positive gating is presented in Fig. 6b,f, whereas MHC2+ gating is presented in Extended Data Fig. 11d,f.  
For Fig. 4k, RBCs were gated out using a FSC/SSC plot; subsequently, single cells were gated using a FCA/FCH plot. Live cells were gated using DAPI. Cells were then gated for CD8-positive cells; tetramer-positive gating is depicted in Fig. 4j. |

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.