IJC
International Journal of Cancer

A focused immune response targeting the homotypic binding domain of the carcinoembryonic antigen blocks the establishment of tumor foci *in vivo*

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Metastatic forms of cancers remain the main cause of death in cancer patients. In this study, we demonstrate that directing a sustained antibody response towards the homotypic binding function of CEA interferes with the implantation and development of tumor foci in CEA-expressing transgenic (CEA.Tg) mice. Specifically, vaccinating CEA.Tg mice with a recombinant, altered self-form of the CEA Ig V-like N domain led to the production of circulating IgG1 and IgG2a antibodies that inhibited CEA-mediated adhesion of murine carcinoma expressing CEA (MC38.CEA) and mediated antibody-dependent lysis of tumor cells. Moreover, vaccinated CEA.Tg mice were resistant to the development of tumor nodules in the lungs and the peritoneal cavity, suggesting that mounting a focused antibody response to the CEA N domain may represent a simple therapeutic strategy to control the establishment of metastatic foci in cancer patients.

There remains a strong need to develop therapies aimed at blocking or preventing the formation of metastatic tumor foci in cancer patients, in light of the fact that most cancer deaths are accounted for by patients with metastatic disease. Aberrantly expressed surface antigens, involved in intercellular adhesion, represent suitable targets for developing antiadhesive or antiaggregative therapies. One such surface marker is the carcinoembryonic antigen (CEA, CEACAM5 and CD66e), a GPI-linked glycoprotein linked to cell transformation and metastasis. CEA is frequently over-expressed on epithelial carcinomas of the intestinal and respiratory tracts, as well as cancers of the breast, pancreas, stomach and ovary.[1–5] From a clinical perspective, high preoperative serum concentrations of CEA correlate with metastasis, treatment failure and poor overall prognosis.[6–10] Specifically, a recent prospective study of CEA levels in the serum of 2,062 breast cancer patients has revealed that a CEA level >7.5 μg/L is associated with a high probability of subclinical metastases and a significant reduction in disease free and overall survival rates.[11]

The association of CEA with cancer progression has led to its use as an immunogen in designing anticancer vaccines.[2,5] Mechanistically, the intercellular homophilic binding property of CEA correlates with cancer invasion and metastasis.[5,12–14] CEA is composed of seven extracellular Ig-like domains (N, A_1, B_1, A_2, B_2, A_3 and B_3) where the binding of N and A_3B_3 Ig-like modules on distinct tumor cells promotes cell aggregation (defined as homotypic binding and homophilic cellular interactions).[13–15] Experimentally, monoclonal antibodies (mAbs) directed at epitopes found in the N domain of CEA,[16,17] or cyclic peptides derived from sequences within the N domain of CEA[15] have been shown to inhibit CEA-specific cellular adhesion *in vitro*. Similarly, the administration of a mAb or $(Fab')_2$, recognizing epitopes located between the N and A_1 domains of CEA, has been shown to increase the survival of nude mice harboring CEA-expressing lung micrometastases.[16,17] These findings suggest that an immune response specifically focused at blocking interactions involving the N domain of CEA may halt or limit the formation of tumor metastases in patients.

Previous attempts at developing CEA-based antitumor vaccines have centered on mounting cell-mediated immune responses using vaccine formulations based either on dendritic cells preloaded with predicted T-cell epitopes or recombinant viruses delivering the full length molecule.[5,18–22] The majority of putative T-cell epitopes have been short sequences located in the central region of this molecule.[23–25] Unfortunately, the lack of immunogenicity of

Key words: cancer vaccine, metastasis, carcinoembryonic antigen, antibody-dependent cytotoxicity
Additional Supporting Information may be found in the online version of this article.
Grant sponsors: The Canadian Breast Cancer Foundation, The University of Toronto Research Program
DOI: 10.1002/ijc.27582
History: Received 2 Sep 2011; Revised 28 Feb 2012; Accepted 27 Mar 2012; Online 11 Apr 2012
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these epitopes coupled with the presence of immuno-suppressive regulatory T (T_{reg}) cells to this self-antigen in tumor microenvironments have been shown to compromise the efficacy of CEA-based antitumor vaccines.[20,26] These limitations have been partly circumvented either through the depletion of T_{reg} cells[20,26] or by co-administering CEA in combination with costimulatory molecules.[21,22] A vaccine aimed at blocking CEA-dependent adhesion events and the establishment of tumor foci may represent a more appropriate and achievable objective. Importantly, the role of CEA in metastasis is linked to its over-expression and self-association which correlates with the early inactivation of caspase-9, the activation of the PI3-K/Akt survival pathway as well as the inactivation of caspase-8[27] presumably by directly binding TRAIL-R2 (DR5) through its PELPK motif (residues 108–112 of the N domain of CEA).[28] This peptide motif is responsible for mediating the lodging of metastasizing cells to the hepatic parenchyma leading to the development of metastatic foci through homophilic interactions involving the IgV-like N- and IgC-like A_3 domains.[5,15,29,30]

In view of the importance of the CEA N domain in tumor metastasis, we generated a folded recombinant form of the molecule (rCEA N) and used it as an immunogen as part of a simple vaccination procedure aimed at eliciting immune responses capable of simultaneously blocking CEA-mediated cellular interactions as well as killing CEA-expressing tumor cells both *in vitro* and *in vivo*.

Material and Methods

Generation of recombinant CEA modules

The human CEA cDNA open reading frame was purchased from Genecopoeia Inc (GermanTown, MD) and was used as a cloning template. Segments corresponding to the CEA N, FLAG-N and A_3B_3 modules were amplified by PCR (using primers listed in Supporting Information Table 1) and subcloned into pET30b (Novagen, Gibbstown, NJ) between the *NdeI* and *XhoI* restriction sites.

The expression of His-tagged recombinant CEA (rCEA) modules in *E. coli* (strain BL21 DE3 Star; Invitrogen, Ontario, Canada) was induced with 1 mM IPTG over a period of 24 hr at 37°C. The modules were subsequently purified by Ni-NTA affinity chromatography under denaturing conditions. The recovered rCEA protein constructs were concentrated by ultrafiltration against a buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl and 10 mM β-mercaptoethanol. The His-tag was subsequently cleaved from individual modules using recombinant Tobacco etch virus (rTEV) protease and the resulting untagged CEA modules recovered in the flow-through of Ni-NTA agarose columns. The extent of cleavage and the purity of the final recombinant products were confirmed by SDS PAGE. Endotoxin contamination was removed from rCEA N preparations using Detoxigel columns (Pierce, Thermo Scientific, Ontario, Canada).

Cell lines and growth conditions

The CEA-expressing human cancer cell lines BxPC-3 (ATCC No. CRL-1687, human pancreatic adenocarcinoma), HT-29 (ATCC No. HTB-38; human colorectal adenocarcinoma) and MCF-7 (ATCC No. HTB22; human breast adenocarcinoma) were used to monitor their sensitivity to complement-dependent cytotoxicity (CDC) in the presence of sera derived from vaccinated mice. The murine colonic carcinoma cell lines MC38.CEA and MC38 were kindly provided by Dr. Jeffrey Schlom (National Cancer Institute, Bethesda, Maryland). The human cervical adenocarcinoma cell line HeLa (ATCC No. CCL-2) as well as MC38 served as CEA^- cell lines for our studies. All cell lines were cultured at 37°C, 5.0% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and dihydrostreptomycin (100 μg/mL).

Animals

Breeder pairs of mice expressing human CEA as a transgene (CEA.Tg) were a gift from Dr. Wolfgang Zimmerman (Tumor Immunology Laboratory, LIFE-Center, Klinikum Grosshadern, Ludwig-Maximilians-University, Germany). CEA-positive litters were generated by backcrossing CEA-positive animals with parental C57BL/6 mice.[31] The genotype of CEA.Tg mice was confirmed by PCR.[31] All animals were bred and kept under standard pathogen-free conditions at the Ontario Cancer Institute animal facility. Experiments were performed under the approval of the local animal welfare committee and in accordance with the rules and regulations of the Canadian Council for Animal Care.

Immunization protocols and tumor challenge

For immunization protocols initiated after tumor implantation, 12–16-weeks-old CEA.Tg mice received 2.0×10^5 MC38.CEA cells subcutaneously (s.c.) in their hind leg. Palpable tumor nodules (1–5 mm^3) occurred in all the implanted animals within 12 days. Animals were then randomly subdivided into three groups: One group was left untreated, the second received an intraperitoneal (i.p.) dose of 100 μg poly I:C alone (referred to thereafter as adjuvant), whereas the last group received (i.p.) 100 μg of endotoxin-free rCEA N domain mixed with 100 μg poly I:C. Animals were vaccinated on days 13, 20 and 28 post-tumor implantation.

For prophylactic immunization studies, CEA.Tg mice were vaccinated (i.p.) on day 1 with 100 μg of endotoxin-free rCEA N domain mixed with 100 μg poly I:C. Two boosts were subsequently administered (i.p.) on days 3 and 10, each containing 50 μg endotoxin-free rCEA N domain and 100 μg poly I:C. The sera from CEA.Tg mice were screened for anti-CEA IgG antibodies and only responders (80–90% of immunized mice) were included in subsequent tumor challenge experiments. MC38.CEA tumor cells were implanted i.p. on

day 28 for peritoneal invasion studies or were injected i.v. (tail vein) for lung colonization studies.

Monitoring tumor growth and tumor burden

The length and width of implanted tumors (hind leg) and tumor nodules (lung, peritoneal cavity) were measured with callipers. Tumor volumes were calculated using a modified ellipsoidal formula where the volume of the tumor (mm^3) equals $[(x^2X\ y)/2]$; where the terms x and y represent the transverse and longitudinal diameters of the tumor, respectively.[32] To enumerate pulmonary tumor nodules, formalin-fixed lung specimens were embedded in paraffin, sectioned at three different depths and 4 μm sections were stained with hematoxylin and eosin (H&E). Images of stained slides were recorded and analyzed for tumor foci using an Aperio slides scanner and ImageScope software (Aperio Technologies Inc, Vista, CA). The number of peritoneal tumor nodules was determined by postmortem analysis of dissected animals.

Preparation and cultivation of spleen leukocytes

Splenocytes were prepared from vaccinated and control CEA.Tg mice as previously described.[33] Following the assessment of cell viability by Trypan blue dye exclusion, cells were suspended to a density of 1×10^6 cells per mL in 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 and maintained at 37°C in a humidified 5.0% CO_2 atmosphere. NK cells were purified from total leukocyte suspensions for ADCC analyses, using the EasySep Mouse NK Cell Enrichment Kit (StemCell Technologies, British Colombia, Canada). Cell viability following harvest was typically $\geq$95%.

Analysis of CEA-specific immune responses

Splenocytes recovered from immunized and control mice were stimulated *ex vivo* with either concanavalin A (ConA; 5 μg per 10^6 splenocytes per mL; Sigma-Aldrich), the full length tumor glycoform of human CEA (1 μg per 10^6 splenocytes per mL; Sigma-Aldrich), rCEA WT N domain (1 μg per 10^6 splenocytes per mL) or left as unstimulated control cells. Quantification of CEA-specific cytokine secreting cells was performed using IFN-γ, IL-10 and IL-4 ELISPOT assay kits, as suggested by the manufacturer (R&D Systems; Minneapolis, MN). The spots were enumerated using an automated ELISPOT plate counter (Cellular Technologies Inc; Shaker Heights, OH). Frequencies of CEA-specific cytokine secreting cells, or spot forming units (SFUs), were calculated by subtracting background values (counted from wells containing unstimulated cells) from measured values derived from tested conditions as previously described.[33,34]

Antibody responses raised in CEA.Tg mice and directed at the N domain of CEA were analyzed by ELISA in 96-well microtiter plates (Falcon) coated with 1 μg per well of rCEA N domain, as previously described.[33]

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 SP label-free, impedance-based cell sensing device (Roche Applied Sciences, Laval, Canada).

The inhibition of CEA-dependent cellular adhesion was monitored using MC38.CEA cells (2.5×10^5 cells per well) suspended in media containing either heat-inactivated sera from immunized, adjuvant-treated or non-immunized CEA.Tg mice (1:100). The cell suspensions were transferred to 96-well microtiter plates incorporating a sensor electrode array (E-plates) and precoated with rCEA N (1μg per well). Cell attachment was measured as a change in relative impedance, termed cell index (CI).[35] The adhesion of non-serum treated MC38.CEA cells served as a positive control for the assay.

Ab-dependent killing of tumor cells (ADCC and CDC) were measured with mid-log phase monolayers of MC38.CEA cells grown in wells of E-plates and subsequently exposed to media supplemented with sera (1:100) and either purified NK cells or complement (1:100). The growth kinetics and viability of MC38.CEA cells were then monitored by measuring changes in impedance as CI values recorded on a xCELLigence RTCA SP 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.[36]

Inhibition of CEA homotypic interactions

The inhibition of FLAG-tagged rCEA N domain binding to the rCEA A_3B_3 domain was assessed using an enzyme-linked immunosorbent assay (ELISA).[37] Briefly, 96-well flat-bottomed Falcon microtiter plates (Becton–Dickinson Biosciences, Franklin Lakes, NJ) were coated with the purified CEA A_3B_3 domain. After blocking with BSA (1% in PBS, 1 hr at room temperature), the plates were incubated for 1 hr at room temperature with FLAG-tagged rCEA N diluted in PBS-Tween (0.05%; 100 μL) supplemented with either immune or control sera (1:1,000 dilution). The presence of bound FLAG-tagged rCEA was detected by incubating the plates for 1 hr at room temperature with horseradish peroxidase (HRP) coupled anti-FLAG monoclonal antibody M2 (1:5,000 dilution; Sigma-Aldrich).

Adoptive transfer of lymphocytes

To assign the role of lymphocytes in protecting mice from developing tumor nodules in the peritoneal cavity, total spleen lymphocytes or purified B cells derived from immunized CEA.Tg mice were injected i.v. (*via* tail vein) into immunologically naïve recipient CEA.Tg mice. B lymphocytes were purified by negative selection (EasySep mouse B cell

enrichment kit; StemCell Technologies) from single cell suspensions of total spleen leukocytes collected from immunized CEA.Tg mice ($n = 6$). Specifically, B cells were separated from other hematopoietic cells as defined by the surface antigens CD4, CD8, CD11b, CD43, CD49b, Ly-6G (GR-1) and TER119. Recipient naïve mice received 2×10^6 B cells per mouse or 4.1×10^6 unfractionated splenocytes. Three days later, 2.0×10^5 MC38.CEA cells were implanted in the peritoneal cavity of treated mice. The proliferation of MC38.CEA cells and the development of tumor nodules were monitored as described above, 21 days post-tumor implantation.

Passive immunization with hyper-immune sera

Sera from immunized CEA.Tg mice ($n = 6$) were collected one day following the last booster injection (day 11 post-immunization), pooled and diluted with PBS (1:10), filter sterilized and stored at -20°C until use. The presence of CEA N domain-specific serum antibodies was verified by ELISA, as described above. Serum samples (200 μL) were injected (i.p.) into immunologically naïve CEA.Tg mice ($n = 5$) on days -5 to 3 and days 10 to 17 post-tumor implantation. On day 0, 2.0×10^5 MC38.CEA cells were implanted in the peritoneal cavity and the development of tumors was monitored as described above.

Statistics and data analysis

Collected data sets were analyzed for significance by ANOVA and individual groups were compared using Student-*t*-test. All statistical analyses and graphs were constructed using PRISM (version 5.01, Graph Pad Software for Science, San Diego, CA). *P* values ≤ 0.05 were considered significant.

Results

Expression of rCEA domains involved in homotypic association

A recombinant form of the IgV-like CEA N domain (corresponding to residues 1–132) was generated in *E. coli*. Its proper folding was confirmed by its ability to bind to the CEA A_3B_3 domain[13] in a yeast two-hybrid survival assay, by their co-immunoprecipitation with a mAb directed at the N domain as well as by ELISA (Supporting Information Fig. 1). Together, the findings suggested that the generated rCEA N domain adopts a folded state that maintains its expected homotypic adhesive function.

Administration of rCEA N retards the growth of subcutaneously implanted tumors

A vaccination protocol using the recombinant CEA N domain as an immunogen was first evaluated to assess if an immune response to CEA in CEA.Tg mice could interfere with the growth of a rapidly expanding primary tumor. The subcutaneous implantation of 2×10^5 MC38.CEA cells resulted in the formation of palpable tumor nodules (1–5 mm^3) in mice within 12 days post-implantation. On day 13 post-tumor implantation, the mice were vaccinated i.p. with the rCEA N domain mixed with poly I:C and boosted on days 20 and 28. As highlighted in Figures 1*b* and 1*c*, vaccination with the rCEA N domain and poly I:C delayed the growth of established hind leg tumors in relation to tumor cells implanted in either non-immunized or adjuvant-treated CEA.Tg mice. In contrast, the implantation of MC38 cells into CEA.Tg mice followed by vaccination did not delay tumor growth (Supporting Information Fig. 2).

Vaccination prevents tumor colonization and nodule formation

A rapidly expending localized tumor mass, as in the case of the s.c. implanted MC38.CEA cells, represents a primary tumor that is typically treated by local surgery and radiation therapy.[5] A more effective use of CEA-based anticancer vaccines would be in the context of adjuvant therapies targeting metastasizing cells. Specifically, the deregulated overexpression of CEA is linked to the process of tumor metastasis,[6,7,28,29] since CEA-expressing adenocarcinomas are known to metastasize to the liver, lungs or the abdominal cavity, in the case of patients with gastric cancer.[38]

We did not observe tumor metastases in distal organs following the implantation of MC38.CEA cells in the hind leg (data not shown). To address this limitation of the s.c. tumor implantation model, vaccinated mice were subsequently challenged with 2×10^5 MC38.CEA tumor cells were either injected directly into the peritoneal cavity or administered intravenously (as outlined in Figs. 2 and 3).

At day 35 post-tumor implantation into the peritoneal cavity, the animals were euthanized and examined for the presence of tumor nodules. No tumor masses were detected outside of the peritoneal cavity. As indicated in Figure 2*b*, vaccinated animals displaying an immune response to CEA were protected against the development of tumor nodules in the peritoneal cavity and remained tumor free. In contrast, non-immunized and adjuvant-treated animals developed large tumor nodules (Figs. 2*b* and 2*c*). These results were specific to CEA, since vaccinated animals rapidly developed tumor nodules, when MC38 cells were implanted into their peritoneal cavities (Supporting Information Fig. 3).

Similarly, CEA.Tg mice were dissected to determine the distribution of tumor nodules in their lungs, 60 days following the i.v. injection of MC38.CEA cells. Non-vaccinated and adjuvant-treated animals developed large tumor masses in their lungs (Figs. 3*b* and 3*c*). Tumor nodules were also observed in the liver in a limited subset of animals (less than 5 percent of untreated mice; data not shown). Histological examination of lung tissues (H&E stained lung sections; $n = 18$; six randomly chosen lungs from each group) derived from control animals (non-immunized and adjuvant treated groups) confirmed that lungs were enlarged as a consequence of the number and size of tumor foci in contrast to lungs taken from either normal age-matched or immunized animals (Fig. 3, panels *b*–*e*). Taken together, these findings suggest that the administration of the rCEA N domain as an

immunogen was effective in preventing the development of CEA^+ tumor nodules.

Analysis of vaccine-engendered CEA-specific immune responses

The sera and splenocytes of age-matched immunized and control CEA.Tg mice were collected to analyze correlates of immune responses and define the mechanisms responsible for the observed positive outcomes (Fig. 4*a*). The presence of CEA-specific cellular responses was first detected using leukocytes stimulated *in vitro* with either rCEA N domain or the full length tumor glycoform of CEA (FL-CEA). Irrespective of the antigen used for stimulation, we did not observe any significant levels of splenocyte proliferation (Supporting Information Fig. 4). This observation suggested that the immunization protocol yielded a modest level of T-cell stimulation. The development of CEA-specific, T_H-cell responses was subsequently assessed by measuring the number of antigen-specific cytokine (IL-4, IL-10 and IFN-γ) secreting cells by ELISPOT assays.[5,39,40] Non-immunized CEA.Tg mice as well as mice given the adjuvant alone did not produce CEA-specific cellular immune responses, since splenocytes derived from these animals did not secrete cytokines in response to antigenic stimulation with either rCEA N domain or the full length tumor glycoform of CEA (Fig. 4*b*). In contrast, stimulation of splenocytes (derived from immunized CEA.Tg animals) with either the rCEA N domain or the full length CEA tumor glycoform yielded a balanced cytokine production profile, as suggested by the comparable numbers of recorded antigen-specific IL-4, IL-10 and IFN-γ secreting cells (as spot forming units from ELISPOT assays; Fig. 4*b*).

The production of circulating anti-CEA antibodies was subsequently analyzed by ELISA. High titers of circulating anti-CEA IgG antibodies were observed only in sera derived from immunized CEA.Tg mice (Fig. 4*c*). Isotype analysis revealed high titers of CEA-specific IgG1 and IgG2a (Fig. 4*c*). These high IgG1 and IgG2a titers were consistently observed in $\geq$90% of individual vaccinated animals derived from independent immunization trials (Fig. 4*d*) and correlated with the observed balanced CEA-specific cytokine response (Fig. 4*d*). Moreover, the vaccination protocol yielded anti-CEA antibodies that specifically reacted with MC38.CEA cell lysates (data not shown), implying that the presence of *N*-linked sugars had no consequence on the recognition of epitopes by rCEA N-domain-specific serum antibodies. Taken together, these observations suggest that the immunization strategy yielded a strong humoral immune response supported by a modest CEA-specific T_H cell response.

N domain-specific antibodies can mediate Ab-dependent killing of tumor cells as well as blocking CEA-dependent intercellular adhesion

The ability of N domain-specific circulating antibodies in mediating antibody-dependent cell killing was first assessed by ADCC and complement-dependent cell killing assays (Fig. 5). Specifically, MC38.CEA cells were incubated with either immune or control sera (1:100 dilution) in the presence of either NK cells or exogenous complement (1:100 dilution) with loss of cell viability being monitored directly as time-dependent changes in impedance signals. As depicted in Figure 5, killing of MC38.CEA target cells only occurred when incubated in the presence of sera derived from vaccinated CEA.Tg mice.

Interference with CEA-mediated homophilic interactions by the vaccine-elicited, anti-CEA N domain antibodies was assessed *via* two methods. First, MC38.CEA cells were premixed with sera derived from immunized or control CEA.Tg mice (1:100 dilution) and the resulting suspensions transferred to impedance-sensing plates (E-plates) coated with rCEA N, and their adhesion monitored over a period of 6 hr. Pretreating MC38.CEA cells with sera from immunized mice significantly reduced CEA-mediated cell adhesion, but not the sera derived from control animals (Fig. 5*d*).

The inhibition of CEA-dependent homotypic interactions at the protein level was subsequently investigated using purified rCEA protein modules. Specifically, an ELISA-based protein binding assay was used to compare the inhibition of interactions between soluble FLAG-tagged rCEA N domain and the immobilized rCEA A_3B_3 protein module following the addition of sera from immunized or control mice (Fig. 5*e*). The addition of sera from control mice had no effect on blocking the homotypic binding between the N and A_3B_3 domain (Fig. 5*e*). In contrast, the addition of sera from immunized mice reduced homotypic binding by $\sim$60% (Fig. 5*e*). In summary, the production of antibodies recognizing the rCEA N domain possesses both cytotoxic and homophilic adhesion blocking properties.

The broad cytocidal property of N domain-specific serum antibodies was further confirmed for their capacity to kill a panel of CEA-expressing human tumor cells by CDC. CEA^+ (MC38.CEA, HT-29, MCF-7 and BxPC3) and CEA^- (MC38, HeLa) cancer cell lines were treated with complement and sera derived from either immunized or control mice. The number of nonsurviving cells was quantified by Trypan blue dye exclusion. As depicted in Figure 5*f*, complement-dependent killing was only observed for CEA^+ MC38.CEA, BxPC-3, HT-29 and MCF-7 cells in the presence of serum derived from vaccinated animals, but not for CEA^- HeLa or MC38 cells. The level of the complement-dependent killing qualitatively correlated with the degree of CEA expression on these cell lines (Fig. 5*f*; Supporting Information Fig. 5).

Passive immunization experiments support the importance of the vaccine-engendered anti-N-domain antibodies as the key effector mechanism against tumor colonization

Adoptive transfer studies were carried out to validate the importance of vaccine-induced anti-CEA antibodies in conferring protection to vaccinated CEA.Tg. Specifically, sera and B lymphocytes were collected from immunized CEA.Tg mice and adoptively transferred to naïve CEA.Tg recipient mice.

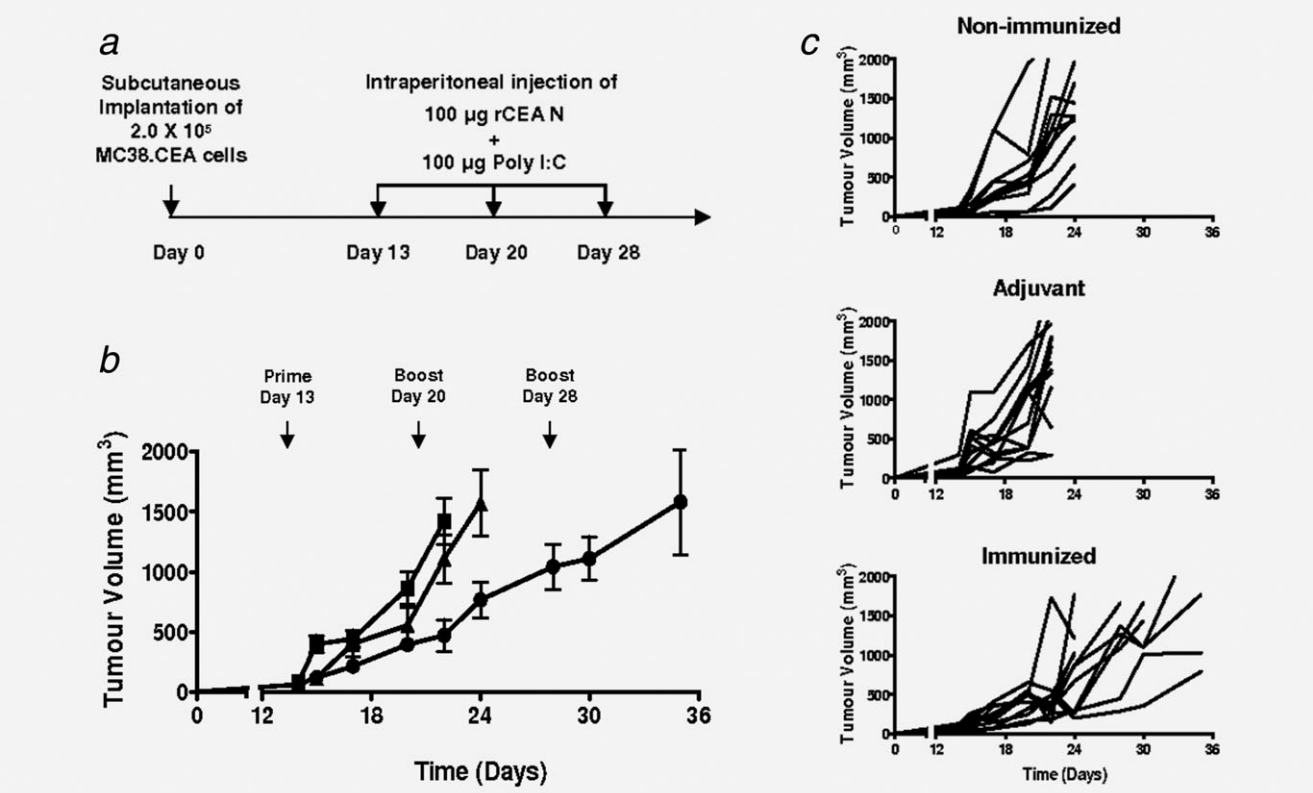

Figure 1. Vaccination of CEA.Tg mice i.p. with the rCEA N domain as an immunogen delays tumor growth in immunized mice. (*a*) Experimental design and immunization schedule. (*b*) Tumor growth kinetics of an established CEA-expressing, murine colonic carcinoma MC38.CEA implanted s.c. in the hind leg of non-immunized CEA.Tg mice (▲; $n = 12$), mice who received the adjuvant poly I:C only (■; $n = 12$), or mice vaccinated with rCEA N domain and adjuvant (●; $n = 12$). (*c*) Collection of individual tumor growth curves observed for every CEA.Tg mice within each experimental treatment group. Each line represents a single mouse. Collected data sets were analyzed for significance by ANOVA and individual groups of animals were compared using Student-*t*-test. The observed growth of tumors in immunized CEA.Tg mice was significantly delayed in comparison to non-immunized and adjuvant alone-treated animals.

Animals were then challenged with an i.p. infusion of 2×10^5 MC38.CEA cells. At day 21 post-tumor implantation, the mice were sacrificed and examined for the early occurrence of tumor nodules in the peritoneal cavity. Small tumor masses were readily observed in all non-immunized mice while immunized mice remained tumor-free (Fig. 6*b*). Similarly, transfer of total leukocytes, purified B cells or immune sera protected recipient animals from developing tumor nodules (Fig. 6*b*). These results support the importance of stimulating CEA N domain-specific antibodies-producing B cells for the observed protection against tumor implantation.

Discussion

CEA is a useful clinical biomarker for monitoring recurrence and the management of metastatic cancers and has been investigated as a candidate cancer vaccine Ag[5–7,18] in light of its association with tumor progression from neogenesis to metastasis.[5,15,27,28] One known biological function of CEA is its role in both homotypic and heterotypic interactions[13,15,41] which strongly correlates with the establishment and growth of tumor metastases in distal sites such as the liver, lung and the peritoneal cavity.[5,13,28,29,38] Structurally, the IgV-like N domain of CEA strongly interacts with the IgC-like A_3B_3 domain, allowing adjacent CEA molecules to homotypically adhere to each other. Such homotypic adhesion events on CEA-expressing cells yield networks of homophilic intercellular interactions that further contribute to lodging additional cells within the context of expanding nascent metastatic foci.[12,13,15,28,29,42]

Previous attempts at developing CEA-based cancer vaccines have traditionally revolved around the idea of mounting cellular (T_H1) immune responses towards the full-length molecule *via* the injection of either recombinant viruses and/or Ag-pulsed dendritic cells with the intention of eradicating primary tumor masses.[5,18–22,43,44] The success of these vaccination strategies has been hampered by central and peripheral tolerance to CEA.[20,26] We hypothesized that mounting a polyclonal antibody response focused on the CEA N domain would yield antibodies capable of blocking homophilic cell adhesion events between CEA-positive cells (that can prevent tumor implantation and growth) as well as antibodies capable of destroying CEA-bearing tumor cells through ADCC and CDC. To that effect, a recombinant CEA N domain was used

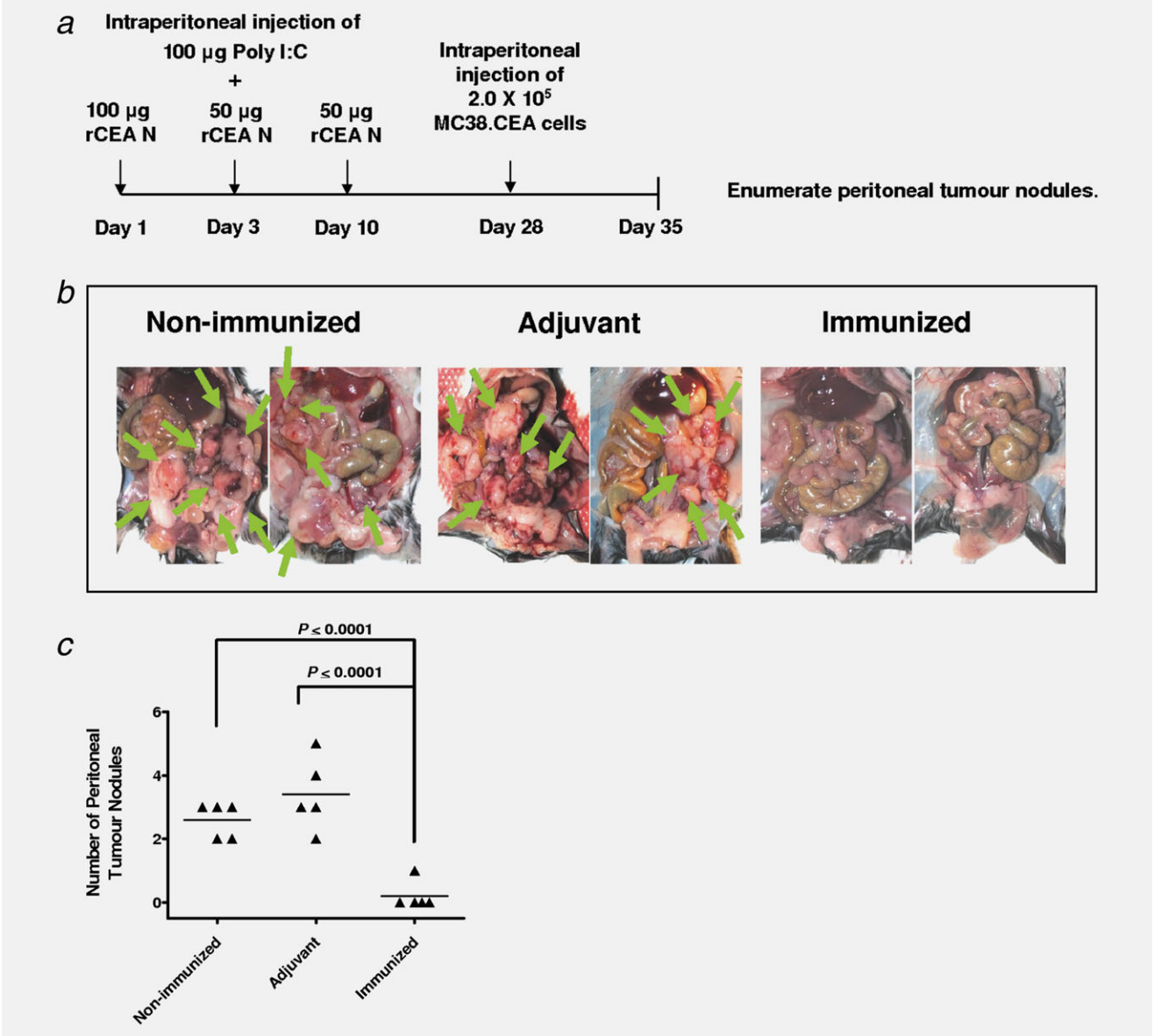

Figure 2. Vaccination of CEA.Tg mice i.p. with the rCEA N domain as an immunogen prevents the development of peritoneal tumor nodules. (*a*) Experimental design and immunization schedule. MC38.CEA cells were injected i.p. into non-immunized, adjuvant-treated or immunized CEA.Tg mice. (*b*) Photographs highlighting the absence and presence of tumor nodules in the viscera of immunized and control mice at day 35 post-tumor injection. The tumor nodules are indicated by green arrows. (*c*) Number of tumor nodules present in the peritoneal cavity of immunized and control mice ($n = 5$). Statistical significance was determined using Student-*t*-test.

to vaccinate CEA.Tg mice. Unlike the full length molecule, the IgV-like rCEA N represents an altered form of a self-antigen since it lacks naturally occurring *N*-linked glycans and displays an unnatural C-terminus; features which we hypothesize would contribute to overcoming immunological tolerance to CEA.[45] Moreover, the use of a single domain of CEA involved in homotypic interactions to serve as the immunogen narrows the immune response to a focused and distinct set of CEA determinants. This CEA module was mixed with Poly I:C and administered to CEA.Tg mice with the view of mounting a protective immune response. Poly I:C was chosen as the adjuvant in view of its capacity to stimulate both Type 1 responses through TLR-3/7 signalling[46] as well as B cell activation,[47] a combination of immune responses shown to positively influence the development of protective antitumor immune responses in both mice and patients.[46] Additionally, this formulation was administered i.p since this route of immunization represents an established route of immunization successfully used to mount anti-CEA antibodies.[48]

MC38.CEA cells were implanted into CEA.Tg mice using three distinct approaches. As a first implantation model, MC38.CEA cells were introduced subcutaneously into the hind leg of CEA-expressing transgenic mice; an approach

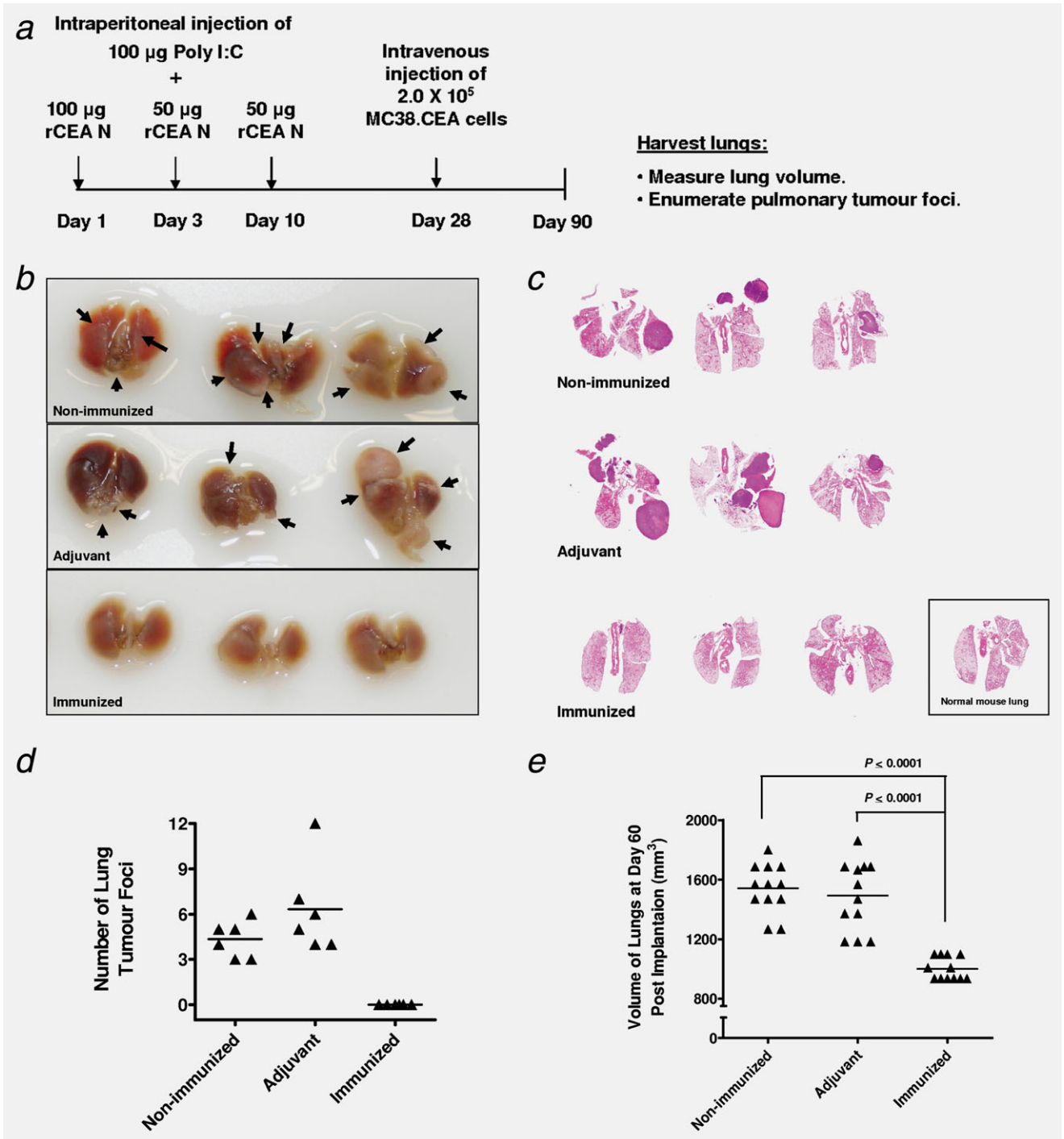

Figure 3. Vaccination of CEA.Tg mice (i.p.) with the rCEA N domain as an immunogen prevents the development of pulmonary tumor nodules. (*a*) Experimental design and immunization schedule. (*b*) CEA-expressing murine colonic carcinoma MC38.CEA cells were injected i.v. (tail vein) into CEA.Tg mice at day 28 post-vaccination. Photographs highlight tumor masses (black arrows) present in lung tissues isolated from immunized and control CEA.Tg mice at day 60 post-tumor injection. (*c*) Hematoxylin and eosin (H&E) stained sections of whole mouse lungs displaying large tumor nodes in the case of non-vaccinated or adjuvant alone-treated animals (dark stained areas). The histological features of lung tissues from immunized mice were similar to that of a normal mouse lung. (*d*) Enumeration of tumor foci in H&E stained lung specimens ($n = 6$, whole lungs from each treatment group). (*e*) Total volume of lung tissues (including tumor masses; $n = 12$) at day 60 post-tumor implantation. Lung volumes were determined by measuring the length, height, and width of whole lungs following postmortem analysis. Statistical significance was determined using Student-*t*-test.

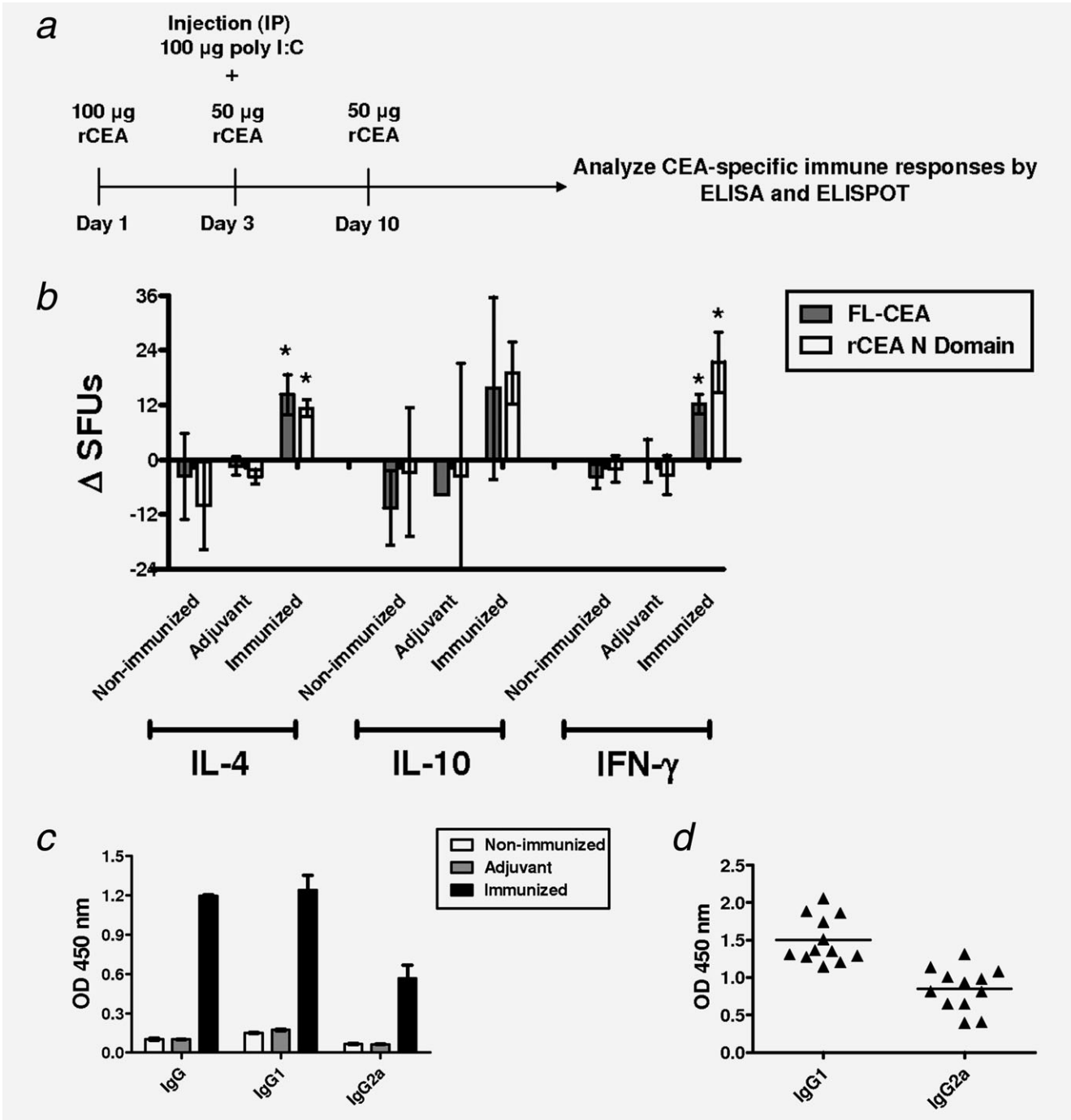

Figure 4. The intraperitoneal administration of rCEA N domain with poly I:C produces a strong CEA-specific humoral response. (*a*) Experimental design and immunization schedule. (*b*) Enumeration of rCEA-specific IFN-γ, IL-10 and IL-4 spot forming units (ΔSFUs) from immunized and control mice as measured by ELISPOT assays. 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. (*c*) Sera of non-immunized, adjuvant-treated or immunized CEA.Tg mice were analyzed by ELISA for the presence of circulating N domain-specific IgG, IgG1, and IgG2a antibody titers. The results represent the mean observed optical density (± SEM) at 450 nm of pooled serum samples ($n = 12$; at a 1:1,000 dilution). (*d*) Comparison of individual mice CEA N domain-specific IgG1 and IgG2a titers as determined by ELISA at a serum dilution of 1:1,000.

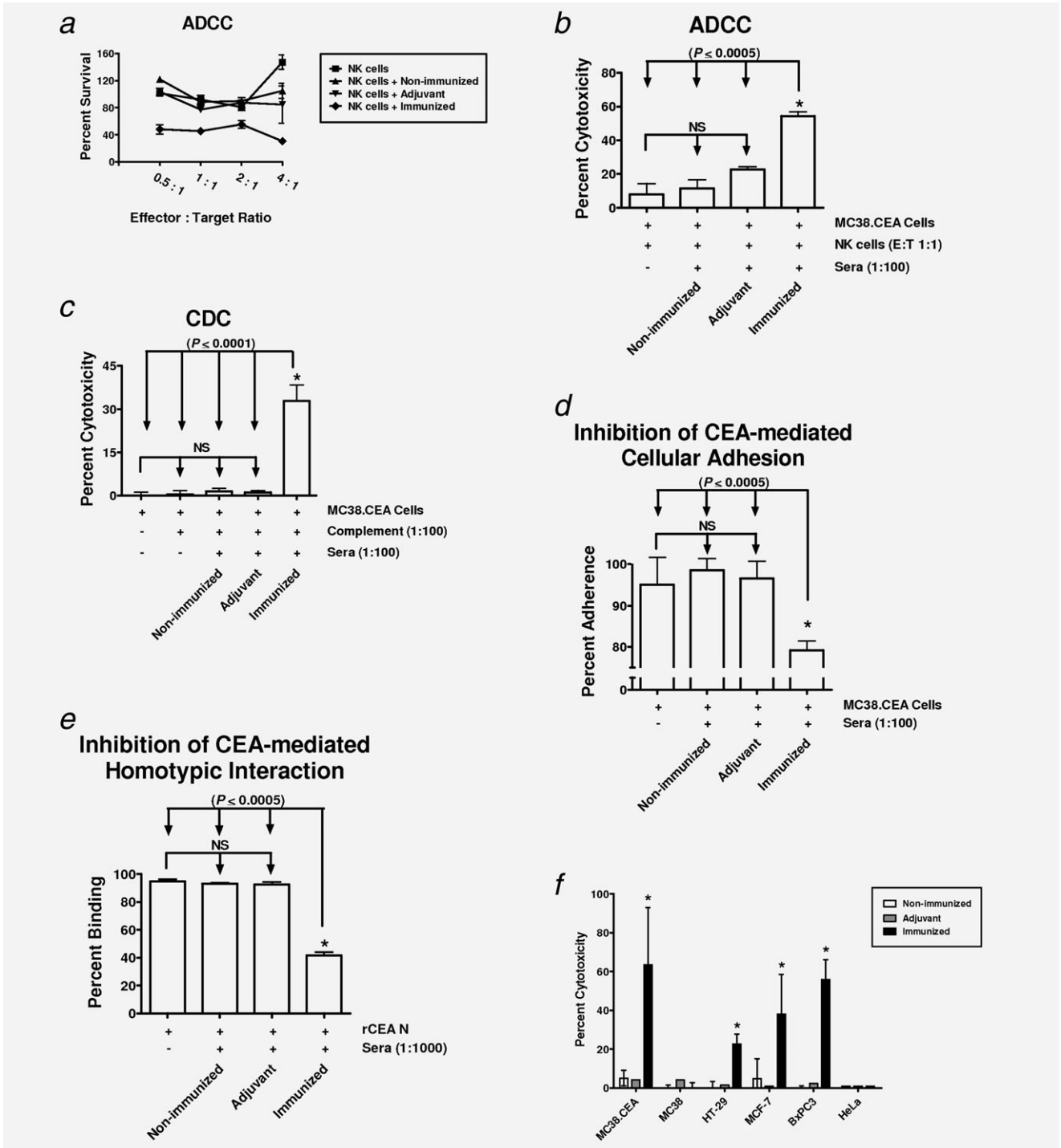

Figure 5. Serum from CEA.Tg mice vaccinated with the rCEA N domain display ADCC and CDC cytotoxic functions towards CEA-expressing cells as well as CEA-specific anti-adhesive properties. Only serum (1:100 dilution) derived from vaccinated CEA.Tg mice can kill CEA-expressing MC38.CEA tumor cells by Ab-dependent cellular cytotoxicity [ADCC; panels (*a*) and (*b*)] as well as complement dependent cytotoxicity [CDC; panel (*c*)]. (*d*) Addition of anti-CEA anti-serum (1:100 dilution) from immunized CEA.Tg mice inhibits CEA-dependent adhesion of MC38.CEA to wells coated with rCEA N. (*e*) Specific inhibition of homotypic binding of pure recombinant rCEA N domain to immobilized A_3B_3 domain by the addition of serum (1:1,000 dilution) derived from mice vaccinated with the rCEA N domain. (*f*) Sensitivity of CEA-expressing human adenocarcinoma cell lines to complement-dependent lysis. CEA^+ (MC38.CEA, HT-29, MCF-7 and BxPC3) and CEA^- (MC38, HeLa) cells were suspended at a density of 1×10^6 cells per mL in a medium supplemented with rabbit complement (1:250 final dilution) and treated with sera derived from immunized or control mice (1:250 final dilution). Experiments were conducted using pooled serum samples ($n = 8$). NS; not statistically significant when compared with untreated cells. Asterisk denotes statistical significance ($P \leq 0.001$) when compared with samples treated with sera from non-immunized CEA.Tg mice; Student-*t*-test.

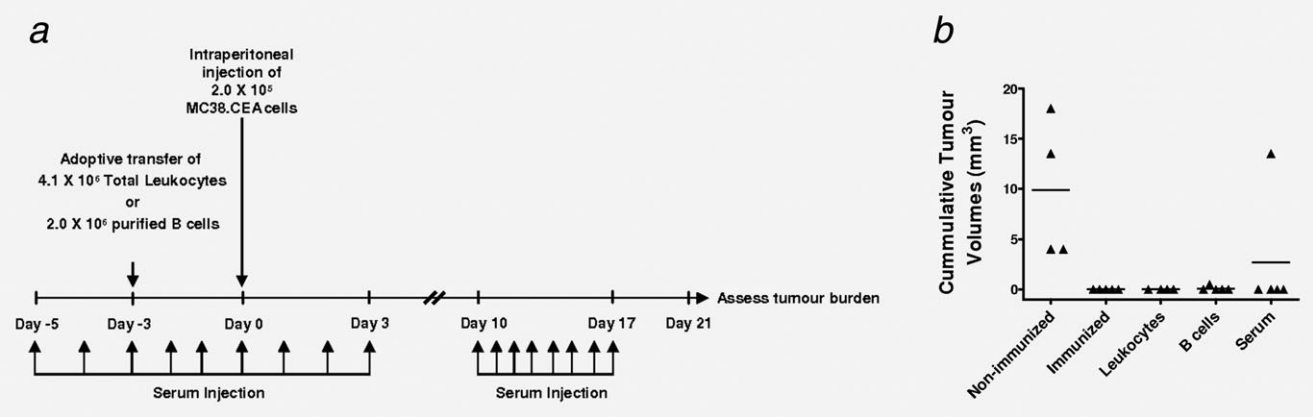

Figure 6. Adoptive transfer of CEA N-domain specific antibodies or B lymphocytes derived from vaccinated CEA.Tg mice into naive CEA.Tg recipients prevents the development of peritoneal tumor nodules. (*a*) Experimental design and treatment schedule. (*b*) Cumulative tumor volumes at day 21 post-MC38.CEA cells implantation into the peritoneal cavity of naïve CEA.Tg mice.

that led to a rapid establishment and growth of localized tumor masses. Vaccination of animals following tumor establishment provided a significant delay in tumor growth (Fig. 1), while displaying no signs of autoimmunity (data not shown). However, we projected that a mounted immune response to the rCEA N domain would serve a more appropriate role in blocking the establishment of new tumor foci or the expansion of micrometastases, rather than in arresting the uncontrolled localized growth of a solid tumor mass. Vaccinated CEA.Tg mice were thus challenged with either i.v. or i.p. injected MC38.CEA tumor cells. In both instances, only vaccinated animals were protected from developing tumor nodules, whereas all non-immunized and adjuvant-treated mice displayed numerous tumor nodules in the lungs or viscera (Figs. 2 and 3), suggesting that vaccination prevented the lodging and establishment of tumor foci.

The present vaccination strategy is appealing over previously published vaccine protocols, since the engendered response targets a narrower range of potentially relevant epitopes, bypassing antigenic competition from irrelevant epitopes[19,22–25] present in full length CEA. One report describes the use of CEA-based subunit vaccine, where a recombinant CEA A_3B_3 domain was mixed with CpG oligonucleotides and subcutaneously injected into C57BL/6 mice.[40] The authors reported that this strategy produced a weak CEA-specific immune response that failed to protect C57BL/6 mice against a lethal tumor implant (when compared with a TAT-fused construct).[40] In contrast, the present study uses the native CEA N domain sequence, mixed with poly I:C to produce an effective CEA-specific immune responses in CEA.Tg mice capable of blocking tumor implantation to the lungs and peritoneal cavity (Figs. 1–6).

The engendered CEA-specific immune response in CEA.Tg mice is dominated by the production of IgG1 and IgG2a antibodies directed at the N domain of CEA (Fig. 4). The overall response to the CEA N domain is thus distinct from most cancer vaccine strategies aimed at producing a CEA-specific cellular immune response[5,43,44] and beneficial in modulating or blocking the growth of implanted tumors (Fig. 2–6), by virtue of inducing Ab-dependent tumor lysis (by both ADCC and CDC) and in interfering with CEA-mediated cellular adhesion (Fig. 5). The importance of B lymphocyte populations in vaccinated mice was further confirmed by adoptively transferring CEA-specific B cells or sera from vaccinated animals into naïve CEA.Tg mice and rescuing them from developing peritoneal tumor nodules (Fig. 6). These observations are supported by Park *et al.*[49] who demonstrated the value of mounting a Her-2/neu-specific polyclonal antibody response in curing mice from pulmonary as well as large established subcutaneous ErbB-2–expressing tumors.[49] Finally, the recent demonstration that a monoclonal antibody directed at the N domain of CEA can suppress the growth of a colorectal tumor xenograft in nude mice further supports the value of mounting a selective antibody response to the N domain.[17]

In summary, the simple (i.p.) injection of an altered-self form of the CEA N domain elicited an antibody response in CEA.Tg mice that prevented tumor colonization and the development of tumor nodules. This antibody-dominated response led to the specific killing of CEA-expressing cells by ADCC and CDC in addition to impeding CEA-dependent intercellular adhesion. Since high circulating levels of CEA in the serum of cancer patients frequently correlate with a higher incidence of metastatic relapse, a vaccine formulation using this rCEA N domain as an immunogen may represent a safe and simple adjuvant therapy delaying or preventing tumor metastasis in cancer patients displaying elevated serum CEA levels prior to surgery.

Acknowledgements

The authors thank Dr. A. McCluskey for help with the yeast two-hybrid assay, Dr. Neil Berinstein for his critical reading of the manuscript, Drs. M. Karimi and J. Carlyle for helpful discussions in performing ADCC experiments, Dr. J. Schlom for providing the MC38.CEA and MC38 cell lines and Dr. W. Zimmerman for providing the CEA.Tg mice. The authors declare no conflict of interest.

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