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Induction of antigen-specific T_H9 immunity accompanied by mast cell activation blocks tumor cell engraftment

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The engraftment of circulating cancer cells at distal sites represents a key step in the metastatic cascade, yet remains an unexplored target for therapeutic intervention. In this study, we establish that a vaccination strategy yielding an antigen-specific T_H9 response induces long term host surveillance and prevents the engraftment of circulating cancer cells. Specifically, we show that vaccination with a recombinant CEA IgV-like N domain, formulated with the TLR3 ligand poly I:C, elicits a CEA-specific T_H9 response, wherein IL-9 secreting T_H cells act in concert with CEA N domain-specific antibodies as well as activated mast cells in preventing tumor cell engraftment. The development of this immune response was dependent on TLR3, since interference with the TLR3-dsRNA complex formation led to a reduction in vaccine-imparted protection and a shift in the resulting immune response toward a T_H2 response. These findings point to the existence of an alternate tumor targeting immune mechanism that can be exploited for the purpose of developing vaccine therapies targeting tumor dissemination and engraftment.

Ninety percent of all cancer-related deaths are associated with the occurrence of metastases,[1–3] with the majority of patients with advanced metastatic cancer given palliative care in the absence of useful curative strategies. Thus, a vaccine strategy designed to induce long term host surveillance to prevent or delay the engraftment of circulating cancer cells, and/or the expansion of micrometastases, would provide an ideal therapy for controlling or limiting relapse in cancer patients.[1,2,4–7]

Aberrantly expressed tumor-associated cell adhesion molecules represent suitable targets for targeting cellular engraftment. One such surface molecule is the carcinoembryonic antigen (CEA, CEACAM5 or CD66e), a well-established tumor biomarker used in the management of cancer patients.[4] Historically, attempts at developing cancer vaccines against tumor-associated self-antigens (TAA), such as CEA, have only yielded modest successes. Such vaccine designs have centered on mounting cell-mediated (T_H1) and CTL immune responses to this TAA using vaccine formulations composed of either dendritic cells preloaded with predicted T-cell epitopes or recombinant viruses delivering the full-length molecule.[4,5,8–12] The lack of efficacy of CEA-based cancer vaccines has been linked to several factors such as the poor immunogenicity of CEA as a self-antigen and the presence of immunosuppressive regulatory T (T_{reg}) cells in tumor microenvironments preventing the development of CEA-specific T_H1 immunity *in vivo*.[5,10,13] Overcoming such limitations has remained challenging despite attempts at depleting T_{reg} cells[10,13] or at administering CEA in combination with co-stimulatory molecules.[11,12] To address these issues, we recently developed an alternate vaccination approach against CEA that results instead in a focused IgG response towards its IgV-like N domain and blocks both homotypic (N and A_3 domains of CEA) and heterotypic (fibronectin, ECM) interactions responsible for the implantation of disseminated tumor cells.[5] This novel vaccine strategy was based on the finding that disrupting CEA N domain-specific interactions with domain-specific antibodies, aptamers or soluble recombinant CEA N (rCEA N) or A_3 modules reduces the engraftment of

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What's new?

The vast majority of cancer deaths are the result of metastasis, yet the integration of circulating tumor cells at sites distant from the tumor of origin remains largely unexplored from the view of therapeutics. A vaccine capable of neutralizing circulating tumor cells, however, could be key to preventing or delaying metastasis, according to this study. A carcinoembryonic antigen (CEA)-based vaccine with a focused response against the IgV-like N domain successfully generated a CEA-specific T_H9 response that blocked the establishment of metastatic tumor nodules in mice. This alternate tumor-eradication mechanism could aid the development of metastasis-preventing immunotherapies.

CEA-expressing tumor cells as well as the formation and expansion of tumor foci *in vivo*.[6,14] Vaccinating CEA transgenic mice (CEA.Tg) with a recombinant, nonglycosylated form of the CEA Ig V-like N domain combined with poly I:C, as an adjuvant, led to the production of circulating antibodies exhibiting anti-adhesive as well as cytocidal properties (ADCC, CDC), which blocked the lodging and formation of CEA-expressing murine tumor foci in the lungs and peritoneal cavity of vaccinated CEA.Tg mice.[5] Remarkably, we now report that administration of this vaccine formulation engenders an antigen-specific T_H9 response, where IL-9 secreting T_H cells act in concert with CEA N domain-specific antibodies as well as activated mast cells in preventing the engraftment of disseminated tumor cells.

Material and Methods

Expression and purification of rCEA N domain

Recombinant His-tagged CEA N domain (residues 1–132) was purified from inclusion bodies under denaturing conditions by affinity chromatography using Ni-NTA agarose beads (Sigma-Aldrich)[5]. The His-tag was subsequently cleaved using recombinant Tobacco etch virus (rTEV) protease and the resulting suspension containing digested as well as undigested His-tagged rCEA N domain and rTEV was mixed with ten volumes of solubilization buffer (50 mM Tris (pH8), 8 M urea, 250 mM NaCl, and 10 mM β-mercaptoethanol) and then subjected to affinity chromatography using Ni-NTA columns. Untagged rCEA N domain was collected in the flow through fraction and refolded as previously described.[5,6] Endotoxin contamination was removed from rCEA N domain preparations using Detoxigel columns (Pierce, Thermo Scientific; Ontario, Canada). The purity of the final recombinant products was confirmed by SDS PAGE and FPLC analysis.

CEA transgenic mice

Mice expressing human CEA as a transgene, thereafter referred to as CEA.Tg, were kindly provided by Dr. Wolfgang Zimmerman (Tumor Immunology Laboratory, LIFE-Center, Klinikum Grosshadern, Ludwig-Maximilians-University; Germany). These transgenic animals as well as C57BL/6 mice were bred and kept under standard pathogen-free conditions at the Sunnybrook Health Sciences Center Comparative Research Animal facility. All experiments were performed following the approval of the local animal welfare committee and in accordance with the rules and regulations of the Canadian Council for Animal Care.

Cells and growth conditions

Murine colon carcinoma MC38.CEA cells were a gift from Dr. Jeffrey Schlom (National Cancer Institute; Bethesda, Maryland). Cells were cultured at 37°C in a humidified 5.0% CO_2 atmosphere in complete media (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and dihydrostreptomycin (100 μg/mL)).

Immunizations and tumor challenge

CEA.Tg mice were vaccinated with formulations containing rCEA N domain as a 1:1 (v/v) mixture with either Alum (Invivogen) or a squalene-based nanoemulsion (AddaVax; Invivogen). We investigated different parenteral routes of administering these formulations and deduced that subcutaneous (SC) administration of Alum-formulated rCEA N domain and intramuscular (IM) administration of Squalene-formulated rCEA N domain produced the best immune responses for these particular adjuvant formulations (Supporting Information Fig. 1). Alternatively, CEA.Tg mice were immunized with the standard regimen previously reported to impart protective prophylactic immunity,[5] whereby mice received an intraperitoneal (IP) injection of a 200 μL formulation containing 100 μg of recombinant CEA N domain mixed with 100 μg Poly I:C (Sigma-Aldrich; Ontario, Canada). Mice were primed on Day 1 followed by two booster shots of the same formulation on days 5 and 10 post-injection.

On Day 15 post-immunization, all animals were challenged with 2×10^5 MC38.CEA tumor cells implanted in their peritoneal cavity. Tumor burdens were assessed by counting the number of tumor nodules.[5] Vaccination was considered protective when vaccinated mice displayed fewer than 15% of the mean number of peritoneal tumor nodules enumerated in non-immunized CEA.Tg mice.

Preparation and cultivation of leukocytes

Spleens were aseptically removed from euthanized mice and cells were collected by gently forcing the organs through a 40 μm cell strainer (Falcon). The cells were subsequently

washed three times with cold wash medium containing RPMI supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) and 1% FBS. Cell viability was determined using Trypan blue dye exclusion assay with cell viability following harvest typically being ≥ 95%. Leukocytes were suspended at a density of 1×10^6 cells per mL in complete medium (RPMI-1640 supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), 2 mM l-glutamine, 1 mM HEPES, 0.05 mM β-mercaptoethanol and 10% FBS).

Peritoneal exudate cells (PECs) were harvested by flushing the abdominal cavities of mice with 10 mL of cold wash medium. Cells were collected from the wash medium by centrifugation, washed twice and suspended at a density 3×10^5 cells per mL in complete medium.

Analysis of CEA-specific T cell responses

CEA-specific cytokine secreting lymphocytes were quantified by cytokine ELISPOT, as previously reported.[5,15] Briefly, splenocytes recovered from immunized and control mice were stimulated *ex vivo* with rCEA N domain (10 μg per well). CEA-specific cytokine secreting cells were quantified using the IFN-γ, IL-2, IL-9 and IL-4 ELISPOT development modules (R&D Systems; Minneapolis, MN, USA) according to the manufacturer's recommendations with the exception that the antibodies and enzyme-coupled streptavidin were diluted at a concentration of 1:100. The spots were enumerated using an automated ELISPOT plate counter (Cellular Technologies Inc; Shaker Heights, OH). Frequencies of CEA-specific cytokine secreting cells were calculated by subtracting background values (calculated from wells containing unstimulated cells) from measured test values as previously described.[5,15]

The production of IL-9 by T_H9 cells was confirmed by intracellular cytokine staining. Splenocytes from immunized and untreated CEA.Tg mice were cultured for 72 hrs. For antigen re-stimulation, splenocytes were cultured in the presence of rCEA N domain (10 μg/mL) for 72 hrs. In the last 12 hrs, monensin was added and cells were harvested and stained for $CD3^+$ $CD4^+$ $CD8^+$ surface expression followed by fixing and permeabilization using the BD Cytofix/Cytoperm Kit (BD Biosciences), according to the manufacturer's instructions. The presence of intracellular IL-9 was detected using Allophycocyanin-conjugated anti-IL-9 mAb (clone RM9A4; Biolegend; San Diego, CA).

Role of TLR3 signaling in inducing a CEA-specific T_H9 response

In order to assess the relevance of TLR3 signaling on the development of protective CEA-specific T_H9 immunity *in vivo*, we co-administered CEA formulated with poly I:C, to CEA.Tg mice with 500 μg of the TLR3/dsRNA complex inhibitor T3RCI ((R)−2–(3-Chloro-6-fluorobenzo [b] thiophene-2-carboxamido)−3-phenylpropanoic acid, EMD Millipore) using the immunization schedule described above. Animals were then subdivided into two groups, where one group was challenged with MC38.CEA tumor cells (IP), as described above and the second group was sacrificed one day following the last injection and their splenocytes recovered for cytokine analyses.

Spleen-derived leukocytes were stimulated *ex vivo* with rCEA N domain (10 μg per well) for 48 hrs and were subsequently recovered for RNA extraction. Quantification of *IL-2* and *IL-4* expression in stimulated lymphocytes was performed using digital PCR. Briefly, total RNA was extracted from 4×10^6 lymphocytes using RNeasy Mini spin columns (Qiagen) as directed by the manufacturer. Complementary DNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific), using oligo $(dT)_{18}$ primer. The cDNA was then used to determine the number of *IL-2* and *IL-4* transcripts by digital PCR, using either a combination of *Il2*-F (ACTTCAAGCTCCACTTCAAG) and *Il2*-R (GAGTCAAA TCCAGAACATGC) or the *Il4*-F (CCAGCTAGTTGTCATCC TGCTCTTCTTTCTCG) and *Il4*-R (CAGTGATGTGGACTT GGACTCATTCATGGTGC) primer pairs. The number of *IL-2* and *IL-4* transcripts was quantified using a QX200 Droplet Digital PCR system (Bio-Rad, Mississauga, ON, Canada), as recommended by the manufacturer.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as previously described,[5] where 96-well microtiter ELISA plates (Falcon) were coated with 1 μg per well of rCEA N domain. Sera derived from immunized or control mice were serially diluted in 1% BSA–PBS–25 mM EDTA and incubated for 1 hr at room temperature with gentle shaking. After a washing step, the plates were incubated with HRP-coupled anti-mouse IgG, IgG1 or IgG2a secondary antibodies (diluted in 0.5% BSA–PBS–EDTA; 1:5,000; Bethyl Laboratories; Montgomery, TX) for 1 hr at room temperature. For analysis of CEA-specific serum IgA levels, plates were prepared as described above, and the presence of bound IgA was detected using anti-mouse IgA (1:800; Bethyl laboratories). The plates were then washed and developed using 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma-Aldrich) for 5 min at room temperature. The chromogenic reactions were stopped using half volume (50 μL) of 0.5 M H_2SO_4.

Analysis of Ab-dependent effector mechanisms

The effects of vaccine-induced anti-CEA antibodies in mediating the blockage of CEA-dependent cell adhesions as well as Ab-dependent cytotoxicities were measured in real-time using an xCELLigence RTCA, impedance-based cell sensing device (ACEA Biosciences; San Diego, CA). The inhibition of CEA-dependent cellular adhesion was monitored using MC38.CEA cells (2.5×10^4 cells per well) suspended in media containing either heat-inactivated sera from immunized, or control CEA.Tg mice (1:100). The cell suspensions were transferred to sensor plates (E-plates) pre-coated with rCEA N domain (1 μg per well). Cell attachment was measured as a change in relative impedance, termed cell index (CI). The adhesion of

non-serum treated MC38.CEA cells served as a positive control for the assay.

Analyses of Ab-dependent killing of tumor cells (ADCC and CDC) were measured using mid-log phase monolayers of MC38.CEA cells grown in wells of E-plates and exposed to medium supplemented with sera (1:100) and either purified leukocytes or complement (1:200 or 1:100, respectively). The growth kinetics and viability of MC38.CEA cells were then monitored by measuring changes in impedance as CI values recorded on an xCELLigence RTCA device. The efficiency of Ab-dependent killing was calculated using the following formula: % Cytotoxicity = [(Experimental − Spontaneous)/(Maximal − Spontaneous)]/100%; where the Experimental, Spontaneous and Maximal values represent changes in CI values as a function of time.[5]

Multiplex analysis of serum cytokine and chemokine levels

Serum cytokine levels were analyzed using sera collected from immunized or control animals. Specific cytokine levels were quantified using the MILLIPLEX MAP Mouse Cytokine/Chemokine Multiplex Assay kits (EMD Millipore; Toronto, Canada), as suggested by the manufacturer.

Analysis of immune serum mast cell modulatory properties

The effect of sera from immunized mice towards mast cells was analyzed by incubating peritoneal leukocytes (3.0×10^5, isolated from naïve mice) in the presence or absence of irradiated MC38.CEA cells (1.0×10^5) and sera (1:200) from either immunized or control CEA.Tg mice. Following the incubation of co-cultures at 37°C for 48 hrs, the cells were harvested and analyzed by flow cytometry for changes in the mast cell population ($Gr1^-$ $Fc\epsilon RI^+$ $CD117^+$).

Relevance of vaccine-induced T_H9 response *in vivo*

The relevance of T cells, IL-9 and mast cells in preventing the implantation of peritoneal tumors was assessed by targeted depletions *in vivo*. Briefly, vaccinated CEA.Tg mice were subdivided into four groups of five mice. The first group of immunized CEA.Tg mice was treated with an IP injection of 20 μg of a T-cell depleting anti CD3 mAb (clone 145-2C11; BioXCell) on Day 14 post-immunization. The second group of vaccinated CEA.Tg mice was treated with an IP injection of 200 μg of an IL-9 neutralizing mAb (clone 9C1; BioXCell) on days 14 and 17 post-immunization; while a third group of vaccinated mice received an IP injection of 4 mg of sodium cromoglycate (Sigma-Aldrich) on days 14, 17 and 19 post-immunization. The last group of vaccinated CEA.Tg mice was left untreated. In a parallel experiment, vaccinated CEA.Tg mice were treated with an IP injection of either 200 μg of an anti-CD117 mAb (clone ACK2) on Day 14 post-immunization, or 100 μg of an anti-CD4 mAb (clone GK1.5) on days 14 and 17 post-immunization.

To assign the role of T lymphocytes in protecting mice from developing tumor nodules in the peritoneal cavity, T cells were purified from immunized CEA.Tg mice and injected into the tail vein of immunologically naïve recipient CEA.Tg mice. T lymphocytes were purified by negative selection from single cell suspensions of total spleen leukocytes, collected from immunized CEA.Tg mice using the EasySep mouse T cell enrichment kit (StemCell Technologies; Vancouver, Canada), as recommended by the manufacturer. All animals were challenged with MC38.CEA (2.0×10^5; IP) on Day 15 post-immunization. Tumor burdens were compared between immunized and control animals, post-mortem, 21 days following tumor challenge.

Statistics and data analysis

Collected data sets were analyzed for significance by ANOVA and the individual groups were compared using Student-*t test*. All statistical analyses and graphs were generated using PRISM (version 5.01; Graph Pad Software for Science, San Diego, CA). Significance was accepted when $p \leq 0.05$.

Results

The vaccine-induced production of IL-9 leads to the efficient prevention of tumor implantation

In a previous study, we reported the prevention of tumor engraftment in the lungs and peritoneal cavity of naïve CEA transgenic (CEA.Tg) mice through the IP vaccination of mice with a recombinant carcinoembryonic antigen (CEA) IgV N domain (rCEA N domain) and the TLR-3 ligand poly I:C.[5] We originally postulated that the generated CEA-specific antibodies were sufficient to prevent the implantation of disseminated tumor cells *in vivo* based on our observation that immunity was produced upon adoptively transferring either B-cells or sera, derived from vaccinated mice, into naïve animals.[5] However, vaccination protocols combining the CEA N domain antigen with alum or squalene yielded comparable CEA N domain-specific antibody levels in CEA.Tg mice displaying anti-adhesive as well as cytotoxic properties (Figs. 1*a*–1*d*), but failed in preventing tumor implantation (Fig. 1*e*). On the other hand, the majority of CEA.Tg mice receiving rCEA N domain mixed with poly I:C (IP) were protected against the development of peritoneal tumor masses (Fig. 1).

Correlation between T_H9 response and the efficient prevention of tumor engraftment

The abovementioned findings led us to investigate differences in vaccine-induced T-cell responses, which would provide a rationale for the observed differences in outcomes of tumor challenge experiments. In preliminary experiments, blood was sampled from vaccinated and control CEA.Tg mice 16 hrs following the last immunization step in order to compare differences in serum cytokine levels. Surprisingly, serum derived from CEA.Tg mice immunized with rCEA N domain mixed poly I:C had substantially higher levels of IL-9 compared to non-immunized or mice immunized with either rCEA formulated with Alum or Squalene (data not shown). The relationship

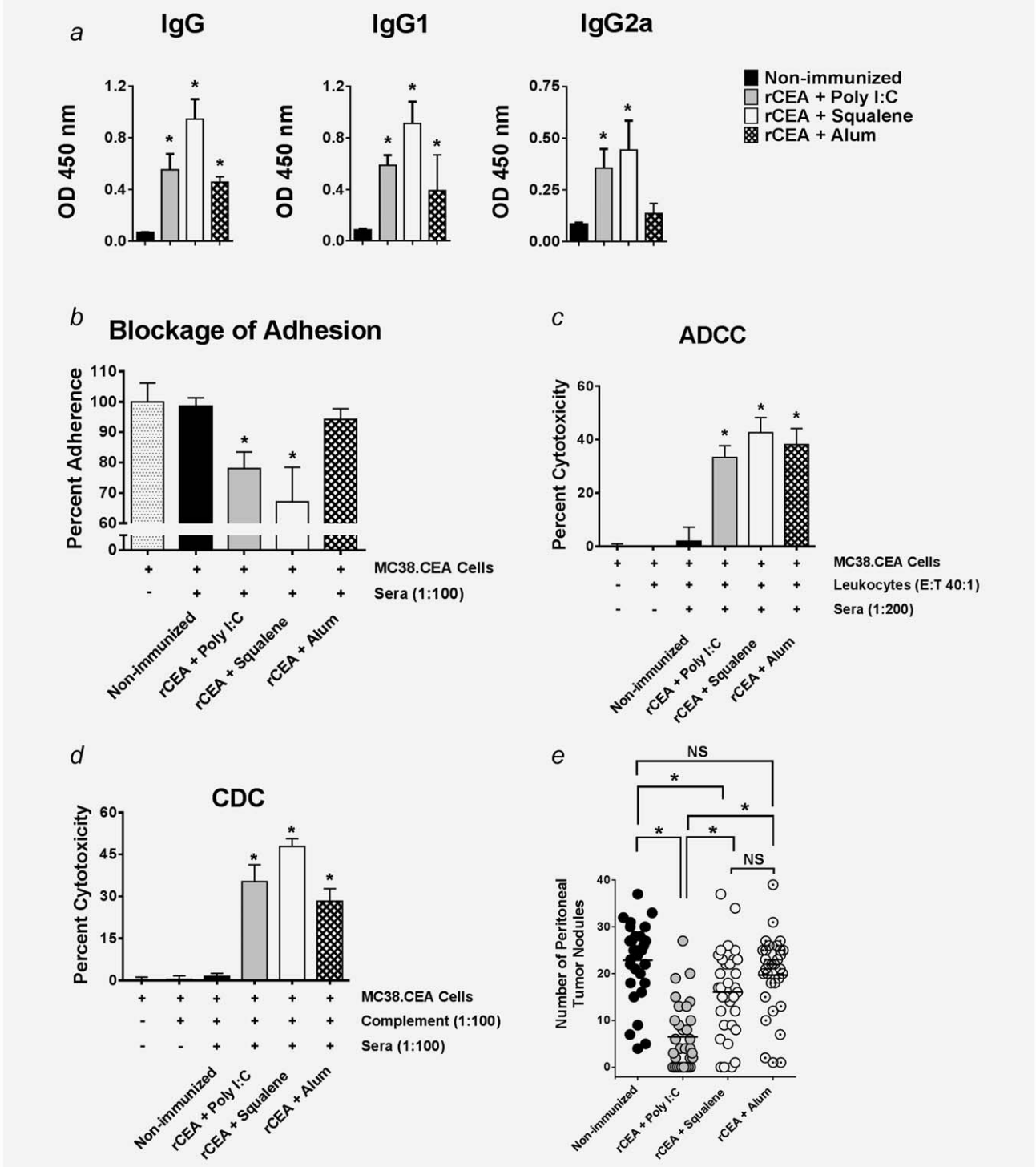

Figure 1. Vaccine-induced CEA N domain-specific antibodies are not sufficient to fully prevent the engraftment of disseminated tumor cells *in vivo*. (*a*) Administration of rCEA N domain formulated with either Poly I:C (IP), a squalene-based oil-in-water nano-emulsion (IM) or Alum (SC) engenders CEA-specific IgG antibodies. (*b*) Sera from mice immunized with either Squalene or poly I:C inhibit CEA-mediated adherence of MC38.CEA cells to CEA-coated surfaces. Asterisk denotes statistical significance ($p \leq 0.05$) when compared with samples treated with sera from non-immunized CEA.Tg mice; Student-*t test*. Sera from animals vaccinated with rCEA N domain mixed with either Alum, squalene or poly I:C display ADCC (panel *c*) and CDC (Panel *d*) toward CEA-expressing murine MC38 colorectal cancer cells (MC38.CEA). (*e*) Vaccination of CEA.Tg mice with rCEA N domain mixed with poly I:C was significantly more effective than rCEA N domain formulated with either Alum (SC) or a squalene-based nano-emulsion (IM) in preventing the establishment of peritoneal tumor nodules. * Denotes statistical significance ($p \leq 0.001$) compared to non-immunized CEA.Tg mice. Statistical significance was determined using one way analysis of variance and individual groups were compared using the Student-*t test*.

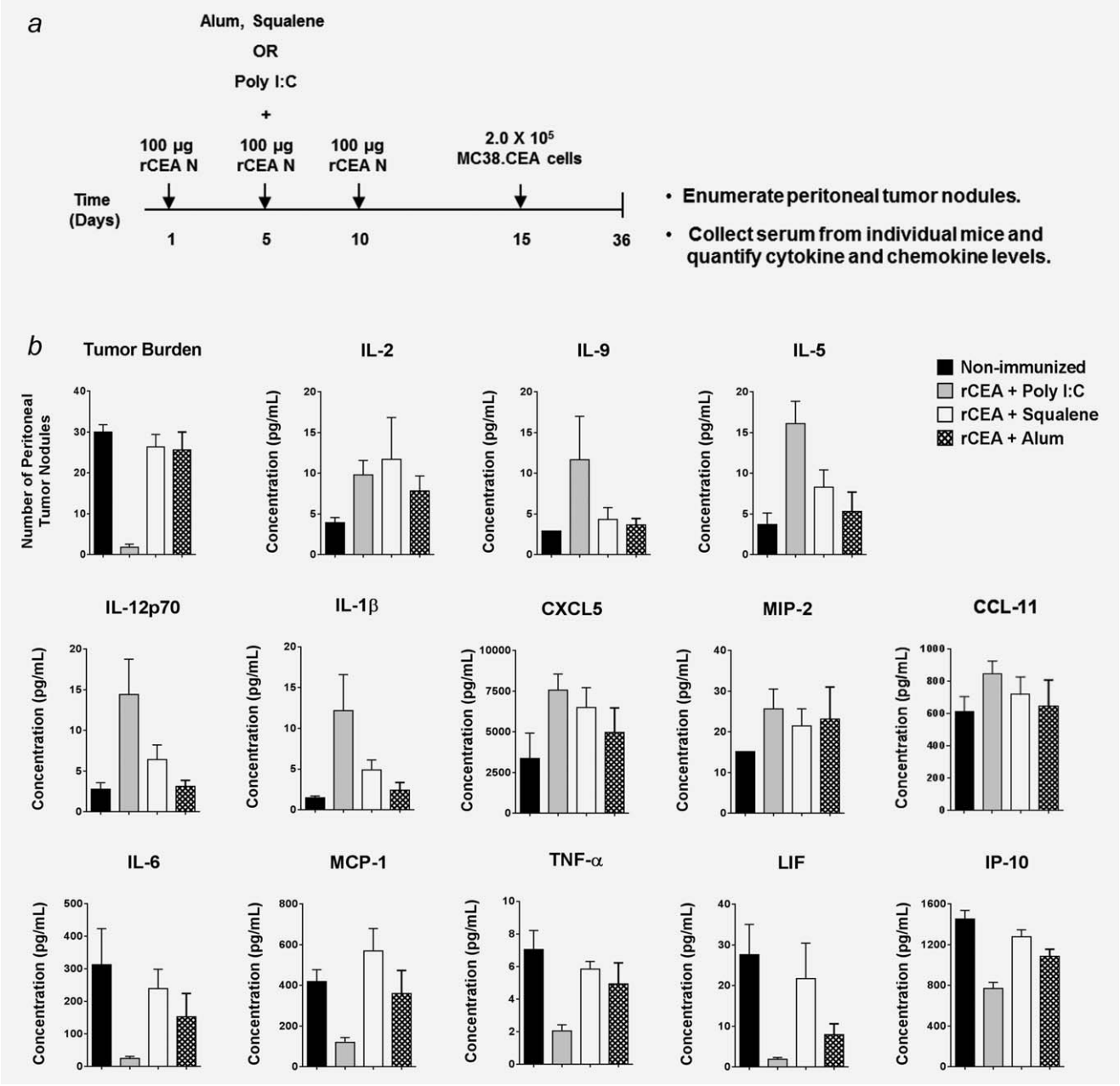

Figure 2. Identification of serum cytokines and chemokines correlating with efficient prevention of tumor engraftment. (*a*) Experimental outline. (*b*) Comparison of peritoneal tumor burdens with the cytokine/chemokine levels in sera of protected (vaccinated with rCEA N domain mixed with poly I:C) and non-protected (vaccinated with rCEA N domain mixed with either Squalene or Alum) immunized CEA.Tg mice with non-immunized animals, 15 days post-tumor challenge. Vaccinated mice protected from tumor implantation show a distinct increase in the levels of serum IL-9, IL-5, IL-12p70, and IL-1β but have a substantially reduced level of MCP-1, TNF-α, LIF, IP-10 and IL-6. Quantification of serum cytokine/chemokine levels was done using the MILLIPLEX MAP 32 cytokine-chemokine Multiplex assay (EMD Millipore). Each histogram bar represents the mean value of individual mice ($n = 5–10$).

between the observed surge in IL-9 production and protection against tumor implantation was subsequently investigated by quantifying serum cytokine and chemokine levels as well as peritoneal tumor burdens in CEA.Tg mice receiving an immunization combining rCEA N domain with either poly I:C (IP), Alum (SC) or Squalene (IM). As shown in Figures 1e and 2b, $\geq$ 60% of animals vaccinated IP with the rCEA N domain and poly I:C displayed no/low number of peritoneal murine MC38.CEA tumor nodules, while the remaining animals receiving either rCEA N domain formulated with either Alum or Squalene or nonvaccinated CEA.Tg mice harbored significant tumor burdens (Fig. 2b). Furthermore, the observed low tumor burden coincided with an increase in serum IL-9, IL-5, IL-12p70, as well as IL-1β; but a substantially reduced level of

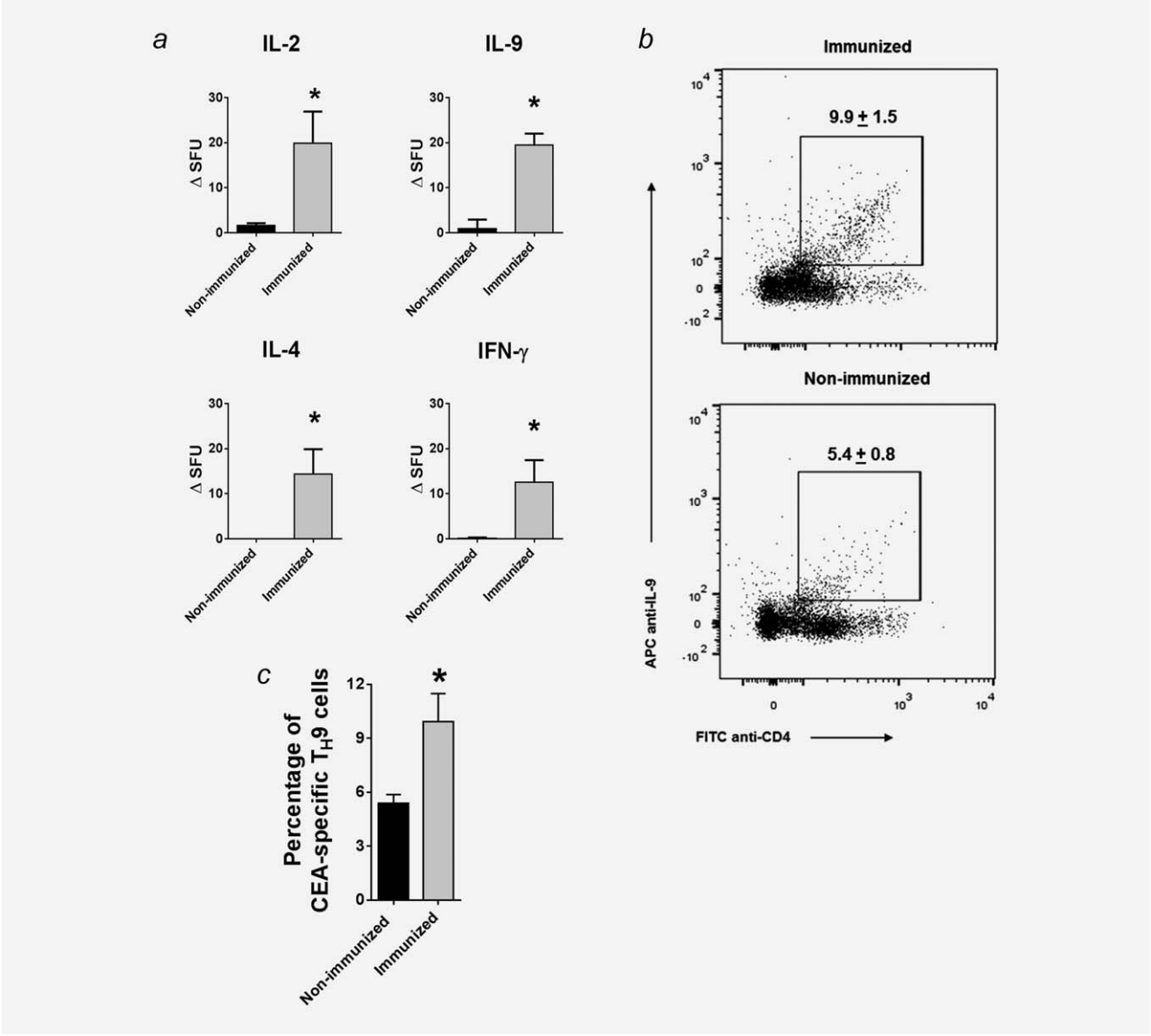

Figure 3. Vaccination of CEA.Tg mice with rCEA N domain mixed with poly I:C results in the production of CEA N domain-specific T_H9 cells. (*a*) Enumeration of rCEA-specific IL-2, IL-9, IL-4 and IFN-γ spot forming units (ΔSFUs) from immunized and control mice as measured by cytokine ELISPOT. Histogram bars represent averaged ΔSFU values measured from two independent immunization trials ($n = 3$ per group). The number of Ag-specific cytokine secreting lymphocytes (ΔSFUs) was calculated by subtracting background values (from wells containing unstimulated cells) from measured values in treated groups. Asterisk denotes statistical significance ($p \leq 0.05$; Student-*t*-test) when compared with the frequency of CEA-specific cytokine secreting cells derived from non-immunized CEA.Tg mice. (*b*) Intracellular cytokine staining of IL-9 in CEA–specific CD3+ CD4+ lymphocytes. Splenocytes from immunized and control mice were stimulated, *in vitro*, with rCEA N domain (10 μg/mL) for 72 hrs, followed by staining for IL-9 production in T_H lymphocyte populations. (*c*) Comparison of the number of CEA-specific IL-9 producing T_H cells between immunized and control mice. * Denotes statistical significance ($p \leq 0.05$) when compared to non-immunized CEA.Tg mice. Statistical significance was determined using the Student-*t test*, with Welch's Correction.

LIF, IP-10, MCP-1, VEGF, TNF-α and IL-6 (Fig. 2*b*). These observations suggested that an intraperitoneal administration of rCEA N domain mixed with poly I:C leads to IL-9 production. However, it has been reported that IL-9 can be produced in a manner, that is, antigen-independent.[16] Therefore, we performed ELISPOT assays to confirm that the above observations were Ag-specific. Consistent with the abovementioned results, immunization of CEA.Tg mice with rCEA N domain mixed with poly I:C generated CEA-specific IL-9 secreting T cells whose frequencies (~20 SFUs) were higher than CEA-specific IL-4 or IFN-γ secreting cells (~10 SFUs; Fig. 3a). In addition, higher numbers of IL-9 producing CD3+ CD4+ T lymphocytes were detected in cultures of splenocytes isolated from vaccinated mice and re-stimulated with rCEA N domain (Figs. 3*b*

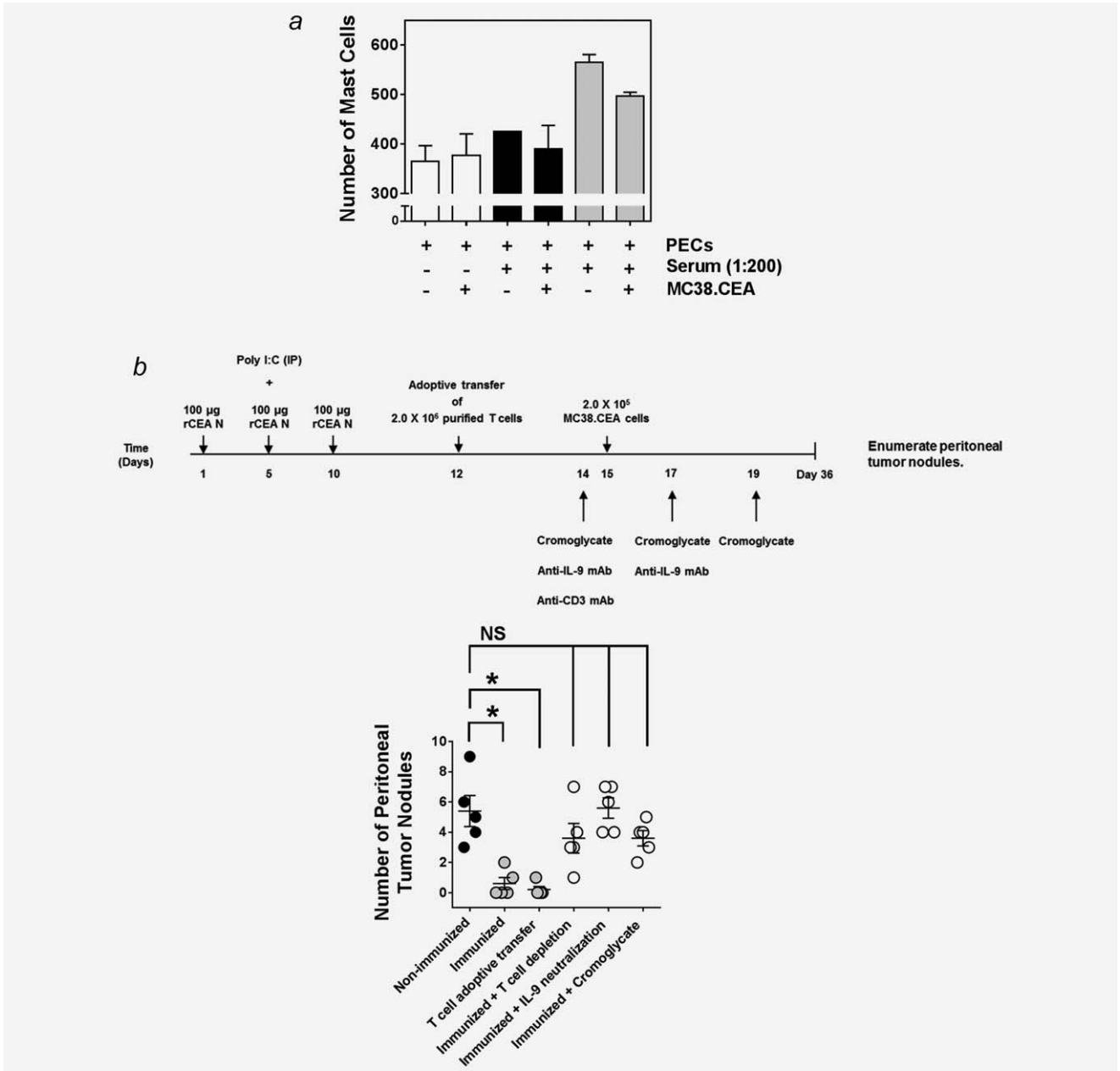

Figure 4. Prevention of tumor engraftment depends on mast cells. (*a*) Addition of serum from immunized mice to peritoneal exudate cells (PECs) is sufficient to induce an expansion of mast cells. PECs were purified from naïve mice and cultured for 48 hrs in the presence of a combination of irradiated MC38.CEA cells and/or pooled sera (1:200) isolated from either immunized or nonimmunized CEA.Tg mice. The number of mast cells ($Gr1^-$ $Fc\epsilon RI^+$ $CD117^+$) was quantified by flow cytometry. (*b*) Reversal of vaccine-imparted protection against tumor implantation through the neutralization of T_H9 immunity. Adoptive transfer of T cells from immunized mice into naïve animals was performed 3 days prior to the injection of 2.0×10^5 MC38.CEA cells (IP). Depleting/neutralizing treatments were initiated one day prior to tumor challenge and continued throughout the first three days post-tumor engraftment. Peritoneal tumor nodules were enumerated in vaccinated and control animals. Mice were divided into groups, wherein a subset received an intravenous bolus of 2.0×10^6 T cells purified from immunized animals. The remaining groups were treated with a CD3-depleting mAb, an IL-9 neutralizing mAb, or injected with a mast cell stabilizer (cromoglycate). **C.** Targeted depletion of either mast cells or T_H cells abrogate vaccine-imparted immunity. Vaccinated CEA.Tg mice were treated with either an anti-CD117 or anti-CD4 mAbs prior to tumor challenge. The number of peritoneal tumor nodules was compared between vaccinated and control animals, revealing the reversal of vaccine-elicited protection to levels comparable to those observed in non-immunized mice. NS, not statistically significant when compared to non-immunized CEA.Tg mice. * Denotes statistical significance ($p \leq 0.001$) compared to non-immunized CEA.Tg mice. Statistical significance was determined using one way analysis of variance and individual groups were compared using the Student-*t test*.

and 3*c*). Together, these findings confirmed that the administration of rCEA with poly I:C led to the development of CEA-specific T_H9 cells.

Vaccine-imparted protection depends upon mast cells

The cytokine/chemokine expression pattern observed in vaccinated animals displaying protection against peritoneal tumor

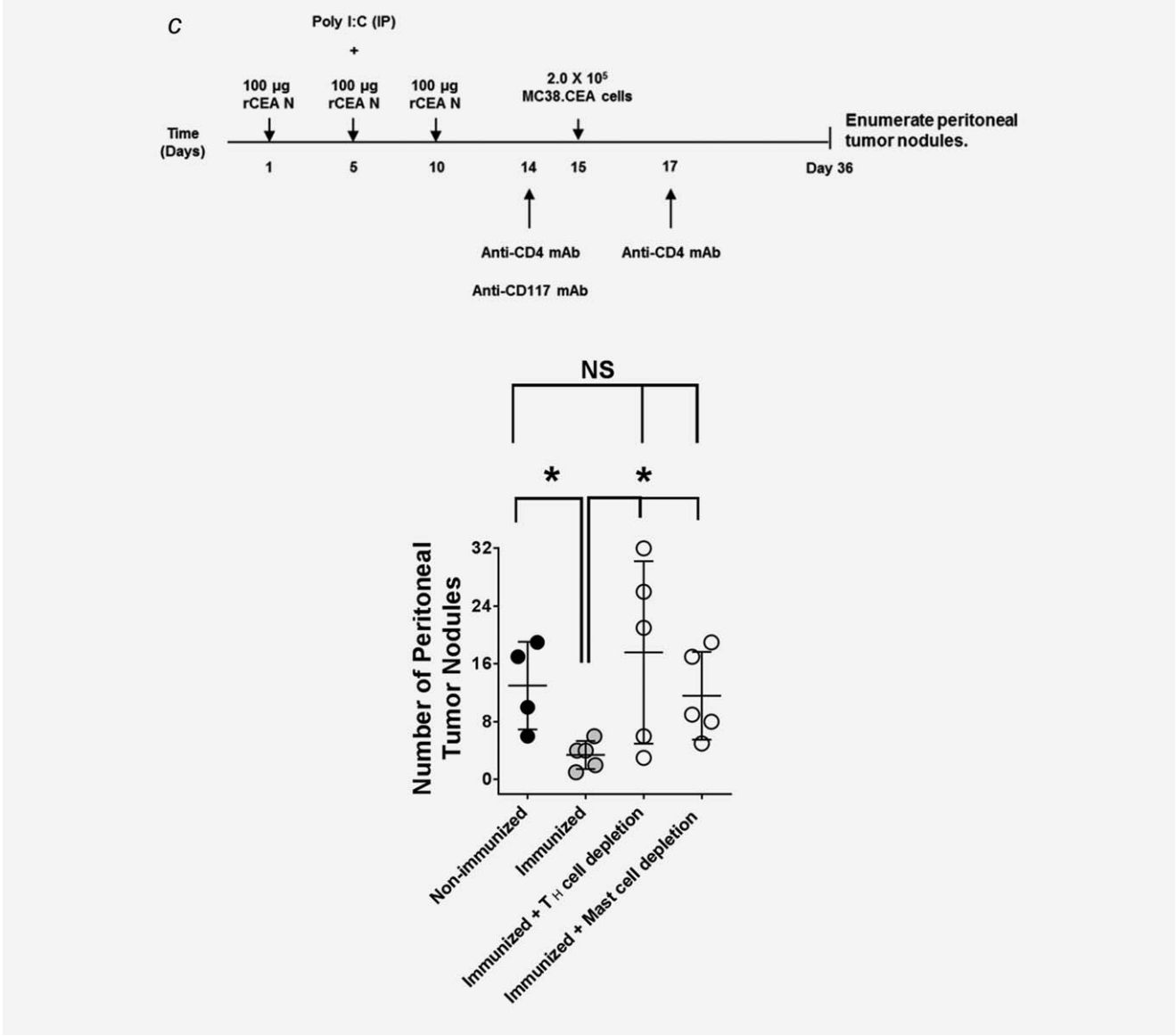

Figure 4. Continued

establishment is reminiscent of immune responses that are normally observed in either mast cell-mediated graft rejections or expulsion of parasitic helminthes.[17–20] As such, we sought to establish the relevance of mast cells in preventing tumor cell implantation. Exposing peritoneal exudate cells (PECs) from naïve mice to pooled sera from (protected, responders) immunized CEA.Tg animals was sufficient to trigger an expansion of mast cells, independently of the presence of murine MC38.CEA target cells, *in vitro* (Fig. 4*a*). We subsequently monitored the importance of vaccine-stimulated T cells, IL-9 and mast cells in protecting CEA.Tg mice from murine MC38.CEA cells implanted in their peritoneal cavity (Fig. 4*b*). Specifically, transferring T lymphocytes from vaccinated animals into naïve CEA.Tg recipients followed by a tumor challenge with 2×10^5 MC38.CEA cells (Fig. 4*b*) led to the absence of tumor nodules or a low tumor burden that was comparable to the vaccinated animals. This finding was confirmed by the reversal of vaccine-imparted protection in immunized mice through the depletion of T cells with an anti CD3 mAb (Fig. 4*b*).

Additionally, vaccinated CEA.Tg mice were subdivided into groups, wherein one group of vaccinated CEA.Tg mice, the circulating levels of IL-9 were decreased through the administration of an anti-IL-9 mAb; while another group of vaccinated animals received cromoglycate administration, in order to pharmacologically inhibit mast cells activity *in vivo* (Fig. 4*b*). These treatments were given before and/or after tumor challenge to monitor their impact on the engraftment of disseminated MC38.CEA tumor cells. As expected, the neutralization of IL-9 (during the initial stages of tumor implantation) in vaccinated CEA.Tg mice resulted in a reversal of vaccine-imparted protection (Fig. 4*b*). Similarly, the administration of cromoglycate to vaccinated CEA.Tg mice

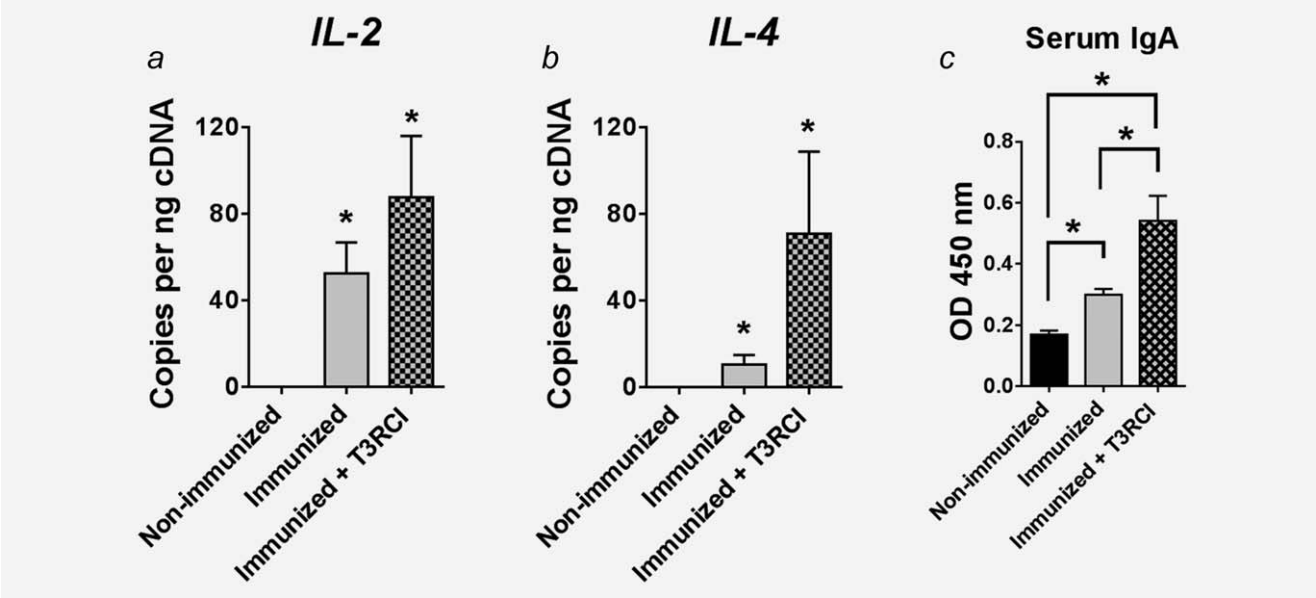

Figure 5. Requirement for TLR3 signaling during vaccination for inducing T_H9 immunity. Pharmacological interference with TLR3 signaling shifts the vaccine generated immune response from a T_H9 towards a T_H2 profile. (*a*) Quantification of CEA-specific *IL-2* expression levels by digital droplet PCR. (*b*) Quantification of CEA-specific *IL-4* expression levels by digital droplet PCR. Splenocytes from immunized and control CEA.Tg mice were stimulated with rCEA N domain (10 μg/mL) for 48 hrs, followed by RNA extraction. (*c*) Quantification of the levels of CEA-specific serum IgA levels confirms the shift of CEA-specific immunity towards a T_H2 profile, following the inhibition of TLR3 signaling. * Denotes statistical significance ($p \leq 0.001$) compared to non-immunized CEA.Tg mice. Statistical significance was determined using the Student-*t test*.

rendered immunized animals susceptible to developing abdominal tumor nodules (Fig. 4*b*). In a parallel experiment, vaccine imparted protection was attenuated in immunized CEA.Tg mice upon the administration of either an anti-CD117 or an anti-CD4 mAbs to deplete mast cells or T_H cells, respectively, prior to tumor challenge (Fig. 4*c*). Together, these observations confirm the importance of mast cells as effector cells in the rejection of implanting tumors.

Dependence of protective T_H9 immunity on the engagement of TLR3 signaling

The detection of protective CEA-specific T_H9 immunity following the administration of rCEA N domain and poly I:C led us to investigate the relative importance of TLR3 signaling during immunization, as poly I:C is known to signal through RLRs as well as TLR3. Pharmacological inhibition of TLR3/dsRNA complex through the co-administration of T3RCI ((R)-2–(3-Chloro-6-fluorobenzo [b] thiophene-2-carboxamido)-3-phenylpropanoic acid) resulted in shifting the vaccine-induced immune response towards a T_H2 response (Figs. 5*a* and 5*b*). As shown in Figure 5 (panels A-C), animals receiving T3RCI had substantially higher CEA-specific IL-4 as well as CEA-specific serum IgA, an Ig marker of T_H2 polarization *in vivo*,[21] than animals vaccinated with rCEA N domain mixed with poly I:C. An additional consequence of including T3RCI in the vaccine formulation was the partial loss of the vaccine-imparted protection (Fig. 6). Specifically, the majority of CEA.Tg mice receiving the vaccine formulation containing T3RCI displayed an increased number of peritoneal tumor nodules as well as a higher cumulative volume of peritoneal tumor nodules (Fig. 6 panels B and C). As summarized by Figure 6*d*, pharmacological interference with TLR3 signaling during vaccination resulted in a significantly lower number of tumor free mice, as compared to vaccinated CEA.Tg mice. Taken together, these findings highlight the importance of TLR3 signaling in inducing a protective T_H9 immune response.

Discussion

In this study, we demonstrated that a specific T_H9 immune response towards a tumor-associated antigen (TAA), namely the IgV N domain of CEA, can be induced in CEA transgenic mice when combined with a TLR3 ligand such as poly I:C, and delivered through a simple vaccination protocol. This response served a pivotal role in preventing the engraftment of disseminated tumor cells in immunocompetent mice. The development of a vaccine-mediated, T_H9 immune response represents a mechanistically distinct approach of inducing an effective anti-tumor response through vaccination than present approaches centered on mounting cell-mediated (T_H1) and CTL immune responses using vaccine formulations composed of either dendritic cells preloaded with predicted T-cell epitopes or recombinant viruses delivering the full-length molecule.[4,5,8–12]

This study provides the first example of how to induce a TAA-specific, T_H9 immune response through vaccination, in generating protective immunity in recipient transgenic mice, as

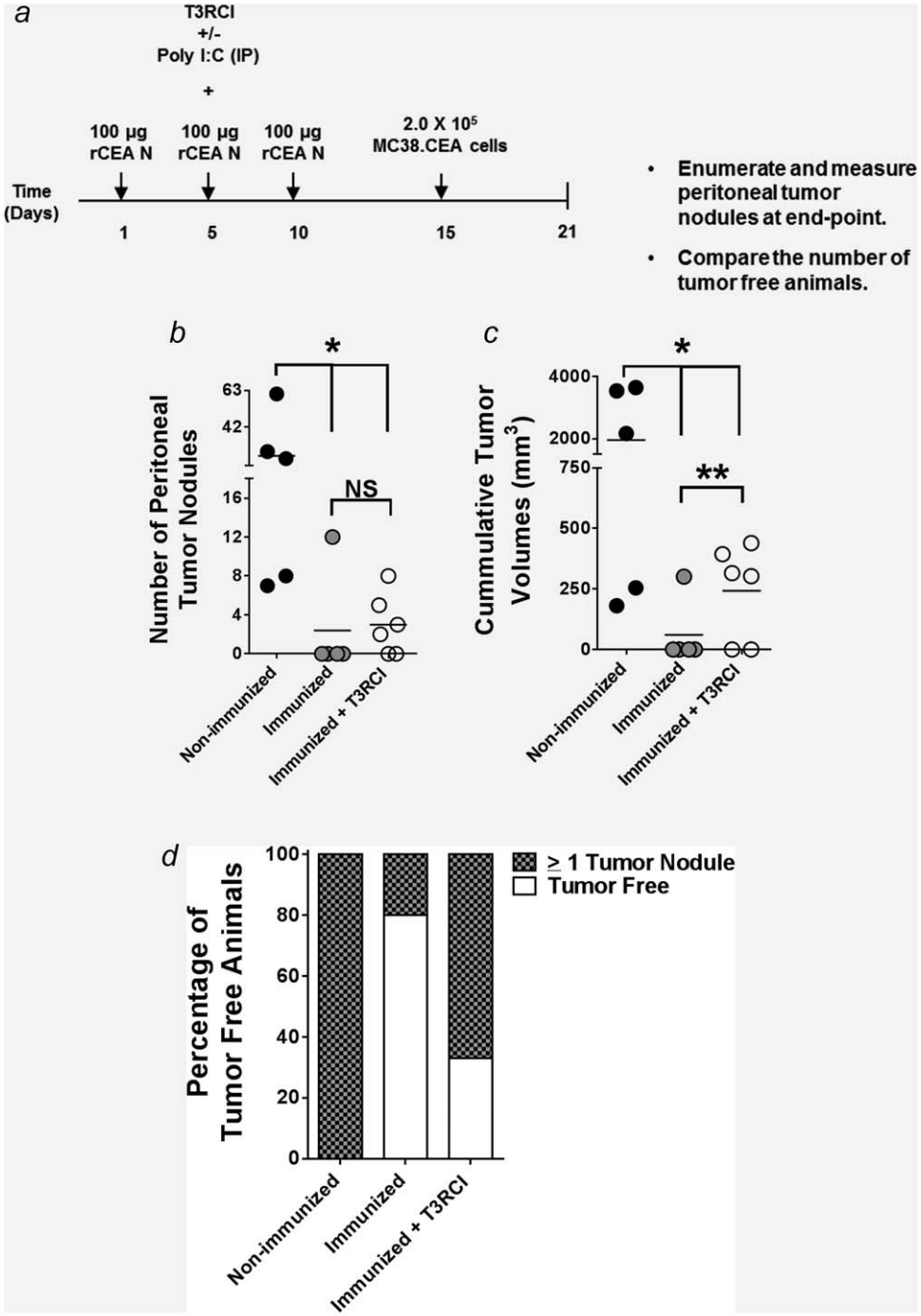

Figure 6. Pharmacological interference with TLR3 signaling hampers vaccine-imparted protection. (*a*) Experimental outline. (*b*) Enumeration of peritoneal tumor nodules in CEA.Tg mice receiving rCEA N domain mixed with either poly I:C alone or poly I:C and T3RCI (a TLR3/dsRNA complex inhibitor). (*c*) Inclusion of T3RCI during vaccination resulted in recipient mice displaying an increased number of peritoneal tumor nodules as well as a higher cumulative volume of peritoneal tumor nodules. (*d*) Bar graph showing the reduction in the percentage of tumor free animals as a consequence of including T3RCI in the vaccine formulation. NS, not statistically significant. * Denotes statistical significance ($p \leq 0.05$) compared to non-immunized CEA.Tg mice. ** Denotes statistical significance ($p \leq 0.05$) compared to vaccinated CEA.Tg mice. Statistical significance was determined using the Student-*t test*.

defined by the blockage of tumor cell implantation and the proliferation of tumor nodules (Figs. 1–5). The expression of IL-9 was originally linked to the development of chronic T_H2 immune responses and often associated with either the clearance of nematode parasites[22–24] or chronic asthma.[24–26] However, IL-9 expressing T helper cells are now described as a

distinct subset of T_H cells (designated T_H9) that contribute to pro-inflammatory conditions through the regulation of *il9* gene expression by IRF4 or PU.1 transcription factors.[24,27,28] The role of T_H9-derived IL-9 remains controversial in the context of tumor immunology. Hoelzinger and colleagues suggested that neutralization of IL-9 helped alleviate tumor burdens[29] while other studies suggested a beneficial (protective) role for IL-9 against B16 melanoma cells through a multivariate effector response.[30,31] Importantly, Purwar *et al.* suggested that T_H9 cells mediate anti-tumor immunity either through a T_H9 effector function, involving Granzyme B, or through mast cell dependent mechanisms.[31] A second study by Lu *et al.* argued that IL-9-dependent tumor rejection is mediated through the T_H9-induced recruitment of CCR6+ CD8+ T cells into the tumor microenvironment, which in turn target tumors through classic CTL mechanisms.[30] The disparity in observations is likely due to differences in the animal models used as well as differences in the effector mechanism(s) responsible for tumor rejection. In this study, we generated a protective CEA N domain-specific T_H9 response through a simple vaccination protocol that blocks the intraperitoneal implantation of murine colorectal MC38.CEA cells expressing CEA as a transgene [Ref. 5; as well as this study]. Our findings support a beneficial role for IL-9 in rejecting tumor cell engraftment in a manner involving the participation of mast cells.[30,31] More importantly, this study provides the first example of a simple method to induce a TAA-specific T_H9 immune response through vaccination.

It is presently unclear how $CD4^+$ T cells develop into T_H9 cells, *in vivo*. A recent report suggested that the development of T_H9 immunity arises from a failure to mount a T_H17 response.[31] *In vitro*, the addition of a combination of TGF-β and IL-4 can yield IL-9 producing CD4+ cells by down regulating the DNA-binding inhibitor Id3, thereby allowing for an enhancement in the binding of the transcription factors E2A and GATA-3 to the *il9* promoter region.[29,30,32] A recent study suggested that the stimulation of glucocorticoid-induced tumor necrosis factor receptor (TNFR)-related protein (GITR) signaling favors the development of T_H9 cells *in vivo*.[33] In both instances, the developed T_H9 cells lack Ag-specificity and are prone to conversion to a different T_H polarity once introduced *in vivo*.[29,30] This study demonstrates for the first time that the vaccine-induced, rCEA N domain-specific T_H9 response was generated through the engagement of TLR3 signaling, since the substitution of the poly I:C with other adjuvants yielded comparable CEA N domain-specific antibody titers but only delayed tumor growth (Fig. 1). For instance, squalene- and alum-based formulations are known to mount Ag-specific T_H2 immunity by inducing the secretion of chemokines (such as MCP-1, MIP-1α, MIP-1β, and IL-8) which recruit and help maturate Ag-presenting cells into MHC II+ CD86+ cells.[34] In contrast, the TLR-3 ligand poly I:C triggers a potent TLR-3/RLR signaling in APCs, which results in the expression of pro-inflammatory signals as well as type I interferons[35,36] that foster an environment conducive to the development of T_H9 cells.[16,24,28] As reported here, the inclusion of T3RCI ((R)-2–(3-Chloro-6-fluorobenzo [b] thiophene-2 -carboxamido)-3-phenylpropanoic acid) in the vaccine formulation, an agent that interferes with the TLR-3-dsRNA complex,[37] resulted in shifting the vaccine-engendered immune response toward a T_H2 response (Figs. 5–*c*) as well as reducing the number of tumor-free CEA.Tg mice (Fig. 6).

The observed surge in IL-5, IL-12p70, CXCL-5, CCL-11, MIP-2 as well as IL-1β in sera taken from protected (vaccinated) CEA.Tg mice (Fig. 2) suggested the involvement of mast cells. A number of reports have indicated a positive correlation between an increase in mast cell infiltration of tumor sites and positive prognosis in cancer.[38–42] In this study, mast cells were found to play a critical role in preventing tumor implantation (Fig. 4). Their association with T_H9-based anti-tumor immunity was further substantiated by the reversal of vaccine-imparted protection through the depletion of $CD4^+$ T cells, mast cells, as well as neutralization of either IL-9 or the pharmacological inhibition of mast cell degranulation, using cromoglycate (Fig. 4). In the context of auto-immunity, the secretion of IL-9 by pro-inflammatory T_H17 cells exacerbates experimental autoimmune encephalitis.[43] We did not detect changes in IL-17 serum levels in vaccine-protected CEA.Tg mice (Supporting Information Fig. 2). Taken together, the findings presented in this study point to the existence of an alternate tumor rejection/expulsion mechanism, involving mast cells, paralleling the mechanisms reported for graft rejection[17,18,44] as well as the IL-9 dependent eradication of parasitic nematodes.[19,20] This study suggests that this T_H9-dependent tumor rejection mechanism could be exploited for the purpose of developing vaccine therapies targeting tumor dissemination and engraftment.

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Author Contributions

A.A.W. and J.G. conceived the study, designed the experiments and wrote the manuscript. AAW performed all experiments. A.A.W., M.C. and M.A. expressed and purified the antigen used in immunization trials. A.A.W. and M.T. managed the transgenic mouse colony and performed the immunization trials. A.A.W., A.P. and E.H. performed post-mortem analysis of vaccinated animals. J.E.S. and S.G.-O. contributed with reagents and help. A.A.W. and M.S. performed the analysis of serum cytokines by Milliplex. A.A.W. and A.P. performed the analysis by flow cytometry.

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