CANCER

Durable anticancer immunity from intratumoral administration of IL-23, IL-36γ, and OX40L mRNAs

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Many solid cancers contain dysfunctional immune microenvironments. Immune system modulators that initiate responses to foreign pathogens could be promising candidates for reigniting productive responses toward tumors. Interleukin-1 (IL-1) and IL-12 cytokine family members cooperate at barrier tissues after microbial invasion, in human inflammatory diseases, and in antitumoral immunity. IL-36γ, in classic alarmin fashion, acts in damaged tissues, whereas IL-23 centrally coordinates immune responses to danger signals. In this study, direct intratumoral delivery of messenger RNAs (mRNAs) encoding these cytokines produced robust anticancer responses in a broad range of tumor microenvironments. The addition of mRNA encoding the T cell costimulator OX40L increased complete response rates in treated and untreated distal tumors compared to the cytokine mRNAs alone. Mice exhibiting complete responses were subsequently protected from tumor rechallenge. Treatments with these mRNA mixtures induced downstream cytokine and chemokine expression, and also activated multiple dendritic cell (DC) and T cell types. Consistent with this, efficacy was dependent on Batf3-dependent cross-presenting DCs and cytotoxic CD8^{+} T cells. IL-23/IL-36γ/OX40L triplet mRNA mixture triggered substantial immune cell recruitment into tumors, enabling effective tumor destruction irrespective of previous tumoral immune infiltrates. Last, combining triplet mRNA with checkpoint blockade led to efficacy in models otherwise resistant to systemic immune checkpoint inhibition. Human cell studies showed similar cytokine responses to the individual components of this mRNA mixture, suggesting translatability of immunomodulatory activity to human patients.

INTRODUCTION

Novel immune-mediated therapies have shifted the clinical cancer treatment paradigm. Systemically administered checkpoint inhibitors (CPIs) have improved metastatic disease survival by targeting the T cell co-inhibitory pathways of CTLA-4 and PD-1/PD-L1 (1). These antibodies have yielded durable outcomes, but many tumors are CPI resistant. Focus has expanded to agonizing T cell costimulators such as B7 immunoglobulin and tumor necrosis factor receptor (TNFR) family members ICOS, GITR, 4-1BB, and OX40 (2). Combinations of systemic antibodies have not only improved patient responses but also increased toxicities such as pruritus, diarrhea, pneumonitis, and hypo- or hyperthyroidism in anti–CTLA-4/anti–PD-1 melanoma therapy (3). Locally administered immunotherapies may improve outcomes with better tolerability and are beginning to show clinical promise, particularly combined with systemic CPIs. Approaches include oncolytic viruses, DNA-based gene delivery, small molecules, antibodies, and more recently mRNA (4, 5).

Long-term benefit of immunotherapy requires T cell memory against cancer-associated epitopes. Optimal T cell responses couple T cell receptor (TCR)–antigen engagement with positive secondary signals such as OX40 costimulation via OX40L to enhance T cell effector function, expansion, and survival (6). Thus, OX40 agonism boosts T cell responses and, for local therapies, may restrict responses to antigens within the tumor microenvironment (TME). Validation of OX40 agonism as an anticancer treatment was reported due to

regressions of metastatic lesions in a phase 1 trial (7), and other trials include intratumorally administered mRNA encoding OX40L (NCT03323398).

Whereas OX40L-dependent efficacy may rely on some basal cancer antigen recognition by T cells, proinflammatory cytokines/chemokines may ignite a productive anticancer response. Interleukin-1 (IL-1) family members are early effectors after pathogenic challenge. Multiple myeloid cell populations respond to IL-36 cytokines (8), and IL-36γ acts on professional antigen-presenting cells and T cells (9, 10). Although IL-1 and IL-18 cytokines correlate with poor prognosis in certain contexts [chronic inflammation (11)], IL-36α correlates with good prognosis in cancer patients (12) and introduced IL-36γ inhibits tumor progression in mouse models with favorable T helper 1 (T_H1)–type TME changes (9).

IL-12 family members instead act as central coordinators of immune responses and bridge innate to adaptive immunity in humans (13). Although IL-23 can be associated with T_H17–type as well as protumorigenic responses in some contexts [sustained cytokine expression (14, 15)], exogenous introduction induces antitumor immunity in multiple preclinical studies (16). IL-23 therapy in mice has resulted in comparable antitumor efficacy to IL-12, yet may have improved tolerability compared to systemic IL-12 protein (17, 18). Furthermore, IL-23 positively modulates cells at the boundary of innate and adaptive immunity [innate lymphoid cells, γδ T cells, and natural killer T (NKT) cells] (19). IL-36γ and IL-23 are both normally involved in the homeostatic protection of epithelial/mucosal borders from pathogens (10, 20, 21). They are both implicated in human inflammatory diseases such as psoriasis and inflammatory bowel disease (10, 22), and IL-1 and IL-12 family members also work in concert in anticancer responses (23, 24).

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This paper describes local delivery of protein-coding mRNAs encapsulated in lipid nanoparticles (LNPs), where modified nucleotides enable evasion of innate sensor triggering that could blunt protein expression (25). Here, we connected a T cell costimulatory approach with inflammatory cytokines in one local mRNA therapy. Intratumoral OX40L mRNA monotherapy induced complete responses (CRs) in tumors partially responsive to systemic CPI treatment, which represent more inflamed TMEs. IL-36γ and IL-23 mRNAs transformed the immune landscape in CPI-refractory mouse cancer models with more suppressive TMEs. These anticancer responses were amplified with co-delivered OX40L mRNA, and triplet mRNA therapy resulted in CRs of treated and distal tumors as well as improved responses in combination with systemic CPIs. This study suggests that local triplet mRNA therapy may be a well-tolerated and broadly efficacious treatment of even multilesional, metastatic cancers.

RESULTS

Expression and cellular distribution of OX40L protein from intratumorally delivered mRNA

To express a functional membrane-bound OX40L in vivo with the same sequence as the naturally occurring homotrimeric protein, mRNA was synthesized and bioactivity was verified in a T cell costimulation assay (fig. S1A). To achieve effective expression within tumors, mRNA was formulated in LNPs for direct tumoral administration. In a subcutaneous mouse MC38 model, intratumoral injection of 5 μg of LNP-formulated mRNA resulted in peak OX40L protein after 6 hours with decreased yet detectable measurements above untreated controls at day 7 (Fig. 1A). OX40L cellular localization was confirmed as predominantly membranous via immunohistochemistry of mRNA-treated A20 tumors (fig. S1B).

mRNA drug exposures in target and distal tissues in mice were evaluated via branched DNA assay after a single injection into MC38 tumors (Table 1). mRNA detection was highest in tumors, followed by spleen and proximal lymph nodes (LNs). The total drug exposure over time [area under the curve (AUC)] in other tissues was ≥500 times lower than in tumors. Maximal mRNA concentrations in tumor and plasma were observed 3 hours after injection and 6 hours in proximal LN. Although measured mRNA amounts were markedly lower in liver than tumor, relatively robust liver expression has been demonstrated from systemic administration of LNP-formulated mRNA (26). MicroRNA-122 (miR122) expression is high in hepatocytes, so all mRNAs included a 3′UTR (untranslated region) miR122 binding site to attenuate potential protein expression in hepatocytes in the event of liver exposure (27). Inclusion of the miR122 binding site did not adversely affect tumor expression of OX40L, whereas elevated liver expression was not observed above the untreated level after intratumoral administration (Fig. 1A) regardless of the miR122 binding element (fig. S1C).

Because the cellular context of an introduced protein may affect its bioactivity, we identified which cells were expressing OX40L by flow cytometry. After intratumoral injection of 5 μg of OX40L-encoding mRNA, 21% of MC38 cancer cells in tumors expressed OX40L 24 hours after dosing (Fig. 1B) and expression on a per-cell basis by mean fluorescence intensity (MFI) was also higher than in controls up to 7 days after treatment. Tumoral CD11b+ myeloid cells expressed OX40L similarly to cancer cells (fig. S1D). The three most abundant myeloid cell types within tumors are macrophages, mono-

cytes, and granulocytes (28); all expressed OX40L above control mRNA–dosed tumors (Fig. 1C), and macrophages were the highest expressers.

Despite detecting OX40L mRNA in the spleen (Table 1), minimal splenic OX40L protein was detected (fig. S1E). In contrast, in the tumor-draining lymph node (TdLN), myeloid cells expressed OX40L, with macrophages again the highest expressers (Fig. 1D), despite low absolute numbers of these cells in this compartment (fig. S1F). T cells expressed OX40L with considerably lower percentages and MFI than myeloid or CD45− cells in tumor and TdLN (fig. S1, G and H). OX40L is normally expressed on professional antigen-presenting cells (6), which can help drive adaptive anticancer immune responses. mRNA-derived OX40L protein expression was therefore assessed on five dendritic cell (DC) populations in tumors and TdLN (29): two classical DCs (cDCs) capable of antigen cross-presentation to CD8+ T cells [peripheral tissue CD103+ DCs, analyzed in both tumors and proximal LN due to migratory capabilities (30), and lymphoid tissue resident CD8a+ DCs] and CD11b+ cDCs, plasmacytoid DCs (pDCs), and inflammatory DCs (iDCs) of monocyte origin. In OX40L mRNA–dosed tumors, all analyzed DCs expressed the protein higher than control-dosed samples, by percentage and by MFI (Fig. 1E). OX40L expression was also seen outside the injected tumor in TdLN on all DC cell types analyzed (Fig. 1F). Expression was higher in TdLN than in tumor by percentage for all DCs and by MFI for four of five DC types (Fig. 1, E and F). Together, intratumoral injection of LNP-formulated mRNA yields robust expression of introduced proteins in cancer and myeloid cells within treated tumors and TdLNs, with particularly notable expression in antigen-presenting cells.

Regression of established tumors in multiple syngeneic models after mRNA treatment

To comprehensively evaluate the therapeutic potential of OX40L-mediated T cell costimulation in models with varied sensitivities to immune-mediated therapies and TMEs, several tumor models were examined for sensitivity to immune checkpoint blockade and extensively characterized. Subcutaneous H22 hepatoma tumors were sensitive to CPIs with a 50% tumor regression rate after anti–PD-1 and tumor growth delay after anti–PD-L1 treatment (fig. S2A). Two separately obtained MC38 colon carcinoma cell lines were verified as MC38-derived via short tandem repeat fingerprint analyses (fig. S2B), yet these tumor models exhibited differential CPI sensitivity; the MC38-S (sensitive) variant was marginally responsive, whereas MC38-R (resistant) was completely insensitive (fig. S2A).

We examined the potential basis for the MC38 variant differential sensitivities. PD-L1 expression was lower on MC38-S cells in vitro before tumor inoculation (fig. S2C). NanoString mRNA profiling revealed that immune cell signatures were more prominent in H22 and MC38-S tumors than in MC38-R tumors, indicating increased infiltration, or a more inflamed TME (fig. S2D). Both MC38 models had lower lymphocyte numbers and contained larger F4/80+ macrophage and Ly6Chi monocyte populations than H22 (Fig. 2A). MC38-S was skewed toward macrophages, which can exert both pro- and antitumorigenic effects. MC38-R tumors contained more Ly6Chi monocytes that can be immunosuppressive given the Ly6Chi marker overlap with monocytic myeloid-derived suppressor cells (31). These data are consistent with reports of large myeloid infiltrates in MC38 tumors (28) and indicate a more immunosuppressive environment in MC38-R tumors.

Delineation of DCs revealed more cross-presenting CD103^{+} DCs in MC38-S tumors and higher total DC numbers than in MC38-R and H22 tumors (Fig. 2A). NanoString profiling showed that DC-related genes were also more highly expressed in MC38-S compared to MC38-R tumors (fig. S2E). These differences indicate that the MC38 variants have diverged with distinct immune infiltrates and CPI sensitivity. These variants, in addition to models with more distinct origins and immune profiles such as H22, enabled mRNA therapeutic testing in varied environments including those refractory to checkpoint blockade.

Anticancer efficacy of OX40L mRNA treatment in H22 tumors resulted in complete tumor regression in 50% of animals (Fig. 2B). The control nontranslating mRNA formulated with the same LNP showed no efficacy, indicating that local OX40L expression could elicit an antitumor response comparable to systemic CPIs. In MC38-S, OX40L treatment induced tumor growth delay in a subset of mice. However, the MC38-R model was as unresponsive to local OX40L therapy as to systemic checkpoint blockade. The relative sensitivities suggested that OX40L mRNA is robustly effective in promoting rejection of inflamed tumors but may be less efficacious in non-inflamed and/or immunosuppressive contexts.

We sought to identify proinflammatory cytokines that could transform such refractory TMEs into a productive anticancer immune response. Several cytokines are involved in initiating and propagating human inflammatory responses, and we tested many as single agents or as mixtures in the MC38-S model. Among the cytokines with monotherapy efficacy, 2.5 μg of IL-23 induced complete tumor regressions in 50% of animals, whereas IL-36γ showed marginal tumor growth delay (Fig. 2C). These mRNA-derived cytokines were secreted, and supernatants of transfected cells had bioactivity in vitro (fig. S3A). Upon intratumoral dosing, both cytokines were detected within tumors (fig. S3B). Remarkably, a 5-μg total dose of IL-23 and IL-36γ doublet mRNA treatment resulted in a strong cooperative antitumor efficacy with complete regression of all treated MC38-S tumors (Fig. 2C).

We tested whether cytokines individually or together with OX40L could induce tumor regression in the CPI-insensitive MC38-R model. Whereas OX40L, IL-23, and IL-36γ monotherapies exhibit little effect in MC38-R, we saw increased efficacy with mRNA mixtures of 5-μg fixed mRNA doses. A single dose of IL-23/IL-36γ and IL-23/OX40L doublet therapies resulted in CRs, and the rate increased to ~50% with a single dose of the triplet RNA mixture including IL-23 and IL-36γ along with OX40L (Fig. 2D). Moreover, tumor escape was further reduced with repeated weekly mRNA injections: four injections of the IL-23/IL-36γ/OX40L triplet led to 73% (Fig. 2D) and 80% (fig. S4A) CR rates in two studies (23 of 30 or 77% average). In addition to improved survival rates from extended triplet mRNA treatment, dose responsiveness was also observed. A single 5-μg dose led to 6 of 15 CRs (13 of 30 or 43% across the two studies) compared to 11 of 15 (73%) from 10 μg (fig. S4A). These treatments were generally well tolerated with no notable body weight loss (fig. S4, B to D), nor no obvious adverse reactions noted in routine clinical observations.

The antitumoral efficacy of mRNA was compared to treatment with recombinant proteins. Total target protein measurements in mRNA-dosed tumors were calculated, and an excess of recombinant proteins was determined to inject into MC38-S tumors (table S1). mRNA injection resulted in far superior antitumor efficacy than administration of the IL-23/IL-36γ/OX40L-Fc proteins (Fig. 2E).

A single injection of the chosen recombinant protein dosing resulted in maximal concentrations of IL-23 in plasma similar to mRNA treatment, yet less durable concentrations (fig. S3C). Together, the mRNA-derived mixture of two proinflammatory cytokines and a T cell costimulator, namely, IL-23/IL-36γ and OX40L, was superior to a protein-based treatment and achieved more than 70% CRs in a model with an immunosuppressive TME that is refractory to checkpoint blockade.

Inflammatory TME and early activation of the innate immune system induced by mRNA treatments

Enhanced efficacy with the IL-23/IL-36γ/OX40L triplet mRNA therapy may be partly due to early innate immune engagement by the introduced inflammatory cytokines, and so we evaluated MC38-R model changes after treatment by cytokine assessment, flow cytometry, and RNA profiling. Untreated samples were first compared to treatment with LNP-encapsulated control nontranslating mRNA. Most cytokine protein measurements did not increase in control mRNA–treated samples above untreated controls (Fig. 3, A and B, and fig. S5), and only 27 of 791 genes were differentially expressed 6 hours after treatment (fig. S6, A and B). Cytokines downstream of RNA sensor triggering and Toll-like receptor (TLR) agonism (25) that could influence antitumor immunity were not induced by control mRNA treatment (fig. S6, C to E). Increases in IL-12, which can enable productive T cell responses (13, 17), were not detected at the mRNA or protein level in response to control mRNA (fig. S6, C to E). Similarly, no to minimal changes in DC maturation or T cell activation were detected with control mRNA compared to untreated animals (fig. S7, A to D). The mRNA itself thus induced minimal changes, and the noted transcriptional changes may be due to the injection itself.

In response to the triplet IL-23/IL-36γ/OX40L mRNA therapy, an early and robust elevation of inflammatory cytokines and chemokines was detected in tumor lysates after single-dose treatment (Fig. 3A and fig. S5A), and all cytokines/chemokines reproducibly elevated by triplet mRNA are shown (Fig. 3, A and B, and fig. S5). Within 6 hours after treatment, tumoral increases were detected in the proinflammatory cytokine IL-6, the neutrophil chemoattractant CXCL1 (GROα), and granulocyte and stem cell–stimulating factor G-CSF (CSF-3). These elevations appeared dependent on IL-36γ (see single mRNA-dosed groups), and they were similarly transiently detected in plasma (fig. S5B). Other cytokines were also elevated by IL-36γ alone or IL-23/IL-36γ/OX40L triplet mRNA in tumor lysates (CCL4, GM-CSF, and CXCL5; fig. S5A) or in plasma (CCL7 and IL-1α; fig. S5B).

A later wave of cytokine release was detected in plasma in both triplet mRNA–treated and IL-23 single-dose samples (Fig. 3B). Maximal IL-22, interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), and IL-1β concentrations were between 48 and 72 hours after treatment and at these times mainly in plasma rather than tumors (fig. S5C). Modest increases in IL-22 and IL-1β were, however, detected early in tumors 6 hours after IL-36γ single-mRNA treatment. In addition, these four cytokines were increased in tumors 7 days after dosing with the most efficacious mixture IL-23/IL-36γ/OX40L (fig. S5C). Because IL-23 is implicated in maintenance of T_H17 cells, defined by IL-17A production (32), we noted that IL-17A was detected in plasma after IL-23 monotherapy. However, IL-17A measurements were much reduced in the triplet mRNA–dosed group, and unlike other IL-23 mRNA-dependent
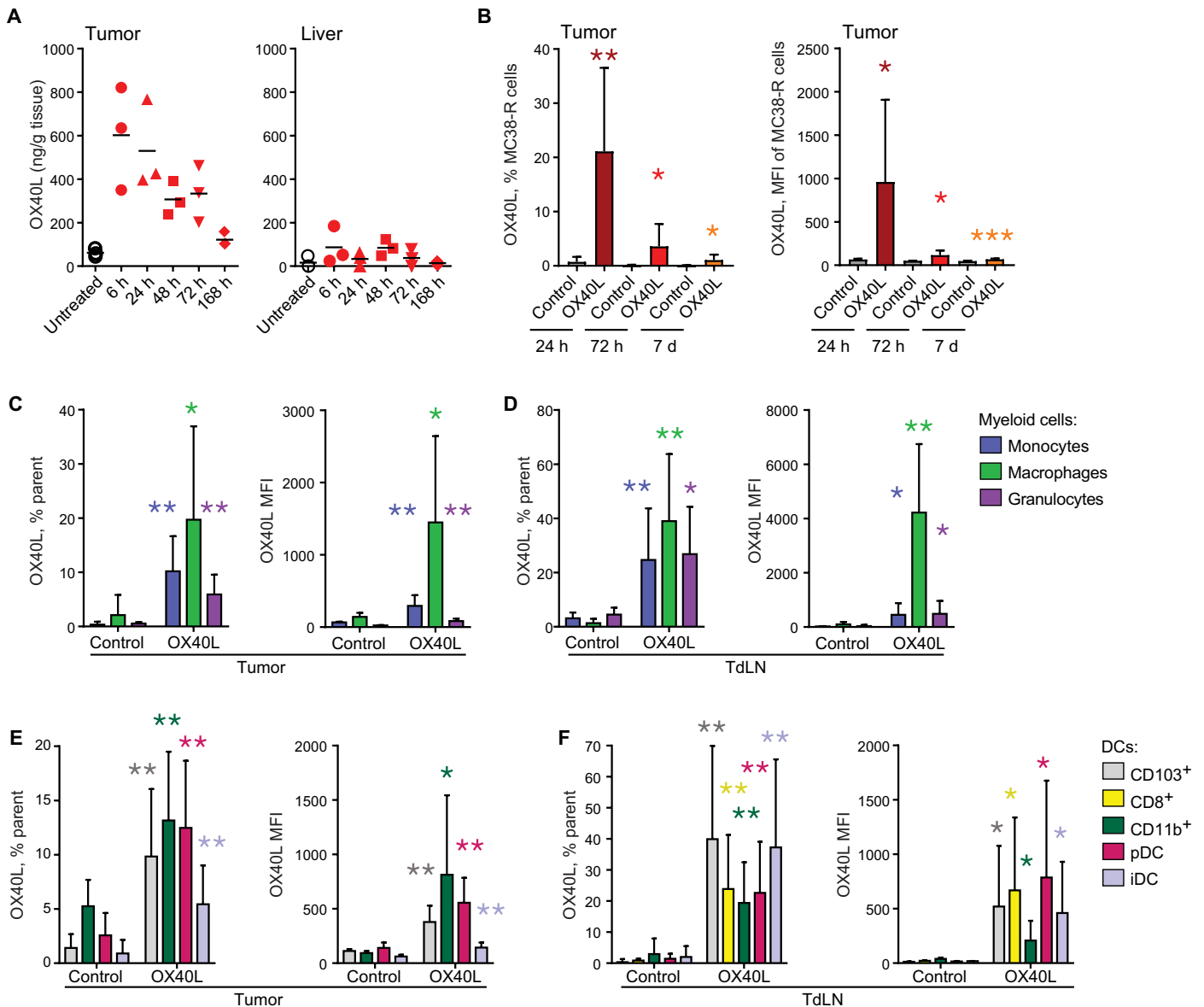
Fig. 1. In vivo OX40L expression detected in tumors and draining LNs after intratumoral mRNA administration. Expression of OX40L in MC38-R tumor–bearing mice after intratumoral injection with 5 µg of LNP-formulated mRNA. (**A**) Time course in tumor and liver lysates by enzyme-linked immunosorbent assay. Each data point is an individual sample (*n* = 3). (**B**) MC38-R cancer cells by flow cytometry by percentage or per cell by total MFI. (**C** and **D**) Myeloid cells within tumors (C) or TdLN (D). (**E** and **F**) Four DC types from tumors (E) or five in TdLN (F). Bars indicate mean with SD (B to F) [*n* = 8 except 3 for monocytes and 4 for macrophages in (D)]. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 by *t* test between control and OX40L mRNA treatment within each cell type (no significance where not shown) (B to F). Data are representative of three (A to D) or two (E and F) independent experiments. Control, control mRNA; h, hour; d, day.

cytokines, IL-17A was not elevated in tumors 7 days after dosing (fig. S5D).

The cellular effects of mRNA treatments and the noted downstream cytokines were analyzed by flow cytometry on treated tumors and proximal TdLNs. We observed an early increase in tumoral Ly6G⁺ granulocytes 24 hours after treatment with triplet and IL-36γ solo treatment (fig. S8A). Granulocytic infiltrate is associated with antitumor efficacy in other preclinical immunotherapies and normally is a first response to pathogenic insult and epithelial barrier disruption (*33*). To investigate granulocytic effects on triplet mRNA–induced efficacy, an intratumoral antibody treatment de-

pleted tumoral granulocytes better than a systemic anti-Ly6G regimen, but was still incomplete particularly at 24 and 48 hours after mRNA treatment (fig. S8B). Across two independent efficacy experiments with intratumoral regimens, the depletion attempts only had a marginal inhibition of triplet-mediated survival (fig. S8C).

DCs can mobilize an effective anticancer T cell response, and our data above demonstrate that DCs express introduced mRNA. We found that CD8⁺ and CD11b⁺ cDCs were activated in TdLN at 24 hours after triplet treatment, as shown by increased expression of the costimulatory molecule and DC maturation marker CD86 (Fig. 3C); these changes were also seen in the IL-36γ single-treatment

Table 1. Biodistribution of OX40L mRNA after intratumoral administration in MC38-R tumor–bearing mice. Quantification of modified mRNA after injection of 12.5 μg of OX40L mRNA. $n = 3$ animals per tissue (except n/a = not applicable, single sample was pooled from three animals).

Tissue	T_{max} (hours)	C_{max} (ng/ml)		AUC_{0-168} (ng*hour/ml)	
		Mean	SE	Mean	SE
Tumor	3	6780	2570	406,000	114,000
Spleen	24	388	94.3	14,800	2920
Proximal LNs	6	149	n/a	3000	n/a
Stomach	3	22.2	20.9	599	306
Distal LNs	3	7.92	n/a	312	n/a
Lung	3	6.46	5.01	211	93.1
Kidney	24	3.41	2.58	146	58.1
Liver	6	9.25	7.75	130	83.1
Plasma	3	3.24	2.94	87.6	15.9
Heart	3	3.78	2.16	70.9	46.6
Brain	24	1.88	1.03	69.6	28.0

group. Seventy-two hours after treatment, maturation of CD8+ and CD11b+ cDCs were seen with the triplet therapy, and independent effects were observed from IL-36γ and IL-23 solo treatments. Milder maturation of CD103+ and iDCs was also detected 72 hours after treatment. Mobilization of CD103+ DCs that are often tissue resident was detected by cell number increases in the TdLN from triplet and IL-23 solo treatments (Fig. 3D), whereas increases or decreases of other DCs were not consistently seen in TdLN (fig. S9A). In vitro analysis with bone marrow-derived DCs (BMDCs) showed an increase in secretion of many cytokines in response to treatment with IL-36γ but not IL-23 (fig. S9B), indicating potential direct effects of IL-36γ on DCs.

Within tumors, increases in many DC populations were seen later at 7 days after IL-23/IL-36γ/OX40L triplet mRNA treatment, including CD103+, CD11b+ cDCs and iDCs (Fig. 3E). This broad response did not extend to either the early activation or later tumoral increase of pDCs (fig. S9C). Many transcripts for DC-associated proteins were increased within tumors in the triplet compared to the control mRNA–dosed group. This included antigen presentation–related transcripts MHCII (*H2-* transcripts) and *Ciita* (C2TA), and the costimulatory ligand *Cd86* and *Flt3* receptor (Fig. 3F). Enriched DC-related transcription factors included *Irf4*, *Irf8*, and *Batf3*, the latter two of which are expressed in cross-presenting DCs (*29*).

We analyzed the impact of cross-presenting DCs on mRNA mixture efficacy using *Batf3*-deficient mice (*34*). We found that the 100% CR rate in wild-type mice of a single dose of triplet mRNA in MC38-S tumors (30 of 30 mice in two studies) fell to 0 and 7% in two studies (1 of 30 CRs across the two studies) in *Batf3*−/− mice (Fig. 3G). These data suggest that triplet mRNA treatment collectively has several effects on DC populations, increasing activation status early in TdLN, CD103+ DC influx into TdLN, and DC abundance later in tumors, and requires cross-presenting DCs to achieve complete tumor regressions.

Activation and proliferation of multiple lymphocyte types, accompanied by increased tumoral immune infiltration with IL-23/IL-36γ/OX40L mRNA treatment

We investigated triplet mRNA's impact on innate lymphocyte cell types that can rapidly carry out effector functions upon activation and can boost or modulate adaptive immune responses (*35*). Both NKT and γδ T lymphocytes were activated 24 hours after treatment in TdLN, as detected by increases in the activation marker CD69 (Fig. 4A). IL-36γ monotherapy induced similar changes, suggesting an early IL-36γ–driven response. Ki-67 analysis indicated a subsequent increase in innate lymphocyte proliferation at 72 hours in TdLN, which was observed with both single-cytokine mRNA treatments and triplet mRNA therapy (Fig. 4B). Correlative with their early activation and proliferation, NKT and γδ T cells increased in tumors at day 7 compared to control-treated animals (Fig. 4C). Analyses of mRNA transcripts revealed that 75% of transcripts used to define a γδ T cell signature, and 88% for NKT cells were elevated with triplet mRNA compared to controls (Fig. 4D).

The wide-ranging responses to triplet mRNA seen with DCs and innate-like lymphocytes may activate the adaptive immune system and fuel specific anticancer responses. Early activation of αβ T cells was detected in TdLN in response to IL-23/IL-36γ/OX40L triplet mRNA and also IL-36γ treatment alone; both CD4+ effector T_H and CD8+ cytotoxic T cells displayed increased CD69 (Fig. 4E). Corresponding increases in proliferation were observed 72 hours after triplet and IL-36γ mRNA treatments (Fig. 4F). In contrast to TdLN, only a small increase in CD8+ T cell activation but not proliferation was detected in tumors (fig. S10A). This suggests that early T cell activation and proliferation were focused in local lymphoid tissues.

CD4+ and CD8+ αβ T cells subsequently increased in tumors 7 days after triplet mRNA treatment compared to negative controls (Fig. 4G and fig. S10B). T cell infiltration was greatest with the triplet mixture compared to any single mRNA (Fig. 4G and fig. S10, B and C). OX40L addition to IL-23/IL-36γ doublet mRNA treatment further increased T cell numbers, most clearly shown for CD8+ T cells at 7 days and CD4+ T cells at 10 days after treatment. Of note, transcripts for costimulatory molecules including OX40 receptor were up-regulated in tumors 7 days after IL-23/IL-36γ treatment (fig. S11A), when mRNA-derived OX40L was still expressed (Fig. 1, A and B). These data suggest that each component of the IL-23/IL-36γ/OX40L triplet mRNA is required to maximally promote αβ T cell infiltration into tumors.

Increased tumoral T cell infiltration may alter the balance of cytotoxic, helper, and suppressor cells. Phenotyping of mature T_H subsets showed that 82% of T_H1-related transcripts were up-regulated in tumors 7 days after treatment (Fig. 4D). Furthermore, 40% of T_H17 and 25% of T_H2-related transcripts were up-regulated. There were no clear T regulatory (T_{reg}) transcript changes, correlated with an increased CD8/T_{reg} cell ratio (Fig. 4H). Transcript analyses indicated up-regulation of some immune-related genes in tumors 6 hours after triplet mRNA treatment, but more comprehensive up-regulation 7 days after treatment (Fig. 4I). Fewer transcripts were increased with IL-23/OX40L doublet mRNA than IL-23/IL-36γ (fig. S11, A and B), suggesting that the two cytokine-encoding mRNAs induce a greater magnitude of transcriptional changes similar to the triplet. In contrast to the immunological changes, angiogenic markers were not up-regulated by triplet mRNA treatment (fig. S11C).
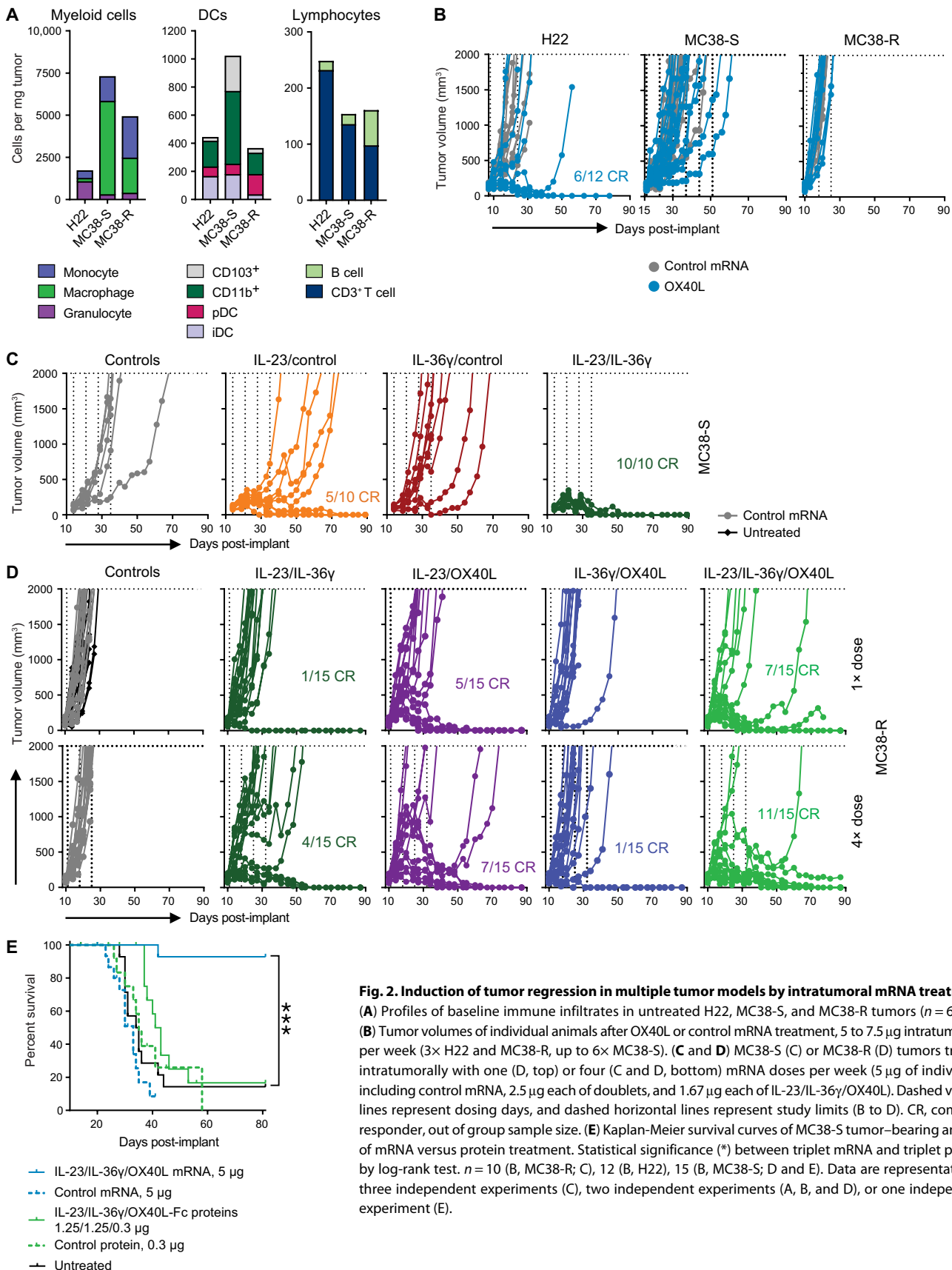
Fig. 2. Induction of tumor regression in multiple tumor models by intratumoral mRNA treatment. (**A**) Profiles of baseline immune infiltrates in untreated H22, MC38-S, and MC38-R tumors ($n = 6$ to 8). (**B**) Tumor volumes of individual animals after OX40L or control mRNA treatment, 5 to 7.5 μg intratumorally per week (3× H22 and MC38-R, up to 6× MC38-S). (**C** and **D**) MC38-S (C) or MC38-R (D) tumors treated intratumorally with one (D, top) or four (C and D, bottom) mRNA doses per week (5 μg of individuals including control mRNA, 2.5 μg each of doublets, and 1.67 μg each of IL-23/IL-36γ/OX40L). Dashed vertical lines represent dosing days, and dashed horizontal lines represent study limits (B to D). CR, complete responder, out of group sample size. (**E**) Kaplan-Meier survival curves of MC38-S tumor–bearing animals of mRNA versus protein treatment. Statistical significance (*) between triplet mRNA and triplet protein by log-rank test. $n = 10$ (B, MC38-R; C), 12 (B, H22), 15 (B, MC38-S; D and E). Data are representative of three independent experiments (C), two independent experiments (A, B, and D), or one independent experiment (E).
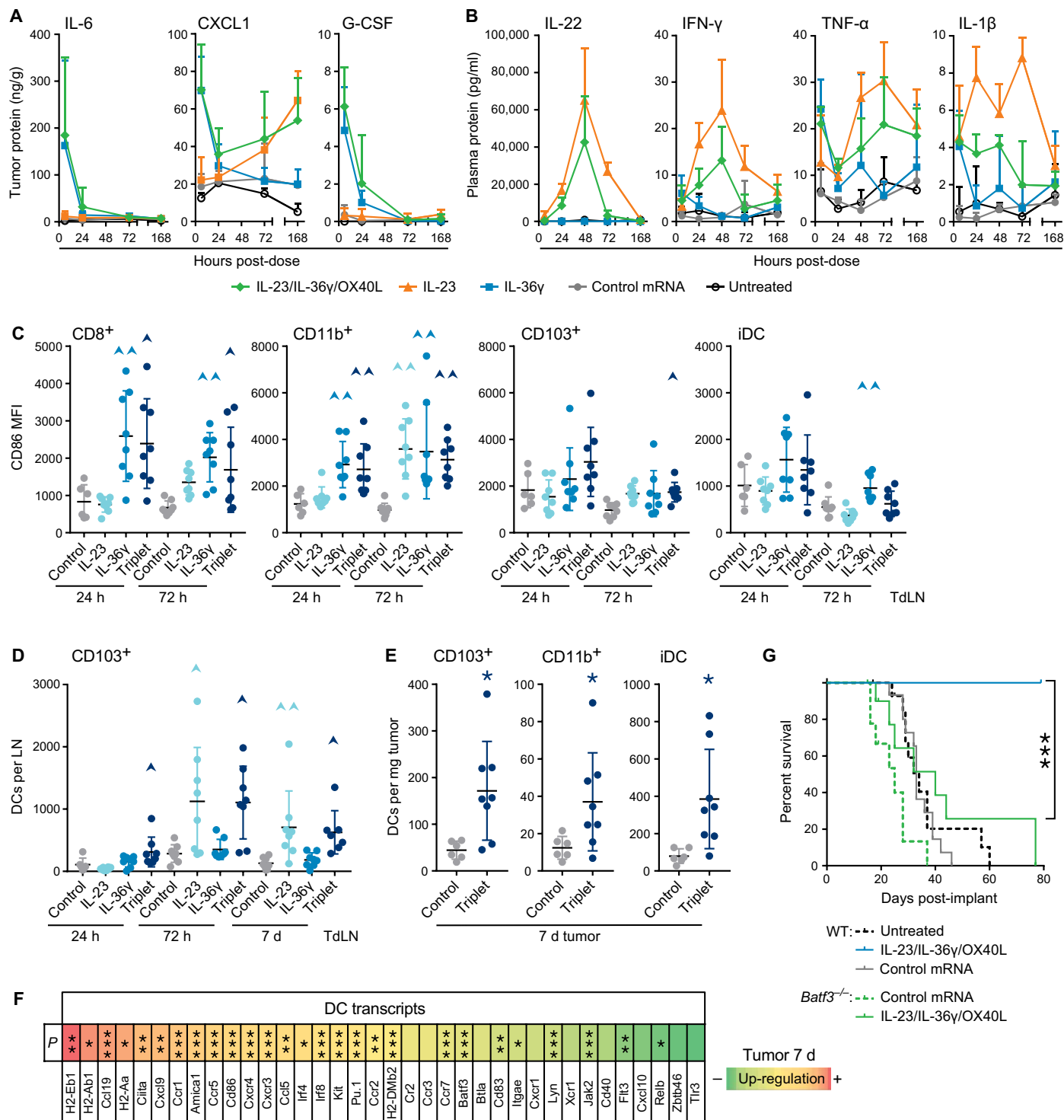
Fig. 3. Inflammatory TME and early activation of the innate immune system induced by mRNA treatments. Response of MC38-R tumor–bearing mice to one 5-μg total dose of mRNA intratumorally (5 μg of individuals and 1.67 μg each of IL-23/IL-36γ/OX40L). (**A** and **B**) Time course of cytokines in tumors (A) and blood plasma (B) (*n* = 6). (**C**) CD86 on DCs in TdLN. Each point indicates one sample. (**D**) CD103^{+} DCs in TdLN, as total cells per LN. (**E**) DCs in tumors 7 days after treatment, per milligram of tumor tissue. *n* = 8 (C to E) [except 6 for control at 24 hours in (C) and (D) and 6 for control in (E)]. (**F**) Differential expression of DC transcripts by NanoString 7 days after treatment with IL-23/IL-36γ/OX40L versus control mRNA (*n* = 6). Statistical significance indicated within each time point between control mRNA and active mRNA (no significance where not shown): by analysis of variance (ANOVA) [^ in (C) and (D)], *t* test [* in (E)], or Benjamini-Yekutieli false discovery rate (FDR) [* in (F)]. (**G**) Survival of wild-type (WT) C57BL/6 or *Batf3*^{−/−} MC38-S tumor–bearing animals treated with one mRNA dose (*n* = 10 for *Batf3*^{−/−} or *n* = 15 for WT). Statistical significance (*) between treated WT and *Batf3*^{−/−} by log-rank test. Data are representative of three independent experiments (E), two independent experiments (A to D and G), or one independent experiment (F).

We carried out depletion experiments with anti-CD4 or anti-CD8 antibodies in both the MC38-S and MC38-R models. Depletion of CD4$^+$ T cells unexpectedly failed to affect triplet mRNA–dependent survival benefit to statistical significance ($P = 0.0985$ in MC38-S, $P = 0.159$ in MC38-R; Fig. 4J). In contrast, CD8 depletion diminished efficacy in both models; CD8$^+$ T cells are critical to this therapeutic approach. Consistent with the dependence on cytotoxic T cells, necrotic tumor domains were detected in MC38-R tumors 7 days after triplet mRNA therapy compared to negative controls (fig. S11D). Diverse changes in the TdLN and tumoral immune landscape point to mobilization of multiple lymphocyte cell types including innate NKT and γδ T cells, and adaptive αβ T cells. The MC38-R TME is subsequently transformed into a predominantly type 1 immune environment with minor type 17 and type 2 signatures, in addition to necrotic tumor detection during the same time frame as increased T cell infiltration.

Intratumoral delivery drives superior efficacy compared to other local routes of administration and inhibits tumor growth at distal sites

Additional local delivery routes were compared to evaluate tumoral versus lymphoid tissue expression and their contributions to anticancer activity. In tumors, the highest OX40L expression was achieved by intratumoral injection as expected, with low expression from subcutaneous injection and almost no expression from intradermal administration close to the LN (fig. S12A). Within TdLN, intradermal injection resulted in the highest OX40L expression in DCs, followed by subcutaneous injection and the lowest from intratumoral injection (fig. S12B). Intratumoral administration of IL-23/IL-36γ/OX40L triplet mRNA resulted in the greatest survival benefit (47 and 87% survival with single or multiple treatments, respectively) compared to either peritumoral delivery route (Fig. 5A). Little antitumoral activity was seen with subcutaneous administration, despite observed expression in both tumors and TdLN, whereas intradermal treatment resulted in 47% survival with multiple doses. This establishes expression both within tumors and local lymphoid tissues as requirements for optimal efficacy; however, higher expression within the tumor itself achieved by intratumoral administration appears to be critical.

Although local mRNA treatment results in high target expression within tumors and reduces drug exposure in normal tissues, success in treating metastatic or multilesional cancer would likely depend on an abscopal effect of soluble factors or immune cells. To evaluate a distal effect, bilateral MC38-S tumors were implanted and mRNA was injected in one tumor. Robust efficacy in both treated and distal tumors was noted with IL-23/OX40L and IL-23/IL-36γ doublet therapies, but remarkably, a single 5-μg triplet mRNA injection led to near-complete control of both treated and distal untreated tumors (100% in Fig. 5B and 34 of 38 or 89% across two studies).

We compared cytokine concentrations and transcriptional profiling after triplet mRNA treatment in the bilateral tumors. IL-23 protein amounts and the IL-36γ downstream cytokine, IL-6, were negligible in distal compared to treated tumors (Fig. 5C). At early time points, transcriptional changes in treated tumors were not reflected in distal tumors and unsupervised transcript clustering separated all distal from treated tumors (Fig. 5D). At 7 days, however, almost all treated and distal tumors clustered together with similar transcript up-regulation. These findings suggest that local triplet mRNA therapy affects distal tumors by a cell-mediated abscopal effect rather than exposure to systemic cytokines.

Development of long-term antitumor memory after triplet mRNA treatment was tested by rechallenging CRs with the same tumor line. All naïve animals succumbed to tumor progression, whereas the triplet responder animals did not display secondary tumor growth in multiple studies with either the MC38-S or MC38-R tumor model (fig. S12, C and D). Cancer cell–specific memory T cell presence was directly assessed by an IFN-γ enzyme-linked immune absorbent spot (ELISPOT) assay, indicating upwards of 300× more MC38-R–reactive CD8$^+$ T cells in responder mice than naïve mice (fig. S12E). Ex vivo stimulation with a peptide from an endogenous retroviral product, p15E, known to be up-regulated in MC38 tumors (36), also exhibited tumor antigen–reactive T cells (fig. S12F). These data indicate that a robust systemic anticancer memory T cell population was generated from this local therapy.

Improved antitumor efficacy with combination of triplet mRNA and checkpoint blockade

Because transformation of MC38-R into a highly inflamed TME by a single dose of triplet mRNA is insufficient to eliminate all tumors, suppression mechanisms may keep tumoral immune responses in check. MC38-R cells express PD-L1 (fig. S2C), and increased inflammation may strengthen the PD-1/PD-L1 inhibitory loop. PD-L1 expression on myeloid and MC38 cancer cells increased 7 days after triplet mRNA treatment (fig. S13A). Many other inhibitory molecules were also up-regulated: PD-1, PD-L2, and CTLA-4 (fig. S13B). Systemic anti–PD-L1 treatment minimally affected MC38-R tumor progression as shown above, whereas a single 5-μg triplet mRNA dose induced rejection of 48% of tumors. Combination triplet mRNA and anti–PD-L1 treatment improved CR rates to 85% (Fig. 6A), similarly to combination with anti–PD-1 (fig. S13C). MC38-R tumors were also refractory to CTLA-4 blockade, whereas a ~40% CR rate with triplet mRNA treatment was improved to >90% with this combination (Fig. 6B). Therefore, antitumor efficacy can be enhanced in immunosuppressive tumors by combining triplet mRNA and CPIs of biologically distinct pathways.

B16F10-AP3, a B16F10 tumor model variant, is classed as immunologically barren with scarce tumoral immune infiltrates (28), and immunotherapeutic efficacy against established parental B16F10 tumors has been challenging (37). Consistent with this, B16F10-AP3 is insensitive to either single systemic CPI monotherapy (Fig. 6C) or even dual anti–PD-1 and anti–CTLA-4 antibodies (fig. S13D). However, a single dose of triplet mRNA combined with a systemic CPI regimen induced long-term tumor regression (Fig. 6C), with an overall 30% (9 of 30) CR rate from two studies. Upon rechallenge with B16F10-AP3 cells, 88% of responder animals were resistant to secondary tumor development compared to 0% of naïve controls. These findings suggest that triplet mRNA combined with CPIs could elicit effective protective immunity in tumors with an extremely unfavorable TME, refractory even to systemic combination anti–PD-1 plus anti–CTLA-4.

Response of human immune cells to IL-23, IL-36γ, and OX40L mRNAs

The translation of a human version of triplet IL-23/IL-36γ/OX40L mRNA to a clinical therapeutic was next explored through analysis of cellular responses to human components. As observed in IL-23–treated mice, human peripheral blood mononuclear cells (PBMCs)
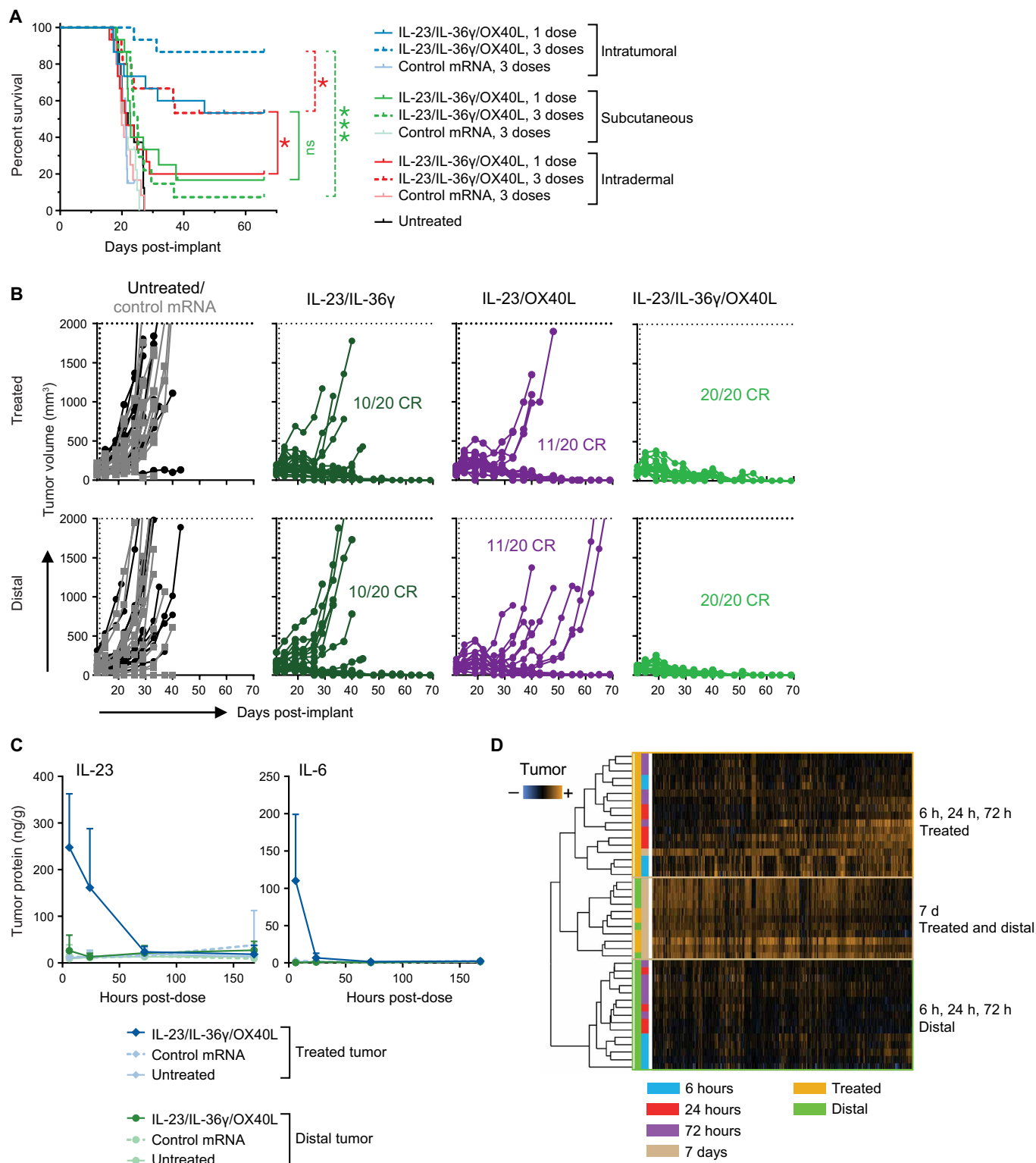
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Fig. 4. Induction of lymphocyte activation and remodeling of the TME by multi-mRNA treatment. Response of MC38-R tumor–bearing mice to one 5-μg total mRNA dose intratumorally (5 μg of individuals, 2.5 μg each of IL-23/IL-36γ, and 1.67 μg each of IL-23/IL-36γ/OX40L). (**A** and **B**) CD69 expression (A) or Ki-67 (B) on NKT and γδ T cells in TdLN. (**C**) Lymphocyte numbers in tumors. (**D**) Differential gene expression in tumors of lymphocyte cell type markers 7 days after IL-23/IL-36γ/OX40L treatment versus control mRNA. $n = 6$. (**E** and **F**) CD69 (E) or Ki-67 (F) on αβ CD4$^+$ and CD8$^+$ T cells in TdLN. $n = 8$ (A to C and E and F) [except 6 for control at 24 hours (A and E) and 5 for triplet at 7 days (C)]. (**G**) Lymphocyte numbers in tumors. EM, effector memory; CM, central memory; N, naïve. (**H**) Ratio of CD8$^+$ T to T$_{reg}$ cells in tumors [$n = 8$ in (G) and (H), except 6 for control]. Statistical significance indicated (no significance where not shown): by ANOVA [^ in (A) to (C) and (E) and (F)], Benjamini-Yekutieli FDR [* in (D)], or t test [* in (H)]. (**I**) Time course of transcripts in IL-23/IL-36γ/OX40L–treated samples. $n = 6$. (**J**) Survival of MC38-S tumor– or MC38-R tumor–bearing animals treated intratumorally with mRNA and intraperitoneally with antibodies to deplete lymphocytes ($n = 15$). MC38-S control mRNA–treated alone, MC38-R with isotype control. Statistical significance by log-rank test (*; ns, not significant). Data are representative of three independent experiments (E to H; J, MC38-R), two independent experiments (A to C; J, MC38-S), or one independent experiment (D and I).

Fig. 5. Intratumoral delivery drives superior efficacy compared to other local routes of administration and inhibits tumor growth at distal sites. (**A**) Survival of MC38-R tumor–bearing animals treated with one or three 5-μg mRNA doses via intratumoral or peritumoral routes of administration ($n = 15$). Statistical significance by log-rank test (*) between intratumoral and subcutaneous/intradermal administration (solid lines, single dose; dashed lines, multiple dose). (**B** to **D**) Abscopal responses of bilateral MC38-S tumors treated once with 5 μg of total mRNA intratumorally into the right flank tumor only. Tumor volumes of both treated and untreated distal tumors (B, $n = 20$), time course of cytokines in tumors (C, $n = 6$), and transcripts in IL-23/IL-36γ/OX40L–treated samples (D, $n = 6$). (A to D) Five micrograms of control mRNA, 2.5 μg each of mRNA administered for doublets, and 1.67 μg each for triplet. Data are representative of two independent experiments (B) or one independent experiment (A, C, and D).
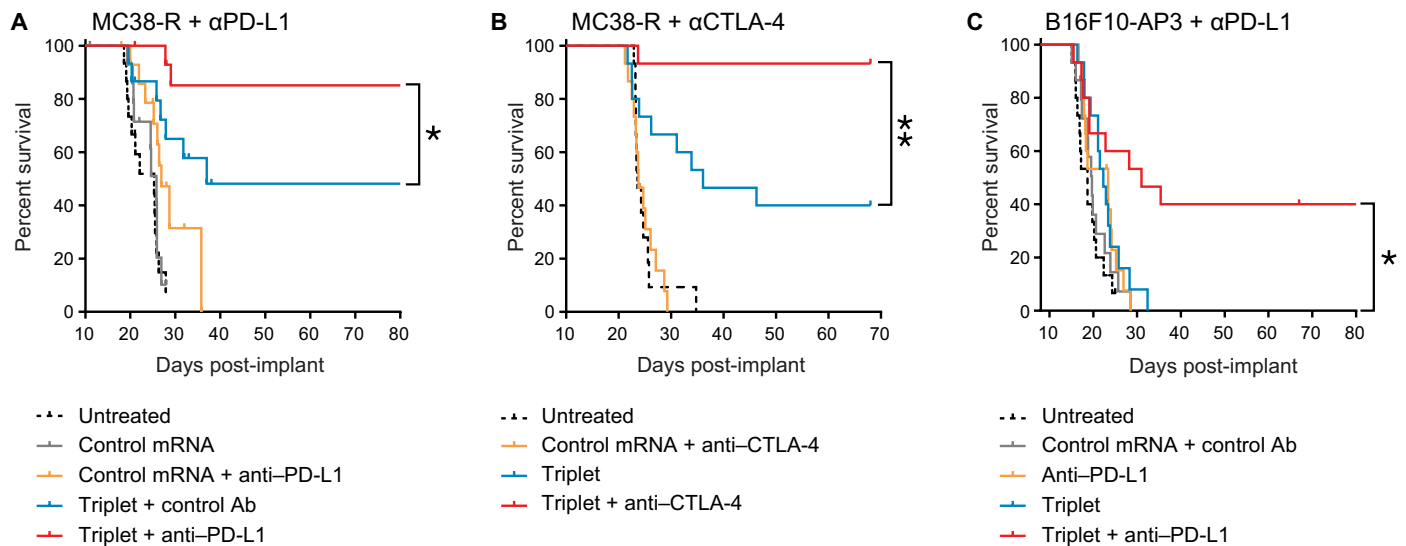
Fig. 6. Improved antitumor efficacy with combination of triplet mRNA therapy and checkpoint blockade. (**A** to **C**) Survival curves of MC38-R (A and B) or B16F10-AP3 (C) tumor-bearing mice after treatment with a single 5-µg dose of IL-23/IL-36γ/OX40L mRNA intratumorally with or without anti–PD-L1 (A and C) or anti–CTLA-4 (B) dosed intraperitoneally. Anti–PD-L1 and anti–CTLA-4 at 10 mg/kg per dose, twice per week for 2 weeks. Statistical significance indicated (*) by log-rank test (*n* = 15). Data are representative of two independent experiments (A and C) or one independent experiment (B).

produced dose-dependent IL-22 in response to recombinant or mRNA-derived human IL-23 (Fig. 7A). In addition, IL-21 was readily detected in IL-23–treated PBMCs, although it was not detected in vivo in triplet mRNA–treated mice. These results were confirmed with IL-23–stimulated human monocyte-derived macrophages (MDMs) (Fig. 7B). Profiling of IL-36γ downstream cytokines in human monocyte-derived DCs (MDDCs) revealed that IL-6 and the neutrophil chemoattractant IL-8 are among the most abundant cytokines induced (Fig. 7C), mirroring elevation of IL-6 and the functional IL-8 homolog CXCL1 in mice. Other cytokines, such as CCL4 (MIP-1β), were similarly induced in human cells and in vivo in mouse (fig. S14). CXCL12a (SDF-1α) was detected in IL-36γ–stimulated human MDDCs, although it is currently unexplored in mice. Human OX40L expressed from mRNA enhanced activation of human CD4+ T cells that were stimulated with suboptimal TCR and CD28 ligation, similar to mouse mRNA–derived OX40L (Fig. 7D). As measured by cytokine responses from responding human cells in culture and mRNA-treated mice in vivo, the bioactivity of mRNA-derived IL-23, IL-36γ, and OX40L appears conserved between human and mouse.

DISCUSSION
To overcome incomplete responses to current immunotherapies, we searched for immunomodulators able to prime a comprehensive immune response and reshape dysfunctional TMEs. Via local mRNA delivery, we ectopically expressed cytokines specialized in initiating local immunity at barrier tissues. We demonstrated that IL-36γ and IL-23 specifically cooperate to mediate antitumoral efficacy, whereas the T cell costimulator OX40L considerably increased response rates. Combination with systemic checkpoint blockade boosted responses in both immunosuppressive and immunologically barren tumor models otherwise refractory to CPIs. IL-23/IL-36γ/OX40L triplet mRNA therapy induced a broad immune response involving several DC types and lymphocytes, ultimately promoting tumoral immune infiltration and tumor regression. This local therapy led to clearance of distal untreated tumors and ignited immune memory to prevent cancer recurrence. These data suggest that triplet mixture mRNA therapy can ameliorate disease burden even in tumors with low preexisting immune infiltrates, suggesting long-term benefit in both primary and metastatic human cancers that encompass different TMEs. One potential limitation of this approach is that lesions must be injectable; however, this does include deep-seated lesions accessible by image-guided technology currently available in the clinic.

Mechanistic analyses outlined the biological effects of the three introduced mRNAs in both the TME and local LNs. Consistent with reported IL-36 classical DAMP (damage-associated molecular pattern) activities and IL-36R expression on DCs (8, 10, 38), both IL-36γ monotherapy and triplet mRNA treatments induced IL-6, CXCL1, G-CSF, and CCL7 release. DC maturation was detected in TdLN and included CD8+ DCs capable of cross-presentation (34). Although chronic elevation of IL-6 and CXCL1 has been associated with protumor effects, transient induction alongside DC activation can positively affect antitumor efficacy (39, 40). IL-23 treatment also corresponded with DC maturation of CD11b+ cDCs and CD103+ DC increase within LNs, which may contribute to critical tumor-specific T cell priming (30). Although the direct activation of CD8+ cDCs has previously been reported (41), the later impact of IL-23 on DCs may imply indirect action by downstream cells or cytokines. Deficiency in Batf3-dependent cross-presenting DCs does reduce T cell priming in general; however, fewer tumor regressions in *Batf3*−/− animals support DC mobilization as an antitumoral mechanism of triplet mRNA therapy.

Triplet mRNA also activated both innate (γδ T and NKT cells) and adaptive lymphocytes (αβ CD4+ and CD8+ T cells), and thus acted as more than a traditional DAMP/alarmin as anticipated from IL-1 family members. IL-23 additionally boosted γδ T and NKT cell proliferation, consistent with IL-23 receptor expression in murine (19, 42) and human cells (43). These innate lymphocytes could initiate early antitumoral responses as they can display rapid
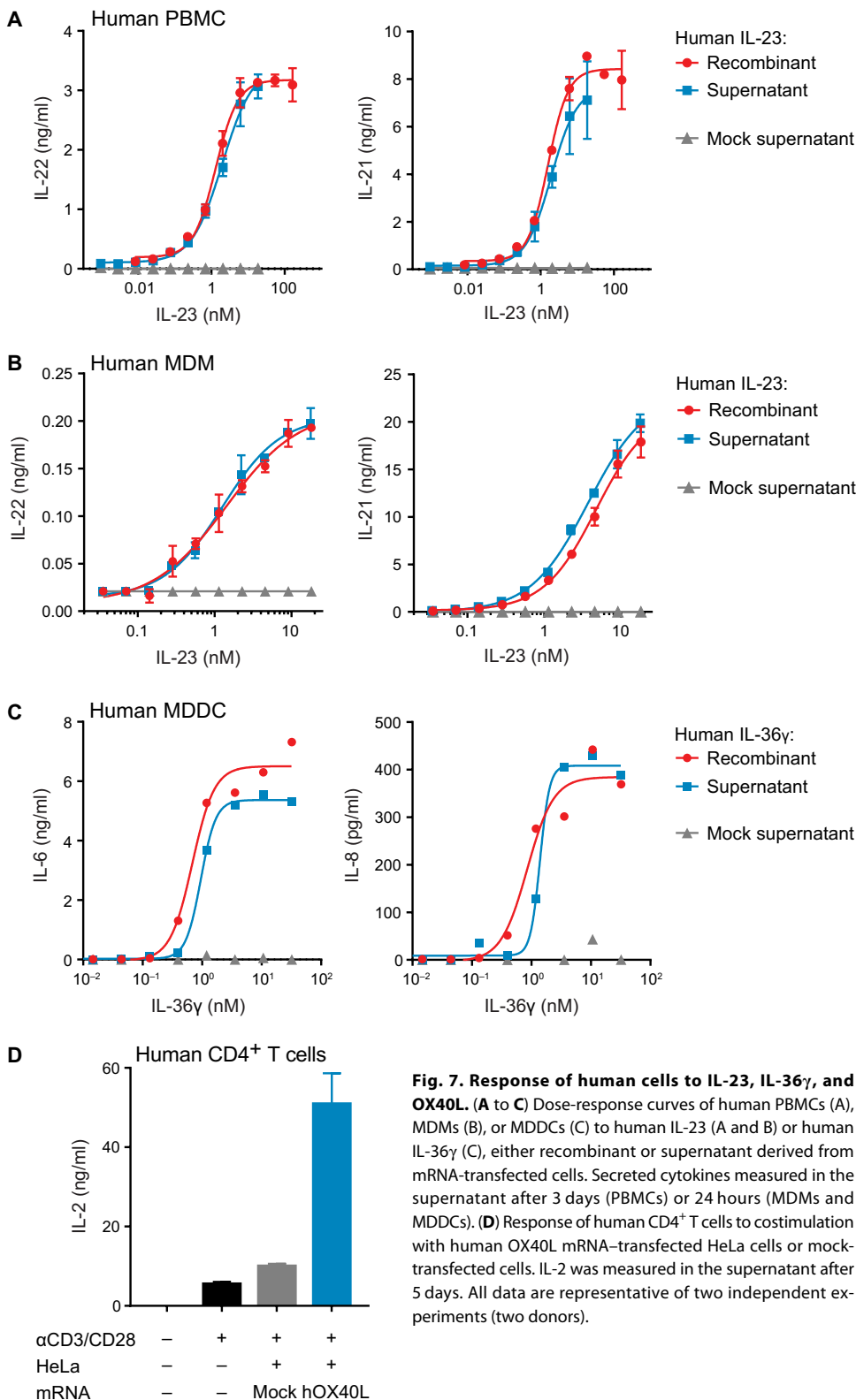
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Fig. 7. Response of human cells to IL-23, IL-36γ, and OX40L. (**A** to **C**) Dose-response curves of human PBMCs (A), MDMs (B), or MDDCs (C) to human IL-23 (A and B) or human IL-36γ (C), either recombinant or supernatant derived from mRNA-transfected cells. Secreted cytokines measured in the supernatant after 3 days (PBMCs) or 24 hours (MDMs and MDDCs). (**D**) Response of human CD4+ T cells to costimulation with human OX40L mRNA–transfected HeLa cells or mock-transfected cells. IL-2 was measured in the supernatant after 5 days. All data are representative of two independent experiments (two donors).

tumors in response to triplet mRNA, more than with any single component. However, only CD8-expressing T cells were required for antitumoral efficacy, although potential CD4+ T cell contributions may be complicated by functional diversity (T_H1, T_H2, T_H17, and T_{reg} cells). IL-23 can polarize T cells to a type 17 phenotype as well as promote long-lasting antitumoral responses, partly through T_H1/T_H17 polyfunctionality and reduction of T_{reg} suppression (46, 47). In contrast to the IL-17A cytokine, T_H17 cells correlate with improved prognosis across human cancers (48). Although an IL-23 response can be mixed, triplet mRNA generated a predominantly type 1 phenotype with minor type 17 and type 2 components and correlated with robust efficacy. Human IL-23 additionally stimulated IL-21 secretion from human cells, and although not detected in mouse triplet mRNA–treated animals, IL-21 can boost adaptive immunity in humans (49).

The cooperative antitumor activity of IL-23 and IL-36γ also counters literature, suggesting that IL-23 and IL-1 family members have tumor-promoting activities (11, 15). Whereas much of the associations with tumor promotion relates to chronic inflammation, mRNA treatment enables acute and transient expression that may mimic an endogenous antimicrobial/damage immune response. There are also differences among IL-1 family members including more restricted expression of the IL-36 receptor compared to the IL-1 receptor (11). Even in pathological conditions clearly implicating IL-36γ, such as psoriasis, no association with tumorigenesis or accelerated tumor growth has been reported. Although both IL-1 cytokines and IL-23, through IL-17A, can be pro-angiogenic, IL-17A induction by IL-23 was reduced in the context of mixture therapy with IL-36γ. Transcriptional up-regulation of angiogenic factors was also not detected, potentially due to anti-angiogenic cytokines such as IL-22 (47). These reports and our data suggest that association of these cytokines with tumorigenesis is context dependent, and our approach with transient and local exposure, as well as utilization of an mRNA mixture beyond IL-23 or IL-36γ alone, induces tumor regression. Addition of OX40L considerably improved efficacy, potentially by amplifying IL-23/IL-36γ–initiated T cell responses, which was indicated by boosted CD4+ and CD8+ T cell numbers in tumors. Similar to OX40

effector functionality, secrete cytokines such as IFN-γ and TNF-α (19, 43), and fuel adaptive immunity (44).

Adaptive immune system engagement is critical for anticancer specificity (45), and both CD4+ and CD8+ αβ T cells increased in

receptor up-regulation by CpG (50), IL-23/IL-36γ up-regulated T cell costimulatory pathways in tumors including OX40 and these changes may be reflected in TdLN. The long duration of OX40L expression potentially provided critical costimulation concurrently with T cell proliferation and may help promote a long-term adaptive response.

Although the idea of locally delivered immunomodulation is not new, indications of clinical benefit are now being observed (e.g., Imlygic, TLR9 agonists, and IL-12 plasmid electroporation) (4). Many of these prospective approaches (oncolytic viruses, other TLR agonists, naked mRNA, or DNA plasmids) may principally be akin to microbial TLR activation, as well as the purposefully expressed proteins. Strong viral antigens can promote local immune responses but may result in immunodominance and lower repeat dosing benefit (51). Nonspecific bacterial or viral mimics could also broadly activate the immune system, signal through cancer cells themselves, or incur toxicity while triggering stronger suppressive feedback mechanisms (52). In contrast, targeted expression through mRNA could use specific receptors, circumventing upstream TLR agonism given that the negative controls described here demonstrated minimal immune stimulation. This platform may also confer benefit over treatment with proteins, as recombinant IL-23/IL-36γ/OX40L-Fc proteins were unable to recapitulate mRNA-driven antitumoral activity. The relatively high tumoral expression coupled with some expression in TdLN from intratumoral delivery, the duration of expression, and where proteins are expressed may all be critical for optimal efficacy.

Together, intratumoral triplet mRNA therapy may avoid systemic toxicities and genomic incorporation while driving in situ vaccination against tumor-derived antigens and conferring long-term benefit. In addition, human cell studies showed similar cytokine responses to mRNA-derived proteins compared to murine contexts. Although this suggests translatability of immunomodulatory activity to human patients, it is currently unknown if the cooperative effects in mice from this mRNA mixture therapy will be recapitulated in humans. The confirmation of bioactivities in human cells alongside the preclinical demonstration of both a specific and ultimately global antitumor immunity, in conjunction with an appropriate safety evaluation, justifies clinical exploration. Intratumoral injections of mRNA encoding human IL-23, IL-36γ, and OX40L are now being evaluated in an ongoing phase 1 trial (NCT03739931).

MATERIALS AND METHODS
Study design
The research objectives were to determine optimal combinatorial efficacy against established tumors in multiple tumor models and evaluate mechanism of action through mRNA tissue distribution, cellular biodistribution of mRNA-derived protein, immune system changes in most cell types at a range of time points, and cytokine/chemokine release profiles.

All animal procedures and experiments were approved by the Institutional Animal Care and Use Committee at Moderna. Mice were randomized into groups when tumor volumes reached ~100 mm^3 for efficacy or ~180 mm^3 for flow cytometry and were dosed 24 hours later. Groups of 15 were designed for efficacy experiments, 10 animals for $Batf3^{-/-}$ efficacy experiments, 20 for abscopal experiments, 8 animals for flow cytometry, and 6 animals for cytokine analyses or NanoString. The protocol endpoint was a total tumor burden of 2000 mm^3. Animals were additionally removed due to ulceration or negative clinical observation per protocol requirements, and were censored from survival rates. CR rates include all animals enrolled per group. Reported flow cytometry data contain all eight samples where available, and five or greater in a minority of tumor analyses when LN was not obtained, or animals removed for ulceration. The exception is OX40L TdLN macrophage analyses in the control mRNA group, where data from three animals are presented due to low macrophage numbers. Main efficacy datasets were carried out three or more times and all repeat numbers are noted in figure legends. One dataset is shown, representing the middle of any variability. Antibodies for in vivo dosing are detailed in table S2. Primary data including main figure repeats are reported in data file S1.

mRNA design, synthesis, and formulation
The coding regions of mouse and human OX40L mRNA were derived from wild-type sequences (NM_009452 and NM_003326). Secreted IL-36γ includes either the signal peptide from mouse IL-2 added to mature mouse IL-36γ or a human immunoglobulin κ chain signal peptide to mature human IL-36γ. Protein sequences were back-translated and optimized for both mRNA synthesis and protein production using an in-house algorithm. IL-23 was constructed using the wild-type nucleotide sequences for the IL12B and IL23A protein subunits (IL-12p40 and IL-23p19; mouse NM_001303244 and NM_031252; human NM_002187 and NM_016584). These were combined in one sequence with linkers (53) between the IL12B and IL23A subunit. The IL12B signal peptide was retained for secretion, and the IL23A signal peptide was removed (first 21 amino acids for mouse and 19 for human, UniProt mouse Q9EQ14 and human Q9NPF7).

mRNA was synthesized in vitro by T7 RNA polymerase–mediated transcription, where the uridine 5′-triphosphate (UTP) was substituted with N1-methylpseudoUTP, from a linearized DNA template, which incorporates the 5′ and 3′UTRs and a polyadenylated tail as described (54). All mRNA constructs incorporated a miR122 binding site in the 3′UTR (27). The final mRNA uses Cap1 to increase mRNA translation efficiency. LNP formulations were prepared using a modified method previously described with the structure and composition of the LNP as described (26).

Cell lines and mouse tumor models
Mouse colon adenocarcinoma model MC38 materials were obtained from the Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis [National Cancer Institute (NCI)], and two variants, MC38-S and MC38-R, were characterized here. Mouse hepatocellular carcinoma line H22 was from the China Center for Type Culture Collection. Female Balb/c, C57BL/6, and $Batf3^{-/-}$ [B6.129(C)-$Batf3^{tm1Kmm}$/J] (34) mice were purchased from The Jackson Laboratory or Charles River Laboratories. B16F10-AP3, a B16F10 subline further passaged in vivo, was provided by MedImmune (28).

NanoString analysis
RNA was analyzed using the NanoString Murine Pan-Cancer Immune Panel on the nCounter MAX Analysis System, allowing differential mRNA expression analysis of 770 genes, including 20 housekeeping genes. An additional 30-probe panel (table S3) was used when analyzing samples from mRNA-treated mice.

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Statistical analysis

Flow cytometry pairwise statistics were calculated in Microsoft Excel by Student's t test with a two-tailed distribution and a two-sample unequal variance (heteroscedastic). For more than two groups within a time point, significance was calculated in GraphPad by one-way ANOVA with Tukey's post hoc test. Kaplan-Meier survival analyses were conducted in GraphPad by log-rank test (Mantel-Cox).

NanoString transcript analyses between IL-23/IL-36γ/OX40L and control mRNA groups were performed on normalized nCounter data. Linear regressions with nSolver investigated differential gene expression between groups in response to the covariate chosen followed by a Benjamini-Yekutieli post hoc FDR correction method. The P value represents the adjusted Benjamini-Yekutieli P value. Linear fold change is represented. All P values less than 0.05 were taken to be significant: $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$.

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/11/477/eaat9143/DC1
Materials and Methods
Fig. S1. In vitro OX40L bioactivity and in vivo cellular and tissue expression of OX40L after intratumoral mRNA administration.
Fig. S2. Characterization of multiple syngeneic tumor models for responses to systemic checkpoint blockade therapies.
Fig. S3. In vitro bioactivity of cytokine targets and in vivo expression of cytokine targets in tumor models.
Fig. S4. Tolerability and dose-dependent efficacy of intratumoral mRNA therapy.
Fig. S5. Induction of cytokines by multi-mRNA treatment.
Fig. S6. Differentially expressed immune-related transcripts in control mRNA–treated tumors.
Fig. S7. Activation status of DCs and T cells in response to local treatment with control mRNA.
Fig. S8. Induction of granulocyte infiltration by multi-mRNA treatment and granulocyte contribution to antitumor efficacy.
Fig. S9. DC profile in TdLN of mRNA-treated tumors and in vitro response of BMDCs to cytokines.
Fig. S10. Induction of lymphocyte activation and remodeling of the TME by multi-mRNA treatment.
Fig. S11. Overall transcript and costimulation focused transcript changes, and remodeling of the TME induced by multi-mRNA treatment.
Fig. S12. Local mRNA therapy: Protein expression by alternate routes of administration, resistance to secondary tumor challenge, and antitumor memory.
Fig. S13. Improved antitumor efficacy with combination of triplet mRNA and checkpoint blockade.
Fig. S14. Response of human MDMs to IL-36γ.
Table S1. Measurement of protein abundance in tumors after mRNA treatment and calculation of recombinant protein dosing.
Table S2. Proteins, antibodies, and controls for in vivo dosing.
Table S3. Probes in NanoString Plus Panel.
Table S4. Flow cytometry staining antibodies.
Data file S1. Primary data.
Reference (55)

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Science Translational Medicine

Durable anticancer immunity from intratumoral administration of IL-23, IL-36γ, and OX40L mRNAs

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Sending tumors a message

T cells need to overcome an immunosuppressive environment for successful cancer immunotherapy. Hewitt *et al.* leveraged a platform for mRNA delivery to devise a combination of factors that would ramp up antitumor immunity. They found that intratumoral injection of mRNAs encoding OX40L, IL-36 γ, and IL-23 led to tumor regression in several cancer models. The triplet therapy also rendered normally resistant tumors susceptible to checkpoint blockade. The specific immune components responsible for protection were also dissected, and they showed that this local therapy led to systemic immunity. This mRNA treatment could one day be translated for patient benefit.

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