Promoting the accumulation of tumor-specific T cells in tumor tissues by dendritic cell vaccines and chemokine-modulating agents

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Published online 18 January 2018; doi:10.1038/nprot.2017.130

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This protocol describes how to induce large numbers of tumor-specific cytotoxic T cells (CTLs) in the spleens and lymph nodes of mice receiving dendritic cell (DC) vaccines and how to modulate tumor microenvironments (TMEs) to ensure effective homing of the vaccination-induced CTLs to tumor tissues. We also describe how to evaluate the numbers of tumor-specific CTLs within tumors. The protocol contains detailed information describing how to generate a specialized DC vaccine with augmented ability to induce tumor-specific CTLs. We also describe methods to modulate the production of chemokines in the TME and show how to quantify tumor-specific CTLs in the lymphoid organs and tumor tissues of mice receiving different treatments. The combined experimental procedure, including tumor implantation, DC vaccine generation, chemokine-modulating (CKM) approaches, and the analyses of tumor-specific systemic and intratumoral immunity is performed over 30–40 d. The presented ELISpot-based *ex vivo* CTL assay takes 6 h to set up and 5 h to develop. In contrast to other methods of evaluating tumor-specific immunity in tumor tissues, our approach allows detection of intratumoral T-cell responses to nonmanipulated weakly immunogenic cancers. This detection method can be performed using basic laboratory skills, and facilitates the development and preclinical evaluation of new immunotherapies.

INTRODUCTION

Recent studies involving cellular immunotherapies and inhibitors of immune checkpoint molecules (checkpoint blockade) have demonstrated the ability of the immune system to control tumor growth[1–6]. To date, immunotherapies have been particularly effective in the therapy of tumors that are already infiltrated with CTLs[6–12].

Here, we describe a protocol to enhance the numbers of tumor-specific type-1 cells in cancer-bearing mice and present strategies to reprogram TMEs for enhanced CTL attraction. The methods include the generation of murine type-1-polarized DCs[13,14] loaded with autologous tumor material[14,15], the induction of tumor-specific CTLs in murine lymphoid organs, and different forms of TME modulation to promote effective CTL entry into tumor lesions[16–18]. We further outline methods to evaluate the changes in the numbers of tumor-specific CTLs in tumor lesions[19] during immunotherapy.

Development of the protocol

Intratumoral accumulation of CTLs is an independent prognostic factor for survival of patients with different cancer types[20–26] and is required for the clinical effectiveness of checkpoint blockade therapies[6–11,27,28]. By contrast, multiple studies have shown that increases in circulating tumor-specific T cells in the course of immunotherapy do not predict clinical responses[29,30]. These observations highlight the importance of tumor-specific T cell entry into the TME as the key limiting factor in the effectiveness of anticancer immunity during natural immune surveillance and cancer immunotherapy[31–34].

New strategies to promote the accumulation of CTLs in tumor lesions. Several strategies have been shown to activate endogenous antigen-presenting cells (APCs) and other TME components, resulting in enhanced cross-priming of tumor-antigen-specific $CD8^+$ T cells, augmented local chemokine production, and enhanced CTL accumulation[34,35]. Productive cross-priming of $CD8^+$ T cells *in vivo* involves early innate immune recognition of cancer cells and induction of type-1 interferons (IFNs) in DCs[36]. The stimulator of interferon genes (STING) pathway detects the presence of a tumor and can drive DC activation and induction of T-cell immunity against tumor-associated antigens (TAAs) *in vivo*[37,38]. Alternative strategies involve targeting exogenous antigens and adjuvants to DCs (reviewed in Kreutz *et al.*[39]), or exposing tumor tissues to combinatorial CKM adjuvants involving exogenous type I IFNs[40] in combination with Toll-like receptor (TLR) ligands or interleukin (IL)-18 (refs. 16,18,41–44), or targeted delivery of CTL-attracting chemokine genes to tumors[45,46].

Dendritic cell therapies. To bypass dysfunction of endogenous DCs in cancer-bearing individuals, *ex vivo*-generated and antigen-loaded DCs can be used as therapeutic agents. The ability of DC vaccines to promote intratumoral CTL infiltration was seen in patients with hormone-refractory prostate cancer receiving Sipuleucel-T (Provenge)[47], the first FDA-approved APC-based cancer vaccine[48].

Modes of DC generation and maturation. Mouse and human DCs can be generated *ex vivo* from bone marrow or blood

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precursors using granulocyte/monocyte colony-stimulating factor (GM-CSF), alone or in combination with IL-4 or tumor necrosis factor (TNF)-α[49–55]. Mouse DCs can also be generated from thymic or splenic progenitor cells[56,57]. Pretreatment of mice with fms-like tyrosine kinase 3 ligand (Flt3L), a factor promoting DC development, can be used to increase the numbers of DC progenitors and DCs in bone marrow, spleen, lymph nodes, and other tissues[58,59].

DC maturation is needed for effective induction of T-cell immunity[60]. Factors that can induce maturation of DCs include TLR ligands (e.g., microbial components such as lipopolysaccharide (LPS), peptidoglycan, cholera toxin, filamentous hemagglutinin, inactivated *Bacillus* Calmette–Guérin (BCG), or double-stranded RNA)[61], cytokines (including type I interferons, IFN-γ, TNF-α, and IL-1β), prostaglandin E2 (PGE_2), damage-associated molecular pattern molecules released by dying or damaged cells (apoptotic bodies, heat shock proteins, and urate crystals)[62], or signals provided by other immune cells (T, natural killer (NK), NKT, or γδT cells)[63]. A maturation cocktail used to generate DCs for early clinical trials involved TNF-α, IL-1β, IL-6, and PGE_2 (refs. 64,65). However, observations that PGE_2-matured DCs produce only very low amounts of IL-12p70 (refs. 13,66–69) prompted a search for alternative DC maturation factors. The ability of DC vaccines to produce IL-12p70 is critical for the induction of Th1/CTL/NK-cell-dominated type-1 immunity[70–74] and is predictive of the positive clinical outcomes in DC-vaccinated cancer patients[75–77]. Among others, we proposed the combination of LPS with IFN-γ to induce highly immunogenic, high-IL-12-producing DCs[66,70,71,78–80]. LPS may be replaced with cytosine-phosphorothioate-guanine (CpG), polyinosinic:polycytidylic acid (poly-IC), or mixtures of more than one TLR ligand to generate DCs with high levels of co-stimulatory molecules necessary for T-cell activation and high production of IL-12, necessary for type-1 immune responses[13,61,81–83].

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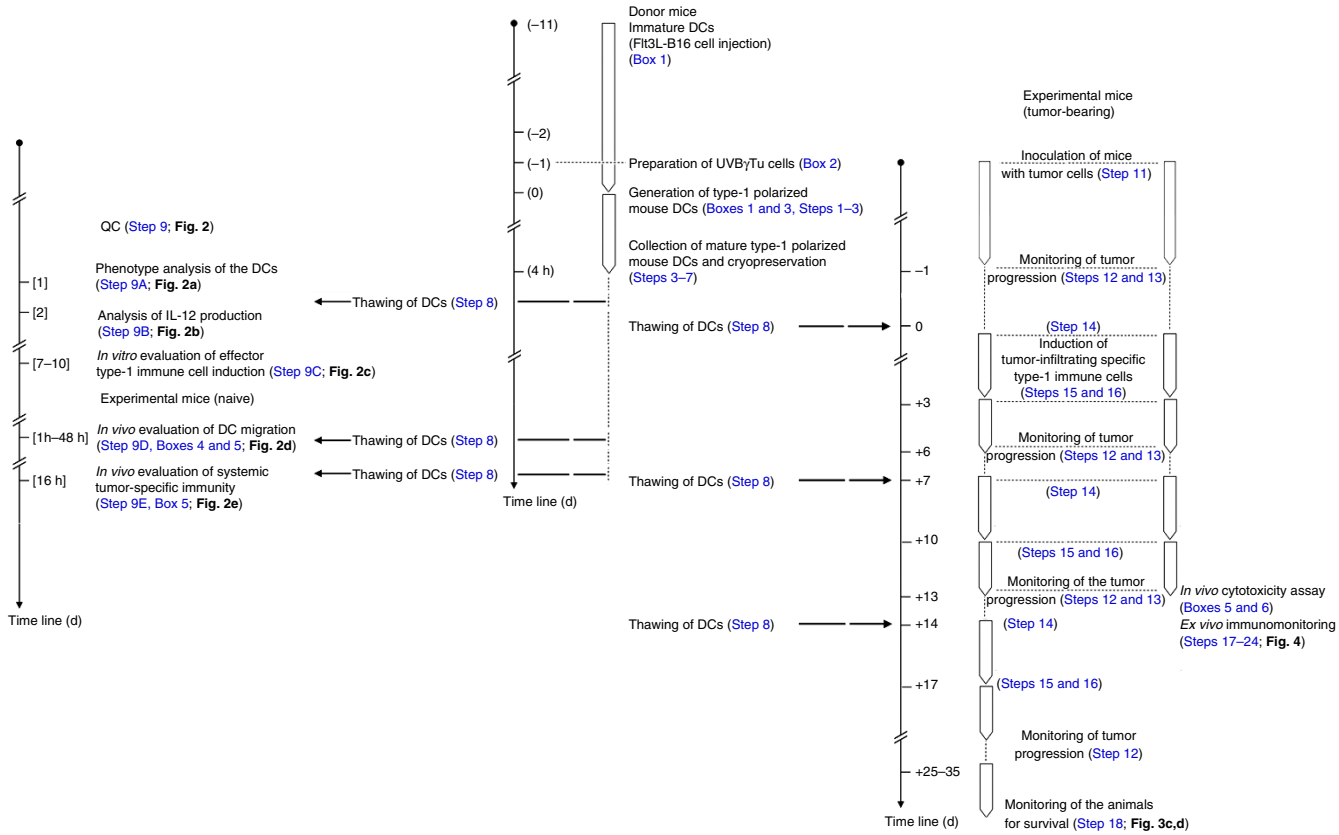

Figure 1 | Overview of the protocol, with associated timing. The three main parts of the protocol are generation of tumor-loaded DCs (Steps 1–7), QC of DCs (Step 9), and monitoring of the therapeutic and immunomodulatory effects (Steps 10–24). Start with Flt3L-B16 injection of C57BL/6J mice on day −11 (**Box 1**), followed by $CD11c^+$ cell isolation and induction of type-1 polarized tumor-loaded DC maturation on day 0 (**Boxes 2** and **3** and Step 1). Proceed to cryopreservation of mature type-1 polarized DCs (Step 6). The thawing of DCs (Step 8) can be performed at any time point; therefore, the QC of DCs and the monitoring of the therapeutic and immunomodulatory effects are time-wise independent parts of the protocol. QC (Step 9) should be performed before administering DCs to experimental mice. Basic QC should involve confirmation of the mature DC phenotype (Step 9A, expression of co-stimulatory molecules, **Fig. 2a**) and the ability of DCs to produce IL-12 upon CD40L stimulation (Step 9B, **Fig. 2b**). Additional tests may involve *in vitro* induction of type-1 immune responses by DCs (Step 9C, **Fig. 2c**), and measurement of their lymph node migratory capacity (Step 9D and **Box 3**, **Fig. 2d**), and ability to induce type-1 immune responses *in vivo* (Step 9E and **Boxes 4** and **5**, **Fig. 2e**). After successful QC, the injection of tumor cells into the experimental mice (Steps 10 and 11) for the monitoring of the therapeutic and immunomodulatory effect can be initiated at any time point. A day before DC vaccination, mice are imaged to determine the baseline intensity of the tumor-related bioluminescence signal in each mouse (Step 12). For optimal results, start vaccinating the mice with tumor-loaded type-1 polarized DCs at day 4 of tumor growth (Step 15). 3 d after DC vaccination, follow up with CKM regimen (Steps 15 and 16; e.g., intratumoral dose of type-1 polarized DCs, combination of IFN-α, Ampligen and COX2 blockers, or chemokine-expressing vaccinia virus) to enhance the ability of the TME to attract the vaccination-induced T cells. Repeat the cycles of DC therapy in combination with CKM regimen at weekly intervals. Use separate sets of experimental mice to evaluate tumor progression/survival (Steps 14–16) and immune monitoring (**Box 6** and Steps 17–24). Analysis of tumor-specific type-1 effector immune cells should be planned at least 4–5 d after CKM regimen, as a shorter period may not suffice for proper migration of DC-induced effector cells into TME. CKM, chemokine modulation.

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Selection of a relevant antigen source is another aspect critical for the therapeutic activity of DC vaccines. One commonly used approach is the use of peptides or recombinant proteins representing defined TAAs[84–86]. An alternative approach is the use of tumor lysates or tumor apoptotic bodies as a source of DC-presented antigens[87–89]. Compared with peptide approaches,

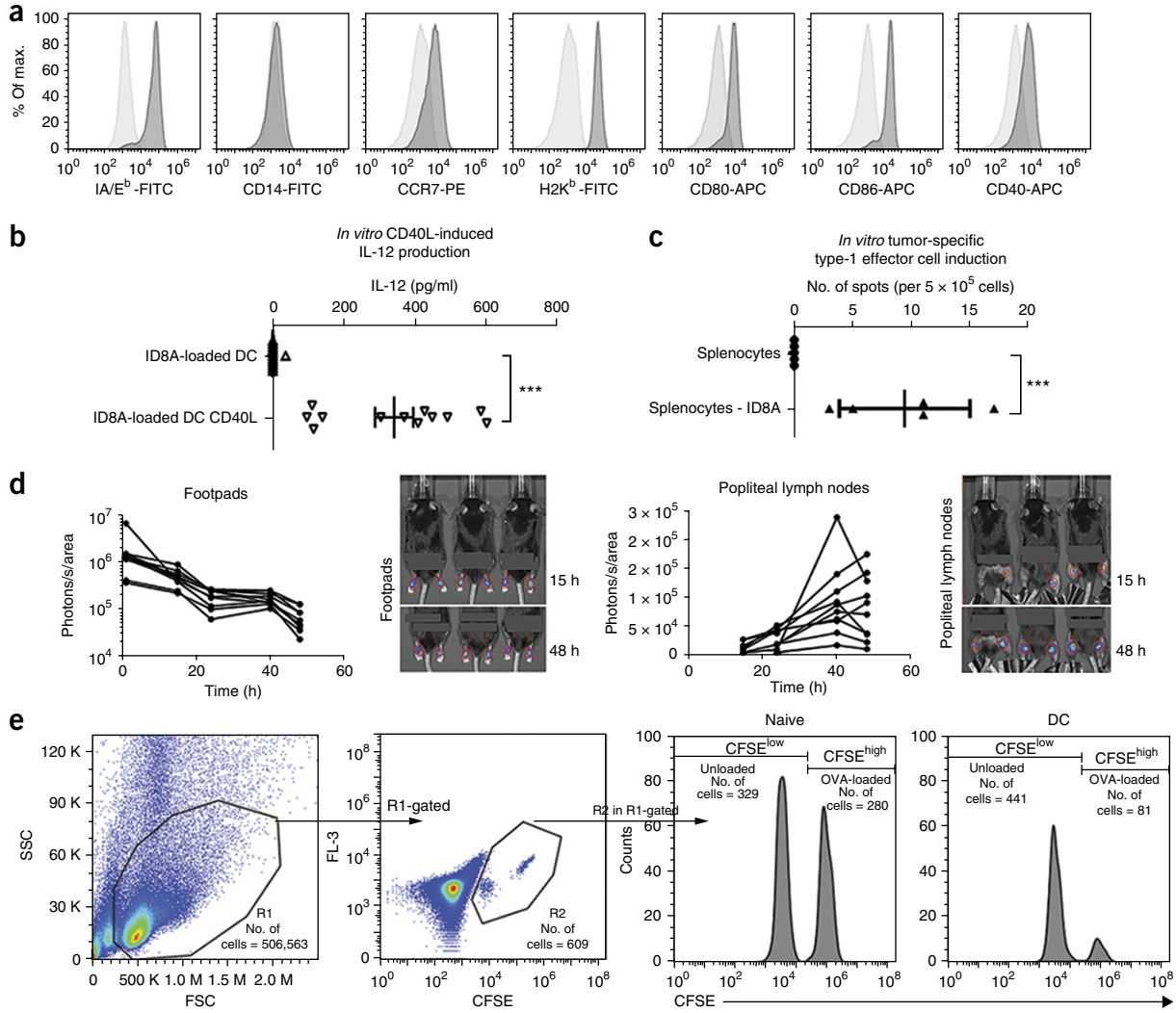

Figure 2 | Key features of type-1 polarized DCs and their functions. (**a**) Flow cytometry overlay histograms showing the surface expression of emblematic co-stimulatory and maturation markers (CD40, CD80, and CD86) and lymph node-driving chemokine receptor CCR7 on DCs. Note the expression of IA/E^b and H2K^b, but the absence of CD14 expression (macrophage marker) in mature DCs. CD14, CD40, CD80, CD86, CCR7, IA/E^b, and H2K^b staining (dark gray) versus isotype control (light gray) of 1 × 10^5 DCs. (**b**) Type-1 polarized DCs produce high levels of IL-12 when re-stimulated with J558-CD40L cells. IL-12 production in ID8A-loaded DC cultures (n = 12) with and without J558-CD40L cells (indicated by + CD40L) measured by ELISA (Step 9B) is shown. Statistical evaluation was performed using Student's unpaired t tests (two-tailed). Results are shown as individual data points (pg/ml) and means (± s.d.). ***P < 0.001. (**c**) *In vitro* induction of tumor-specific type-1 effector cells by DCs. DCs matured in the presence of IFN-γ, TNF-α, IL-1β, IFN-α, and GM-CSF, and loaded with ID8A tumor cells were set up in *in vitro* cultures with splenocytes (n = 5) to induce tumor-specific effector cells. On day 7 of activation, splenocytes were harvested and analyzed by ELISpot assay for their ability to produce IFN-γ in the absence or presence of target ID8A tumor cells (Step 9C). Statistical evaluation was performed using Student's unpaired t tests (two-tailed). Results are shown as individual data points (no. of spots per 0.5 × 10^6 cells) and means (± s.d.). ***P < 0.001. (**d**) *In vivo* DC migration assay. DCs were generated from firefly luciferase (CAG-luc-eGFP) transgenic mice (as described in DC generation section, Steps 1–5) and injected into the footpads of the C57BL/6J mouse (n = 10). Mice were imaged by IVIS after 1, 15 , 24 , 40, and 48 h. Each mouse was imaged twice: after obtaining the first image (luminescence signal in the footpads), the footpads of the imaged mice were covered with black masking tape and re-imaged, in order to detect the signal from the draining popliteal lymph nodes. The luminescence signal was gated (region of interest is marked by red circles) and the intensity recorded as photons/s/area. Results are shown as individual data points (photons/s/area) at separate time points. Representative BLI of footpads and popliteal lymph nodes of three mice at 15 and 48 h are shown. (**e**) *In vivo* specific cytotoxicity assay. Example of the evaluation of the cytolytic capacity of effector type-1 cells, induced by DCs *in vivo*. Gating strategy and evaluation histograms are presented. A histogram including CFSE^{low} + CFSE^{high} cells should be made and gates set for CFSE^{low} and CFSE^{high}, respectively. Only 0.05–0.5% of the cells are expected to be CD45.1$^+$. Numbers of recorded events for the presented flow cytometry data of splenocytes from naive mice were 704,397 (ungated), 506,563 (R1, 71.9% of ungated), 609 (R2, 0.12% of R1), 329 (CFSE^{low}) and 280 (CFSE^{high}), and for DC-vaccinated mice, 441 (CFSE^{low}) and 81 (CFSE^{high}). Percentage of specific killing is defined as (100 – (ratio loaded:unloaded in experimental mouse/average ratio loaded:unloaded in naive mice)) × 100. CD40L-transfected J558 (J558-CD40L) (Balb/c mouse strain) cells[148] were a gift from P. Lane, University of Birmingham, UK. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh. FSC, forward scatter; SSC, side scatter.

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Box 1 | Generation of immature DCs ● TIMING 1 h

Procedure

1. Inject 1 × 10^6 Flt3L-expressing B16 cells s.c. into C57BL/6J mice.

▲ CRITICAL STEP Flt3L-expressing B16 cells should be passaged at least one to two times before injection into mice.

2. After 10–12 d (the CD11c$^+$ cells should be isolated on day 11, ±24 h), euthanize mice per IACUC protocol, followed by cervical dislocation.

3. Make a cut into the left flank of the mouse to enter the peritoneal cavity and collect the spleen for CD11c$^+$ cell isolation[147], then follow the procedure outlined in **Box 3** to prepare the splenocyte suspension.

4. Resuspend the splenocytes in magnetic-activated cell sorting (MACS) buffer for subsequent CD11c$^+$ cell isolation using mouse CD11c microbeads, following the manufacturer's instructions. Splenocytes from two spleens can be combined and loaded onto a single column.

5. Once eluted from the column, resuspend the CD11c$^+$ cells in 10 ml of culture medium and count by Trypan blue dye exclusion. **Table 1** provides information on the strain and number of mice required for the generation of DCs for specific QC and experimental testing.

▲ CRITICAL STEP Determine the number of DCs needed for vaccination and the number of CD11c$^+$ cells that should be started in culture; also determine the number of mice needed for injection of Flt3-expressing B16 cells. The number of CD11c$^+$ cells depends on the number of vaccine cycles, number of mice treated, and number of injections per cycle. The expected number of CD11c$^+$ cells isolated from one spleen is 30–40 × 10^6, and the expected number of DCs generated from one spleen is 15–20 × 10^6.

whole-tumor-based vaccines can provide a range of antigens and induce polyclonal immune responses[90]. Especially for vaccination against tumor types that do not have well-characterized common tumor-rejection antigens, UV beta and gamma (UVBγ)-irradiated tumor (UVBγ-Tu) cells[15,91] or freeze–thaw tumor lysates[14] are a convenient source of multiple TAAs, including patient-specific unique tumor antigens. DCs can also be transfected with total mRNA or DNA from tumors to induce CTLs against unique tumor antigens[92].

Routes of DC administration. DC entry into lymph nodes is important for their ability to effectively activate tumor-specific CTLs[93,94]. Although the intravenous route is the standard mode of administration of the APC-based Sipuleucel-T (Provenge)[47], DCs administered intradermally have been shown to migrate to the lymph nodes better than intravenously or intraperitoneally injected DCs[95–97]. Ultrasound-guided intranodal injection or intralymphatic administration can also be used to deliver DCs to lymph nodes[97–99]. Alternatively, intratumoral injection of DCs can be used for the induction of antitumor immune responses[97,100,101]. Although intratumorally injected immature DCs take up tumor antigens and migrate to the draining lymph nodes, intratumorally injected mature DCs induce local priming of CD8$^+$ T cells in the tumors, rather than in lymphoid tissues[101,102].

Approaches to enhance local effectiveness of tumor-infiltrating CTLs. In addition to inducing high numbers of tumor-specific T cells and promoting their entry into tumors, the effectiveness of anti-tumor immunity benefits from counteracting tumor-associated immunosuppression and blocking immune checkpoints[103,104]. Preclinical models have shown that immune checkpoint blockade (anti-CTLA-4 and anti-PD-1/PD-L1 mAbs) can synergize with vaccines[105–108]. T-cell co-stimulatory factors[109] (e.g., CD40, CD70, LIGHT, OX40L, B7H3, and 4-1BBL), functional inhibition of T regulatory (T_{reg}) cells, and/or myeloid-derived suppressor cell (MDSC) activity by cyclooxygenase-2 (COX-2)

Box 2 | Preparation of apoptotic tumor cells for DC loading using UVB and gamma irradiation (UVBγTu cells) ● TIMING 1.5 h

Procedure

1. Remove the culture medium from the tumor cell culture plate, wash the cells with 10 ml of 1 mM EDTA in PBS by tilting the culture flask. Discard the 1 mM EDTA in PBS and add a new 10 ml of 1 mM EDTA in PBS. Put the cell culture plate into the incubator at 37 °C for 5–10 min, until the cells detach. Harvest the tumor cells and centrifuge at 400*g* and room temperature (20–23 °C) for 3–5 min.

▲ CRITICAL STEP Ensure the that cancer cell line that will be injected into the experimental mice is used for DC loading.

2. Resuspend tumor cells at 1 × 10^6 cells per ml of PBS and plate 1 ml per well of a six-well culture plate.

3. Place the plate under the UVBγ light for 20 min. Harvest the cells in a centrifugation tube and centrifuge at 800*g* for 3–5 min at room temperature (the high speed is required to pellet the apoptotic bodies).

4. Resuspend the pellet in 10 ml of culture medium and irradiate the cells with 200 Gy (the specific time depends on the type of the gamma irradiator).

5. Resuspend the tumor cells at 1 × 10^6 UVBγ-Tu cells per ml in culture medium. The estimated number of UVBγTu cells needed for DC loading is indicated in **Table 1**. The cells can be stored overnight in the cell incubator at 37 °C and 5% (vol/vol) CO_2.

6. Prepare an extra aliquot of cells (take a 100–200 µl of 1 × 10^6 UVBγ-Tu cells per ml) to test for the efficacy of irradiation.

▲ CRITICAL STEP It is critical that the tumor cells prepared in this step be apoptotic before their use for vaccine production. Annexin V and propidium iodide (PI) staining should be performed to confirm the irradiation was effective (i.e., the tumor cells were successfully killed with UVB and gamma irradiation). Staining will determine if the cells should be re-irradiated or a new aliquot of tumor cells should be used.

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Box 3 | Preparation of the splenocyte suspension ● TIMING 1 h

Procedure

1. Euthanize the mouse per IACUC protocol, followed by cervical dislocation.
2. Make a cut into the left flank of the mouse to enter the peritoneal cavity and collect the spleen.
3. Transfer the spleen onto a 100-µm strainer placed on top of a 50-ml conical centrifuge tube. Fill a 10-ml syringe with PBS and inject it through a22-gauge needle into the spleen; repeat three to five times to thoroughly flush the cells. Mash the spleen using a rubber piston from a 3-ml syringe while continuously washing the strainer with PBS.

? TROUBLESHOOTING

4. Centrifuge the cells at 400*g* and room temperature (20–23 °C) for 3–5 min, discard the supernatant, and resuspend the cells in 1 ml of ammonium–chloride–potassium (ACK) lysis buffer to remove red blood cells. Add an additional 3 ml of ACK lysis buffer on top and rotate the conical centrifuge tube to mix. Incubate the cells at room temperature for 4 min.

? TROUBLESHOOTING

5. Add 30 ml of PBS and centrifuge the cells at 400*g* and room temperature for 3 min, then resuspend in 20 ml of PBS and transfer to a 70-µm strainer.
6. Count the cells using a hemocytometer before use in specific experiments.

(refs. 110–112) and indoleamine 2,3-dioxigenase 1 (IDO1) (ref. 113) inhibitors also predispose the TME toward effective antitumor immunity. The combinatorial approaches combining DC or viral therapies with COX-2 blocker described in this protocol have a dual role, promoting both CTL influx into tumor lesions and their local antitumor activity.

Applications of the current protocol

This protocol is designed to enhance the numbers of tumor-specific CTLs within tumor lesions and to evaluate their changing numbers in the course of immunotherapy. The approaches involve systemic immunization using DC vaccines and different forms of systemically or locally applied modulation of TME, such as systemic or local application of combinatorial adjuvants, intratumoral injection of DCs, or viral therapies.

The presented DC-based approaches can be used to efficiently induce CTLs recognizing different tumor-relevant antigens and their entry into tumors. We anticipate that they may be combined with the blockade of tumor survival pathways[114], oncolytic virus therapies[115], TNF superfamily ligands[116], and blockers of inhibitory factors in the TME (e.g., immune checkpoints, IDO or COX2 blockers). The application of combinatorial adjuvants involving type I IFNs in combination with TLR ligands to promote the entry of the CTLs into the tumor lesions can be used as self-standing therapies following tumor resection, or can be combined with vaccines, checkpoint blockade, or adoptive T-cell therapies to enhance their effectiveness against advanced unresectable tumors.

Comparisons with alternative techniques

Common methods of evaluating the numbers of tumor-specific T cells in tumor tissues involve the use of T cells from genetically manipulated mice with defined T-cell receptors (e.g., OT-I or OT-II), and genetically modified tumor cell lines that uniformly express highly immunogenic model antigens, such as ovalbumin (OVA) (reviewed in Dranoff[117]). As the artificially high immunogenicity of such genetically manipulated tumor cells is known to substantially affect both the magnitude and character of immune responses (reviewed in Ngiow *et al.*[118]), such models may not be fully relevant to the immunotherapy of human tumors, which rarely express strong antigens and are antigenically heterogenous.

Advantages of the current protocol

The ability to evaluate polyclonal T-cell responses to multiple tumor-related weak antigens (mutated or overexpressed 'self' antigens) provides a more direct translatable readout, by eliminating the need for surrogate antigens or genetically manipulated T cells with artificial T-cell receptors (TCRs) and allowing the evaluation of T cells recognizing different TAAs expressed by cancer cells. It eliminates the need for known cancer antigens and an arbitrarily selected threshold of antigen specificity, reflected in the design of tetramers or defined α/β TCR combinations. Our protocol allows for enumeration of spontaneously arising and immunotherapy-induced T cells that are able to recognize any cancer cell. Thus, the key advantages of the current technique are its ability to evaluate responses to multiple TAAs without the need for prior identification of the relevant immunogenic epitopes, alleviating the bias related to the narrow focus on high-affinity TCR-antigen interactions, and its immediate general applicability to multiple tumor models and mouse strains.

Limitations of the current protocol and comparison with other methods

Our method relies on the detection of cytokine-producing cells (IFN-γ and potentially other mediators of effector and suppressive T cells) upon stimulation of tumor-isolated lymphocytes with relevant cancer cells. Cytokine-producing cells can be detected by flow cytometry or ELISpot analysis, and potentially by single-cell analyses of $CD8^+$ T-cell degranulation (such as CD107 assay)[119] and target cell killing[120].

Our approach allows detection of antigen-specific cells that are capable of infiltrating tumor lesions *in vivo* and that specifically recognize cancer cells. However, isolated tumor-infiltrated lymphocytes (TILs) and/or tumor-associated lymphocytes (TALs) must be rechallenged *in vitro* with the original cancer cells (non-professional APCs) to detect tumor-specific responses, which may result in apoptosis of a proportion of antigen-specific cells.

In terms of the ability to detect very low cell numbers, ELISpot assay and detection by tetramers are very similar (1 in 3–10 tetramer-positive cells are positive in ELISpot analysis)[121]. The advantage of detection by tetramers is that cells can be additionally stained for various markers and subsets of tetramer-positive cells can be analyzed. However, as T-cell responses to tumor epitopes are known to be weaker than antiviral responses or

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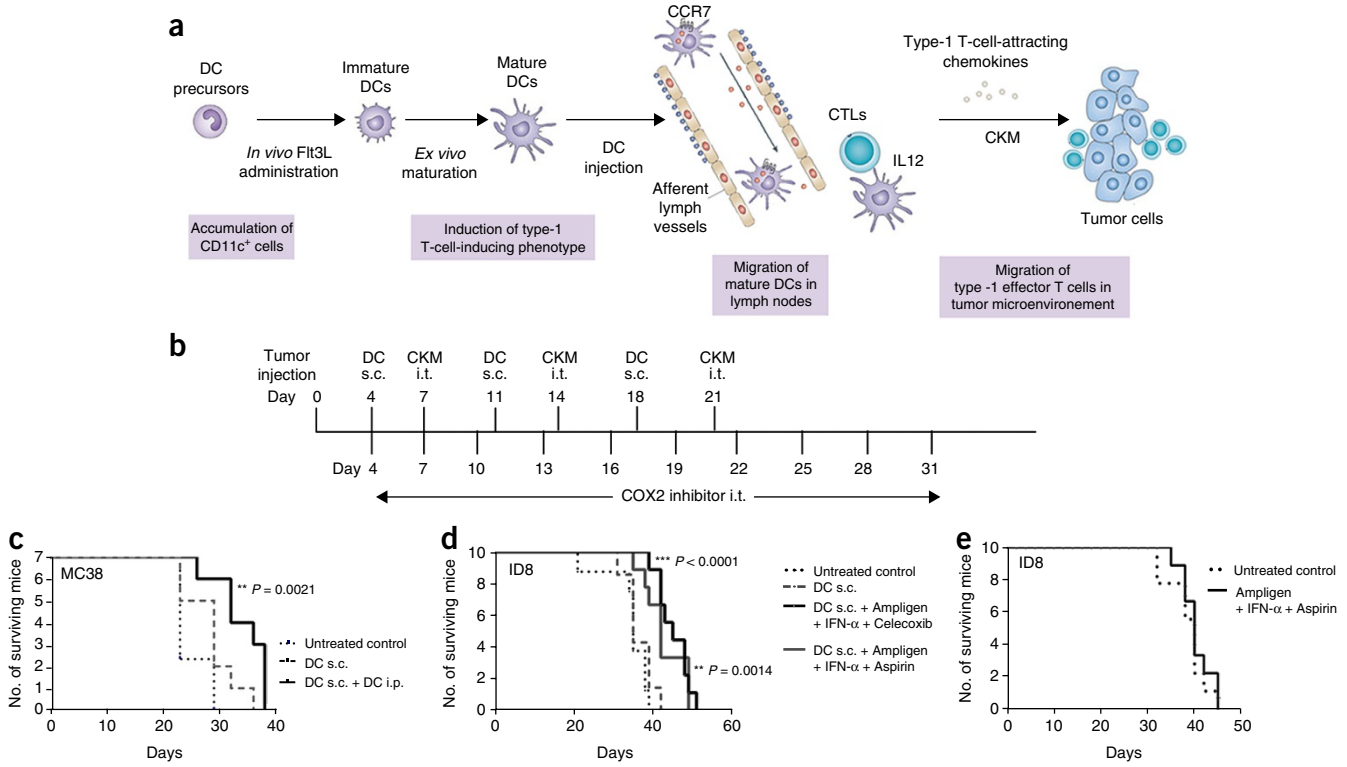

Figure 3 | Combinatorial strategy to increase the numbers of tumor-specific TILs. (**a**) A schematic overview of the induction of tumor-specific TILs following DC vaccination and chemokine modulation. *In vivo* administration of Flt3L results in the intrasplenic accumulation of immature DCs ($CD11c^+$ cells). Maturation cocktail consisting of IL-1β, IFN-α, TNF-α, IFN-γ, poly-IC, and GM-CSF induces DCs producing IL-12, their migration into lymph nodes, and activation of type-1 effector CTLs. Besides expressing relevant co-stimulatory molecules and chemokine receptor CCR7 (to effectively migrate into lymph nodes), mature DCs must produce IL-12 upon stimulation with CD40L (**Fig. 1**) in order to induce type-1 effector CTLs. Following DC vaccination, modulation of TME is required to promote tumor infiltration of DC-induced tumor-specific type-1 effector T cells. IL-12 (produced by DCs), a combination of IFNα and Ampligen[16], or chemokine-expressing oncolytic virus[115,156] enhances trafficking of CTLs into tumors (**Fig. 4**). Further optimization of therapeutic effect may be obtained by combinatorial approaches targeting immunosuppressive T_{reg} cells and MDSC (e.g., COX-2 blockers[112,157]; celecoxib or aspirin) and/or immune checkpoints (e.g., antibodies blocking PD1, PDL1, CTLA4). (**b**) Recommended time line for combinatorial therapeutic approach, involving weekly doses of DCs injected s.c., followed by CKM regimen for TME modulation. COX-2 inhibitors should be administered frequently (every 2–3 d) during combinatorial therapy. (**c**–**e**) Anticipated prolongation of survival following induction of tumor-infiltrating specific type-1 immune responses. (**c**) MC38 tumor bearing mice (seven per group) were left untreated (control), treated with matured type-1 effector T cell-inducing DC (DC, 3×10^5 s.c., days 5 and 12) only or with additional dose of 3×10^5 DC injected i.p. on days 8 and 15, and followed up for survival. ($P = 0.0021$ for control versus DC s.c. + DC i.p.) (**d**) ID8A tumor-bearing mice (ten per group) were left untreated (control), treated with matured type-1 effector T cell-inducing DC (DC, 3×10^5 s.c., days 5, 12, and 19) only, or in combination with i.p. injection of IFN-α and Ampligen and COX-2 inhibitor (celecoxib or aspirin) on days 8, 15 and 22, and followed for survival. ($P < 0.0001$ for control versus DC s.c. + IFN-α + Ampligen + celecoxib; $P = 0.0014$ for control versus DC s.c. + IFN-α + Ampligen + aspirin). (**e**) ID8A tumor bearing mice (ten per group) were left untreated (control) or treated with i.p. injection of IFN-α and Ampligen and aspirin on days 8, 15, and 22, and followed for survival (not significant, $P = 0.469$ for control versus IFN-α + Ampligen + aspirin). i.t., intratumoral. ID8A (luciferase-expressing), a cell line derived from spontaneous *in vitro* malignant transformation of C57BL/6 mouse ovarian surface epithelial cells (MOSECs), was a generous gift from T.J. Curiel, University of Texas Health Science Center at San Antonio. Animal experiments were approved by the IACUC of the University of Pittsburgh. CKM, chemokine modulator.

responses to model antigens, such as OVA, *ex vivo* analysis by tetramers allows for detection of only a relatively narrow range of the potential tumor epitopes available.

Overview of the protocol

The three main parts of the protocol are generation of DCs (**Fig. 1**, Steps 1–7), quality control of the DCs (QC: **Fig. 1**, Step 9), and monitoring of the therapeutic and immunomodulatory effect of DCs in mice inoculated with tumor cells (**Fig. 1**, Steps 10–24). Each of these parts can be performed at separate time points, starting with DC generation, and followed by QC and monitoring of the effectiveness of DC vaccine on tumor progression. DC generation starts with administration of Flt3L to C57BL/6J mice on day −11. It is followed by $CD11c^+$ cell isolation and induction of mature type-1 polarized DCs on day 0, including optional cryopreservation of DCs. QC involves confirming the mature DC phenotype, detecting expression of co-stimulatory molecules (**Fig. 2a**), and determining the ability to produce IL-12 upon CD40L stimulation (**Fig. 2b**). Additional analyses include *in vitro* induction of type-1 immune responses by DCs (**Fig. 2c**), assessment of the capacity of DCs to migrate to lymph nodes (**Fig. 2d**), and *in vivo* induction of type-1 immune responses (**Fig. 2e**). Our tumor model involves intraperitoneal injection of cancer cells into experimental mice. A day before DC vaccination, mice are imaged to determine the baseline intensity of the bioluminescence signal. Mice receive vaccination with tumor-loaded DCs at day 4 after cancer cell inoculation. 3 d after DC vaccination, CKM adjuvants are applied (e.g., intratumoral DC injections; combination of IFN-α, Ampligen, and COX2 blockers; or chemokine-expressing vaccinia virus) to modulate the TME. The

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Box 4 | Extraction of cells from the lymph nodes of mice to investigate DC migration by flow cytometry ● TIMING 2 h

Procedure

1. Thaw DCs, as described in Step 8, and adjust the cell density to 1 × 10^6 cell per ml in culture medium.
2. Inject 50 µl of CFSE-labeled DCs (**Box 5**) or DCs from Cd45.1 mice (5 × 10^5) in PBS into the footpad of the mouse.
3. At 1, 15, 24, 40, and 48 h after DC injection (**Fig. 2d**), euthanize mice per IACUC protocol and remove the skin from the legs of the mice, starting at the foot and pulling it toward the body.

▲ CRITICAL STEP Special attention should be paid to avoiding disruption of the underlying muscle tissue.

4. Holding the carcass firmly by the foot and tail, stretch out the leg with the back facing up. Using a scalpel, make a shallow cut along the center of the back of the leg. Squeeze the knee joint area and the popliteal lymph node should pop out from the cut.
5. Place lymph node in 50 µl of serum-free RPMI in a 96-well V-bottom plate.
6. Using a scalpel, cut the lymph node into small pieces in the well, add 50 µl of collagenase to the well, and incubate at 37 °C for 15 min.
7. Passage remaining pieces through an 18-gauge needle and transfer/filter the cells into a 15-ml conical tube.
8. Wash the cells with 10 ml of serum-free RPMI medium, centrifuge at 400*g* for 5 min at room temperature, and resuspend in 200 µl of blocking buffer to count and analyze by flow cytometry.
9. *Flow analysis*. In the case that DCs from Cd45.1 mice were injected into the footpad, stain the cells for CD45.1 with CD45.1-APC in blocking buffer. After 20–30 min of incubation at 4 °C, wash the cells with 200 µl of blocking buffer and resuspend in 100–200 µl of 4% (wt/vol) paraformaldehyde.
10. Perform the flow cytometry. Analyze by gating on $CD45.1^+$ (DCs from Cd45.1 mice) or $CFSE^+$ (CFSE-labeled DCs from C57BL/6J mice) to determine the frequencies of migrated DCs.

cycles of DC therapy and CKM regimen should be repeated weekly to prolong the survival of the tumor-bearing mice. Separate sets of experimental mice are used for tumor progression/survival monitoring and immune monitoring. Enumeration of tumor-specific type-1 immune cells is typically performed 4–5 d after CKM adjuvant application. Although the current protocol has been developed using the C57BL/6 mice, other mouse strains in combination with the relevant MHC-compatible tumor models may be used instead.

Experimental design

DCs should be generated from mice of the same genetic background as that of the experimental mice to avoid allogeneic immune responses. In this protocol, we use $CD11c^+$ splenocytes isolated from Flt3L-expressing B16 tumor-bearing donor mice (**Box 1**) to generate DCs. During the maturation, we expose the isolated $CD11c^+$ cells to UVBγTu cells (e.g., MC38 or ID8A cells) and use the generated DCs to induce tumor-specific TILs into the experimental mice injected with matching tumor cells.

DC generation. In this immunotherapy protocol, we replaced the *in vitro* generation[14,82] of bone marrow-derived DC with *in vivo* induction of intrasplenic DC accumulation after injection of B16-Flt3L cells to enhance the efficiency and reduce the costs[115]. We inject Flt3L-expressing B16 cells into subcutaneous tissue in the right flank of C57BL/6 mice[115] to avoid the need for daily injections and the cost of recombinant Flt3L. Alternatively, daily injection of 10 µg of Flt3L subcutaneously to mice for 9–11 consecutive days can be done instead. Administration of either recombinant Flt3L or Flt3L-expressing B16 cells results in expansion of both $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets ($CD11c^+$ class II^+)[58,122,123]. $CD11c^+$ immature DCs can then be isolated from the spleens of mice injected with Flt3L-B16 cells or the recombinant Flt3L-treated animals and exposed to cytokines and/or TLR ligands *ex vivo* to generate type-1 polarized DCs.

Vaccine generation. To effectively promote anti-tumor immunity, DCs must be mature and produce high levels of IL-12 (refs. 13,61,70–77,81–83). The manner in which DCs are matured will affect the phenotype and functional activity (i.e., IL-12-producing capacity) of the mature DCs. To achieve translational analogy to our clinical DC vaccines[76], the LPS-based maturation method[82] was replaced with the mouse variant of our previously developed maturation cocktail[13]. Our DC maturation cocktail

Box 5 | Labeling of the cells with CFSE ● TIMING 30–45 min

Procedure

1. Resuspend the cells (from Step 9E(iii); or from step 1 of **Box 6**) in 2 ml of PBS.
2. Add 1 µl of CFSE reagent and incubate the cells at 37 °C for 10 min.

▲ CRITICAL STEP To perform staining with 20× diluted CFSE, first dissolve 1 µl of CFSE reagent in 1 ml of PBS and add 50 µl of 20× diluted CFSE reagent to the cells.

3. Add 10 ml of cold culture medium and incubate cells for an additional 10 min in the refrigerator.
4. Centrifuge the cells at 400*g* for 3 min at 4 °C, then resuspend the cells in 10 ml of culture medium, and centrifuge again at 400*g* for 3 min at 4 °C to wash the cells.
5. Resuspend the CFSE-labeled cells in 2 ml of culture medium and keep at 37 °C for no longer than 30–60 min before use in specific experiments.

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Box 6 | *In vivo* immunomonitoring: *in vivo*-specific cytotoxicity assay

● TIMING 2–3 h

Procedure

1. Isolate splenocytes from a B6 CD45.1 mouse as described in **Box 3**. Resuspend the splenocytes in culture medium at 5 × 10^6 cells per ml and plate 2 ml per well in two 12-well culture plates. Immediately add 100 µl per well of the prepared UVBγ-Tu cells (see **Box 2** for preparation of UVBγ-Tu cells) to the wells of one culture plate only. Leave the cells in the other plate unloaded. Gently rock the loaded plate back and forth to ensure even distribution of cells. After 4 h of incubation at 37 °C, harvest tumor-loaded splenocytes as described in Step 3.
2. Label the tumor-loaded splenocytes with CFSE and the unloaded splenocytes with 20× diluted CFSE as described in Box 5. Mix the unloaded $CFSE^{low}$- and loaded $CFSE^{high}$-labeled splenocytes at a 1:1 ratio in PBS to 2.5 × 10^7 cells per ml and inject 200 µl i.v. into immunized mice (3–5 d after Step 14) and untreated (control) tumor-bearing mice and at least 2 naive (non-tumor bearing) mice.
▲ CRITICAL STEP The number of injected cells is not critical, although higher cell numbers facilitate their detection by flow cytometry. We recommend 5 × 10^6 cells per mouse.
3. Harvest spleens 16 h after injection and isolate the splenocytes as described in **Box 3**.
4. Perform the flow analysis. Make a histogram of live cells expressing low and high levels of CFSE to determine the specific killing (**Fig. 2e**).
5. (Optional) Stain the cells for CD45.1 as described in step 9 of **Box 4**. Make a histogram of live $CD45.1^+$ cells and set gates on $CFSE^{low}$ and $CFSE^{high}$ cells. Only 0.05–0.5% of the cells are expected to be $CD45.1^+$.

contains the recombinant murine cytokines rmGM-CSF, rmIL-1β, rmIFNα, rmIFNγ, rmTNFα, and poly-IC to convert DCs into type-1 polarized DCs.

We observed that, compared with our human protocol[13,66,124,125], the maturation period of mouse DCs needed to be reduced from 16–48 to 4 h for optimal DC stimulatory capacity. The 4 h–matured DCs can secrete IL-12p70 while showing high levels of expression of key co-stimulatory and maturation markers (e.g., CD40, CD80, CD86, and CD83) and the lymph node-homing chemokine receptor CCR7 (Giermasz *et al.*[14]; **Fig. 2a**,**b**).

We use a UVBγ irradiation-based technique to prepare apoptotic tumor cells (UVBγ-Tu cells) for antigen loading[15,91,99,126] (**Box 2**). The ability to take up UVBγ-Tu cells is reduced in DCs that have completed the maturation process[127]. Thus, we load DCs with UVBγ-Tu cells during their maturation to generate type-1 polarized DCs that produce high levels of IL-12p70 and cross-present tumor antigens[15,91].

In a single process using ten donor mice, we can generate up to 150 million DCs, which, after QC testing, will suffice for ~500 doses of a single batch of vaccine.

QC of tumor-loaded DCs. Before vaccine administration in tumor-bearing mice, we test each batch of DC vaccines for consistency in phenotypic and functional characteristics. We perform flow-cytometry analysis for expression of markers such as IA-E^b, $H2K^b$, CCR7, CD14, CD40, CD80, CD83, and CD86 in DCs that have been matured *in vitro* with Th1-inducing cocktail. Apart from expressing co-stimulatory molecules and CCR7 (**Fig. 2a**), type-1 polarized DCs produce IL-12p70 upon stimulation with CD40L[128] (**Fig. 2b**). Consistent with the key role of IL-12p70 in the induction of type-1 immunity, tumor-loaded DCs matured in the presence of type-1-inducing cocktail induce high numbers of tumor-specific T cells[129] in *in vitro* cultures of syngeneic splenocytes (**Fig. 2c**). Another important functional characteristic of DCs is their ability to migrate to draining lymph nodes (**Box 3**). *In vivo* migration of DCs generated from firefly luciferase (CAG-luc-eGFP) transgenic mice can be traced by bioluminescence imaging (BLI) after injection. *In vivo* migration of DCs from B6 CD45.1 mice or carboxyfluorescein succinimidyl ester (CFSE)-labeled DCs from C57BL/6J mice[130] can be detected by flow cytometry of lymph node-infiltrating cells. The advantage of using CAG-luc-eGFP DCs is that migration can be monitored in the same mice over time (**Fig. 2d**). Generation of $CD45.1^+$ DCs avoids the need for DC labeling, as the cells can be detected by staining for the CD45.1 marker. Furthermore, the functional activity of DCs can most comprehensively be evaluated by the *in vivo* induction of tumor-specific type-1 immune response assay (**Fig. 2e**).

Mouse tumor models. The evolving role of animal models in cancer vaccine development has been recently reviewed[118,131]. The assessment of immunotherapy using inducible tumor models in genetically manipulated mice or carcinogen-induced tumor models is particularly relevant to the physiological situation, as they involve chronic inflammation, development of immune tolerance, tumor immune editing, and immunosuppressive events. At the same time, transplantable models (we have used ovarian cancer ID8A and colorectal cancer MC38 cells) offer multiple advantages, especially regarding their costs and feasibility to address specific mechanistic questions (e.g., manipulation of antigenicity, suppressive activity, resistance to apoptosis, chemokine production), as recently summarized by Mac Keon *et al.*[132]. In orthotopic tumor models, the tumor cell lines are implanted into the relevant organ and are allowed to form metastases, reflecting the human disease[133]. The ID8A tumor-bearing mice develop massive malignant ascites in the late stages, mirroring the characteristics of human epithelial ovarian cancer. As different sites of tumor inoculation can give rise to different immune microenvironments, these can affect tumor progression and therapeutic outcomes[134]. Therefore, our protocol involves two alternative sites of MC38 tumor implantation (subcutaneous and intraperitoneal) in syngeneic C57BL/6J mice, which are both similarly susceptible to the described immunotherapies.

Promoting the infiltration of tumor-specific T cells into tumor tissues. Homing of effector type-1 immune cells into the TME is critical for effective immunotherapy. To promote the migra-

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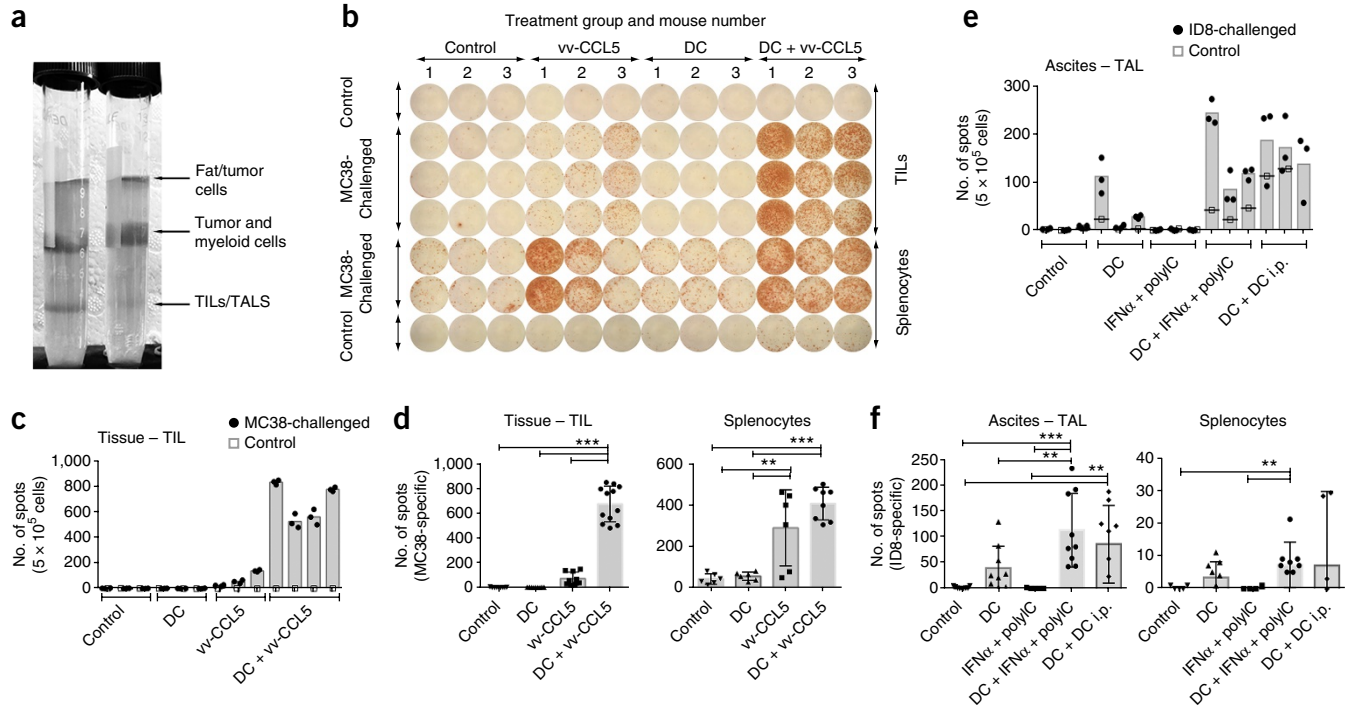

Figure 4 | Monitoring of the immunotherapy-associated changes in the numbers of tumor-specific T cells in TME versus the spleen. (**a**) Isolation of TIL/TALs by gradient centrifugation for IFN-γ ELISpot assay (Step 18). Following Percoll gradient centrifugation, the bottom interface of lymphocytes must be collected for further ELISpot analysis. Top and middle interfaces are enriched in myeloid cells and tumor cells. (**b**) Representative image of ELISpot plate readout analyzing lymphocytes (splenocytes—bottom three rows and TILs—top four rows) from 12 mice (three mice per treatment group). Tumor cells were added to the wells marked MC38-challenged, whereas the lymphocytes in the top and bottom margin wells of each sample were left unchallenged. (**c**,**d**) ELISpot data showing the induction of tumor-specific TILs in MC38-tumor-bearing mice following DC vaccination in combination with CCL5-expressing vaccinia virus therapy. MC38-tumor-bearing mice (1×10^6 s.c., 3–4 per group) were left untreated (control), treated with type-1 polarized DCs (DC, 3×10^5 s.c., opposite flank, days 5 and 8) only, treated with intratumoral injection of CCL5-expressing vaccinia virus (1×10^7 PFU) on day 11, or with the combination of both. Tumors and spleens were harvested on day 14 and lymphocytes were analyzed for IFN-γ production by ELISpot. The cells were either left unstimulated (control) or challenged with MC38 tumor cells. (**c**) Results are shown as individual data points (number of spots in each well) and bars (means ± s.d.) of TILs from each mouse, evaluated in triplicates, as shown in **Figure 4b**. (**d**) To determine tumor specific responses, the average value of spots from control wells were subtracted from the number of spots in MC38-challenged wells. Histograms represent pooled data of the triplicates from all the mice in the group. Results are shown as individual data points (number of spots in each well) and bars (means ± s.d.). Statistical evaluation was done with ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$. (**e**,**f**) ELISpot data showing the induction of tumor-specific TALs in ID8A-tumor-bearing mice following DC vaccination in combination with CKM treatment. ID8A-tumor-bearing mice (4×10^6, 3 per group) were left untreated (control), treated with type-1 polarized DCs (DC, 3×10^5 s.c., day 5) only, treated with i.p. injection of IFN-α and Ampligen and COX-2 inhibitor celecoxib on day 8, treated with 3×10^5 DCs s.c., day 5 and additional dose of 3×10^5 DCs injected i.p. on day 8, or a combination of 3×10^5 DCs s.c., day 5 and i.p. injection of IFN-α and Ampligen and COX-2 inhibitor celecoxib on day 8. Peritoneal washes/ascites and spleens were harvested on day 13 and lymphocytes were analyzed for IFN-γ production by ELISpot. The cells were either left unstimulated (control) or challenged with ID8A tumor cells. (**e**) Results are shown as individual data points (number of spots in each well) and bars (means ± s.d.) of TALs from each mouse evaluated in triplicates, as shown in **Figure 4b**. (**f**) To determine tumor-specific responses, the average values of spots from control wells were subtracted from the number of spots in ID8A-challenged wells. Histograms represent pooled data of the triplicates from all the mice in the group. Results are shown as individual data points (number of spots in each well) and bars (means ± s.d.). Statistical evaluation was done with ANOVA. $^{**}P < 0.01$, $^{***}P < 0.001$. Combination of DC therapy with CKM regimen results in an increase in tumor specificity. ID8A (luciferase-expressing), a cell line derived from spontaneous *in vitro* malignant transformation of C57BL/6 mouse ovarian surface epithelial cells (MOSECs), was a generous gift from T.J. Curiel, University of Texas Health Science Center at San Antonio. Animal experiments were approved by the IACUC of the University of Pittsburgh. ANOVA, analysis of variance; PFU, plaque-forming unit; vv-CCL5, CCL5-expressing *Vaccinia* virus.

tion of CTLs to tumors, we developed two strategies of TME modulation (**Fig. 3a**). Our basic protocol combines two individual doses of DC injection. In the first subcutaneous vaccine is followed 3 d later by a second intratumoral booster (**Fig. 3b**,**c**). Alternatively, the vaccination may be combined with the selective induction of effector-attracting chemokines using combinatorial CKM adjuvants[16]. Multiple chemokines can be targeted to manipulate and alter the TME, in order to correct the local balance of immunosuppressive and effector cells (reviewed in Devaud *et al.*[135]). A potent way to manipulate TME is the use of TLR ligands (TLR-Ls). TLR-Ls trigger broad inflammatory responses that elicit rapid innate immunity and promote the activation of the adaptive immune reaction[136]. Examples of commonly used TLR-Ls are poly-IC (TLR3 agonist) and CpG (TLR9 agonist)[137,138]. Although systemic application of the individual TLR-Ls can induce a substantial nonselective systemic response composed of both the effector cell-attracting chemokines and T_{reg}/MDSC attractants, their combination with cytokines and blockers of the COX2 pathway allows the focus of their activity on tumor tissues and selectively induces desirable chemokines[16–18]. Therefore, we use the combination of type-1 interferons, TLR ligands (such as poly-IC), and COX2 inhibitors (such as celecoxib or aspirin) (**Fig. 3**).

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Another approach to selectively target tumor lesions[139] is the introduction of effector cell–attracting chemokines by administration of genetically manipulated viruses[136,140]. Oncolytic viruses are known to stimulate multiple TLRs[141] and predominantly infect cancer cells[139]. Directing cytokines (e.g., IFN-α and IL-12) specifically to tumors, using intratumoral administration of viral vectors or DC vaccines, has been shown to reprogram the TME and increase efficacy of additional immunotherapeutic agents[76,142–144].

The presented DC vaccination and CKM approaches may be used as individual modalities or in combinatorial approaches to enhance efficacy of intratumoral tumor-specific T cells to achieve optimal therapeutic outcomes[145,146].

Detection and quantification of tumor-specific immune responses in the tumor tissues compared with evaluation of systemic immunization. The *in vivo* tumor-specific cytotoxicity assay using i.v. injection of a 1:1 mixture of nonloaded $CFSE^{negative/dim}$ and antigen-loaded $CFSE^{high}$ splenocytes[115] (**Boxes 4** and **5**) is a straightforward method to determine the magnitude of the systemic tumor-specific immune response in tumor-bearing mice (**Box 6** and **Fig. 2e**). However, this strategy does not allow evaluation of the effectiveness of new cancer therapies to promote the increase in the numbers of tumor-specific T cells within tumor tissues. To address this issue, we developed a highly sensitive *ex vivo* IFN-γ ELISpot-based protocol allowing us to compare the numbers of tumor-specific cells in splenocytes[19] and TILs. Our protocol involves the isolation of immune cells from cancerous tissues (e.g., solid primary tissues and malignant ascites) using tissue digestion and subsequent Percoll gradient centrifugation, followed by *ex vivo* exposure of the isolated immune cells to the relevant antigens, such as whole cancer cells (**Fig. 4**) or, potentially, defined peptide-loaded target cells.

MATERIALS

REAGENTS

- RPMI-1640 medium (Gibco, Invitrogen, cat. no. 61870-010)
- Recombinant mouse IL-1β (research grade; Miltenyi, cat. no. 130-094-053)
- Recombinant mouse TNF-α (research grade; Miltenyi, cat. no. 130-094-085)
- Recombinant mouse IFN-γ (research grade; Miltenyi, cat. no.130-094-048)
- Recombinant mouse GM-CSF (research grade; Miltenyi, cat. no. 130-094-043)
- Recombinant mouse IFN-α (research grade; Miltenyi, cat. no.130-093-131)
- Recombinant mouse IL-2 (research grade; Miltenyi, cat. no.130-094-055)
- Recombinant mouse IL-7 (research grade; Miltenyi, cat. no. 130-094-636)
- Poly-IC (Sigma-Aldrich, cat. no. P9582-5MG)
- Ampligen (polyI:polyC$_{12}$U; Hemispherx Biopharma) ▲ **CRITICAL** Ampligen is safer and more specific in comparison with poly-IC.
- Celecoxib (Biovision, cat. no. 1574100)
- Aspirin (Sigma-Aldrich, cat. no. A5376-100G)
- Trypan blue (0.4% (wt/vol); Corning Cellgro, cat. no. MT-25-900-CI)
- L-Glutamine (L-glu; Gibco; Invitrogen, cat. no. 25030-024)
- Penicillin–streptomycin (10,000 U/ml; Gibco; Invitrogen, cat. no. 15140-114)
- Non-essential amino acids (NEAAs; Gibco; Invitrogen, cat. no. 11140-035)
- Sodium pyruvate (Gibco; Invitrogen, cat. no. 11360-039)
- 2β-mercaptoethanol (2βME; Sigma-Aldrich, cat. no. M3148) ! **CAUTION** β-mercaptoethanol is toxic upon inhalation, upon contact with skin, and if swallowed, and it is hazardous to the aquatic environment. Avoid contact with skin, eyes, and clothing, and handle it with gloves in a chemical fume hood.
- FBS (Gemini Foundation B, cat. no. 900-208)
- ACK lysis buffer (Life Technologies, cat. no. A10492-01)
- Mouse CD11c microbeads (Miltenyi Biotec, cat. no. 130-052-001)
- Collagenase (10% (wt/vol) stock; Sigma-Aldrich, cat. no. C0130-100MG)
- Tumor Dissociation Kit (enzymes D, R, and A; Miltenyi Biotec, cat. no. 130-096-730)
- Percoll (Sigma-Aldrich, cat. no. P1644)
- PBS (Cellgro Cell Culture PBS (1×); Corning, cat. no. MT-21-040-CV)
- EDTA (500 mM; Life Technologies, cat. no. 15575-020)
- Carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, cat. no. C34554) ▲ **CRITICAL** Protect the reagent from light.
- Ethanol (diluted 70% (vol/vol); Pitt Pharmco, cat. no. 111000200CS) ! **CAUTION** Ethanol is flammable, and it may cause skin and eye irritation. Avoid contact with skin, eyes, and clothing, and handle it with gloves in a chemical fume hood.
- Luciferin (Gold Biotechnology, cat. no. Luck-1g) ▲ **CRITICAL** Protect the luciferin from light.
- OVA peptide SIINFEKL (Genescript, cat. no. rp10611)
- R4-6A2 anti-IFN-γ biotinylated detecting antibody (Mabtech, cat. no. 3321-6-250)
- AN18 anti-IFNγ-coating antibody (Mabtech, cat. no. 3321-3-1000)
- ABC reagent (Vector Laboratories, cat. no. PK-6100)
- ELISpot AEC substrate kit (Vector Laboratories, cat. no. SK-4200) (Vector Laboratories, cat. no. PK6100) ! **CAUTION** ELISpot Substrate reagent is flammable and is harmful upon contact with skin, of if swallowed or inhaled. Avoid contact with skin and eyes and handle it with gloves.

Antibodies for flow cytometry

- CD80-APC (Armenian hamster IgG, 0.2 mg/ml; BioLegend, cat. no. 104714)
- CD40-APC (IgG_{2a}, 0.2 mg/ml; BioLegend, cat. no. 124612)
- CD86-APC (IgG_{2a}, 0.2 mg/ml; BioLegend, cat. no. 105012)
- CD14-FITC (IgG_1, 0.5 mg/ml; BD Biosciences, cat. no. 553739)
- CCR7-PE (IgG_{2a}, 0.2 mg/ml; BioLegend, cat. no.120106)
- IA/E^b-FITC (IgG_{2a}, 0.5 mg/ml; BioLegend, cat. no.114406)
- H2K^b-FITC (IgG_{2a}, 0.5 mg/ml; BioLegend, cat. no. 116505)
- IgG_1-FITC (IgG_1, 0.5 mg/ml; BD Biosciences, cat. no. 553953)
- IgG_{2a}-FITC (IgG_{2a}, 0.5 mg/ml; BD Biosciences, cat. no. 554688)
- IgG-PE (Armenian hamster IgG, 0.2 mg/ml; BioLegend, cat. no. 400908)
- $IgG2_a$-APC (IgG_{2a}, 0.2 mg/ml; BioLegend, cat. no. 400219)
- IgG_1-APC (IgG_1, BD Biosciences, cat. no. 555751)
- CD45.1-APC (IgG_{2a}, 0.2 mg/ml; BioLegend, cat. no. 110714) ▲ **CRITICAL** Protect the antibodies from light.
- IL-12 ELISA Kit (R&D, cat. no. DY419)
- BSA (Sigma-Aldrich, cat. no. A9647)
- dH_2O (Life Technologies, cat. no. 10977)
- Tween 20 (Fisher Scientific, cat. no. BP337-500)
- 10× PBS (Cellgro PBS (1×); Corning, cat. no. MT-46-013-CM)
- NaCl (Sigma-Aldrich, cat. no. S7653)
- $Na_2HPO_4{\cdot}2H_2O$ (Sigma-Aldrich, cat. no. 71643)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P5655)
- ELISA substrate (Fisher Scientific, cat. no. ENN301) ! **CAUTION** ELISA substrate is flammable and is harmful upon contact with skin, or if swallowed or inhaled. Avoid contact with skin and eyes, and handle it with gloves.
- H_2SO_4 (Sigma-Aldrich, cat. no. 339741) ! **CAUTION** Sulfuric acid is highly flammable, and it may cause severe skin burns and eye damage. Avoid contact with skin, eyes, and clothing, and handle it with gloves in a chemical fume hood.
- Paraformaldehyde, 32% (wt/vol) solution (PFA; Electron Microscopy Sciences, cat. no. 15714-S) ! **CAUTION** PFA is a hazardous reagent; use a chemical fume hood and wear protective gloves and a mask when you are working with it.

Cells

- Flt3L-expressing B16 melanoma cells[147] (C57BL/6J mouse strain), a gift from M. Kronenberg, La Jolla Institute for Allergy and Immunology ▲ **CRITICAL** The cell lines should be regularly checked to ensure they are authentic and are not infected with mycoplasma. ▲ **CRITICAL** B16-Flt3L cells can be replaced with recombinant Flt3L (Invitrogen, cat. no. 14-8001-80) as described in the Experimental design section.
- CD40L-transfected J558 (J558-CD40L) (Balb/c mouse strain) cells[148], a gift from P. Lane, University of Birmingham. Alternatively, CD40L-expressing EL-4-B5 cells are available from Kerafast (cat. no. EVU301);

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however, we have never used them. ▲ CRITICAL Soluble CD40L (MEGACD40L; Enzo Life Sciences, cat. no. ALX-522-120-C010) can be substituted for the CD40L-expressing cells, as described in Step 9B(iv).
- ▲ CRITICAL The cell lines should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- MC38 (C57BL/6J mouse strain) colorectal cancer cells (luciferase-expressing)[149] (available from Kerafast, cat. no. ENH204) ▲ CRITICAL The cell lines should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- ID8A (luciferase-expressing), a cell line derived from spontaneous *in vitro* malignant transformation of C57BL/6 mouse ovarian surface epithelial cells, was a gift from T.J. Curiel, University of Texas Health Science Center at San Antonio ▲ CRITICAL The cell lines should be regularly checked to ensure they are authentic and are not infected with mycoplasma.

▲ CRITICAL Cancer cell lines should be of the same genetic background as the mice into which the cells will be injected, to avoid any allogeneic immune responses and tumor rejection.

Mice

▲ CRITICAL Donor and experimental mice used in separate steps of the protocol are indicated in **Table 1** and listed below.
- C57BL/6J (The Jackson Laboratory, cat. no. 000664) ! CAUTION All procedures involving animal experiments should follow approved institutional and governmental animal protocols and comply with the relevant guidelines and regulations of the local animal ethics committee. The animal studies reported here were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh Cancer Institute (protocol 14063339).
- FVB-Tg(CAG-luc,-GFP)L2G85Chco/J—backcrossed on the C57BL/6 line for >14 generations[150]—can be obtained from A. Beilhack, University Hospital of Wurzburg, or from the Jackson Laboratories (cat. no. 008450) ! CAUTION All procedures involving animal experiments should follow approved institutional and governmental animal protocols and comply with the relevant guidelines and regulations of the local animal ethics committee. The animal studies reported here were approved by the IACUC at the University of Pittsburgh Cancer Institute (protocol 14063339).
- B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ (C57BL/6 CD45.1; The Jackson Laboratory, cat. no. 002014) ! CAUTION All procedures involving animal experiments should follow approved institutional and governmental animal protocols and comply with the relevant guidelines and regulations of the local animal ethics committee. The animal studies reported here were approved by the IACUC at the University of Pittsburgh Cancer Institute (protocol 14063339).

EQUIPMENT
- 2-ml Pipettes (sterile, individually wrapped; Denville, cat. no. p7132)
- 5-ml Pipettes (sterile, individually wrapped; Denville, cat. no. p7133)
- 10-ml Pipettes (sterile, individually wrapped; Denville, cat. no. p7134)
- 50-ml Pipettes (sterile, individually wrapped; Denville, cat. no. p7135)
- Alcohol prep pads (Fisher Scientific, cat. no. 22-363-750)
- 12 × 75-mm Polystyrene tubes (Falcon, cat. no. 14-959-11B)
- Sterile cell culture plates (12 well; Denville, cat. no. t1012)
- Sterile cell culture flasks (75 mm^2; Falcon, cat. no. 13-680-65)
- Sterile cell culture plates (96 well, flat-bottom; Denville, cat. no. t1096)
- Sterile cell culture plates (96 well, V-bottom; Corning Costar, cat. no. CLS3894)
- High-binding 96-well plates for ELISA (Costar 96-well EIA/RIA plates; Corning, cat. no. 07-200-35)
- Cell strainers (100 µm; Falcon, cat. no. 08-771-19)
- Cell strainers (70 µm; Falcon, cat. no. 08-771-2)
- Conical centrifuge tubes (50 ml; Denville, cat. no. C1060-p)
- Conical centrifuge tubes (15 ml; Denville, cat. no. c1018-p)
- Eppendorf centrifugation tubes (2 ml; Sigma-Aldrich, cat. no. T2795-1000EA)
- ELISpot plates (Millipore, cat. no. MAHAS4510)
- Forceps (Fisher Scientific, cat. no. 08-880)
- Scissors (Fisher Scientific, cat. no. 08-951-20) ! CAUTION Scissors are sharp. Handle them with care.
- Needles, 18 gauge, 22 gauge, 27 gauge (BD Biosciences, cat. nos. 305195, 305155, 305109) ! CAUTION Needles are sharp. Handle them with care.
- 0.5-ml Syringes (BD Biosciences, cat. no. 305620)
- 3-ml Syringe (BD Biosciences, cat. no. 309585)
- 10-ml Syringe (BD Biosciences, cat. no. 309604)
- Pipette tips (10, 200, 1,000 µl; MidSci, cat. nos. AV10R-H, AV200, AV1000)
- Cryovials (Thermo Scientific, cat. no. 12-565-164N)
- Sterile beakers (Pyrex, cat. no. 02-540M)
- Pasteur pipettes (Denville, cat. no. p0458-9)
- Countess cell counter (Invitrogen) or Neubauer hemocytometer (Fisher Scientific, cat. no. 02-671-10)
- Microscope (Carl Zeiss, model no. Axiovert 200)
- FACS instrument (Accuri or Fortessa; BD Biosciences)
- MACS MultiStand (Miltenyi Biotec, cat. no. 130-042-303)
- QuadroMACS separator (Miltenyi Biotec, cat. no. 130-090-976)
- Cell incubator (temperature = 37 °C, CO_2 level = 5% (vol/vol); Thermo/Kendro, model no. Heracell 150)
- Gamma irradiator (Nordion International) ! CAUTION Gamma irradiation is hazardous. Appropriate safety measures should be taken while working with a gamma irradiator.
- UVBγ light (Spectroline, model no. EB-160C) ! CAUTION UV light is hazardous. Appropriate personal protective equipment should be worn while using a UV source.
- ELISpot reader (ImmunoSpot, Cellular Technology)
- ELISA reader (Perkin Elmer, model no. Victor 2)
- *In vivo* bioluminescence imager (IVIS; Perkin Elmer)
- Temperature-controlled freezing container (Mr Frosty; Thermo Scientific, cat. no. 51000001)
- Centrifuge (Eppendorf, model no. 5810 R)
- Pipetman (P10-P100; Gilson, cat. no. F123615G)
- Pipetman (P100-P1000; Gilson, cat. no. F123602G)
- Pipet-Aid (Drummond Scientific)
- Pipettes (0.5–10 µl, 20–200 µl, 100–1,000 µl; Eppendorf, cat. nos. 3121.000.020, 3121.000.054, 3121.000.062)
- Multichannel pipette (20–300 µl; Thermo Scientific, cat. no. 4661140)

REAGENT SETUP

FBS We purchase the serum in frozen 500-ml bottles and store them directly at −20 °C. Individual bottles should be slowly thawed (at 4 °C overnight) and heat-inactivated for 30 min at 56 °C, and then serum aliquots should be prepared in 50-ml tubes and frozen again. Freeze–thaw cycles should be avoided. The serum can be stored for up to 2 years at −20 °C.

Standard isotonic Percoll solution Prepare 10× acidic (pH: 4.6, 1.051 g/ml) PBS from 13.5 g NaCl, 0.125 g of $Na_2HPO_4{\cdot}2H_2O$, 2.1 g KH_2PO_4, and 200 ml dH_2O. Sterilize acidic PBS by 0.22-µm filtration, and store at 4 °C in 4-ml aliquots. The maximum recommended storage time is 3 months. Percoll must be mixed with acidic PBS 10:1 and further diluted with culture medium to prepare 60% (vol/vol), 45% (vol/vol), and 34% (vol/vol) standard isotonic Percoll solution (SIP). Freshly prepare SIP before use and keep it at 37 °C.

Cell culture medium To prepare cell culture medium, supplement RPMI-1640 medium with 1% (vol/vol) (100 U/ml) penicillin–streptomycin, 1% (vol/vol) (1 mM) sodium pyruvate, 1% (vol/vol) NEAAs, 0.1% (vol/vol) (14.3 mM) 2βME, 1% (vol/vol) (2 mM) L-glu, and 10% (vol/vol) FBS and store at 4 °C. The maximum recommended storage time is 1 month.

Cytokines Many cytokine concentrations are indicated in IU/ml, which is the correct form for comparison on the basis of functional activity. However, for some cytokines, this information is not available. Values can differ greatly from company to company, suggesting that measurement of activity may be challenging. We provide information referring to specific cytokines from specific companies. Adjustments must be made when cytokines from different vendors are used. We prepare all cytokines as 100× or 1,000× concentrated stock solutions and store the aliquots at −20 °C. The maximum recommended storage time is 6 months. The concentrations vary, as indicated in **Table 2**.

DC maturation medium To generate effector type-1 polarized DCs, prepare maturation medium before setting up the DC maturation. The volume needed depends on the number of isolated $CD11c^+$ cells—~10 ml of maturation medium is required for maturation of DCs isolated from one spleen. Supplement culture medium with cytokines as indicated in **Table 2**. The cytokines are stored at −20 °C and should be completely thawed at RT before adding to the medium. Allow the prepared medium to equilibrate to 37 °C before adding to the cultures. ▲ CRITICAL Freshly prepare the DC maturation medium before use.

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TABLE 1 | Donor and experimental mice used in separate steps of the protocol.

Experimental purpose	Donor mice		Experimental mice (and recommended number of mice per experimental group)
	[a]DC vaccine generation	Induction of type-1 effector cells	
Phenotype analysis of the DCs (Step 9A; **Fig. 2a**)	C57BL/6J mouse (UVBγ-Tu-loaded DCs)		
Analysis of IL-12 production (Step 9B; **Fig. 2b**)	C57BL/6J mouse (UVBγ-Tu-loaded DCs)		
In vitro evaluation of effector type-1 immune cell induction (Step 9C; **Fig. 2c**)	C57BL/6J mouse (UVBγ-Tu-loaded DCs)	C57BL/6J mouse	
In vivo evaluation of DC migration (Step 9D; **Box 4**; **Fig. 2d**)	CAG-luc-GFP or B6 Cd45.1 mouse (UVBγ-Tu-loaded DCs)		3–5 C57BL/6J mice + 2 control (no injection of DCs) C57BL/6J mice
In vivo evaluation of systemic tumor-specific immunity (Step 9E; **Fig. 2e**)	C57BL/6J mouse (OVA-loaded DCs)	B6 Cd45.1 or C57BL/6J mouse (OVA-loaded $CFSE^+$ splenocytes)	3–5 C57BL/6J mice + 2 control (without DC vaccination) C57BL/6J mice
Monitoring of the tumor progression and survival (Steps 12, 13, and 18; **Fig. 3c**,**d**)	C57BL/6J mouse (UVBγ-Tu-loaded DCs)		8–10 C57BL/6J mice (1 s.c. vaccine + 1 i.p. vaccine/cycle; 3 cycles)
In vivo cytotoxicity assay (**Box 6**)	C57BL/6J mouse (UVBγ-Tu-loaded DCs)	B6 Cd45.1 or C57BL/6J mouse (UVBγ-Tu loaded $CFSE^+$ splenocytes)	7–10 C57BL/6J mice (1 s.c. vaccine + 1 i.p. vaccine/cycle; 1–2 cycles) + 2 naive C57BL/6J mice
Ex vivo immunomonitoring (Steps 17–24; **Fig. 4**)	C57BL/6J mouse (UVBγ-Tu-loaded DCs)		3–5 C57BL/6J mice (1 s.c. vaccine + 1 i.p. vaccine/cycle; 1–2 cycles)

[a]One donor mouse is expected to yield ~30–40 × 10^6 $CD11c^+$ cells. ~1 × 10^6 UVBγ-Tu cells are needed for loading of 30–40 × 10^6 $CD11c^+$ cells, which should yield ~15 × 10^6 type-1 polarized antigen-loaded DCs. When using 0.3 × 10^6 type-1 polarized DCs per injection, one donor mouse allows for generation of DCs for ~35–40 vaccinations.

Cell-freezing medium For freezing medium 1 (FM1), supplement RPMI-1640 medium with 50% (vol/vol) FBS. Freezing medium 2 (FM2) contains RPMI-1640 medium (40% (vol/vol)), FBS (40% (vol/vol)), and DMSO (20% (vol/vol)). Store both media at 4 °C. The maximum recommended storage time is 1 month.

CKM adjuvants for modulation of the TME We prepare a solution of 5 × 10^4 IU/ml IFN-α and 0.25 mg/ml Ampligen in PBS for injecting 200 µl i.p. per mouse (for i.p. tumor models) and 2 × 10^5 IU/ml IFN-α and 1.0 mg/ml Ampligen in PBS for injecting 50 µl intratumorally per mouse (for s.c. MC38 tumor models). ▲ **CRITICAL** Freshly prepare the CMK adjuvant solutions before use.

COX-2 inhibitors We prepare a 4 mg/ml stock solution of celecoxib and a 200 mg/ml stock solution of aspirin in DMSO and store aliquots at −20 °C. The maximum recommended storage time is 6 months. We make 500 µg/ml (12.5% (vol/vol) DMSO, celecoxib) and 2.5 mg/ml (1.25% (vol/vol) DMSO, aspirin) working solutions in PBS immediately before administration (for injecting 200 µl per mouse). ▲ **CRITICAL** Freshly prepare the COX-2 inhibitor solutions before use.

CFSE reagent Prepare CFSE reagent in the dark by adding 18 µl of DMSO to the vial of lyophilized CFSE. Store CFSE reagent at −20 °C. The maximum recommended storage time is 1 month.

EDTA in PBS For detachment of tumor cells, prepare 1 mM EDTA in PBS by adding 1 ml of 500 mM EDTA to 500 ml PBS and sterile-filter the solution through a 0.22-µm filter. Store at 4 °C. The maximum recommended storage time is 3 months.

MACS buffer For MACS buffer (0.5% (wt/vol) BSA and 2 mM EDTA in PBS), supplement 500 ml of PBS with 2.5 g of BSA and 2 ml of 500 mM EDTA and sterile-filter the solution through a 0.22-µm filter. Store at 4 °C. The maximum recommended storage time is 3 months.

Blocking buffer Prepare blocking buffer (3.0% (wt/vol) BSA in PBS) by adding 1.5 g of BSA to 50 ml of PBS and sterile-filter the solution through a 0.22-µm filter. Store at 4 °C. The maximum recommended storage time is 1 month.

Wash buffer We prepare 10 liters of wash buffer (0.05% (vol/vol) Tween 20 in PBS) by diluting 1 liter of 10× PBS with 9 liters of dH_2O and adding 5 ml of Tween 20. Store at room temperature (20–23 °C). The maximum recommended storage time is 3 months.

ABC reagent Prepare avidin–peroxidase complex (Vector Laboratories) by adding one drop each of reagents A and B to 10 ml of wash buffer. ▲ **CRITICAL** Freshly prepare ABC reagent before use.

Digestion buffer Thaw digestion enzymes D, R, and A from the Tumor Dissociation Kit at room temperature (see manufacturer's instructions for reconstitution information). Per 2.5 ml of digestion medium, 100 µl of enzyme D, 50 µl of enzyme R, and 12.5 µl of enzyme A are required. ▲ **CRITICAL** Freshly prepare the digestion buffer before use.

Luciferin Thaw luciferin at room temperature. Reconstitute 1 g of luciferin in the dark with 33.3 ml of cold PBS and freeze at −80 °C in 1-ml aliquots. The maximum recommended storage time is 6 months.

Paraformaldehyde 4% (wt/vol) solution Dilute 5 ml of paraformaldehyde 32% (wt/vol) solution with 35 ml of PBS. Store at 4 °C. The maximum recommended storage time is 6 months.

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TABLE 2 | DC growth factors and DC maturation factors.

Cytokine	Diluent	Stock concentration	Maturation medium (2× final concentration)	Volume/ml of maturation medium
GM-CSF	RPMI-1640	1×10^6 IU/ml	2×10^3 IU/ml	2 µl
Poly-IC	dH_2O	1 mg/ml	20 µg/ml	20 µl
IFN-α	0.1% (wt/vol) BSA in PBS	1×10^6 IU/ml	2×10^3 IU/ml	2 µl
TNF-α	0.1% (wt/vol) BSA in PBS	5 µg/ml	10 ng/ml	2 µl
IL-1β	dH_2O	25 µg/ml	50 ng/ml	2 µl
IFN-γ	20 mM acetic acid	5×10^5 IU/ml	1×10^3 IU/ml	2 µl

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PROCEDURE

Generation of type-1 polarized mouse DCs ● TIMING 4–5 h

1| Resuspend the $CD11c^+$ cells (see **Box 1** for $CD11c^+$ cell isolation) in cell culture medium at 4×10^6 per ml and add 1 ml of cell suspension to each well of a 12-well culture plate. Add 1 ml of DC maturation medium to each well. Examine the DC cultures for the density and general appearance of the cells, using an inverted microscope. Immediately after addition of DC maturation medium, add 100 µl of 1×10^6 UVBγ-Tu cells per ml per well (40:1 $CD11c^+$ cells (DCs)/tumor cell ratio).
▲ **CRITICAL STEP** Ensure that the same cancer cell line that will be injected into the experimental mice is used for DC loading (see **Box 2** for preparation of UVBγTu cells). To generate control unloaded type-1 polarized DCs, do not add UVBγ-Tu cells to the maturing DCs.
▲ **CRITICAL STEP** Add UVBγ-Tu cells within 10 min after addition of DC maturation medium to ensure optimal take up of tumor antigens by maturing DCs.

2| Gently rock the plate back and forth to ensure even distribution of cells. Place the culture plates in the cell incubator at 37 °C and 5% (vol/vol) CO_2 for 4 h. The target maturation time is 4 h before harvesting, but the maturation period can be extended to 4.5 h in the case that many plates must be harvested.

3| Transfer the plates to a refrigerator for 5–10 min. Depending on the number of plates, you may perform harvesting of the DCs in batches so that the plates are not kept at 4 °C for longer than 30 min, as this might affect DC function.
▲ **CRITICAL STEP** In contrast to the classic cytokine-based maturation cocktail (supplemented with PGE_2), type-1 polarized mature DCs are more adherent to the plastic, displaying more elongated morphology. Incubation of the plates at 4 °C is important to reduce the adherence to the plastic and recover high numbers of viable DCs.

Collection of mature type-1 polarized mouse DCs ● TIMING 30–60 min

4| Detach DCs by vigorous pipetting and collect them in a 15-ml conical centrifuge tube. Refill each well with 1 ml of cold PBS and check for remaining adherent cells using a microscope. If some cells remained attached, vigorously pipette again and collect the cells in the 15-ml tube. Repeat until all cells are collected.
? TROUBLESHOOTING

5| Centrifuge the cells at 400*g* for 5 min at 4 °C. Resuspend cell pellets in all tubes with 1-ml of cold PBS and combine the UVBγ-Tu-loaded DCs (or unloaded DCs) into one tube. Assess viability of the cells by Trypan blue exclusion. If the viability is below 50–70%, we recommend discarding the batch of DCs and repeating Steps 1–5.

Cryopreservation and thawing of type-1 polarized mature DCs ● TIMING 40–50 min

6| Keep the harvested type-1 polarized mature DCs at 4 °C while preparing for cryopreservation. The number of DCs frozen per cryovial depends on the number of planned vaccine doses and the number of mice receiving the vaccine. Prepare to freeze the DCs in at least as many cryovials as the number of planned vaccine doses plus an additional vial for QC. For example, if mice need to receive three cycles of vaccination and two vaccine doses per cycle, then freeze the DCs in at least 7 (2 × 3 + 1) cryovials. Freeze at least 1×10^6 DCs per cryovial.

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7| Centrifuge the type-1 polarized mature DCs at 400*g* for 5 min at 4 °C and resuspend in FM1 (0.5 ml per cryovial). Place the cells on ice for 10 min and add the same volume of FM2. Place the cells in a temperature-controlled freezing container (e.g., Nalgene, Mr Frosty) and store at −80 °C overnight before their transfer to liquid nitrogen.
■ **PAUSE POINT** DCs can be frozen for up to 1 year of cryopreservation, after which the viability will decrease.

8| To thaw the cells, place the vials in a water bath at 37 °C and transfer the cells to cold PBS (20 ml) immediately after thawing. Spin down the DCs (5 min, 400*g*, 4 °C), resuspend them in 20 ml of fresh PBS and centrifuge again (5 min, 400*g*, 4 °C). Resuspend the cells in 1 ml of culture medium and count the cells.

Quality control of the generated DCs

9| To check the quality of the DCs, follow option A for phenotype analysis of the DCs, option B for analysis of IL-12 production, option C for *in vitro* evaluation of effector type-1 immune cell induction, option D for *in vivo* evaluation of DC migration, and option E for *in vivo* evaluation of systemic tumor-specific immunity. Although the *in vivo* induction of tumor-specific type-1 immunity represents the most definitive test of DC function, the analysis of IL-12 production is a very strong predictor of their overall functional status.

(A) Phenotype analysis of the DCs ● TIMING 40–50 min for staining and ~20 min for flow analysis

(i) Adjust the cell density of the type-1 polarized mature DCs to 1 × 10^6 cell per ml in culture medium.
(ii) Prepare five staining mixes by adding the following antibodies to 100 µl of blocking buffer: 1 µl of CD14-FITC, 5 µl of CCR7-PE, and 2.5 µl of CD80-APC (mix 1);
1 µl of IA/E^b-FITC and 2.5 µl of CD40-APC (mix 2);
1 µl of H2K^b-FITC and 1 µl of CD86-APC (mix 3);
1 µl of IgG_1-FITC, 5 µl of IgG-PE, 2.5 µl of $IgG2_a$-APC (mix 4); and
1 µl of IgG_{2a}-FITC and 10 µl of IgG_1-APC (mix 5).
(iii) Add 200 µl of cell suspension (2.0 × 10^5 cells per well) to 5 wells of a V-bottom 96-well plate and centrifuge at 400*g* for 5 min at 4 °C.
(iv) Discard the supernatant, add 200 µl of blocking buffer, and centrifuge at 400*g* for 5 min and at 4 °C.
(v) Discard the supernatant and add 100 µl of each staining mix prepared in the previous step to one well with cells in a 96-well plate and mix. After 20–30 min incubation at 4 °C in the dark, wash the cells with 100–200 µl of blocking buffer and resuspend in 100 µl of 4% (wt/vol) PFA.
(vi) Perform flow analysis following the instructions of the flow cytometer manufacturer (**Fig. 2a**).

(B) Analysis of IL-12 production ● TIMING 20–30 min for culture set-up and ~6 h for ELISA

(i) Start cultures of J558-CD40L cells in cell culture medium at 2 × 10^5 cells per ml and maintain between 1 × 10^5 and 1 × 10^6 cells per ml for at least 3–5 d after thawing before their use, to ensure their high viability and potency.
(ii) Adjust the cell density of the type-1 polarized mature DCs to 1 × 10^6 cell per ml in culture medium.
(iii) Add 100 µl of type-1 polarized mature DCs to 6 wells in a sterile 96-well flat-bottom plate.
(iv) Prepare 5 × 10^5 cells per ml of J558-CD40L cells in culture medium and add 100 µl of cell suspension to the 3 wells with DCs. Add 100 µl of culture medium to the remaining 3 wells for controls. Place the culture plate in an incubator (37 °C, 5% (vol/vol) CO_2) and collect the media from the cells after 24 h. Alternatively, soluble CD40L with enhancer (MEGACD40L, 1 µg/ml; Enzo Life Sciences, cat. no. ALX-522–120-C010) (ref. 151) can be used instead of J558-CD40L cells.
■ **PAUSE POINT** Supernatants may be stored at −20 °C until analysis. The maximum recommended storage time is 1 month.
(v) Perform IL-12 ELISA following the manufacturer's instructions (**Fig. 2b**).

(C) *In vitro* evaluation of effector type-1 immune cell induction ● TIMING 1–2 h for setting up the IVS culture, ~2 h for setting up the ELISpot plate, and ~5 h for developing the ELISpot plate

(i) Adjust the cell density of the type-1 polarized mature DCs to 1 × 10^6 cell per ml in culture medium.
(ii) Add 0.25 ml of tumor-loaded DCs in cell culture medium to each well of a 48-well plate (4.0 × 10^5 cells per well).
(iii) Prepare the splenocyte suspension (**Box 3**) and resuspend the splenocytes in cell culture medium at a density of 4.0 × 10^6 cells per ml. Add 0.25 ml of splenocytes to DC cultures to induce *in vitro* stimulation (IVS) and incubate at 37° C and 5% (vol/vol) CO_2 for 7–10 d.
(iv) On day 4 of culture, add IL-2 (25 U/ml) and IL-7 (0.5 ng/ml) to the DC–splenocyte co-cultures.
(v) Prepare the ELISpot plate. To do this, add 100 µl of 15 µg/ml AN18 anti-IFNγ-coating antibody to each well of an ELISpot plate in PBS, wrap the plate with Parafilm, and incubate overnight at 4° C.
(vi) Wash the wells by adding 200 µl of RPMI-1640 and wait 3–5 min before removing the media. Repeat the wash three times.

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(vii) Block the wells by adding 200 µl of culture medium for 30 min at 37 °C.
▲ **CRITICAL STEP** The IFNγ ELISpot plate must be pre-coated with IFNγ capture antibody at least 1 d before harvesting the splenocytes in the next step to ensure maximal efficiency. Alternatively, the plate may be coated for 2 h at 37° C; however, such incubation is less efficient.
■ **PAUSE POINT** The maximum recommended storage time for an anti-IFN-γ antibody precoated and blocked ELISpot plate is 72 h at 4° C.

(viii) Harvest the DC-stimulated splenocyte co-cultures on days 7–10 in a 15-ml conical centrifuge tube. Centrifuge the cells at 400*g* at room temperature for 3–5 min, discard the supernatant, and resuspend the cells in 5 ml of PBS. Centrifuge the cells at 400*g* and room temperature for 3 min, then resuspend the cells in 0.5 ml of cell culture medium and count the cells using a hemocytometer.

(ix) Discard the culture medium in the anti-IFN-γ antibody precoated and blocked plate (from Step 9C(vii)) and plate 100 µl of 1 × 10^5 splenocytes in six wells of the plate.

(x) Harvest the tumor cells (use the tumor cell line that was used for loading of DCs) in culture with 1 mM EDTA in PBS as described in step 1 of **Box 2** and centrifuge at 400*g* and room temperature for 5 min.

(xi) Resuspend the tumor cells at 1 × 10^6 cells per ml in culture medium and irradiate with 20 Gy. Centrifuge at 400*g* and room temperature for 5 min and resuspend the irradiated tumor cells at 4 × 10^5 cells per ml.

(xii) Add 100 µl of irradiated tumor cells (4 × 10^4 cells) to 3 wells with DC-stimulated splenocytes. Add 100 µl of culture medium to 3 wells of control DC splenocytes. Incubate the cultures at 37° C, 5% (vol/vol) CO_2 for 24–48 h.

(xiii) *Develop the ELISpot plate*. To do this, first wash the plate five times by soaking 3–5 min in 200–300 µl of wash buffer (PBS/0.05% (vol/vol) Tween 20). After the washes, add 100 µl of 1 µg/ml R4-6A2 biotinylated secondary antibody in PBS/0.5% (wt/vol) BSA and incubate at room temperature for 2 h. 10 min before the incubation is finished, prepare ABC reagent according to the manufacturer's instructions.

(xiv) Mix the ABC reagent and let it stand at room temperature for 30 min.

(xv) Wash the plate five times as in Step 9C(xiii), add 100 µl of ABC reagent, and incubate the plate at room temperature for 1 h.

(xvi) Wash the plate three times by soaking 3–5 min in wash buffer, followed by an additional two washes in PBS. While washing the plate, prepare AEC substrate according to the manufacturer's instructions.

(xvii) Add 100 µl of AEC reagent per well. Spots appear after 5–20 min, depending on the assay. Once spots are clearly visible, but before background appears, rinse the plate thoroughly in dH_2O. Remove the plastic lid from the plate and allow the plate to dry. Read the plate using an ELISpot reader (**Fig. 2c**).

(D) *In vivo* evaluation of DC migration ● TIMING 10–20 min for DC injection and ~15 min for imaging per five mice

▲ **CRITICAL** To conduct this analysis, generate the DCs from firefly luciferase (CAG-luc-eGFP) transgenic mice (FVB-Tg(CAG-luc,-GFP)L2G85Chco/J; The Jackson Laboratory, cat. no. 008450) (ref. 150). If you generate the DCs from B6 Cd45.1 mice, or from C57BL/6J mice, you must perform evaluation of DC migration by flow cytometric analysis instead of imaging (**Box 4**).

(i) Adjust the cell density of UVBγ-Tu-loaded DCs to 1 × 10^6 cell per ml in PBS.

(ii) Inject 50 µl of DCs (5 × 10^5) in PBS with a 1-cc syringe and a 27-gauge or 30-gauge needle into the footpad of the C57BL/6J mouse.

(iii) Image mice by IVIS after 1, 12, 24, and 48 h (**Fig. 2d**). To do this, inject luciferin (100 µl of 30 mg/ml i.p. with a 1-cc syringe and a 27-gauge or 30-gauge needle) before imaging. Follow the instructions outlined in Step 12 for animal handling and care to image the mice.

(E) *In vivo* evaluation of effector type-1 immune cell induction ● TIMING 8–10 h for preparation and injection of OVA-loaded DCs, 3–4 h for preparation of target cells, and 3–4 h for flow analysis of harvested splenocytes

(i) Resuspend the unloaded DC cells (from Step 8) in culture medium at 1 × 10^7 per ml and add OVA peptide to cells at 1.0 µg per 10^6 cells. Incubate in a 50-ml conical centrifugation tube at 37 °C for 1.5–2.0 h. Fill the tube with PBS to 35–40 ml and centrifuge at 400*g* for 5 min at room temperature.

(ii) Resuspend the cells in PBS at 3 × 10^6 cells per ml in PBS and inject 3–4 × 10^5 OVA-loaded DCs/0.1 ml of PBS s.c. with a 1-cc syringe and a 27-gauge or 30-gauge needle into the flank of naive mice.

(iii) *Antigen loading of target cells*. 3–5 d after vaccination with OVA-loaded DCs, isolate splenocytes from a B6 Cd45.1 mouse as described in **Box 3**. Resuspend splenocytes at 2 × 10^7 cells per ml in culture medium and divide into two 15-ml conical centrifugation tubes. Add OVA peptide to splenocytes in one of the tubes to load at 0.5 µg per 10^6 cells. Add nothing or a control peptide to the splenocytes in the other tube. Incubate both tubes at 37 °C for 1.5–2.0 h. Fill the tubes with PBS to 12–15 ml and centrifuge at 400*g* for 5 min at room temperature.

(iv) Label loaded splenocytes with CFSE and unloaded splenocytes with 20× diluted CFSE as described in **Box 5**. Mix unloaded $CFSE^{low}$- and loaded $CFSE^{high}$-labeled splenocytes at a 1:1 ratio in PBS to 2.5 × 10^7 cells per ml and inject

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200 µl i.v. into DC-vaccinated mice (3–5 d after vaccination with OVA-loaded DCs) and into at least two naive (unvaccinated) mice.

▲ CRITICAL STEP For the i.v. injections, keep the mouse warm under a heat lamp (or other heating device), making sure not to overheat the animal. After placing the animal in a restraint device, hold the tail between the thumb and forefinger and clean it with 70% (vol/vol) ethanol. Keep the tail under pressure and place the needle with the bevel facing upward at the middle or slightly distal part of the tail. Insert the needle at least 3 mm into the tail, while keeping the needle parallel to the vein. Inject the material in a slow, smooth motion, without aspirating. If swelling occurs or if there is resistance to the injection, remove the needle from the tail and repeat the procedure slightly above the initial site of injection.

▲ CRITICAL STEP The number of injected cells is not critical, although more cells are better, as the cells will be detected by flow cytometry. We aim for 5×10^6 cells per mouse.

(v) Harvest spleens 16 h afterward and isolate splenocytes as described in **Box 3**.

(vi) *Flow analysis*. Determine the specific killing by the comparative analysis of live $CFSE^{low}$ and $CFSE^{high}$ cells as presented in **Figure 2e**.

(vii) (Optional) Stain the cells for CD45.1 as described in step 9 of **Box 4**. Make a histogram of live $CD45.1^+$ cells and gate on $CFSE^{low}$ and $CFSE^{high}$ cells.

Inoculation of mice with tumor cells ● TIMING 5–10 min per five mice

10| Culture the relevant tumor cells for at least two passages after thawing before harvesting. Harvest the tumor cells as described in step 1 of **Box 2**. Wash the cells twice with 10–20 ml of PBS and resuspend in PBS for subsequent injection (5×10^6 per ml for MC38Luc or 20×10^6 per ml for ID8A cells).

11| Inject the tumor cells. Follow option A to perform i.p. injection (disseminated peritoneal tumor model) or option B for s.c. injection (localized/solid tumor).

(A) Intraperitoneal injection

(i) Use a 1-cc syringe and a 27-gauge needle to inject 1×10^6 MC38Luc or 4×10^6 ID8A cells in 200 µl of PBS i.p. into 6–8-week-old B6 mice for colorectal or ovarian cancer models, respectively.

▲ CRITICAL STEP To make the injection into lower left abdominal quadrant (the preferred injection site for i.p. injections), manually restrain the mice with dorsal recumbence (abdomen side up) and the cranial end of the animal pointed down. Enter the abdominal cavity with the needle bevel-side up and slightly angled (15–20 degrees). After penetrating the abdominal wall (~4–5 mm), attempt to aspirate (pulling back slightly on the needle) to ensure that abdominal organs, such as the bladder or colon, have not been penetrated.

(B) Subcutaneous injection

(i) Inject 0.5×10^6 MC38Luc cells in 100 µl PBS s.c. into the lower right flank.

▲ CRITICAL STEP For s.c. injections, shave the right flank of the mice and then manually restrain the mice with the right side pointed up. When penetrating the skin, hold the needle bevel-side up and angled 15–25 degrees. Advance the needle ~2–3 mm below the skin surface so that the tip is visible under the skin. When injecting, a bleb should develop, otherwise withdraw the needle slightly.

Monitoring of tumor progression ● TIMING ~15 min per five mice

12| Inject mice i.p. with luciferin (100 µl of 30 mg/ml using a 1-cc syringe and a 27-gauge or 30-gauge needle) before imaging. Wait 8 min after the luciferin injection before transferring the mice to a 5% (vol/vol) isoflurane chamber before imaging (firefly luciferase signal peaks after 10 min and then declines).

▲ CRITICAL STEP Optimal *in vivo* imaging time should be determined by taking images over incremental periods of time. We image mice every 5–7 d.

13| Use the control panel to manually set the imaging parameters or use the software's Imaging Wizard (under 'sequence setup') to automatically set the imaging parameters. Adjust the field of view depending on number of mice imaged. The field of view size affects the sensitivity. Note that longer exposure time, higher binning, and a lower F/Stop (a wider aperture allows more light to reach the charge-coupled device) increase sensitivity. Images should take no longer than 5 min of exposure time.

▲ CRITICAL STEP Luciferase signal can decrease at later time points (owing to ascites accumulation in the ID8A model or because of ulceration in the MC38 s.c. model), therefore, tumor progression should be concomitantly monitored by increase in mouse weight or circumference (for i.p. tumors) or by assessing tumor size with a caliper (for MC38 s.c. tumors).

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Induction of tumor-infiltrating specific type-1 immune cells ● TIMING 20–30 min for vaccine preparation and ~10 min for injection into five mice

▲ **CRITICAL** We recommend imaging and weighing mice 1 d before therapy initiation and grouping mice randomly into untreated and treated sets.

14| Thaw DCs as described in Step 8. Refer to **Table 1** to determine the number of vials needed. Dilute the cells to 3×10^6 cells per ml in PBS and inject $3–4 \times 10^5$ DCs per 0.1 ml of PBS s.c. into the left flank of tumor-bearing mice (as in Step 10B(i)), on days 3–7 after injecting tumor cells as a repeat injection two to four times, with a 1-week interval between treatments.

▲ **CRITICAL STEP** If a s.c. tumor model is investigated, DCs should be injected s.c. into the opposite flank.

15| Perform TME modulation 3 d after each s.c. DC vaccination. Follow option A to modulate the TME by i.p. injection of the type-1 polarized mature DCs or option B for CKM adjuvant-mediated modulation.

(A) DC injection

(i) Inject $3–4 \times 10^5$ type-1 polarized mature DCs in 0.2 ml of PBS i.p. (for the i.p. model) or $3–4 \times 10^5$ type-1 polarized mature DCs in 0.05 ml intratumorally (for the s.c. model).

(B) CKM adjuvant injection

(i) Inject 1×10^4 IU of IFN-α and 50 μg Ampligen (**Fig. 3b**) in 0.2 ml of PBS i.p. (for the i.p. model) or 1×10^4 IU of IFN-α and 50 μg Ampligen in 0.05 ml intratumorally (for the s.c. model).

16| Inject 0.2 ml of 500 μg/ml celecoxib (in 12.5% (vol/vol) DMSO) or 0.2 ml of 2.5 mg/ml (1.25% (vol/vol) DMSO) aspirin i.p. every 2–3 d starting as early as day 4 after tumor inoculation.

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***Ex vivo* immunomonitoring ● TIMING 8–10 h for setting up an ELISpot plate and ~6 h for developing the ELISpot plate** ▲ **CRITICAL** Initiate Step 17 before the mice from Step 18 approach the relevant time point.

17| Add 100 μl of 15 μg/ml AN18 anti-IFNγ coating antibody in PBS to each well of an ELISpot plate and wrap the plate with Parafilm to precoat the plate. Incubate overnight at 4 °C. Alternatively, the plate may be coated for 2 h at 37° C; however, such incubation is less efficient.

18| Monitor animals in accordance with institutional guidelines. Kill mice by CO_2 inhalation, followed by cervical dislocation when one of the following end points is met: weight exceeds 140% of initial body weight, mice appear to be in distress (anorexic, emaciated, lethargic), changes in behavior occur (immobile and/or non-responsive), tumor size exceeds 2 cm in diameter, or ulceration occurs (for s.c. tumors).

▲ **CRITICAL STEP** Do not allow mice to die due to high tumor burden. Typically, the untreated mice must be euthanized before day 30 (MC38 model) or day 40 (ID8A model).

19| Isolate the TILs/TALs from the tumors (in the case of the s.c. tumor model; option A) or peritoneal washes/cancer ascites (in the case of the i.p. tumor model; option B).

(A) Harvesting TILs from tumors

(i) Make a cut to enter the peritoneal cavity and collect the tumor tissue into 15-ml conical tubes filled with 2-ml of PBS.

(ii) Add 2 ml of PBS to a Petri dish to cover the bottom. Transfer the harvested tumor tissue and mince it with two scalpels to <0.5-mm pieces. Transfer the tumor pieces to a new 15-ml conical centrifugation tube and add enough digestion buffer to cover the tumor pieces.

▲ **CRITICAL STEP** If the tumor pieces are very small, transfer them directly to a 2-ml Eppendorf centrifugation tube, add 500 μl of digestion buffer, and then mash with the rubber piston of the syringe.

(iii) Incubate tumors at 37 °C for 20–45 min on a rotator. After the tissue digestion, transfer the tumors to a pre-wetted 100-μm strainer placed on top of a 50-ml conical centrifuge tube and mash with the rubber piston of the syringe and continuously wash with PBS to a final volume of 10–20 ml.

(iv) Centrifuge the cells at 400*g* for 3–5 min at room temperature, discard the supernatant, and resuspend the cells in 1 ml ACK lysis buffer to remove the red blood cells. Add an additional 3 ml of ACK lysis buffer on top and rotate the conical centrifuge tube to mix. Incubate the cells at room temperature for 4 min.

(v) Add 30 ml of PBS and centrifuge the cells at 400*g* for 3 min at room temperature, then resuspend them in 20 ml of PBS and transfer the mixture to a 70-μm strainer.

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(vi) Count the cells using a hemocytometer.
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(vii) Centrifuge the cells at 400*g* for 3–5 min at room temperature, resuspend the cells in 3 ml of 60% (vol/vol) SIP, and transfer them to 15-ml conical tube.

(B) Harvesting TALs from peritoneal washes/cancer ascites

(i) Use a 10-ml syringe and an 18-gauge needle to obtain the peritoneal washes/cancer ascites. Fill the syringe with 3–5 ml of PBS. After penetrating the abdominal wall (~4–5 mm) into the lower left abdominal quadrant, inject PBS into the i.p. cavity and then aspirate as much peritoneal fluid as possible. Ensure that the abdominal organs, such as the bladder or colon, have not been penetrated. In such case, try to readjust the needle by pulling it backward for a few millimeters and changing the angle. Blood or fecal material may indicate that the intestine has been ruptured. If fecal material or blood is aspirated, discard the collected sample. Transfer the peritoneal fluid to a 50-ml conical tube.

(ii) Centrifuge the cells at 400*g* for 3–5 min at room temperature, discard the supernatant, and resuspend the cells in 1-ml of ACK lysis buffer to remove the red blood cells. Add an additional 3 ml of ACK lysis buffer on top and rotate the conical centrifuge tube to mix. Incubate the cells at room temperature for 4 min.

(iii) Add 30 ml of PBS and centrifuge the cells at 400*g* for 3 min at room temperature, then resuspend them in 20-ml of PBS, and transfer the mixture to a 70-µm strainer.

(iv) Count the cells using a hemocytometer.

(v) Centrifuge the cells at 400*g* for 3 min at room temperature, resuspend the cells in 3 ml of 60% (vol/vol) SIP, and transfer them to a 15-ml conical tube.

(vi) Layer 3 ml of 45% (vol/vol) SIP slowly on top of the 60% (vol/vol) SIP layer by expelling the 45% (vol/vol) SIP at a shallow angle down the side of the centrifuge tube. Take care not to mix with the bottom layer. Repeat with a similar technique to layer 3 ml of 34% (vol/vol) SIP on top of the 45% (vol/vol) SIP layer.

(vii) Centrifuge the tubes at 2,400*g* for 30 min at room temperature without applying the brake.

(viii) Collect the bottom interface (**Fig. 4a**) (~2–3 ml) into a new 15-ml conical centrifugation tube, fill the tube to 15 ml with PBS, and centrifuge at 600*g* for 10 min at room temperature. Resuspend the cell pellet in 0.5 ml of the cell culture medium.
▲ CRITICAL STEP High centrifugation speed and dilution of transferred TILs/TALs with PBS is necessary to allow effective centrifugation.
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20| Adjust the cell concentration to 1×10^6 per ml with cell culture medium. If the cell number is $<1 \times 10^6$ per ml, resuspend the samples in 0.7 ml of the cell culture medium.
▲ CRITICAL STEP When analyzing the ELISpot data, normalize the number of spots to the number of cells loaded into the wells.

21| Isolate splenocytes as described in **Box 3** 4–5 d after TME modulation (Step 15) and resuspend to 5×10^6 per ml in culture medium.

22| Wash the ELISpot plate from Step 17 three times by adding 200 µl of RPMI-1640 and waiting 3–5 min before removing media. Block the plate by adding 200 µl of culture medium for 30 min at 37° C.

23| Discard the culture medium from the plate and dispense 100 µl (5×10^5) splenocytes and 1×10^5 TILs/TALs into six wells of the ELISpot plate. Harvest the tumor cells in culture with 1 mM EDTA in PBS and centrifuge at room temperature as described in step 1 of **Box 2**. Resuspend the tumor cells at 8×10^5 cells per ml of culture medium and irradiate with 20 Gy. Add 100 µl of (4×10^4) tumor cells to three wells with splenocytes and TIL/TALs (**Fig. 4b**). Add 100 µl of culture medium to make three control wells of splenocytes and TIL/TALs (**Fig. 4b**). Incubate the cultures at 37° C, 5% (vol/vol) CO_2 for 24–48 h.
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24| Develop the ELISpot plate as in Step 9C(xiii).
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Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
step 3 of **Box 3**	Low splenocyte count	The cell strainer is clogged	Make sure to mash the splenocytes thoroughly and wash extensively with PBS
step 4 of **Box 3**	Low splenocyte count/ viability	Prolonged exposure to ACK buffer compromises cell viability	Decrease incubation time in ACK buffer; decrease centrifugation time. Dilute cells with culture medium after incubation in ACK buffer
Step 4	Low yield of DCs	Insufficient washing after maturation of $CD11c^+$ cells	Application of the correct pressure for washing the cells off takes practice. Increase the washing steps and control frequently using a microscope
		Adherence to plastic of cells differs depending on the plate manufacturer. If plates from other manufacturers are used, adherence and DC recovery may vary	Adapt the DC-harvesting technique (washing intensity and number of washing steps) when changing the plate manufacturer
Step 13	Images take >5 min of exposure time	There are no luciferase-expressing tumor cells growing in the mice	Verify that the luciferin reagent is working and that tumor cells express luciferase by imaging them in the presence of luciferin in a cell culture plate
			Image mice after 2–3 d, allowing the tumors to grow bigger
Step 16	Precipitation of celecoxib	The suspension was left too long at room temperature	Make sure to prepare celecoxib prior to administration or consider making the final suspension with a higher DMSO content
Step 19A(vi)	Low TIL count/viability	Prolonged exposure to digestion buffer compromises cell viability, whereas an incubation time that is too short can limit effective cell isolation	Adjust the incubation time in the digestion buffer
			In the case that the incubation time must be prolonged, add FBS or BSA (1–2% (vol/vol)) to the digestion buffer to increase the viability of isolated cells
Step 19B(viii)	No layers visible	Brake was applied during centrifugation	Make sure that the brake is not applied during centrifugation and that the acceleration is slow
		Mixing of layers while adding SIP	Add SIP with a lower concentration, using caution to avoid disturbing the bottom layer
Steps 23 and 24	High background and/or no distinct dots visible	Tumor cells are overgrown	Consider irradiating tumor cells with a higher dose or decreasing the incubation time
		Too few lymphocytes were plated	Consider combining lymphocytes from two to three mice of the same group

● TIMING

This protocol covers a minimum of 30 d. A workflow schema is provided in **Figure 1**

Generation of tumor-loaded DCs

Box 1, Flt3L-B16 cell injection into donor mice, day −11 ± 1: 1 h
Box 2, preparation of UVBγ-Tu cells, day −1: 1.5 h
Box 3, preparation of the splenocyte suspension, day 0: 1 h
Box 4, extraction of cells from the lymph nodes of mice, days 1–3: 2 h
Box 5, labeling of the cells with CFSE (several time points): 30–45 min
Box 6, flow analysis of the spleens for *in vivo* specific cytotoxicity assay, days 13–15: 2–3 h
Steps 1–8, generation, collection, cryopreservation, and thawing of type-1 polarized DCs from donor mice, day 0: 10 h

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QC of DCs

Step 9, phenotype analysis, *in vitro* evaluation of type-1 immune cell induction, and J558-CD40L restimulation of DCs from donor mice, day 1: 6 h
Step 9A, phenotype analysis of the DCs, day 2: 40–50 min for staining and ~20 min for flow analysis
Step 9B, ELISA to evaluate the capacity of DCs from donor mice to produce IL-12, day 2: 20–30 min for culture set-up and ~6 h for ELISA
Step 9C, *in vitro* evaluation of effector type-1 immune cell induction, day 2: 1–2 h for setup of the IVS culture, ~2 h for setup of the ELISpot plate, and ~5 h for developing the ELISpot plate
Step 9D, *in vivo* evaluation of DC migration, day 2: 10–20 min for DC injection and ~15 min for imaging per five mice
Step 9E, *in vivo* evaluation of effector type-1 immune cell induction, day 2: 8–10 h for preparation and injection of OVA-loaded DCs, 3–4 h for preparation of target cells, and 3–4 h for flow analysis of harvested splenocytes
Steps 10 and 11, inoculation of experimental mice with tumor cells, day −4: 5–10 min per five mice
Steps 12 and 13, monitoring of tumor progression (BLI) of experimental mice, weekly from day −1: ~15 min per five mice
Steps 14–16, induction of tumor-infiltrating-specific type-1 immune responses in experimental mice with type-1 polarized DCs (and CKM), weekly from days 0 and 3 : 20–30 min for vaccine preparation and ~10 min for injection into five mice

Monitoring of the therapeutic and immunomodulatory effects

Step 17, preparation of ELISpot plate to detect intratumoral type-1 immune cells from experimental mice, day 12: 10 min
Steps 18–23, isolation of splenocytes and TILs/TALs from experimental mice and setup of the ELISpot assay, days 13–15: 8–10 h
Step 24, developing of ELISpot plate to detect intratumoral type-1 immune cells from experimental mice, days 16/17: 6 h

ANTICIPATED RESULTS

Typical results of immunotherapy-induced changes (following DC therapy and combinatorial approaches with TME modulation) in animal survival are shown in **Figure 3c**,**d**. It may be expected that the s.c. injections of tumor-loaded type-1 polarized DCs (3×10^5 cells on days 5 and 12), used as a single treatment, will not significantly prolong the survival of MC38 intraperitoneal tumor–bearing mice. The data from this therapeutic model are in contrast to prevention models[14] and the therapy of genetically manipulated tumors with DCs loaded with strong antigens[81,152]. However, additional intraperitoneal administration of DCs (on days 8 and 15) significantly prolongs survival of the mice (median survival = 29 d versus 23 d; $P = 0.0021$; $n = 10$ mice per group) (**Fig. 3c**). Furthermore, we evaluated the effect of substituting additional intraperitoneal administration of DCs with intraperitoneal injection of CKM (IFN-α and poly-IC) and COX-2 inhibitor (celecoxib or aspirin) on prolongation of mice survival (Steps 15 and 16). Typically, mice treated with s.c. injection of DCs only- (**Fig. 3d**) or a CKM regimen alone (**Fig. 3e**) do not demonstrate an advantage in survival. However, the combinatorial approach shows significant prolongation of mouse survival (median survival = 45 d (celecoxib); $P < 0.0001$ and 42 (aspirin); $P = 0.0014$ versus 35 d for control; $n = 10$ mice per group)) (**Fig. 3d**).

The current protocol is designed to enhance the infiltration of tumor-specific CTLs into the TME (**Fig. 4**). High tumor production of CCL5/RANTES (ligand for CCR5) and CXCL9/MIG, CXCL10/IP10, and CXCL11/ITAC (three known ligands for CXCR3) is associated with high CTL infiltration in cancers[153–155]. Our own studies show strong correlations between the intratumoral production of CCL5, CXCL9, and CXCL10 and local infiltration of $CD8^+$ granzyme B^+ CTLs[16]. To achieve the antitumor effectiveness of immune responses in the cancer microenvironment, TME modulation is critical and can be induced by administration of a CKM regimen, e.g., IL-12-producing DCs; combination of IFN-α, poly-IC and COX2 blockers; and chemokine-expressing *Vaccinia* viruses. However, additional combinatorial approaches are likely to be effective for selective attraction of specific subsets of immune cells. In this regard, the currently presented protocol can be modified to include additional elements and readouts, for example, ELISpot assays that detect T cells that produce alternative cytokines, and thus are able to detect T cells with different functions.

ACKNOWLEDGMENTS We dedicate this article to Eva Wieckowski (who died unexpectedly on 14 December 2015). We thank T.J. Curiel for providing the ID8A ovarian cancer cell line, P. Lane for CD40L-expressing J558 cells, and M. Kronenberg for Flt3-L-expressing B16 cells. We thank K. Lemon for assistance with monitoring the mice and BLI, and P. Bailey for critical review and editorial comments. This work was supported by NIH grant CA132714 (to P.K.), by a 2015 CRI Clinical Strategy Team Grant (to P.K.), and by the David C. Koch Regional Therapy Cancer Center (D.L.B.).

AUTHOR CONTRIBUTIONS N.O., J.U., and R.R. participated in data generation; N.O. and J.U. selected and evaluated the experimental data; N.O., J.U., R.M., R.R., E.W., P.K., and D.L.B. participated in the design of the protocol; N.O. and P.K. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare competing financial interests: details are available in the online version of the paper.

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1. Johnson, L.A. & June, C.H. Driving gene-engineered T cell immunotherapy of cancer. *Cell Res.* **27**, 38–58 (2017).
2. Kalos, M. & June, C.H. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity* **39**, 49–60 (2013).

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3. Maus, M.V. *et al.* Adoptive immunotherapy for cancer or viruses. *Annu. Rev. Immunol.* **32**, 189–225 (2014).
4. Palucka, K. & Banchereau, J. Cancer immunotherapy via dendritic cells. *Nat. Rev. Cancer* **12**, 265–277 (2012).
5. Topalian, S.L. *et al.* Immunotherapy: the path to win the war on cancer? *Cell* **161**, 185–186 (2015).
6. Tumeh, P.C. *et al.* PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature* **515**, 568–571 (2014).
7. Herbst, R.S. *et al.* Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* **515**, 563–567 (2014).
8. Brahmer, J.R. *et al.* Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. *N. Engl. J. Med.* **366**, 2455–2465 (2012).
9. Taube, J.M. *et al.* Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin. Cancer Res.* **20**, 5064–5074 (2014).
10. Topalian, S.L. *et al.* Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N. Engl. J. Med.* **366**, 2443–2454 (2012).
11. Ji, R.R. *et al.* An immune-active tumor microenvironment favors clinical response to ipilimumab. *Cancer Immunol. Immunother.* **61**, 1019–1031 (2012).
12. Gajewski, T.F., Schreiber, H. & Fu, Y.X. Innate and adaptive immune cells in the tumor microenvironment. *Nat. Immunol.* **14**, 1014–1022 (2013).
13. Mailliard, R.B. *et al.* Alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. *Cancer Res.* **64**, 5934–5937 (2004).
14. Giermasz, A.S. *et al.* Type-1 polarized dendritic cells primed for high IL-12 production show enhanced activity as cancer vaccines. *Cancer Immunol. Immunother.* **58**, 1329–1336 (2009).
15. Lee, J.J., Foon, K.A., Mailliard, R.B., Muthuswamy, R. & Kalinski, P. Type 1-polarized dendritic cells loaded with autologous tumor are a potent immunogen against chronic lymphocytic leukemia. *J. Leukoc. Biol.* **84**, 319–325 (2008).
16. Muthuswamy, R. *et al.* NF-kappaB hyperactivation in tumor tissues allows tumor-selective reprogramming of the chemokine microenvironment to enhance the recruitment of cytolytic T effector cells. *Cancer Res.* **72**, 3735–3743 (2012).
17. Muthuswamy, R., Corman, J.M., Dahl, K., Chatta, G.S. & Kalinski, P. Functional reprogramming of human prostate cancer to promote local attraction of effector CD8(+) T cells. *Prostate* **76**, 1095–1105 (2016).
18. Muthuswamy, R., Wang, L., Pitteroff, J., Gingrich, J.R. & Kalinski, P. Combination of IFNα and poly-I:C reprograms bladder cancer microenvironment for enhanced CTL attraction. *J. Immunother. Cancer* **3**, 6 (2015).
19. Downs-Canner, S. *et al.* Complement inhibition: a novel form of immunotherapy for colon cancer. *Ann. Surg. Oncol.* **23**, 655–662 (2016).
20. Bach, S. *et al.* A yeast-based assay to isolate drugs active against mammalian prions. *Methods* **39**, 72–77 (2006).
21. Galon, J., Fridman, W.H. & Pages, F. The adaptive immunologic microenvironment in colorectal cancer: a novel perspective. *Cancer Res.* **67**, 1883–1886 (2007).
22. Zhang, L. *et al.* Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N. Engl. J. Med.* **348**, 203–213 (2003).
23. Sato, E. *et al.* Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc. Natl. Acad. Sci. USA* **102**, 18538–18543 (2005).
24. Fridman, W.H. *et al.* Prognostic and predictive impact of intra- and peritumoral immune infiltrates. *Cancer Res.* **71**, 5601–5605 (2011).
25. Fridman, W.H., Pages, F., Sautes-Fridman, C. & Galon, J. The immune contexture in human tumours: impact on clinical outcome. *Nat. Rev. Cancer* **12**, 298–306 (2012).
26. Pages, F. *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N. Engl. J. Med.* **353**, 2654–2666 (2005).
27. Llosa, N.J. *et al.* The vigorous immune microenvironment of microsatellite instable colon cancer is balanced by multiple counter-inhibitory checkpoints. *Cancer Discov.* **5**, 43–51 (2015).
28. Le, D.T. *et al.* PD-1 blockade in tumors with mismatch-repair deficiency. *N. Engl. J. Med.* **372**, 2509–2520 (2015).
29. Astsaturov, I. *et al.* Amplification of virus-induced antimelanoma T-cell reactivity by high-dose interferon-alpha2b: implications for cancer vaccines. *Clin. Cancer Res.* **9**, 4347–4355 (2003).
30. Rosenberg, S.A. *et al.* Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J. Immunol.* **175**, 6169–6176 (2005).
31. Fox, B.A. *et al.* Defining the critical hurdles in cancer immunotherapy. *J. Transl. Med.* **9**, 214 (2012).
32. Apetoh, L. *et al.* Consensus nomenclature for CD8+ T cell phenotypes in cancer. *Oncoimmunology* **4**, e998538 (2015).
33. Kalinski, P., Muthuswamy, R. & Urban, J. Dendritic cells in cancer immunotherapy: vaccines and combination immunotherapies. *Expert Rev. Vaccines* **12**, 285–295 (2013).
34. Gajewski, T.F. The next hurdle in cancer immunotherapy: overcoming the non-T-cell-inflamed tumor microenvironment. *Semin. Oncol.* **42**, 663–671 (2015).
35. Tang, H. *et al.* Facilitating T cell infiltration in tumor microenvironment overcomes resistance to PD-L1 blockade. *Cancer Cell* **29**, 285–296 (2016).
36. Fuertes, M.B., Woo, S.R., Burnett, B., Fu, Y.X. & Gajewski, T.F. Type I interferon response and innate immune sensing of cancer. *Trends Immunol.* **34**, 67–73 (2013).
37. Woo, S.R. *et al.* STING-dependent cytosolic DNA sensing mediates innate immune recognition of immunogenic tumors. *Immunity* **41**, 830–842 (2014).
38. Ohkuri, T. *et al.* STING contributes to antiglioma immunity via triggering type I IFN signals in the tumor microenvironment. *Cancer Immunol. Res.* **2**, 1199–1208 (2014).
39. Kreutz, M., Tacken, P.J. & Figdor, C.G. Targeting dendritic cells—why bother? *Blood* **121**, 2836–2844 (2013).
40. Yang, X. *et al.* Targeting the tumor microenvironment with interferon-β bridges innate and adaptive immune responses. *Cancer Cell* **25**, 37–48 (2014).
41. Wong, J.L., Muthuswamy, R., Bartlett, D.L. & Kalinski, P. IL-18-based combinatorial adjuvants promote the intranodal production of CCL19 by NK cells and dendritic cells of cancer patients. *Oncoimmunology* **2**, e26245 (2013).
42. Wong, J.L., Berk, E., Edwards, R.P. & Kalinski, P. IL-18-primed helper NK cells collaborate with dendritic cells to promote recruitment of effector CD8+ T cells to the tumor microenvironment. *Cancer Res.* **73**, 4653–4662 (2013).
43. Kalinski, P. & Gingrich, J.R. Toward improved effectiveness of bladder cancer immunotherapy. *Immunotherapy* **7**, 1039–1042 (2015).
44. Lu, H. TLR agonists for cancer immunotherapy: tipping the balance between the immune stimulatory and inhibitory effects. *Front. Immunol.* **5**, 83 (2014).
45. Liu, Z. *et al.* CXCL11-armed oncolytic poxvirus elicits potent antitumor immunity and shows enhanced therapeutic efficacy. *Oncoimmunology* **5**, e1091554 (2016).
46. Francis, L. *et al.* Modulation of chemokines in the tumor microenvironment enhances oncolytic virotherapy for colorectal cancer. *Oncotarget* **7**, 22174–22185 (2016).
47. Kantoff, P.W. *et al.* Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N. Engl. J. Med.* **363**, 411–422 (2010).
48. Fong, L. *et al.* Activated lymphocyte recruitment into the tumor microenvironment following preoperative sipuleucel-T for localized prostate cancer. *J. Natl. Cancer Inst.* **106** http://dx.doi.org/10.1093/jnci/dju268 (2014).
49. Inaba, K., Young, J.W. & Steinman, R.M. Direct activation of CD8+ cytotoxic T lymphocytes by dendritic cells. *J. Exp. Med.* **166**, 182–194 (1987).
50. Caux, C., Dezutter-Dambuyant, C., Schmitt, D. & Banchereau, J. GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* **360**, 258–261 (1992).
51. Inaba, K. *et al.* Identification of proliferating dendritic cell precursors in mouse blood. *J. Exp. Med.* **175**, 1157–1167 (1992).
52. Bender, A., Sapp, M., Schuler, G., Steinman, R.M. & Bhardwaj, N. Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. *J. Immunol. Methods* **196**, 121–135 (1996).
53. Schuler, G. & Steinman, R.M. Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J. Exp. Med.* **186**, 1183–1187 (1997).
54. Sallusto, F. & Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J. Exp. Med.* **179**, 1109–1118 (1994).
55. Peters, J.H. *et al.* Signals required for differentiating dendritic cells from human monocytes *in vitro*. *Adv. Exp. Med. Biol.* **329**, 275–280 (1993).
56. Brasel, K., De Smedt, T., Smith, J.L. & Maliszewski, C.R. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. *Blood* **96**, 3029–3039 (2000).
57. Saunders, D. *et al.* Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J. Exp. Med.* **184**, 2185–2196 (1996).

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58. Maraskovsky, E. *et al.* Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. *J. Exp. Med.* **184**, 1953–1962 (1996).
59. Brasel, K. *et al.* Hematologic effects of flt3 ligand *in vivo* in mice. *Blood* **88**, 2004–2012 (1996).
60. Albert, M.L., Jegathesan, M. & Darnell, R.B. Dendritic cell maturation is required for the cross-tolerization of CD8+ T cells. *Nat. Immunol.* **2**, 1010–1017 (2001).
61. Sparwasser, T. *et al.* Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. *Eur. J. Immunol.* **28**, 2045–2054 (1998).
62. Binder, R.J., Anderson, K.M., Basu, S. & Srivastava, P.K. Cutting edge: heat shock protein gp96 induces maturation and migration of CD11c+ cells *in vivo*. *J. Immunol.* **165**, 6029–6035 (2000).
63. Kalinski, P. *et al.* Natural killer-dendritic cell cross-talk in cancer immunotherapy. *Exp. Opin. Biol. Ther.* **5**, 1303–1315 (2005).
64. Jonuleit, H. *et al.* Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. *Eur. J. Immunol.* **27**, 3135–3142 (1997).
65. Anguille, S., Smits, E.L., Lion, E., van Tendeloo, V.F. & Berneman, Z.N. Clinical use of dendritic cells for cancer therapy. *Lancet Oncol.* **15**, e257–e267 (2014).
66. Vieira, P.L., de Jong, E.C., Wierenga, E.A., Kapsenberg, M.L. & Kalinski, P. Development of Th1-inducing capacity in myeloid dendritic cells requires environmental instruction. *J. Immunol.* **164**, 4507–4512 (2000).
67. Kalinski, P., Schuitemaker, J.H., Hilkens, C.M. & Kapsenberg, M.L. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. *J. Immunol.* **161**, 2804–2809 (1998).
68. Kalinski, P., Vieira, P.L., Schuitemaker, J.H., de Jong, E.C. & Kapsenberg, M.L. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. *Blood* **97**, 3466–3469 (2001).
69. Whittaker, D.S., Bahjat, K.S., Moldawer, L.L. & Clare-Salzler, M.J. Autoregulation of human monocyte-derived dendritic cell maturation and IL-12 production by cyclooxygenase-2-mediated prostanoid production. *J. Immunol.* **165**, 4298–4304 (2000).
70. Hilkens, C.M., Kalinski, P., de Boer, M. & Kapsenberg, M.L. Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood* **90**, 1920–1926 (1997).
71. Kalinski, P., Hilkens, C.M., Snijders, A., Snijdewint, F.G. & Kapsenberg, M.L. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J. Immunol.* **159**, 28–35 (1997).
72. Butterfield, L.H., Gooding, W. & Whiteside, T.L. Development of a potency assay for human dendritic cells: IL-12p70 production. *J. Immunother.* **31**, 89–100 (2008).
73. Gustafsson, K. *et al.* Recruitment and activation of natural killer cells *in vitro* by a human dendritic cell vaccine. *Cancer Res.* **68**, 5965–5971 (2008).
74. Wesa, A., Kalinski, P., Kirkwood, J.M., Tatsumi, T. & Storkus, W.J. Polarized type-1 dendritic cells (DC1) producing high levels of IL-12 family members rescue patient TH1-type antimelanoma CD4+ T cell responses *in vitro*. *J. Immunother.* **30**, 75–82 (2007).
75. Carreno, B.M. *et al.* IL-12p70-producing patient DC vaccine elicits Tc1-polarized immunity. *J. Clin. Invest.* **123**, 3383–3394 (2013).
76. Okada, H. *et al.* Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with α-type 1 polarized dendritic cells and polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. *J. Clin. Oncol.* **29**, 330–336 (2011).
77. Zitvogel, L. *et al.* IL-12-engineered dendritic cells serve as effective tumor vaccine adjuvants *in vivo*. *Ann. N. Y. Acad. Sci.* **795**, 284–293 (1996).
78. Xu, S. *et al.* Rapid high efficiency sensitization of CD8+ T cells to tumor antigens by dendritic cells leads to enhanced functional avidity and direct tumor recognition through an IL-12-dependent mechanism. *J. Immunol.* **171**, 2251–2261 (2003).
79. Ten Brinke, A., Karsten, M.L., Dieker, M.C., Zwaginga, J.J. & van Ham, S.M. The clinical grade maturation cocktail monophosphoryl lipid A plus IFNgamma generates monocyte-derived dendritic cells with the capacity to migrate and induce Th1 polarization. *Vaccine* **25**, 7145–7152 (2007).
80. Chiang, C.L. *et al.* Optimizing parameters for clinical-scale production of high IL-12 secreting dendritic cells pulsed with oxidized whole tumor cell lysate. *J. Transl. Med.* **9**, 198 (2011).
81. Hokey, D.A., Larregina, A.T., Erdos, G., Watkins, S.C. & Falo, L.D. Jr. Tumor cell loaded type-1 polarized dendritic cells induce Th1-mediated tumor immunity. *Cancer Res.* **65**, 10059–10067 (2005).
82. Budiu, R.A. *et al.* Immunobiology of human mucin 1 in a preclinical ovarian tumor model. *Oncogene* **32**, 3664–3675 (2013).
83. Schreibelt, G. *et al.* Commonly used prophylactic vaccines as an alternative for synthetically produced TLR ligands to mature monocyte-derived dendritic cells. *Blood* **116**, 564–574 (2010).
84. Zitvogel, L. *et al.* Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J. Exp. Med.* **183**, 87–97 (1996).
85. Mayordomo, J.I. *et al.* Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nat. Med.* **1**, 1297–1302 (1995).
86. Paglia, P., Chiodoni, C., Rodolfo, M. & Colombo, M.P. Murine dendritic cells loaded *in vitro* with soluble protein prime cytotoxic T lymphocytes against tumor antigen *in vivo*. *J. Exp. Med.* **183**, 317–322 (1996).
87. Fields, R.C., Shimizu, K. & Mule, J.J. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **95**, 9482–9487 (1998).
88. Schnurr, M. *et al.* Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and gammadelta T cells. *Cancer Res.* **62**, 2347–2352 (2002).
89. Strome, S.E. *et al.* Strategies for antigen loading of dendritic cells to enhance the antitumor immune response. *Cancer Res.* **62**, 1884–1889 (2002).
90. Chiang, C.L., Coukos, G. & Kandalaft, L.E. Whole tumor antigen vaccines: where are we? *Vaccines* **3**, 344–372 (2015).
91. Wieckowski, E. *et al.* Type-1 polarized dendritic cells loaded with apoptotic prostate cancer cells are potent inducers of CD8(+) T cells against prostate cancer cells and defined prostate cancer-specific epitopes. *Prostate* **71**, 125–133 (2010).
92. Specht, J.M. *et al.* Dendritic cells retrovirally transduced with a model antigen gene are therapeutically effective against established pulmonary metastases. *J. Exp. Med.* **186**, 1213–1221 (1997).
93. Mullins, D.W. *et al.* Route of immunization with peptide-pulsed dendritic cells controls the distribution of memory and effector T cells in lymphoid tissues and determines the pattern of regional tumor control. *J. Exp. Med.* **198**, 1023–1034 (2003).
94. Okada, N. *et al.* Administration route-dependent vaccine efficiency of murine dendritic cells pulsed with antigens. *Br. J. Cancer* **84**, 1564–1570 (2001).
95. Engleman, E.G. & Fong, L. Induction of immunity to tumor-associated antigens following dendritic cell vaccination of cancer patients. *Clin. Immunol.* **106**, 10–15 (2003).
96. Fong, L., Brockstedt, D., Benike, C., Wu, L. & Engleman, E.G. Dendritic cells injected via different routes induce immunity in cancer patients. *J. Immunol.* **166**, 4254–4259 (2001).
97. Bedrosian, I. *et al.* Intranodal administration of peptide-pulsed mature dendritic cell vaccines results in superior CD8+ T-cell function in melanoma patients. *J. Clin. Oncol.* **21**, 3826–3835 (2003).
98. Grover, A. *et al.* Intralymphatic dendritic cell vaccination induces tumor antigen-specific, skin-homing T lymphocytes. *Clin. Cancer Res.* **12**, 5801–5808 (2006).
99. Radomski, M. *et al.* Prolonged intralymphatic delivery of dendritic cells through implantable lymphatic ports in patients with advanced cancer. *J. Immunother. Cancer* **4**, 24 (2016).
100. Hu, J. *et al.* Induction of potent antitumor immunity by intratumoral injection of interleukin 23-transduced dendritic cells. *Cancer Res.* **66**, 8887–8896 (2006).
101. Nishioka, Y., Hirao, M., Robbins, P.D., Lotze, M.T. & Tahara, H. Induction of systemic and therapeutic antitumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin 12. *Cancer Res.* **59**, 4035–4041 (1999).
102. Thompson, E.D., Enriquez, H.L., Fu, Y.X. & Engelhard, V.H. Tumor masses support naive T cell infiltration, activation, and differentiation into effectors. *J. Exp. Med.* **207**, 1791–1804 (2010).
103. Melero, I. *et al.* Evolving synergistic combinations of targeted immunotherapies to combat cancer. *Nat. Rev. Cancer* **15**, 457–472 (2015).
104. Vasaturo, A., Verdoes, M., de Vries, J., Torensma, R. & Figdor, C.G. Restoring immunosurveillance by dendritic cell vaccines and manipulation of the tumor microenvironment. *Immunobiology* **220**, 243–248 (2015).
105. Pardoll, D.M. The blockade of immune checkpoints in cancer immunotherapy. *Nat. Rev. Cancer* **12**, 252–264 (2012).

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106. Ribas, A. *et al.* Dendritic cell vaccination combined with CTLA4 blockade in patients with metastatic melanoma. *Clin. Cancer Res.* **15**, 6267–6276 (2009).
107. Rosenblatt, J. *et al.* PD-1 blockade by CT-011, anti-PD-1 antibody, enhances *ex vivo* T-cell responses to autologous dendritic cell/myeloma fusion vaccine. *J. Immunother.* **34**, 409–418 (2011).
108. Wilgenhof, S. *et al.* Phase II study of autologous monocyte-derived mRNA electroporated dendritic cells (TriMixDC-MEL) plus ipilimumab in patients with pretreated advanced melanoma. *J. Clin. Oncol.* **34**, 1330–1338 (2016).
109. Melero, I., Hervas-Stubbs, S., Glennie, M., Pardoll, D.M. & Chen, L. Immunostimulatory monoclonal antibodies for cancer therapy. *Nat. Rev. Cancer* **7**, 95–106 (2007).
110. Fujita, M. *et al.* COX-2 blockade suppresses gliomagenesis by inhibiting myeloid-derived suppressor cells. *Cancer Res.* **71**, 2664–2674 (2011).
111. Wong, J.L., Obermajer, N., Odunsi, K., Edwards, R.P. & Kalinski, P. Synergistic COX2 induction by IFNγ and TNFα self-limits type-1 immunity in the human tumor microenvironment. *Cancer Immunol. Res.* **4**, 303–311 (2016).
112. Obermajer, N., Muthuswamy, R., Lesnock, J., Edwards, R.P. & Kalinski, P. Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells. *Blood* **118**, 5498–5505 (2011).
113. Vacchelli, E. *et al.* Trial watch: IDO inhibitors in cancer therapy. *Oncoimmunology* **3**, e957994 (2014).
114. Vanneman, M. & Dranoff, G. Combining immunotherapy and targeted therapies in cancer treatment. *Nat. Rev. Cancer* **12**, 237–251 (2012).
115. Li, J. *et al.* Chemokine expression from oncolytic *Vaccinia* virus enhances vaccine therapies of cancer. *Mol. Ther.* **19**, 650–657 (2011).
116. Jensen, S.M. *et al.* Signaling through OX40 enhances antitumor immunity. *Semin. Oncol.* **37**, 524–532 (2010).
117. Dranoff, G. Experimental mouse tumour models: what can be learnt about human cancer immunology? *Nat. Rev. Immunol.* **12**, 61–66 (2011).
118. Ngiow, S.F., Loi, S., Thomas, D. & Smyth, M.J. Mouse models of tumor immunotherapy. *Adv. Immunol.* **130**, 1–24 (2016).
119. Betts, M.R. *et al.* Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. *J. Immunol. Methods* **281**, 65–78 (2003).
120. Zaritskaya, L., Shurin, M.R., Sayers, T.J. & Malyguine, A.M. New flow cytometric assays for monitoring cell-mediated cytotoxicity. *Expert Rev. Vaccines* **9**, 601–616 (2010).
121. Klenerman, P., Cerundolo, V. & Dunbar, P.R. Tracking T cells with tetramers: new tales from new tools. *Nat. Rev. Immunol.* **2**, 263–272 (2002).
122. Karsunky, H., Merad, M., Cozzio, A., Weissman, I.L. & Manz, M.G. Flt3 ligand regulates dendritic cell development from Flt3+ lymphoid and myeloid-committed progenitors to Flt3+ dendritic cells *in vivo*. *J. Exp. Med.* **198**, 305–313 (2003).
123. Pulendran, B. *et al.* Distinct dendritic cell subsets differentially regulate the class of immune response *in vivo*. *Proc. Natl. Acad. Sci. USA* **96**, 1036–1041 (1999).
124. Kalinski, P., Schuitemaker, J.H., Hilkens, C.M., Wierenga, E.A. & Kapsenberg, M.L. Final maturation of dendritic cells is associated with impaired responsiveness to IFN-gamma and to bacterial IL-12 inducers: decreased ability of mature dendritic cells to produce IL-12 during the interaction with Th cells. *J. Immunol.* **162**, 3231–3236 (1999).
125. Watchmaker, P.B. *et al.* Independent regulation of chemokine responsiveness and cytolytic function versus CD8+ T cell expansion by dendritic cells. *J. Immunol.* **184**, 591–597 (2010).
126. Hoffmann, T.K., Meidenbauer, N., Dworacki, G., Kanaya, H. & Whiteside, T.L. Generation of tumor-specific T-lymphocytes by cross-priming with human dendritic cells ingesting apoptotic tumor cells. *Cancer Res.* **60**, 3542–3549 (2000).
127. Albert, M.L. *et al.* Immature dendritic cells phagocytose apoptotic cells via alphavbeta5 and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* **188**, 1359–1368 (1998).
128. Snijders, A., Kalinski, P., Hilkens, C.M. & Kapsenberg, M.L. High-level IL-12 production by human dendritic cells requires two signals. *Int. Immunol.* **10**, 1593–1598 (1998).
129. Wieckowski, E. *et al.* Type-1 polarized dendritic cells loaded with apoptotic prostate cancer cells are potent inducers of CD8(+) T cells against prostate cancer cells and defined prostate cancer-specific epitopes. *Prostate* **71**, 125–133 (2011).
130. MartIn-Fontecha, A. *et al.* Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J. Exp. Med.* **198**, 615–621 (2003).
131. Wei, W.Z., Jones, R.F., Juhasz, C., Gibson, H. & Veenstra, J. Evolution of animal models in cancer vaccine development. *Vaccine* **33**, 7401–7407 (2015).
132. Mac Keon, S., Ruiz, M.S., Gazzaniga, S. & Wainstok, R. Dendritic cell-based vaccination in cancer: therapeutic implications emerging from murine models. *Front. Immunol.* **6**, 243 (2015).
133. Westwood, J.A., Darcy, P.K. & Kershaw, M.H. The potential impact of mouse model selection in preclinical evaluation of cancer immunotherapy. *Oncoimmunology* **3**, e946361 (2014).
134. Devaud, C. *et al.* Tissues in different anatomical sites can sculpt and vary the tumor microenvironment to affect responses to therapy. *Mol. Ther.* **22**, 18–27 (2014).
135. Devaud, C., John, L.B., Westwood, J.A., Darcy, P.K. & Kershaw, M.H. Immune modulation of the tumor microenvironment for enhancing cancer immunotherapy. *Oncoimmunology* **2**, e25961 (2013).
136. Rakoff-Nahoum, S. & Medzhitov, R. Toll-like receptors and cancer. *Nat. Rev. Cancer* **9**, 57–63 (2009).
137. Lou, Y. *et al.* Antitumor activity mediated by CpG: the route of administration is critical. *J. Immunother.* **34**, 279–288 (2011).
138. Amos, S.M. *et al.* Adoptive immunotherapy combined with intratumoral TLR agonist delivery eradicates established melanoma in mice. *Cancer Immunol. Immunother.* **60**, 671–683 (2011).
139. John, L.B. *et al.* Oncolytic virus and anti-4-1BB combination therapy elicits strong antitumor immunity against established cancer. *Cancer Res.* **72**, 1651–1660 (2012).
140. Bartlett, D.L. *et al.* Oncolytic viruses as therapeutic cancer vaccines. *Mol. Cancer* **12**, 103 (2013).
141. Kirn, D.H. & Thorne, S.H. Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. *Nat. Rev. Cancer* **9**, 64–71 (2009).
142. Kerkar, S.P. *et al.* IL-12 triggers a programmatic change in dysfunctional myeloid-derived cells within mouse tumors. *J. Clin. Invest.* **121**, 4746–4757 (2011).
143. Zhang, L. *et al.* Improving adoptive T cell therapy by targeting and controlling IL-12 expression to the tumor environment. *Mol. Ther.* **19**, 751–759 (2011).
144. Lotze, M.T. *et al.* Cytokine gene therapy of cancer using interleukin-12: murine and clinical trials. *Ann. N. Y. Acad. Sci.* **795**, 440–454 (1996).
145. Schlom, J. Therapeutic cancer vaccines: current status and moving forward. *J. Natl. Cancer Inst.* **104**, 599–613 (2012).
146. Datta, J. *et al.* Rationale for a multimodality strategy to enhance the efficacy of dendritic cell-based cancer immunotherapy. *Front. Immunol.* **6**, 271 (2015).
147. Mach, N. *et al.* Differences in dendritic cells stimulated *in vivo* by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res.* **60**, 3239–3246 (2000).
148. Lane, P., Burdet, C., McConnell, F., Lanzavecchia, A. & Padovan, E. CD40 ligand-independent B cell activation revealed by CD40 ligand-deficient T cell clones: evidence for distinct activation requirements for antibody formation and B cell proliferation. *Eur. J. Immunol.* **25**, 1788–1793 (1995).
149. Thirunavukarasu, P. *et al.* A rationally designed A34R mutant oncolytic poxvirus: improved efficacy in peritoneal carcinomatosis. *Mol. Ther.* **21**, 1024–1033 (2013).
150. Wilhelm, K. *et al.* Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. *Nat. Med.* **16**, 1434–1438 (2010).
151. Kaka, A.S., Foster, A.E., Weiss, H.L., Rooney, C.M. & Leen, A.M. Using dendritic cell maturation and IL-12 producing capacity as markers of function: a cautionary tale. *J. Immunother.* **31**, 359–369 (2008).
152. Nakamura, Y. *et al.* Helper function of memory CD8+ T cells: heterologous CD8+ T cells support the induction of therapeutic cancer immunity. *Cancer Res.* **67**, 10012–10018 (2007).
153. Musha, H. *et al.* Selective infiltration of CCR5(+)CXCR3(+) T lymphocytes in human colorectal carcinoma. *Int. J. Cancer* **116**, 949–956 (2005).
154. Kunz, M. *et al.* Strong expression of the lymphoattractant C-X-C chemokine Mig is associated with heavy infiltration of T cells in human malignant melanoma. *J. Pathol.* **189**, 552–558 (1999).
155. Ohtani, H., Jin, Z., Takegawa, S., Nakayama, T. & Yoshie, O. Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma. *J. Pathol.* **217**, 21–31 (2009).
156. Sampath, P. *et al.* Crosstalk between immune cell and oncolytic vaccinia therapy enhances tumor trafficking and antitumor effects. *Mol. Ther.* **21**, 620–628 (2013).
157. Obermajer, N., Muthuswamy, R., Odunsi, K., Edwards, R.P. & Kalinski, P. PGE(2)-induced CXCL12 production and CXCR4 expression controls the accumulation of human MDSCs in ovarian cancer environment. *Cancer Res.* **71**, 7463–7470 (2011).

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