Overcoming primary and acquired resistance to anti-PD-L1 therapy by induction and activation of tumor-residing cDC1s

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The ability of cancer cells to ensure T-cell exclusion from the tumor microenvironment is a significant mechanism of resistance to anti-PD-1/PD-L1 therapy. Evidence indicates crucial roles of Batf3-dependent conventional type-1 dendritic cells (cDC1s) for inducing antitumor T-cell immunity; however, strategies to maximize cDC1 engagement remain elusive. Here, using multiple orthotopic tumor mouse models resistant to anti-PD-L1-therapy, we are testing the hypothesis that in situ induction and activation of tumor-residing cDC1s overcomes poor T-cell infiltration. In situ immunomodulation with Flt3L, radiotherapy, and TLR3/CD40 stimulation induces an influx of stem-like Tcf1+ Slamf6+ CD8+ T cells, triggers regression not only of primary, but also untreated distant tumors, and renders tumors responsive to anti-PD-L1 therapy. Furthermore, serial in situ immunomodulation (ISIM) reshapes repertoires of intratumoral T cells, overcomes acquired resistance to anti-PD-L1 therapy, and establishes tumor-specific immunological memory. These findings provide new insights into cDC1 biology as a critical determinant to overcome mechanisms of intratumoral T-cell exclusion.

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Despite unprecedented clinical activity across multiple types of cancer with programmed death 1 (PD-1)/ligand-1 (PD-L1) blockade therapy, the majority of patients do not respond (primary resistance) or develop resistance after initial tumor regression (acquired resistance). This is due, at least in part, to poor T cell infiltration into the tumor, which is negatively correlated with treatment response. Therefore, the development of novel approaches to increase T cell infiltration within the tumor microenvironment (TME) is of paramount importance and likely would markedly increase the number of patients benefiting from immunotherapy.

To this point, compelling evidence indicates critical roles for tumor-resident Batf3-dependent conventional type-1 dendritic cells (cDC1s) (migratory CD103+t and lymphoid CD8α+ DCs in mice, and CD141+ DCs in humans) in priming and expansion of tumor-specific CD8+ T cells and their recruitment to the TME. Unique among the various DC subsets, cDC1s display enhanced abilities to phagocytose dead cells, and to cross-present exogenous antigens onto major histocompatibility complex (MHC) class I molecules. Although sparse in the TME, cDC1s can be recruited by systemic or, preferentially, intratumoral injection of Fms-like tyrosine kinase 3 ligand (Flt3L) and can be employed for enhancing Flt3L-induced cDC1 function in the tumor. Indeed, there is preclinical and clinical data from T cell-infiltrated tumors showing that in situ vaccination with Flt3L, RT, and TLR3 agonist facilitates cross-priming of tumor-specific T cells, and synergizes with PD-1 blockade. Thus, enhancing cDC1 function in the tumor in situ holds promise for patients with locally accessible unresectable cancer. Since much of what is known about the importance of cDC1s in antitumor immunity has been accumulated in T cell-infiltrated tumor models, the significance and precise role of cDC1s in converting poorly T cell-infiltrated tumors to inflamed ones remain to be elucidated.

One method to potentially maximize the engagement of tumor-residing cDC1s is providing CD40 signaling. A growing body of evidence shows that engagement of CD40 on cDC1s (migratory CD103+ and lymphoid CD8α+ DCs) provides potent maturation and anti-apoptotic signals to DCs, augments priming of antigen-specific cytotoxic T lymphocytes (CTLs), induces interleukin 12 (IL-12) production for the development of Th1 helper 1 (Th1) cells, and overcomes peripheral T cell tolerance. Combined TLR/CD40 stimulation synergistically enhances CD8+ T cell expansion, and mediates potent antitumor immunity in multiple syngeneic mouse models.

Here, we aim to overcome resistance to PD-L1 blockade therapy in TMEs characterized by poor T cell infiltration. We are testing the overarching hypothesis that the induction and activation of tumor-residing cDC1s would facilitate the priming, expansion, and infiltration of tumor-specific CD8+ T cells into the poorly T cell-infiltrated TME, and overcome resistance to anti-PD-L1 therapy. To this end, we employ a combinatorial in situ immunomodulation (ISIM) regimen comprising in situ administration of: (1) Flt3L to mobilize cDC1s to the TME; (2) RT to promote immunogenic death of cancer cells and maturation of DCs; and (3) dual TLR3/CD40 stimulation to activate antigen-loaded cDC1s for priming and expansion of tumor-specific CD8+ T cells.

Using multiple orthotopic mouse models of poorly T cell-infiltrated and anti-PD-L1-resistant tumors, our data demonstrate that this strategy significantly increases the infiltration of CD8+ T cells that mediate the regression not only of primary but also untreated distant poorly T cell-infiltrated tumors. Such CD8+ T cell effector responses are characterized by novel clonotypes and stem-like Tc1+ Slam6+ phenotypes expressing Tox, further bolstering antitumor efficacy through use of anti-PD-L1 antibody (Ab). Altogether, our study identifies a previously unrecognized role for cDC1s as well as a combinatorial platform to engage them for fostering efficient T cell infiltration within poorly infiltrated TMEs for robust anti-PD-L1-based immunotherapy.

**Results**

**Sensitivity to PD-L1 blockade correlates with a density of CD8+ T cell infiltrates.** Antitumor effect of anti-PD-L1 therapy was assessed in four syngeneic mouse tumor models: MC38 colon adenocarcinoma, B16 melanoma, and two triple-negative mammary cancers: AT-3 derived from the PyMT-MMTV model on an H-2d background and 4T1 on an H-2b background. Treatment delayed growth of MC38 but not AT-3, B16, or 4T1 tumors (Supplementary Fig. 1a). Immunohistochemistry (IHC) analysis revealed sparse CD8+ T cells but abundant CD163+ tumor-associated macrophages (TAMs) in AT-3, B16, and 4T1 tumors compared to MC38 tumors (Supplementary Fig. 1b), consistent with a positive correlation between CD8+ tumor-infiltrating lymphocytes (TILs) frequency and response to anti-PD-L1 therapy, and validating AT-3, B16, and 4T1 tumors as models for poorly T cell-infiltrated tumors refractory to anti-PD-L1 therapy.

ISIM with Flt3L, RT and TLR3/CD40 stimulation facilitates priming, expansion and infiltration of tumor-specific T cells in poorly T cell-infiltrated tumors. Using three orthotopic mouse models of poorly T cell-infiltrated tumors (AT-3, B16, and 4T1), we evaluated the efficacy of the combinatorial therapeutic regimen, ISIM, comprised of intratumoral sequential administration of Flt3L (to recruit cDC1s to the TME), RT (to induce immunogenic death of cancer cells and maturation of DCs), and TLR3/CD40 agonists (to stimulate antigen-loaded cDC1s for priming and expansion of tumor-specific CD8+ T cells) (Fig. 1a).

ISIM-induced TME changes were evaluated by flow cytometry (Supplementary Fig. 2) as previously described by others. In situ daily administration of Flt3L for 9 days increased CD103+ DCs in AT-3 tumors, peripheral blood (PB), and lymph nodes (LN) including tumor-draining LN (TDLN) and mesenteric LN (mLN) in a line with a recent study in lymphoma. Tumor CD11b+DCs also increased, but the higher CD103+CD11b+ DC ratio suggests preferential mobilization of CD103+ DCs. Notably, Flt3L did not alter the frequency of CD8+ T cells or CD11b+ myeloid cells including TAMs nor did it impact on tumor growth (Supplementary Fig. 3b, c). Local irradiation with a single dose of 9 Gy increased expression of CD40 and CD86 on CD103+ DCs, suggesting maturation of Flt3L-induced tumor-residing CD103+ DCs (Fig. 1c).

Migratory CD103+ DCs are the dominant cells that carry antigen to the TDLN, but how this process can be enhanced is unknown. To this end, mice bearing AT-3 tumors expressing GFP (AT-3-GFP) were treated with in situ injections of TLR3/CD40 agonists or phosphate-buffered saline (PBS) following Flt3L administration and RT, and tumors and TDLN were harvested after 24 h. In situ TLR3/CD40 stimulation decreased intratumoral GFP CD103+ DCs (Fig. 1d). Concomitantly, imaging flow cytometry analysis of TDLN revealed increased numbers of GFP+CD103+ DCs in treated mice compared to the PBS controls (Fig. 1e), consistent with efficient trafficking of tumor-associated antigen (TAA)-loaded cDC1s to TDLN by TLR3/CD40 stimulation. Moreover, we found an increased...
presence of GFP+ CD103+ DC/CD8+ T cell doublets (Supplementary Fig. 4a, b), suggesting a cross-talk between TAA-loaded cDC1s and CD8+ T cells. In agreement, significant upregulation of CD69 was observed on both CD4+ and CD8+ T cells in the TdLN, indicating effective activation of T cells (Supplementary Fig. 5). These findings prompted us to assess whether ISIM facilitated priming of tumor-specific CD8+ T cells using a tetramer (Tet) to identify H-2Ld-restricted MuLV gp70-specific CD8+ T cells in mice bearing 4T1 tumor, which harbor gp70 epitopes as an endogenous TAA25. In TdLN, ISIM promoted generation of Tet+ CD8+ T cells (Fig. 1f) that downregulated CD62L (L-selectin) and were capable of producing IFN-γ and TNF-α (Supplementary Fig. 6), consistent with an effector phenotype.

Analysis of circulating CD8+ and CD4+ T cells demonstrated that the combination of either Flt3L/RT or TLR3/CD40 stimulation increased CD44+ CD62L+ central memory T cell (TCM) subsets (Supplementary Fig. 7a, b). However, generation of CD44+ CD62L− effector memory T cell (TEM) subsets and upregulation of PD-1 expression were greatly enhanced by combining all ISIM

**Fig. 1b, Fig. 1c, Fig. 1d, e, f, Fig. 1g, h, i**
In situ immunomodulation (ISIM) with Flt3L, radiotherapy (RT), and TLR3/CD40 stimulation facilitates priming, expansion, and infiltration of tumor-specific T cells in poorly T cell-infiltrated tumors. a Experimental set-up. Mice bearing AT-3, B16, or 4T1 tumors were treated with intratumoral Flt3L injection followed by RT and in situ TLR3/CD40 stimulation. The timing of sample take down in each experiment was indicated. b Representative flow cytometric plots showing CD103+ and CD11b+ expression on Ly6c+ class II$^+$ CD11c$^+$ CD24$^+$ F4/80$^+$ DCs, and frequency of total DCs, CD11b$^+$ DCs, CD103$^+$ DCs among CD45+$^+$ cells, and CD103$^+$/DC/CD11b$^+$ DC ratio in AT-3 tumors. n = 5 mice per group. c Representative flow cytometric plots and the frequency of CD45$^+$ CD8$^+$ CD68$^+$ cells among CD103$^+$ DCs in AT-3 tumors. n = 6 mice per group. d Frequency of GFP$^+$ CD103$^+$ DCs among CD45$^+$ cells in AT-3 tumors treated with Flt3L+RT or Flt3L+RT+TLR3/CD40 agonists. n = 5 mice per group. e Representative images of CD11c$^+$ CD103$^+$ GFP$^+$ cells in tumor-draining lymph nodes (TDLN) of AT-3-GFP-bearing mice treated with ISIM obtained by imaging flow cytometry. Data panel shows the frequency of GFP$^+$ CD11c$^+$ CD103$^+$ cells (CD103$^+$ DCs) out of all cells in TDLN of mice treated with Flt3L+RT or Flt3L+RT+TLR3/CD40 agonists. n = 5 mice per group. All panels are the same magnification, scale bar = 10 μm. f Representative flow cytometric plots and the frequency of gp70 tetramer (Tet)+ CD8$^+$ T cells among CD8$^+$ T cells in TDLN from 4T1 tumor-bearing mice, n = 5 mice (NT) and 13 mice (ISIM). g Frequency of CD8$^+$ T cells among CD45$^+$ cells in AT-3, B16, and 4T1 tumors. n = 4 mice (ISIM of B16 tumors) and 5 mice (any other groups). h, i Representative flow cytometric plot and the frequency of Tet$^+$ CD8$^+$ T cells (h) and Pmel-1 T cells (CD90.1$^+$CD8$^+$) (i). n = 8 mice (NT) and 5 mice (ISIM). i, n = 3 mice (NT) and 4 mice (ISIM). Data shown are representative of two (h, i) or three (g) independent experiments. Two-tailed t-test (b–i). Mean ± SEM. Source data are provided as a Source Data file.

ISIM reverses CD8$^+$/CD11b$^+$ cell ratio in the tumor, and renders poorly T cell infiltrated tumors responsive to anti-PD-L1 therapy. High densities of myeloid cells and TAMs associate with poor prognosis, and resistance to PD-1 blockade29,30. Phenotypic analysis of AT-3, B16, and 4T1 tumors revealed that an ISIM-mediated increase of CD8$^+$ TILs was associated with a concomitant decrease of CD11b$^+$ myeloid cells and TAMs, resulting in a reversal of a CD8$^+$/CD11b$^+$ cell ratio (Fig. 3a, b). Activation of the local PD-1/PD-L1 immune inhibitory axis has been associated with the presence of CD8 TILs2. Having observed an ISIM-induced increase of CD8$^+$ T cell infiltration, we evaluated expression of PD-1 on CD8$^+$ TILs. The frequency of PD-1-expressing CD8$^+$ TILs increased in AT-3, B16, and 4T1 tumors (Fig. 3c) and PD-1 expression was more prevalent in Tet$^+$ CD8$^+$ T cells than Tet$^-$ CD8$^+$ T cells (Fig. 3d), suggesting a higher susceptibility to PD-L1-mediated inhibition of the ISIM-induced tumor-specific CD8$^+$ T cell infiltrates. Upregulation of PD-L1 in tumors and tumor-infiltrating immune cells represents an adaptive immune resistance mechanism which correlates with response to anti-PD-1/PD-L1 therapy31,32. Therefore, we evaluated PD-L1 expression on tumor cells of AT-3-GFP tumor-bearing mice treated with or without ISIM. ISIM increased PD-L1 expression on CD45$^+$ GFP$^+$ tumor cells (Fig. 3e). Furthermore, ISIM increased PD-L1 expression on TAMs in all three tumor models (Fig. 3f).

Having achieved conversion of poorly T cell-infiltrated tumors and activation of the PD-1/PD-L1 axis in the TME, we sought to test whether ISIM augments antitumor efficacy of anti-PD-L1 therapy. ISIM-treated tumors became sensitive to anti-PD-L1 therapy, resulting in delayed tumor growth and improved survival in all three tumor models (Fig. 3g). Collectively, these findings provide a proof-of-concept that in situ induction and activation of tumor-residing cDC1s converts poorly T cell-infiltrated tumors, and renders them responsive to anti-PD-L1 therapy.

Conversion of poorly T cell-infiltrated tumors requires dual TLR3/CD40 stimulation of Flt3L-induced DCs. Next, we examined the role of combined TLR3/CD40 stimulation of Flt3L-induced DCs for the conversion of poorly T cell-infiltrated tumors.
tumors. We treated AT-3 tumor-bearing mice with agonistic anti-CD40 Ab, poly(I:C), or their combination following Flt3L administration and RT. While mice treated with either CD40 or TLR3 agonist responded with modest tumor growth delay and increased survival, combined TLR3/CD40 stimulation resulted in superior tumor control and survival in all three tumor models (Fig. 4a and Supplementary Fig. 8). Dual TLR3/CD40 stimulation was required to upregulate CD40, CD70, and CD86 on CD103+ DCs in TdLN (Fig. 4b). Phenotypic analysis of circulating T cells indicates that stimulation with TLR3 agonist increased $T_{CM}$

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**Fig. 2 In situ immunomodulation (ISIM) triggers rapid regression of established poorly T cell-infiltrated tumors and improves survival.**

- **a** Individual tumor volume curves of AT-3 (upper) and B16 (lower) tumor-bearing mice treated with PBS (NT) or ISIM. $n=7$ mice per group (AT-3) and $9$ mice per group (B16).
- **b** Bioluminescence imaging of 4T1-luc tumor-bearing mice treated with PBS (NT) or ISIM ($n=5$ mice per group) on day 14. The data panel shows mean radiance at each time point as indicated. Statistical significance was determined by a two-tailed t-test.
- **c** Waterfall plots (c), tumor volume curves (d), and survival curves (d) in AT-3 tumor-bearing mice in different treatment as indicated. Waterfall plots show maximal change of tumor volume at the day when TLR3/CD40 agonists were administered. $n=7$ mice (PBS, Flt3L, RT, TLR3/CD40) and 8 mice (any other groups). *$p<0.05$, **$p<0.01$, ***$p<0.001$ by a log-rank test.
- **e** Tumor volume curves (mean) and tumor weight (mg) in AT-3 tumor-bearing mice. Flt3L was administrated intraperitonially (i.p.) or intratumorally (i.t.) followed by RT and TLR3/CD40 agonists. $n=5$ mice (NT) and 6 mice (any other groups). Primary tumors were harvested 7 days after TLR3/CD40 stimulation (e, f). Data shown in a-d are representative of three independent experiments. **$p<0.01$, ***$p<0.001$ by one-way ANOVA with Tukey’s multiple comparisons (e, f). Source data are provided as a Source Data file.
CD8+ and CD4+ T cells; however, CD40 agonist was required for their differentiation into T_{EM} and upregulation of PD-1, 4-1BB, and CX3CR1, which were further enhanced by dual TLR3/CD40 stimulation (Supplementary Fig. 9). Importantly, combined TLR3/CD40 stimulation was needed to increase the frequency of CD8+ TILs in AT-3 tumors, and tumor-specific Tet+ CD8+ T cells in the PB and 4T1 tumors (Fig. 4c). In addition, alteration of the immune cell composition of the TME (reduction of myeloid infiltrates, reversal of a CD8+/CD11b+ cell ratio, and upregulation of PD-L1 in TAMs) was observed only with combined
Fig. 3 In situ immunomodulation (ISIM) reverses a CD8+/CD11b+ cell ratio, and renders poorly T cell-infiltrated tumors responsive to anti-PD-L1 therapy. a Representative flow cytometric plots showing CD11b and CD8 expression of total CD45+ cells in AT-3 tumors. Data panels show frequency of CD11b+ cells of CD45+ cells and CD8+ T cell/CD11b+ cell ratio in AT-3, B16, and 4T1 tumors. n = 4 mice (ISIM of B16) and 5 mice (any other groups). b Frequency of Ly6c- class II+ CD24+/F4/80+ tumor-associated macrophages (TAMs) among CD45+ cells in AT-3, B16, and 4T1 tumors. n = 4 mice (ISIM of B16) and 5 mice (any other groups). c Representative histogram showing PD-1 expression and percentage of PD-1+ cells in CD8+ TILs in AT-3, B16, and 4T1 tumors. n = 5 mice (all groups of AT-3, ISIM of AT-3, 6 mice (NT of 4T1), 7 mice (NT of B16), and 8 mice (ISIM of B16). d Percentage of PD-1+ cells in gp70-specific Tett or Tet1+ CD8+ T cells in ISIM-treated 4T1 tumors. n = 5 mice per group. e Representative histogram showing PD-L1 expression and median fluorescence intensity (MFI) of PD-L1 in CD45− gp70+ AT-3 tumor cells (e), and TAMs in AT-3, B16, and 4T1 tumors treated with or without ISIM (f). n = 4 mice (ISIM of B16) and 5 mice (any other groups). Tumors were harvested 5–7 days after TL3R/CD40 stimulation (a–f). Two-tailed t-test (a–f). g Tumor growth curves (mean) and survival curves in AT-3, B16, and 4T1 tumor-bearing mice in different treatment groups as indicated. n = 5 mice (all groups of AT-3, dpD-L1 of B16, and ISIM + isotype Ab of B16) and n = 6 (any other groups) *p < 0.05, **p < 0.01, ***p < 0.001 by a log-rank test. Data shown are representative of two (d, e, g) or three (a–c, f) independent experiments. Mean ± SEM. Source data are provided as a Source Data file.

ISIM of primary tumors triggers the immune remodeling and regression of untreated distant tumors. To determine whether ISIM-induced adaptive T cell immunity could generate systemic antitumor immunity, the abscopal effect of ISIM performed only on one tumor in mice bearing bilateral AT-3 tumors was examined. ISIM resulted in rapid regression not only of treated tumors but also of the untreated distant tumors (Fig. 5a). Furthermore, ISIM-induced immune remodeling of the TME was evident in distant tumors, as well as in treated tumors (Fig. 5b) as confirmed by qualitative IHC analysis (Fig. 5c). These results indicate that ISIM generates potent systemic antitumor immunity, causing immune remodeling and regression of untreated distant tumors.

ISIM induces stem-like Tcfa7+Slamf6+ T cells into poorly T cell-infiltrated tumors. From the initial analysis of total cell populations, we isolated annotated lymphoid clusters by extracting cells expressing Cd3e and/or Ncr1, and reanalyzed the data. This approach yielded 13 distinct lymphoid clusters (Ly_C) (Fig. 7a, Supplementary Fig. 14a, b, and Supplementary Data 3). In line with a recent study35, some clusters were identified on the basis of functional markers rather than their cellular lineage. Ly_C4 and C7 comprised CD4+, CD8+, and NK cells in control and anti-PD-L1-treated mice, and largely CD8+ T cells in ISIM- and ISIM + anti-PD-L1-treated mice (Fig. 7b), but were notable for elevated expression of cell-cycle genes and pathways: Mki67, Cenpa, and Ks1b in Ly_C4 and Mcm3, Mcm5, and Ung in Ly_C7 (Supplementary Figs. 15, 16a, and Supplementary Data 3). Ly_C2 expressed Il7r, Sell, Klf2, Lef1, and Tcf7 (encoding Tcf-1), and high levels of ribosomal subunit genes and pathways, but did not express Pdcd1, Lag3, and Haver2 (encoding Tim3) (Fig. 7b, Supplementary Figs. 15 and 16a, and Supplementary Data 3), consistent with naïve CD8+ T cells36,37. This cluster was found mainly in the control tumors, and substantially decreased in ISIM-treated tumors (Fig. 7c).

In contrast, a massive influx of Slamf6 (encoding Ly108)-expressing cells (Ly_C0, C6, and C10) was observed in ISIM-treated tumors (Fig. 7a–c). These clusters also expressed Tcfa7, Lef1, stem cell/memory markers (Il7r, Klf2, Cxcr3, and Cd28), intermediate levels of Pdcd1, but were relatively negative for Haver2 and cell-cycle-related pathways (Fig. 7b, Supplementary Figs. 15, 16a, and Supplementary Data 3), suggesting stem-like progenitor-exhausted T cells37–42. Ly_C0 was the predominant ISIM-induced Tcfa7+Slamf6+ Pdcd1+ CD8+ T cell population expressing high levels of nuclear factor Tox (Fig. 7b and Supplementary Data 3), a transcription factor also expressed on terminally exhausted T cells, but critical for sustaining CD8+ T cell responses during chronic infection and cancer13–47. This cluster was notable for strong enrichment of T cell receptor (TCR)/CD3 signaling (Supplementary Fig. 16a). Similar to Ly_C0, Ly_C6 also expressed Tcfa7, Lef1, Slamf6, Pdcd1, and Tox, but was enriched with IFN-stimulated genes (ISGs) such as Ift11, Ift3 and Isg15, and increased IFN signaling (Fig. 7b, Supplementary Figs. 15 and 16a, and Supplementary Data 3). Ly_C10 expressed higher levels of Tcfa7, Lef1, Slamf6, Klf2, S1pr1, and Cd28 and increased in frequency in response to anti-PD-L1 therapy (Fig. 7b, c, Supplementary Fig. 15, and Supplementary Data 3). This cluster was uniquely enriched with hypoxia, HIF-1α signaling, and glucose metabolism pathways (Supplementary Fig. 16a).

Conversely, Ly_C5 expressed high levels of Haver2, Pdcd1, Lag3, Entpd1 (encoding CD39), Cd38, and Gemb, but lacked Tcfa7, Lef1, and Slamf6 (Fig. 7b, Supplementary Fig. 15, and Supplementary Data 3), suggesting terminally exhausted CD8+ T cells37–42. Flow cytometric analysis confirmed significantly

TLR3/CD40 stimulation (Fig. 4d, e and Supplementary Fig. 10). Together, these results indicate critical roles of in situ dual TLR3/CD40 stimulation of induced CD103+ DCs in remodeling the poorly T cell-infiltrated TME.

ISIM decreases M2-like macrophages and increases IL-12+ DCs in the TME. Next, we performed single-cell RNA sequencing (scRNAseq) to assess transcriptional and functional changes of immune cell populations infiltrating AT-3 tumors treated with ISIM, anti-PD-L1 Ab, or both. Control mice received isotype Ab (NT), scRNAseq analysis of CD45+ tumor-infiltrating cells yielded data for 2470–3768 high-quality cells per group (12,228 total cells) (Supplementary Fig. 11). Unsupervised clustering analysis and subsequent cell type annotation and differential expression procedures revealed 12 lymphoid clusters, 2 macrophage clusters (cluster C1 and C11), and small clusters of monocytes/DCs (C14), DCs (C15, C16 and C18), neutrophils (C17), and B cells (C19) (Fig. 6a, Supplementary Figs. 12 and 13a, and Supplementary Data 1). In ISIM-treated tumors the frequency of macrophages was substantially decreased while T cells were increased compared to control tumors (Fig. 6b, c and Supplementary Fig. 13b) consistent with flow cytometry (Fig. 3b) and IHC (Fig. 5c) results. Macrophage clusters (C1 and C11) expressed Arg1, Mrc1, and Ccl8 but were largely negative for Nos2, consistent with M2-like TAMs (Fig. 6d)29,30,33.

Two DC clusters (C15 and C16) that expressed Baff3, Flt3, and Zbtb46 (Fig. 6e) were identified. C16 expressed markers of CD1cIs (Igαe (encoding CD103), Xcr1, Tlr3, and Clec9a), and high levels of MHC class II-related genes (Ciita, H2-Dmb1, and H2-aa). In ISIM-treated tumors there is an increased frequency of C15, which expressed Fscn1, Ly75 (DEC-205), Il12b (IL-12p40), key non-canonical NF-κB pathway genes (Cd40, Birc2, Map3k1, Nfkβ2, and Relb), and high levels of maturation markers (Cd70, Cd80, and Cd83) (Fig. 6c, e, f and Supplementary Data 2), consistent with IL-12-producing DCs (IL-12 DC) that are critical for effective anti-PD-1 therapy34.

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Fig. 4 Conversion of poorly T cell-infiltrated tumors requires dual TLR3/CD40 stimulation of Flt3L-induced DCs. a Tumor volume curves (mean) and survival curves in AT-3 tumor-bearing mice in different treatment groups as indicated. n = 7 mice in all groups. **p < 0.01, ***p < 0.001 by a log-rank test.

b Median fluorescence intensity (MFI) of CD40, CD70, and CD86 in CD103+ DCs in tumor-draining lymph nodes (TdlN) of AT-3 tumor-bearing mice. n = 5 mice (NT), 7 mice (Flt3L + RT + TLR3, Flt3L + RT + CD40), and 8 mice (Flt3L + RT + TLR3/CD40).

c Frequency of CD8+ T cells of CD45+ cells in AT-3 tumors, and frequency of gp70-specific Tet+ cells among CD8+ T cells in peripheral blood and numbers (per gram) of gp70-specific Tet+ cells in tumors of 4T1 tumor-bearing mice. n = 5 mice (NT), 8 mice (Flt3L + RT + TLR3, Flt3L + RT + CD40), and 9 mice (Flt3L + RT + TLR3/CD40) for CD8+ T cells in AT-3 tumors. n = 5 mice (NT) and 6 mice (any other groups) for Tet+ cells. Frequency of CD11b+ cells of CD45+ cells, CD8+/CD11b+ cell ratio, and frequency of tumor-associated macrophages (TAMs: Ly6c– class II+ CD24– F4/80+) of CD45+ cells (d), and MFI of PD-L1 in TAMs (e) in AT-3 tumors. n = 5 mice (NT) and 8 mice (any other groups). Tumors were harvested 5–7 days after TLR3/CD40 stimulation (b–e). NS not significant, *p < 0.05, **p < 0.01, ***p < 0.001 by one-way ANOVA with Tukey’s multiple comparisons (b–e). Data shown are representative of three independent experiments. Mean ± SEM. Source data are provided as a Source Data file.
increased Tcf1+ and Slam6+ CD8+ TILs in ISIM-treated tumors compared to control tumors (Fig. 7d). Furthermore, CD8+ TILs in control tumors were mainly Slam6+ CD8+ with dichotomous expression of PD-1; PD-1− (Ly_C2) and PD-1hi (Ly_C5), while ISIM-treated tumors contained large numbers of Slam6+ PD-1int CD8+ TILs (Ly_C0, C6 and C10) (Fig. 7e).

Anti-PD-L1 therapy increased expression of genes associated with effector cytokine (Ifng, Gzmb, and Gzma), chemokine (Ccl4, Ccl5, Ccl8, Ccl11, Ccl12, Ccl15, and Ccl19), and immune checkpoint (Pdgaln1, Pdgaln2, Pdgaln3, Tigit, Pdga, Pdgb, Pdgc, Pdgd, and Pdge).
and Ccl5), inhibitory receptors (Lag3 and Ctla4), and transcription factors (Tox, Nr4a2, and Bhlhe40), and decreased expression of the memory-associated gene Il7r in Tcf7+ Slamf6+ Ly_C0 of ISIM-treated tumors (Fig. 8a, b, Supplementary Fig. 16b, and Supplementary Data 4). Gene set enrichment analysis (GSEA) of differential gene expression (DGE) between ISIM and ISIM + anti-PD-L1-treated tumors revealed a strong upregulation of gene signatures associated with TCR, NFAT, IFNG, TNF, IL2, hypoxia, HIF-1α, and/or glycogenesis/glycolysis in the majority of infiltrating CD8+ T cell clusters except for Tcf7− Havcr2+...
Fig. 6 Clustering of tumor-infiltrating immune cells and analysis of myeloid cell populations by scRNAseq. a UMAP plots of tumor-infiltrating immune cells (CD45+ cells) in AT-3 tumors. Cluster 1 (C1), 11 (C11), and 15-18 (C15-18) were identified as myeloid cells for further analysis (c-f). b Distribution of cell type in AT-3 tumors treated with PBS + isotype Ab (NT), PBS + anti-PD-L1 Ab (qPD-L1), in situ immunomodulation (ISIM) + isotype Ab (ISIM), or ISIM + anti-PD-L1 Ab (ISIM + qPD-L1). Cell types on UMAP plots are highlighted by matching colors on data panel. c Frequency of each myeloid cluster in AT-3 tumors in different treatment as indicated. Neutrophil, pDC plasmacytoid dendritic cell. d Expression plots of Arg1, Mrc1, Ccl5, and Nox2 in myeloid cell clusters. e Heatmap displaying normalized expression of selected genes in each myeloid cell cluster. Mono monocyte. f Volcano plot showing enrichment differentially expressed genes between C15 (IL-12 DC) and C16 (cDC1). Each red and blue dot denotes an individual gene with Benjamini-Hochberg-adjusted p value <0.05 and log fold change >0.25. Source data are provided as a Source Data file.

Ly_C5 (Fig. 8c, Supplementary Fig. 17, and Supplementary Data 5), suggesting the minimal effect of anti-PD-L1 therapy on terminally exhausted CD8+ T cells in ISIM-treated tumors.

PD-L1 blockade increases the frequency of Th1-polarized CD4+ T cells in ISIM-treated tumors. Ly_C8 expressed Gata3 and genes linked to Th2 type cytokine including Il13, Il5, Il4, and Il6, consistent with Th2 CD4+ T cells while Ly_C11 expressed Tbx21, and genes linked to Th1-type cytokine including Ifng and Tnf (encoding TNF-α) consistent with Th1 CD4+ T cells (Fig. 8a and Supplementary Data 3). Regulatory T cells (Tregs) expressing Foxp3 were identified in Ly_C9, while a distinct cluster for Th17 cells was not observed. The percent of three distinct clusters of CD4+ T cells (Ly_C8, C9, and C11) decreased in ISIM-treated mice; however, anti-PD-L1 therapy increased them, most notably Ly_C11 Th1 cells in ISIM-treated mice (Fig. 7c). Moreover, anti-PD-L1 therapy increased Ifng expression in Ly_C11, and decreased Il13 and Il5 expression in Ly_C8 in ISIM-treated tumors (Fig. 8a and Supplementary Fig. 16b), suggesting a skewing toward a Th1-type response. In agreement with this, anti-PD-L1 therapy upregulated pathways associated with TCR, NFAT, IFNG, IL-2, hypolysis, and glycosylation in Ly_C11 (Fig. 8c and Supplementary Data 5). Taken together, scRNAseq analysis revealed significant ISIM-induced alterations in both lymphoid and myeloid cell compartments of poorly T cell-infiltrated tumors.

ISIM elicits IFN-γ and IL-12-dependent de novo adaptive T cell immunity mediated by Batf3-dependent DCs. To determine whether cDC1s were key to ISIM therapeutic efficacy, we generated Batf3−/− bone marrow chimera mice into C57BL/6 wild-type (WT) host mice that lack myeloid-derived Batf3-dependent cells including CD103+ DCs. Therapeutic efficacy of ISIM decreased to the level of RT alone in Batf3−/− bone marrow chimera mice but not in mice reconstituted with WT bone marrow, demonstrating that the immunomodulatory effect of ISIM depends on Batf3-dependent bone marrow-derived cells (Fig. 9a). Depletion of CD8+ or CD4+ T cells but not NK1.1+ cells decreased antitumor efficacy of ISIM (Fig. 9b and Supplementary 18a). Intriguingly, depletion of CD4+ T cells abrogated ISIM-induced reduction of TAMs and their PD-L1 upregulation while depletion of CD8+ T cells or NK1.1+ cells did not (Fig. 9c, d). This suggests that both the increase of CD8+ TILs and CD4+ T cell-dependent decrease of myeloid cells contributed to the ISIM-induced reversal of a CD8+/CD11b+ cell ratio in the TME.

Supported by co-stimulatory signals on DCs, engagement of CD4+ T cell help enhances CD8+ T cell response in secondary lymphoid organs (SLOs)48–50. To determine the role of SLOs in ISIM therapy, mice were treated with vehicle control or FTY720 to block new T cell trafficking from SLOs51. Treatment with FTY720 compromised antitumor efficacy in ISIM-treated mice (Fig. 9e and Supplementary 18b) and decreased the frequency of CD8+ TILs (Fig. 9f), indicating the importance of SLOs as sites for ISIM-induced priming.

Considering that scRNAseq analysis showed increased TILs and DCs expressing Ifng and Il12b, respectively, within ISIM-treated tumors, we tested whether ISIM-mediated antitumor efficacy depended on IFN-γ and IL-12. Neutralization of IFN-γ or IL-12 resulted in markedly reduced CD8+ T cell infiltrates and antitumor efficacy of ISIM (Fig. 9g, h). As seen with depletion of CD4+ T cells, both IFN-γ and IL-12 neutralizing Ab blocked ISIM-induced reduction of TAMs and their PD-L1 upregulation (Fig. 9i, j). Taken together, these results demonstrate that ISIM elicits IFN-γ- and IL-12-dependent de novo adaptive T cell immunity that is orchestrated by Batf3-dependent DCs.

Serial ISIM reshapes intratumoral TCR repertoires, and overcomes acquired resistance. While we observed synergistic antitumor efficacy of ISIM and anti-PD-L1 therapy (Fig. 3g), all treated mice eventually acquired resistance to anti-PD-L1 therapy and succumbed to the disease. To determine whether a prime-boost approach could overcome resistance, we repeated TLR3/CD40 stimulation following ISIM. Additional weekly TLR3/CD40 stimulation once or twice following ISIM did not improve tumor response compared to mice treated with ISIM once in AT-3 tumor-bearing mice (Supplementary Fig. 19a). In contrast, we found repeating all components of ISIM, but not combination of any two components of ISIM, mediated additional tumor regression and improved survival following the initial ISIM (Fig. 10a).

Hypothesizing that the additional regression of tumors resistant to immunotherapy by serial ISIM is associated with restoring CD103+ DCs in the tumor, and facilitating oligoclonal infiltration of novel TCR repertoires, we examined the frequency of CD103+ DCs before and after ISIM. While their frequency returned to the baseline after ISIM, likely due to the trafficking of tumor-residing CD103+ DCs to TdLN (Fig. 1d, e), a second cycle of Flt3L administration restored CD103+ DCs in the tumor (Supplementary Fig. 19b, c), resulting in significantly increased CD8+ TILs, decreased CD11b+ cells, and increased CD8+/CD11b+ cell ratio after a second ISIM treatment (Supplementary Fig. 19d).

To evaluate changes of TCR repertoires during serial ISIM, longitudinal incisional tumor biopsies were taken from mice treated with PBS alone (PBS → PBS), ISIM followed by PBS (ISIM → PBS), or ISIM twice (ISIM → ISIM), for TCR sequencing analysis. Similarities of intratumoral TCR repertoires were compared between samples from the same mice by the Morisita TCR overlap index52, and the ImmunoMap platform, which allows analysis of the sequence similarity of different CD3R regions53. PBS-treated tumors (PBS → PBS) contained similar TCR repertoires with high TCR overlap index throughout the course; however, ISIM-treated tumors were enriched for novel clonotypes characterized by a higher frequency of gained/lost clones and lower TCR overlap index in between the first (d33) and second (d45) biopsies, suggesting the clonal expansion of novel T cell clones (d33 vs d45 in Fig. 10b, Supplementary Fig. 20 and Supplementary Tables 1 and 2). Importantly, second ISIM treatment (ISIM → ISIM) further reshaped the intratumoral TCR
repertoires while single ISIM-treated (ISIM → PBS) tumors maintained high TCR overlap index with similar clonally expanding T cells in between the second (d45) and third (d55) biopsies (d45 vs d55 in Fig. 10b, Supplementary Fig. 20, and Supplementary Table 2), suggesting continuous clonal replacement of TILs by serial ISIM.

Serial ISIM in combination with PD-L1 blockade eradicates poorly T cell-infiltrated tumors and establishes tumor-specific systemic immunological memory. Finally, we investigated whether serial ISIM could overcome acquired resistance to anti-PD-L1 therapy. Effective tumor regressions were seen after each cycle of ISIM in combination with anti-PD-L1 therapy,
**Fig. 7 Identification of tumor-infiltrating lymphoid cell populations by scRNAseq.** a UMAP plots of lymphoid subsets in AT-3 tumors. Right panels show plots of tumors treated with PBS + isotype Ab (NT), PBS + anti-PD-L1 Ab (αPD-L1), in situ immunomodulation (ISIM) + isotype Ab (ISIM), or ISIM + anti-PD-L1 Ab (ISIM + αPD-L1). b Expression plots of indicated genes in lymphoid cell clusters in AT-3 tumors. Expression levels are color-coded: gray, not expressed; orange, expressed. c Frequency of each lymphoid cluster in AT-3 tumors in different treatment as indicated. γδT: γδ T cells, ILC: Innate lymphoid cells. d Frequency of Tcf1+CD8+ T cells and Slamf6+CD8+ T cells among CD45+ cells in untreated (NT) and ISIM-treated AT-3 tumors. n = 5 mice per group. e Representative flow cytometric plots showing PD-1 and Slamf6 expression of CD8+ T cells in untreated (NT) and ISIM-treated AT-3 tumors, and percentage of PD-1− Slamf6−, PD-1int Slamf6+, PD-1hi Slamf6− subsets among CD8+ T cells. n = 5 mice per group. Tumors were harvested 7 days after TLR3/CD40 stimulation. Two-tailed t-test (d, e). Mean ± SEM. Data shown (d, e) are representative of two independent experiments. Source data are provided as a Source Data file.

**Fig. 8 PD-L1 blockade reshapes in situ immunomodulation (ISIM)-induced tumor-infiltrating lymphocytes.** a Heatmap displaying normalized expression of select genes in each lymphoid cell cluster in tumors treated with PBS + isotype Ab (NT), PBS + anti-PD-L1 Ab (αPD-L1), ISIM + isotype Ab (ISIM), or ISIM + anti-PD-L1 Ab (ISIM + αPD-L1). b Volcano plot showing enrichment differentially expressed genes in lymphoid cluster (Ly_C) 0 between that treated with ISIM and with ISIM + αPD-L1. Each red and blue dot denotes an individual gene with Benjamini-Hochberg-adjusted p value <0.05 and log fold change >0.25. c Heatmap of gene set enrichment analysis (GSEA) of ISIM vs NT and ISIM + αPD-L1 vs ISIM in Ly_C 0, 2, 4, 5, 6, 7, 10, and 11 showing normalized enrichment score (NES). Gene sets of REACTOME, PID, HALLMARK, and KEGG are examined.
resulting in durable complete responses in all treated mice after the fourth cycle (Fig. 10c). These surviving mice rejected the same AT-3 tumors rechallenged in the contralateral mammary fat pad, but not unrelated B16 tumors inoculated on the back, suggesting the development of tumor-specific immunological memory (Fig. 10d). In line with this, we also observed curative potential of serial ISIM and anti-PD-L1 therapy (Supplementary Fig. 21a), and generation of systemic immunological memory against B16 melanoma (Supplementary Fig. 21b). Taken together, these findings suggest that in situ induction and activation of cDC1s could reshape intratumoral T cell type, density and repertoires, convert poorly T cell-infiltrated tumors, and overcome primary and acquired resistance to anti-PD-L1 therapy.
**Discussion**

Primary and acquired resistance remain major limitations of anti-PD-1/PD-L1 therapy. Although the mechanisms behind resistance to therapy are diverse, T cell infiltration into the TME is a critical determinant of response to immunotherapy. The work described herein defined a crucial role for cDC1s in converting poorly T cell-infiltrated tumors into heavily infiltrated TMEs, and identified a combinatorial immunotherapeutic regimen to engage them for robust CD4+ and CD8+ T cell-mediated tumor regression, resulting in the reversal of resistance to anti-PD-L1 therapy. Evidence from multiple orthotopic murine tumor models of poorly T cell-infiltrated tumors resistant to anti-PD-L1 therapy demonstrated that: (1) in situ induction and activation of cDC1s elicited IFN-γ- and IL-12-dependent de novo adaptive T cell immune responses that facilitated priming and effector differentiation of tumor-specific CD8+ T cells, promoted an influx of stem-like Tcf7+ Slamf6+ T cells committed to persistence, triggered rapid regression of primary tumors as well as untreated distant tumors, and rendered poorly T cell-infiltrated tumors responsive to PD-L1 blockade; (2) dual TLR3/CD40 stimulation of Flt3L-induced DCs was required to facilitate trafficking of TAA-loaded CD103+ DCs expressing high levels of CD40/CD70/CD86 to TdLN, and to induce CD4+ T cell-mediated remodeling of the myeloid-enriched TME, resulting in a reversal of intratumoral CD8+/CD11b+ cell ratio; and (3) serial ISIM reshaped clonally expanding TCR repertoires in tumors which have been...
Fig. 10 Serial in situ immunomodulation (ISIM) reshapes intratumoral TCR repertoires, eradicates non-immunogenic tumors with PD-L1 blockade, and establishes tumor-specific immunological memory. a. Tumor growth curves (mean) and survival curves in AT-3 tumor-bearing mice in different treatment groups as indicated (n = 5 mice in all groups). *p < 0.01 by a log-rank test. For second and third treatment after first ISIM, Fik3L or PBS was injected daily from one day after TLR3/CD40 stimulation for five consecutive days. RT and TLR3/CD40 agonists were given in order. b. AT-3 tumor-bearing mice were treated with PBS → PBS, ISIM → PBS, or ISIM → ISIM. Serial tumor biopsies were done before treatment at d33, after first treatment (PBS or ISIM) at d45, and after second treatment (PBS or ISIM) at d55. DNA from tumor tissue was extracted, and TCRseq was performed. Significantly increased (gain) and decreased (loss) clonotypes are shown in red and blue bubbles, respectively, in representative scatter plots from three independent experiments. Data panels show the percent of significantly increased (gain) or decreased (loss) clones out of total clones (left) and TCR overlap score (right): d33 vs d45 (upper), d45 vs d55 (lower). Each dot represents an individual animal. Two-tailed t-test (d33 vs d45) or one-way ANOVA with Tukey’s multiple comparisons (d45 vs d55). NS not significant. Mean ± SEM. c. Tumor growth curves (mean and individual) and waterfall plots in AT-3 tumor-bearing mice treated with serial ISIM and anti-PD-L1 therapy. Waterfall plots show maximal change of tumor volume at the day when the first TLR3/CD40 agonists were administered. Anti-PD-L1 Ab (ap-PD-L1) or Isotype Ab were given from the day first RT performed until the treated mice developed obvious resistance to PD-L1 blockade. n = 6 mice (ISIMx2 + ap-PD-L1) and 5 mice (any other groups). *p < 0.01 by a one-way ANOVA with Tukey’s multiple comparisons vs any other treatments. d. Naïve mice and surviving mice from the experiment (c) were rechallenged with AT-3 and B16 in the contralateral flank at d88 (AT-3) (left) and on back at d102 (B16) (right), respectively. Mean ± SEM. Data shown (a, c) are representative of two independent experiments. Source data are provided as a Source Data file.

destined to become resistant to immunotherapy, eradicated established poorly T cell-infiltrated tumors, and developed tumor-specific systemic immunological memory. cDC1s play a central role in cross-priming CTLs10, but also are important for the induction of cross-tolerance to self-antigens under normal conditions54. Therefore, if TAA s are presented by cDC1s in the absence of co-stimulation, this may cause tolerance rather than immunity against cancer. This is especially relevant to tumor-residing DCs, which are functionally immature due to the highly immunosuppressive TME, and such immature DCs in turn promote immune tolerance to the tumor29,30. DC activation by TLR ligands alone is insufficient to break peripheral cross-tolerance in the absence of specific CD4+ T cell help49,55, however, engagement of CD40 on DCs overcomes peripheral CD8+ T cell tolerance19,20. Although targeting these proteins may break self-tolerance to TAA s and cause undesirable autoimmune damage, we did not observe overt toxicities even with repetitive TLR3/CD40 stimulation of Flt3L-induced DCs.

High-dimensional single-cell profiling of tumor-infiltrating immune cells uncovered significant remodeling of the myeloid and lymphoid compartments in poorly T cell-infiltrated tumors by ISIM. The most notable change was an induction of Slamf6+ subsets among CD8+ TILs, which was subsequently validated by flow cytometric analyses. Unlike control tumors which contained naïve and terminally exhausted CD8+ T cells but lacked Slamf6+ subsets, ISIM-treated tumors responded to anti-PD-L1 therapy. These findings are in line with recent evidence that particular phenotypes of T cells but not the mere presence of CD8+ TILs correlate with response to immune checkpoint blockade in mouse models and patients38,39. While Tc1 and Slamf6 are highly co-expressed by progenitor-exhausted T cells37–39, Tc1 cells are also expressed by naive and memory T cells36,57. We found that the frequency of Tc1+ cells within CD8+ T cells decreased (data not shown), although total numbers of Tc1+ CD8+ T cells increased in the ISIM-treated tumors compared with control tumors (Fig. 7d). Our scRNAseq and flow cytometric analyses suggest that Slamf6 expression may distinguish progenitor-exhausted T cells from naïve and terminally exhausted T cells, and thus Slamf6 might be a valuable marker to predict response to immunotherapy.

Understandably, considerable interest is focused on CD8+ T cells for effective immunotherapy control due to their direct role in tumor cell destruction. CD4+ T cells have received less attention, but are nonetheless important drivers of antitumor immunity38–41. Our CD4+ T cell depletion study revealed an important role played by CD4+ T cells in ISIM-mediated antitumor efficacy. Further detailed studies are required; however, to determine how ISIM-prime CD4+ T cells contribute to the antitumor efficacy against poorly T cell-infiltrated tumors. A possible explanation is that dual TLR3/CD40 stimulation facilitated DCs to generate tumor-specific Th1 cells42 that enhanced effector function of tumor-specific CD8+ T cells39 and IFN-γ production in the TME35,60. Although we did not directly test this hypothesis, ISIM-induced upregulation of PD-L1 in TAMs was not observed in the absence of CD4+ T cells even with agonistic anti-CD40 Ab. Furthermore, scRNAseq analysis identified a cluster of tumor-infiltrating Th1 cells in ISIM-treated tumors which increased its frequency and Ifng expression in response to anti-PD-L1 therapy. In line with our findings, a recent study identified a critical role of cDC1s in the priming of CD4+ T cells against tumor-derived antigens43.

Our experiments with FTY720 revealed that ISIM-mediated antitumor efficacy was associated with newly activated rather than pre-existing T cells, and that ISIM had the ability to mount a de novo adaptive immune response in poorly T cell-infiltrated tumors, which then became responsive to anti-PD-L1 therapy. These data are in line with emerging evidence that the expansion of novel TCR clones rather than pre-existing exhausted T cells associates with response to anti-PD-1 therapy44. Our study further identified that serial ISIM reshaped TCR repertoires in the TME and rendered tumors continuously responsive to anti-PD-L1 therapy, resulting in durable complete response. These findings underscore the importance of developing strategies to persistently attract new T cells to the TME for overcoming resistance to the treatment.

We identified several requirements for ISIM to mediate regression of poorly T cell-infiltrated tumors. Immunomodulatory effect of ISIM was lost in the absence of Batf3-dependent cells, CD8+ T cells, IFN-γ, or IL-12. Furthermore, scRNAseq analysis showed increased TILs and DCs expressing Ifng and Il12b within ISIM-treated tumors. These findings align with recent evidence that successful anti-PD-1 therapy relies on the interplay between effector T cells and DCs involving IFN-γ and IL-12 in a model of a T cell-inflamed TME34. It is noteworthy that ISIM augmented IFN-γ and IL-12 signaling in the absence of anti-PD-L1 therapy in poorly T cell-infiltrated tumors. Of note, scRNAseq analysis identified a cluster of IL-12 DCs (C15) distinct from cDC1s and cDC2s. While the ontology of this DC subset remains to be determined, recent work has demonstrated that the subset resembling C15 in our study differentiates from both cDC1s and cDC2s, and expresses PD-L1 in the TME uptake of tumor antigens45. Consistent with our findings, this DC subset was enriched in maturation (Cd40, Cd70, Cd80, Cd83, Il12b, and Relb), immune regulatory (Cd274, Pdcd11g2, Socs2, and Cd120) and Th2 response genes (Il4i1 and Ccl22), and was named...
"mature DCs enriched in immune regulatory molecules" (mregDCs) by Maier et al.65. Future studies are needed for a better understanding of how this subset contributes or regulates ISIM-mediated efficacy. We recognize that the use of implantable tumor models, such as those tested in this study, do not fully recapitulate the complexity of the host-tumor dynamic of autochthonous/spontaneous tumor models. Thus, the potential of ISIM in overcoming anti-PD-L1 resistance under those settings1 remains to be fully understood. While ISIM might be effective in poorly T cell-infiltrated tumors caused by a reduced recruitment of cDC1s into the TME such as that seen in β-catenin-positive tumors5, the impact of ISIM against tumors unresponsive to T cells caused by mutations in the IFN-γ signaling pathway or mutations altering antigen expression or presentation also remains to be elucidated. Future studies are warranted to assess ISIM for its ability to overcome other mechanisms of anti-PD-L1 resistance in genetically engineered mouse tumor models.

An in situ vaccination strategy involving Flt3L, RT, and Poly-ICLC was shown to be feasible, safe, and induced regression of primary and untreated distant tumors ("abscopal effect") in patients with lymphoma14. Systemic anti-CD40 Ab has been investigated clinically for more than a decade20, and is now being tested in combination with Flt3L or PD-1 blockade (NCT03329950). Our results suggest that the addition of anti-CD40 Ab to Flt3L, RT, and Poly-ICLC clinical platform may overcome other mechanisms of anti-PD-L1 resistance in genetically engineered mouse models.

In summary, the results of this study demonstrate that in situ induction and activation of cDC1s reshapes the type, density, and repertoires of intratumoral T cells, and warrant investigating this mechanism-driven rationally combined treatment regimen in patients with poorly T cell-infiltrated tumors refractory to anti-PD-1/PD-L1 therapy.

Methods

Mice. Female C57BL/6 mice, Pmel-1 TCR-transgenic mice (B6.Cg Tbt1-Tg (Tcrα Tcrβ) 39 Rest/J), and Batf3−/− mice on C57BL/6 background were purchased from Charles River Laboratories. All mice were age matched (7–10-week-old) at the beginning of each experiment and kept under specific pathogen-free conditions and housed in the Laboratory Animal Resources. All animal studies were conducted in accordance with and approved by the Institute Animal Care and Use Committee (IACUC) at Roswell Park Comprehensive Cancer Center.

Cell lines. The AT-3 tumor cell line was established from a primary mammary gland carcinoma of the PyMT-MMTV transgenic mice on a B6 strain and was maintained as described66. The 4T1 breast cancer and B16-F10 (B16) melanoma cell lines were purchased from the American Type Culture Collection (ATCC), authenticated at ATCC, and maintained. The MC38 colon adenocarcinoma cell line was gift from Dr. Weiping Zou (University of Michigan). AT-3 cells were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Sigma), 1% NEAA (Gibco), 2 mM L-glutamine (Gibco), 0.5% penicillin/streptomycin (Gibco), and 1 μg/ml-2-mercaptoethanol (Gibco). 4T1, MC38, and B16 cells were cultured in RPMI (Gibco) supplemented with 10% FBS, 1% NEAA, 2 mM L-glutamine, 0.5% penicillin/streptomycin, and 5 μg ml-2-mercaptoethanol. For generation of mouse AT-3 cells expressing GFP (AT-3-GFP), AT-3 cells were infected with retroviruses encoding GFP (mEGFP-N1, Addgene plasmid #54767 a gift from Michael Elowitz) and were bred in-house. Female BALB/C-AnnCr mice were purchased from Charles River Laboratories. All mice were age matched (7–10-week-old) at the beginning of each experiment and kept under specific pathogen-free conditions and housed in the Laboratory Animal Resources. All animal studies were conducted in accordance with and approved by the Institute Animal Care and Use Committee (IACUC) at Roswell Park Comprehensive Cancer Center.

Tumor inoculation. B16 (5 × 10⁴) or MC38 (8 × 10⁴) tumor cells were injected subcutaneously on the left flank. AT-3 (5 × 10⁴), AT-3-GFP (5 × 10⁴), 4T1 (1 × 10⁶), and 4T1-luc (1 × 10⁵) tumor cells were orthotopically implanted (injected or surgically implanted under anesthesia with isoflurane) into the fourth mammary gland of female mice.

In situ immunomodulation. Tumor-bearing mice were treated with hFlt3L (30 μg/dose; Celldex Therapeutics, Inc.) in 30 μl PBS or control PBS intratumorally for nine consecutive days. For radiation therapy, the mice were anesthetized with isoflurane and positioned under a 2-mm-thick lead shield with small apertures limiting exposure to the tumors. Radiation (9 Gy) was performed with an orthovoltage X-ray machine (Philips RT250, Philips Medical Systems) at 75 kV using a 1 × 2 cm cone.

One day after RT, mice were treated with injection of agonistic anti-CD40 Ab (50 μg/dose; clone FGK4.5, BioXcell), high molecular weight poly(LC) (50 μg/dose; InvivoGen), or combination of these at the peritumoral site subcutaneously. For Pmel-1 CD8+ T cell injection, Pmel-1 splenocytes were sorted with CD8α-PE-Cy7 (Ly-2, Miltenyi Biotec) according to the manufacturer’s protocol. 1 × 10⁶ sorted Pmel-1 CD8+ T cells were administered via tail vein injection on the day when RT was given. In the serial ISIM experiments, for the second and subsequent ISIM treatments, Flt3L or PBS was injected for five consecutive days starting one day after TL3/CD40 stimulation. RT and TL3/CD40 agonists were given 1 and 2 days, respectively, after completion of Flt3L or PBS injection (Supplementary Fig. 16).

Tumor growth was measured 3–7 times a week, and the volumes were calculated by determining the length of short (l) and long (d) diameters (volume = l² × d/2). Experimental endpoints were reached when tumors exceeded 20 mm in diameter or when mice died due to moribund and showed signs of lateral recumbency, cachexia, lack of response to noxious stimuli, or observable weight loss. In the experiments described in Fig. 10c, experimental endpoints were reached when the treated mice developed obvious resistance to PD-L1 blockade and continuous tumor growth was observed.

In vivo antibody treatment. For PD-L1 blockade, anti-PD-L1 Ab (clone 10 F.9G2, Bioxcell), or rat IgG2b (clone LTF-2, BioXcell) were given intraperitoneally (i.p.) every third day from the day RT was given. For in vivo depletion of lymphocytes, 200 μg of anti-CD4 (clone GK1.5, BioXcell), anti-CD8 (clone Lyt 3.2, Bioxcell), anti-NK1.1 (clone PK136, Bioxcell) Abs, or rat IgG2b (clone LTF-2, Bioxcell) Abs were given i.p. every third day for three times from the day when RT was given. For in vivo depletion of IL-12 and IFN-γ, 1 mg of anti-IL-12p40 (clone C17.8, BioXcell) and anti-IFN-γ (clone R4-6A2, Bioxcell) Abs were administered by i.p. injection at the day when RT was performed, with follow-up doses of 500 μg for five consecutive days.

Treatment with FTY720. FTY720 was given to mice to inhibit lymphocyte migration out of SLOs. FTY720 stock solution (10 mg/ml in water) was diluted to a 0.2 mg/ml in 3% Tween 20 directly before administration. Mice received a dose of 20 μg FTY720 or vehicle (3% Tween 20) i.p. as a control. Therapy was initiated one day before RT and was given daily for 9 days.

In vivo bioluminescence imaging. Ten minutes after D-luciferin (1.5 mg/20 g body weight) i.p. injection, images were obtained by in vivo bioluminescence imaging (IVIS Spectrum imager) with 1 min exposure. Quantification of bioluminescence signal was determined using the Living Image (PerkinElmer Inc.) and average radiance (Total Flux cm²/sr) was calculated, implementing standard region of interest (ROI) drawn over the tumor site.

Flow cytometry, cell sorting, and imaging flow cytometry. Single-cell suspensions of mouse blood, LN, and tumors were prepared for flow cytometric analysis. Red blood cells in blood were lysed using ACK Lysis Buffer (Life Technologies). Cells were blocked with anti-mouse CD16/32 (Biolgend) and surface stained with indicated markers. Live/dead cell discrimination was performed using LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies). For analyzing antigen-specific T cells in 4T1 tumor-bearing mice, a tetramer specific for H-2Ld restricted epitopes gp70 amino acids 423-431, referred to as AH1 (SPSYVYHQF) was obtained from the NIH Tetramer Core Facility. To evaluate change in the myeloid and lymphoid compartment of TME by ISIM, we used the 12-color flow cytometry gating strategy (Supplementary Fig. 2) as described by others9. Following gating to exclude doublets and dead cells, DCs were identified as Ly6c+MHC class II+/CD24+/CD11c+ cells. Based on CD11b and CD103 expression, DCs were divided into two subsets, CD11b+ DCs and CD103+ DCs. TAMs were identified by expression of CD24+ and F4/80+ within Ly6c+ MHC class II+ cells. Intracellular IL-12 p40 and IFN-γ assays were performed using Fixation/Permeabilization Solution Kit (BD Biosciences) according to the manufacturer’s recommendations. Prior to intracellular staining, cells were co-cultured with 1 μM/L of AH1 peptide (SPSYVYHQF) (GenScript) in the presence of Protein Transport Inhibitor (Brefeldin A) (BD Biosciences) for 5 h. Samples acquired on LSRII or Fortessa (BD Biosciences) cytometers were analyzed with FlowJo software. Tumors imaged with ImageStream-MKII was used for imaging flow cytometry (Luminex Corporation), which enables spatially correlated image analysis of spectrally separated cell imaging. Data were analyzed using IDEAS software (Luminex Corporation). Doublets of CD103+ DCs and CD8+ T cells were identified by a low bright gate (BD) around the values of the presence of CD103, CD11c, and CD8. For identified doublets of GFP+ CD103+ DC-D8+ T cell, an ROI was created based on the location of the CD103 and CD11c stains and the GFP
fluorescence intensity restricted to that ROI for each doublet was then quantified. Antibodies used in this study are listed in Supplementary Table 3.

**IHC staining.** Tissues were fixed for 24 h in 10% formalin and stored in 70% ethanol until further processing. Five micrometer sections of formalin fixed, paraffin-embedded tissues were deparaffinized with xylene, rehydrated, and subjected to antigen retrieval with heated antigen unmasking solution (1.0 mM EDTA, 0.05% Tween-20, pH 8.0). After 1 h in horse serum blocking buffer, primary antibodies applied for 3 h at room temperature or overnight at 4 °C. Anti-mouse antibodies included CD8a (1:400, Clone D4W2Z, Cell Signaling Technologies) and CD163 (1:300, Clone EPR19518, Abcam).

For IHC, the ABC reagent was used with DAB chromogen, followed by counterstaining with hematoxylin QS (all from Vector Labs). Images were acquired with a Zeiss Axios Imager Z1. The number of cells showing CD8a or CD163 in each high-power field (HPF) (>200) was quantitated.

**Bone marrow chimeras.** To generate bone marrow chimeras, bone marrow–recipient C57BL/6 mice were irradiated with two equal doses of 550 cGy 3 h apart. To obtain donor bone marrow (B6 wild type, Braf–/–, femurs and tibiae were harvested and the bone marrow flushed out. After washing, total 5 × 10^6 bone marrow cells were resuspended in PBS for transfer into mice after irradiation. After 8–12 weeks, recipients were used for the experiments.

**Incidental tumor biopsy, DNA isolation, TCR sequencing, and repertoire analysis.** The skin overlying the AT-3 tumor was clipped, and a small piece of tumor measuring approximately 5 mm in maximal diameter was removed aseptically under anesthesia with isoflurane. The incision was closed with wound clips. DNA from tumor tissue was extracted using the QIAamp DNA Mini kit (Qiagen). TCRβ chain CDR3 variable region sequencing was performed using the illumina MiSEQ assay at the survey level (Adaptive Biotechnologies). T cell repertoires, comprising all detected CDR3 sequences with annotated V and J gene segment identifications, were downloaded directly from the immunoSEQ Analyzer from Adaptive Biotechnologies. Metrics of the complete TCR repertoire in each sample, including numbers of productive rearrangements, productive clonality, and clonal frequencies were determined using the immunoSEQ Analyzer software and confirmed using the LymphoSeq package (Supplementary Table 1). Only data from productive rearrangements were exported from the immunoSEQ Analyzer for further analysis. On average, 11,685 TCR sequences were detected from tumor samples (range 7193–54,671), representing an average of 2950 unique tumor CDR3 sequences (range 577–9,853). All other analyses were performed using the LymphoSeq package and custom scripts in the R statistical software environment. The level of similarity between the different TCR repertoires was measured using the Morisita–Horn Index\(^2\), using the vegan package. This unitless index ranging from 0 to 1 takes into account the number of shared sequences between two repertoires as well as the contribution of those shared sequences to each repertoire. TCR similarity measures within each repertoire were compared using an unpaired two-tailed Student’s t-test (two groups) or a one-way ANOVA with Tukey’s multiple comparisons (three groups or more). \(P < 0.05\) was considered statistically significant. Significant potential differential clonality between samples was determined using the Fishers’s exact test with multiple test correction (Holm method). Only those clones with at least five observed read counts in one sample were considered (the remaining pool of clones were considered “excluded” from differential analysis), and significance threshold set at \(q < 0.05\) for final determination of productive vs. non-productive and lost clones in each binary comparison or repertoires. TCR repertoires were visualized as weighted dendrograms using Immunoseq\(^3\). Only productive sequences with a frequency >0.1% in the tumor were considered for analysis. Sequence distances were calculated based on sequence alignments scores using a PAM10 scoring matrix and gap penalty of 30. Clones are overlaid at the end of the branches corresponding to the CDR3 sequences with diameters proportional to the frequency of the sequences observed in the samples.

**Single-cell RNA sequencing.** Sample preparation: For the scRNAseq experiments, we harvested AT-3 tumors from 2 to 3 C57BL/6 mice treated with or without ISLM and/or anti-PD-L1 therapy (Supplementary Fig. 11a). Single-cell suspensions of digested tumor were stained for 5 min at room temperature with 500 ng of Fc block (anti-Cd16/32, Biologend), and then stained with Pacific Orange anti-CD45 Ab (Thermo Fisher Scientific) and Live/Dead Fixable Near-IR Dead Cell Stain Kit (Life Technologies). Sorting of live CD45+ tumor-infiltrating cells were performed on a BD FACSAria II (BD Biosciences) (Supplementary Fig. 11b).

Single-cell RNA sequencing library generation: Droplet-based 3′ end massively parallel sequencing was performed by encountering sorted live CD45+ tumor infiltrating cells into droplets and libraries were prepared using Chromium Single Cell 3′ Reagent Kits v3 according to manufacturer’s protocol (10x Genomics). To minimize batch effect, library preparation was performed on all captured cells simultaneously, and all libraries sequenced on a single Novaseq S1 flow cell on an Illumina Novaseq 6000 instrument.

**Data availability** Raw scRNAseq data supporting the findings of this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) under accession number GSE154879 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154879. The TCRseq data are available at https://github.com/mdlong-rpccc/Immunoseq. Additional information included in the supplementary materials is freely available to the reader upon reasonable request. Databases used for collecting gene and/or functional pathway information include the Mouse Genome Informatics database (http://www.informatics.jax.org/) and the Molecular Signatures Database (https://www.gsea-msigdb.org/gsea/). Source data are provided with this paper.

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