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Induction of Immunogenic Apoptosis by Blockade of Epidermal Growth Factor Receptor Activation with a Specific Antibody

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Despite promising results in the use of anti-epidermal growth factor receptor (EGFR) Abs for cancer therapy, several issues remain to be addressed. An increasing emphasis is being placed on immune effector mechanisms. It has become clear for other Abs directed to tumor targets that their effects involve the adaptive immunity, mainly by the contribution of Fc region-mediated mechanisms. Given the relevance of EGFR signaling for tumor biology, we wonder whether the oncogene inhibition could contribute to Ab-induced vaccine effect. In a mouse model in which 7A7 (an anti-murine EGFR Ab) and AG1478 (an EGFR-tyrosine kinase inhibitor) displayed potent anti-metastatic activities, depletion experiments revealed that only in the case of the Ab, the effect was dependent on CD4+ and CD8+ T cells. Correspondingly, 7A7 administration elicited a remarkable tumor-specific CTL response in hosts. Importantly, experiments using 7A7 F(ab′)2 suggested that in vivo Ab-mediated EGFR blockade may play an important role in the linkage with adaptive immunity. Addressing the possible mechanism involved in this effect, we found quantitative and qualitative differences between 7A7 and AG1478-induced apoptosis. EGFR blocking by 7A7 not only prompted a higher pro-apoptotic effect on tumor metastases compared with AG1478, but also was able to induce apoptosis with immunogenic potential in an Fc-independent manner. As expected, 7A7 but not AG1478 stimulated exposure of danger signals on tumor cells. Subcutaneous injection of 7A7-treated tumor cells induced an antitumor immune response. This is the first report, to our knowledge, of a tumor-specific CTL response generated by Ab-mediated EGFR inhibition, suggesting an important contribution of immunogenic apoptosis to this effect. The Journal of Immunology, 2011, 187: 000–000.

Among the molecular targets that are currently in clinical evaluation for cancer treatment, the epidermal growth factor receptor (EGFR) has been widely validated. EGFR is a cell membrane growth factor receptor characterized by tyrosine kinase activity that plays a crucial role in the control of key cellular transduction pathways in both normal and tumor cells. EGFR is overexpressed in a variety of human epithelial tumors (1). Ligand binding to EGFR results in stabilization of the receptor active conformation that allows its dimerization and autophosphorylation (2). EGFR tyrosine kinase activation ultimately leads to stimulation of cell proliferation, invasion, angiogenesis, and the blocking of apoptosis (3). In addition, recent studies have demonstrated that EGFR signaling is involved not just in the malignant behavior of tumor cells, but also in the modification of the tumor microenvironment to favor cancer progression (4). Two pharmacological approaches have been successfully used in cancer treatment to inhibit EGFR functions: neutralizing mAbs and small-molecule tyrosine kinase inhibitors (TKI) (5).

Anti-EGFR Ab therapy has been reported to mediate tumor regression by interrupting oncogenic signals and inducing Fc-mediated innate mechanisms (6). However, the capacity of anti-EGFR mAbs to activate cell populations of adaptive immunity has not yet been explored. Recent evidence has shown the possibility that a vaccine effect could be generated in animals and patients with tumors after Ab-based immunotherapy. DC101, an Ab specific for vascular endothelial growth factor receptor-2, induced T cell-dependent tumor regression in mice in addition to mediating an antiangiogenic effect (7). Immunotherapy using anti-CD20 Abs provoked the generation of tumor-specific T cell response in mice (8) and patients (9) with hematological malignances. Also, dependence on adaptive immunity for the therapeutic effect of an anti-HER2 Ab has been recently documented (10, 11). The capacity of some Abs to induce a specific antitumor immunity, not restricted to the target Ag but against several unknown tumor-derived Ags, reinforces the relevance of exploring the connection between EGFR targeting and adaptive immunity activation. Considering that preclinical reports of the mechanisms involved in an Ab-induced vaccine effect have demonstrated an Fc-dependence (8, 10), the most relevant question would be whether EGFR blockade contribute to this effect.
Absence of appropriate preclinical models has constituted an important limitation to determine the involvement of adaptive immune response in the antitumor activity of anti-EGFR Ab-based therapies. Because anti-human EGFR Abs do not cross-react with the murine molecule, in vivo experiments studying their antitumor effect have been conducted with xenograft tumors in nude mice. Use of immunocompetent mice in a complete autologous scenario is required to evaluate this phenomenon. Description of murine tumor models with EGFR expression has been quite limited. In that sense, we have generated an Ab specific for the extracellular domain of murine EGFR named 7A7 (12). Preliminary experiments using a syngeneic metastasis model have suggested that 7A7 mAb, in addition to conventional mechanisms associated with the anti-EGFR Ab effects, is able to induce a T cell response (CD4+ and CD8+) that is indispensable to its antitumoral effect (13).

The goal of the current study was to determine the role of EGFR signal interference in the generation of an antitumor adaptive immunity. We characterized tumor-specific T cell response after 7A7 treatment and explored whether this ability was exclusive for anti-EGFR Abs or a TKI specific for this receptor was also capable of inducing a T cell response. For this purpose, we used AG1478 as an inhibitor of EGFR tyrosine kinase activity, which has demonstrated its therapeutic potential in preclinical studies (14). We conducted experiments to evaluate whether the apoptosis induced by EGFR inhibition contributes to T cell response generation.

### Materials and Methods

**Cell line and reagents**

C57BL/6-derived D122 metastatic clone of the Lewis lung carcinoma (15) and B16F10 metastatic clone of the B16 melanoma (16) were cultured in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin (Life Technologies). AG1478, specific for the extracellular domain of murine EGFR (12), was obtained at the Center of Molecular Immunology (Havana, Cuba). 7A7 (Fab)2 was obtained by pepsin digestion (17). AG1478, a small-molecule EGFR TKI, was purchased from LC Laboratories (Woburn, MA). AG1478 was dissolved in DMSO for in vitro studies and in 0.05% (v/v) Tween 80 solution for in vivo use. Abs to phospho-eukaryotic initiation factor 2α (p-eIF2α) and total eIF2α were used for immunoblot experiments and obtained from Cell Signaling Technology (Beverly, MA). Abs specific for mouse molecules CD11c, I-A/E, CD40, CD80, CD86, CD3, CD8, D4, CD69, CD44, granzyme B, and IFN-γ were used for flow cytometry analyses and purchased from eBioscience (San Diego, CA) or BD Biosciences (San Francisco, CA). Abs specific for calcineurin (CRT), ERp57 (Abcam, Paris, France), cleaved caspase-3, heat shock protein (HSP) 70, and HSP 90 (Cell Signaling Technology) were also used for flow cytometry studies.

**Experimental and spontaneous metastasis models**

All animal studies were done according to protocols approved by the Institutional Animal Care and Use Committee of the Center of Molecular Immunology. D122 cells were grown at 70–85% confluence before being harvested for cell counting. For experimental metastasis assays, 2.5 × 105 cells were injected into tail lateral veins of 6–8-wk-old female C57BL/6 mice (Center for Laboratory Animal Production, Havana, Cuba). 7A7 mAb (56 μg i.v.), 7A7 (Fab)2 (573.3 μg i.v.), and AG1478 TKI (1 mg orally) administration began on day 6 after tumor challenge and continued at three doses per week. On day 21, mice were euthanized, and lungs were removed. The number of D122 lung metastases was counted. Cell population depletion were achieved by i.p. injections of 0.1 mg anti-CD8+ or anti-CD4+ Abs [obtained from rat anti-mouse hybridomas YTS169 or YTS191 (American Type Culture Collection, Manassas, VA) and purified as previously described (18)]. In some scenarios, depletion schedule began at the same time as anti-EGFR agents and continued every 4 d. Mice injected with PBS or 0.05% (v/v) Tween 80 in water were used as controls.

**Assessment of specific T cell response**

Mice receiving six doses of 7A7 mAb, 7A7 F(ab)2, or AG1478 TKI as described above were euthanized 16 d after tumor inoculation by i.v. injection. Cells from axillary lymph nodes were isolated and restimulated in vitro during 7 d with IL-2 (200 U/ml; R&D Systems, Minneapolis, MN) in the presence of irradiated D122 cells (previously treated with 120 U/ml IFN-γ to increase H-2Kb expression). Cytotoxic activity was determined in 4 h in vitro lactate dehydrogenase assay using IFN-γ−treated D122 or IFN-γ−treated B16F10 as target cells. CTL response against D122 tumor-associated Ag H-2Kb−restricted peptide [Mut 1 (FEQNTAQP) (19)] and EGFR H-2Kb−restricted peptide [DLHAFENL; SYFPEITHI score: 22 (20)] was determined. H-2Kb−binding OVA257–264 peptide (SIINFEKL) was used as irrelevant. Percentage of specific lysis was calculated as: ([experimental release − effector cell release − spontaneous release]/[maximum release − spontaneous release]) × 100. Maximum release was obtained by adding 1% Triton X-100 to target cells, and spontaneous release was determined by incubating target cells with medium alone.

**Analyses of lung mononuclear cells**

To evaluate lung-infiltrating CD8+ T cells, D122 experimental metastasis-bearing mice treated with 7A7 mAb or AG1478 TKI were euthanized 16 d after tumor injection. Lung mononuclear cells were isolated by flow cytometry using the specific Abs described above. Granzyme B and IFN-γ intracellular detection was performed using BD Cytofix/Cytoperm Plus kit with BD GolgiPlug kit following the protocol recommended by the manufacturer (BD Biosciences).

**Apoptosis measurements**

D122 cells were plated (5 × 103) in 10% FCS RPMI 1640 in six-well plates (Costar, Cambridge, MA). Twenty-four hours later, 7A7 mAb (10 μg/ml), 7A7 F(ab)2 (6.66 μg/ml), or AG1478 TKI (10 μM) were added in 1% FCS RPMI 1640 and cells were incubated for an additional 24, 48, or 72 h. Control cells were grown without treatment or cultured in medium with the appropriate concentration of DMSO. To measure phosphatidylserine exposure, Annexin V-propidium iodide (PI) double staining was carried out according to manufacturer’s protocol (Bender MedSystems, Vienna, Austria). To detect caspase activation (48 h), permeabilized cells were stained with an Ab specific to cleaved form of caspase 3. To analyze DNA fragmentation (72 h), cells were fixed with ice-cold methanol/acetic (4:1) and stained with incubation with a solution containing 100 μg/ml PI (Life-Aldrich, St. Louis, MO) and 50 U/ml RNase (Sigma-Aldrich). All analyses were performed on a FACScan flow cytometer (BD Biosciences) by collecting a minimum of 20,000 events and analyzed using WinMDI 2.8 and ModFit 3.0 software packages.

**Immunogenic apoptosis assays**

A total of 1.5 × 106 D122 cells treated with 7A7 mAb, 7A7 F(ab)2, AG1478 TKI, MC, or DX during 24 h as described above were inoculated s.c. into one flank of mice. Despite the fact that ~75% of the 7A7, 7A7 F(ab)2, and AG1478-treated cells and ~30% of the MC, DX-treated cells were still nonapoptotic (PT An−), treated cells injected rarely induced tumors, presumably because these nonapoptotic cells were already preprogrammed to die later. Seven days later, mice received a challenge with D122 or B16F10 live cells (5 × 105) onto the contralateral flank. The evolution of these tumors was monitored. In mice that did not receive the
second challenge, cells from afferent lymph nodes were isolated 10 d after injection of dying cells and restimulated in vitro for 7 d as described above. Cytotoxic activity was determined in 4 h in vitro lactate dehydrogenase assay using IFN-γ–treated D122 as target cells. Depletion of CD8+ or CD4+ cells was achieved by i.p. injection of specific Abs 3 d before challenge with dying [7A7, 7A7 F(ab′)2, DX-treated] tumor cells and 3 d before injection with live tumor cells. Animals that bore tumors in excess of 20–25% of the body mass or that were necrotic were killed.

Flow cytometric analysis of chaperones on the cell surface
Flow cytometry was used to detect CRT, Erp57, HSP 70, and HSP 90 exposure in cells treated (preincubated or not with 10 μM brefeldin A) with 7A7 mAb, 7A7 F(ab′)2, AG1478 TKI, MC, or DX. D122 cells were collected after treatment at several times (4, 6, 12, and 48 h), washed twice with PBS, and fixed with 0.25% paraformaldehyde in PBS for 5 min. After washing twice in cold PBS, cells were incubated with primary Abs followed by washing and incubation with the FITC-conjugated secondary Ab. Isotype-matched IgG Abs or second Ab alone were used as controls. For CRT and Erp57 exposure, cells analyzed were gated from PI-negative cells.

Immunoblot analyses
Serum-starved D122 cells were treated with 7A7 mAb or DX for 6 h. Cell lysates were prepared in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) with 50 mM NaF, 1 mM Na3VO4, 5 mM EDTA, and 1 mM PMSF that were freshly added to the lysis solution before each experiment. Cell extracts were applied to SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Gelman, Ann Arbor, MI). Membranes were blocked with NEGT buffer (0.15 M NaCl, 5 mM EDTA, and 0.02% paraformaldehyde in PBS for 5 min. After washing twice in cold PBS, cells were incubated with primary Abs followed by washing and incubation with FITC-conjugated secondary Ab. Protein content was visualized using HRP-conjugated secondary Ab (Cell Signaling Technology) followed by Chemiluminescent Substrate (Pierce, Rockford, IL).

Generation of bone marrow-derived dendritic cells and culture with apoptotic cells
As previously described (22), bone marrow cells were harvested from femurs and tibias of normal C57BL/6 mice and filtered through a Falcon 40 μm cell strainer (Thomas Scientific, Swedesboro, NJ). Whole bone marrow cells were seeded (0.6 × 106) in six-well plates in RPMI 1640 supplemented with 10% FCS and 20 μg/ml recombinant murine GM-CSF (R&D Systems). Cultures were fed at day 3, adding an equal amount of fresh, growth factor-supplemented medium. On day 6 of culture, immature dendritic cells (DC) were incubated with apoptotic cells for 16 h at a 1:1 ratio. DC surface phenotype was analyzed by flow cytometry using the specific Abs described above. Changes in cell size were also studied. LPS stimuli were used to monitor the maturation potential of DC generated in our experimental conditions. DX- and MC-treated D122 cells were used as positive and negative controls of DC maturation, respectively.

Statistical analyses
All statistical analyses were performed using SPSS software (SPSS). To analyze data from DC maturation experiments, parametric statistical methods (unpaired t test and one-way ANOVA with Dunnet posttest) were used. For comparisons from CTL experiments, a two-way ANOVA was performed using treatment group and target as factors, followed by mean multiple comparisons by Bonferroni test. These statistical methods were also used for tumor volume comparisons (factors: treatment group and day). Log-rank tests were used for Kaplan–Meier curves of tumor-free survival. When variables studied were not normally distributed (number of lung metastases and percentage of Tdt+, cleaved caspase-3+, CRT+, and Erp57+ cells), nonparametric statistical methods were applied. Mann–Whitney U test was used to compare variables between two groups. When three or more groups were compared, the Kruskal–Wallis test with Dunn posttest was used. Data were considered significant when p < 0.05.

Results

T cells are essentials for 7A7 mAb but not for AG1478 TKI therapeutic effect
Previous experiments had shown that treatment with 7A7 mAb inhibited cellular functions associated with EGFR signaling on D122 cells and impaired the metastatic spread of this tumor in an experimental metastasis model (13). Therefore, we examined the ability of EGFR-specific TKI AG1478 to induce an antimetastatic effect on D122 cells. First, we analyzed the capacity of this drug to inhibit EGFR activation. AG1478 inhibited EGFR-induced EGFR tyrosine phosphorylation (Supplemental Fig. 1A). We also determined the effect of AG1478 on D122 cell cycle progression. An increase in the proportion of cells in G0-G1 phase with the corresponding decrease of cells in S and G2-M phases was found after AG1478 treatment (Supplemental Fig. 1B). When we conducted cell viability experiments, AG1478 was able to reduce the percentages of viable D122 cells in a dose-dependent manner (Supplemental Fig. 1C). To determine the in vivo effect of AG1478 on D122 tumor, we first carried out an experimental metastasis assay in which 7A7 used in therapeutic setting demonstrated a potent antimetastatic effect (13). Administration of AG1478 provoked a significant reduction in D122 lung metastases number similar to that induced by the treatment with the Ab (Fig. 1A).

Our previous results had also suggested a dependence on CD8+ and CD4+ T cells for the antimetastatic effect of 7A7 on D122 experimental metastasis assays (13). Thus, the next step was to define the contribution of T cell response for the AG1478 effect on this model. With this aim, anti-EGFR agent-treated mice were inoculated with CD8- or CD4-depleting Abs. Neither CD4 nor CD8 depletion affected the potent antimetastatic effect of AG1478 (Fig. 1B, upper panel). However, in accordance with our previous report, in vivo injection of CD4/CD8-specific Abs completely abolished 7A7 antimetastatic effect (Fig. 1B, lower panel). Additional data suggesting the differential contribution of T cells for 7A7 and AG1478 antimetastatic activity were obtained when the administration frequency of anti-EGFR agents was decreased in the experimental metastasis assay. A single 7A7 dose given at day 6 after tumor cell inoculation or two doses at days 6 and 13 retained the same effect of six doses of the Ab (Fig. 1C, left panel). In contrast, the reduction of AG1478 administration frequency provoked a significant decrease in its antimetastatic effect (Fig. 1C, right panel).

Relevance of CD8+ T cells for the antimetastatic activity of 7A7 was also verified using a D122 spontaneous metastasis model as described in Fig. 2A. In this scenario, 7A7 and AG1478 decreased both primary tumor growth as well as the appearance of metastases (Fig. 2B, 2C). Depletion of CD8+ cells had no detectable impact on AG1478 action. In contrast, this treatment provoked the abrogation of 7A7 antimetastatic activity (Fig. 2C). In primary tumors, a complete dependence on CD8+ cells for 7A7 effect was not found. In mice treated with anti-CD8 depleting Ab together with 7A7, an effect on tumor growth was achieved when compared with control group. This effect was similar to that induced by AG1478 groups (Fig. 2B).

7A7 mAb treatment induces a potent CD8+ T response on D122 metastasis-bearing mice
We explored the capacity of 7A7 versus AG1478 to generate a tumor-specific CTL activity on D122 experimental metastasis-bearing mice. First, we examined whether D122 cells express a sufficient amount of H-2Kb molecules that could stimulate CD8+ tumor-specific CTL activity on D122 experimental metastasis-bearing mice. First, we examined whether D122 cells express a sufficient amount of H-2Kb molecules, which could be increased by IFN-γ treatment (data not shown). Thus, IFN-γ–treated D122 cells were used for in vitro restimulation of lymphocytes from axillary lymph nodes and as target cells in cytotoxicity experiments. C57BL/6 mice were i.v. inoculated with D122 cells and treated with anti-EGFR passive agents. Sixteen days after tumor inoculation, in vitro CTL assays were performed (Fig. 3A). Previously, we had detected that 7A7 treatment promotes a significant mobilization of T cells into metastatic sites.
between days 12 and 17 after tumor inoculation (13). As expected, administration of AG1478 did not stimulate measurable lytic activity against IFN-γ-treated D122 cells. In contrast, 7A7 treatment stimulated a potent CTL activity that eliminated a high percentage of IFN-γ-treated D122 target cells (∼75% using an E:T ratio of 100:1). This response was not present when the IFN-γ-treated murine melanoma cell line B16F10, which is negative for EGFR expression (23), was used as target cell.

To determine the presence of EGFR-specific clones in the 7A7-induced CTL repertoire, in vitro CTL assays were carried out using as a target D122 cells pulsed with an EGFR-derived peptide (Fig. 3B). Mut 1, a peptide described as immunodominant for MHC class I in Lewis lung carcinoma (19), was used as a positive control. We found that 7A7-stimulated CD8+ T cells recognized and killed not only mut 1-pulsed D122 cells, but also EGFR-derived peptide-pulsed D122 cells. In contrast, no CTL activity was detected in 7A7-treated mice against D122 cells pulsed with an irrelevant OVA peptide.

To exert a therapeutic antimetastatic effect, effector T cells must infiltrate lungs to destroy implanted metastases. At day 16 after tumor inoculation, we found a 5-fold increase in CD8+ T cell infiltration into lungs following 7A7 administration when compared with AG1478-treated mice (Fig. 3C, left panel). The phenotype of these CD8+ T cells corresponded with mature and Ag-experienced lymphocytes, measured by CD44 and CD69 cell markers, which were accordingly augmented in lung-infiltrating CD8+ T cells from 7A7-treated mice when compared with mice that received AG1478 (Fig. 3C, right panel). Given the low percentage of CD8+CD69+ T cells in lungs from AG1478-treated mice, we did not analyze the effector phenotype of these cells. Instead, CD8+CD69+ T cells from 7A7-treated mice were positive for granzyme B and IFN-γ staining, suggesting the functional activity of these cells in the metastatic site (Fig. 3D).

We also evaluated the induction of an antitumor humoral response in 7A7-treated mice comparing to control mice. Sera of 7A7-treated mice did not show IgG binding to D122 cells (Sup-
bination. We tested the capacity of 7A7 F(\(\text{ab}^\prime\))\(_2\) to recognize the murine EGFR extracellular domain recombinant protein by ELISA. Using 7A7 F(\(\text{ab}^\prime\))\(_2\), similar to 7A7-treated D122 cells (Supplemental Fig. 2A). IgM binding to tumor cells was detected in the Ab-treated group at lower sera dilution (1:100), but a similar recognition capacity was achieved in control and naive mice (Supplemental Fig. 2B), suggesting that 7A7 administration was not able to induce a tumor-specific humoral response.

7A7 F(\(\text{ab}^\prime\))\(_2\) elicits a tumor-specific cytotoxic T cell response

To evaluate whether the EGFR blockade by 7A7 mAb was involved in its capacity to stimulate antitumor CD8\(^+\) T cells, we conducted in vivo experiments as described in Fig. 1A using 7A7 F(\(\text{ab}^\prime\))\(_2\). First, we demonstrated in vitro biological activity of this molecule. We tested the capacity of 7A7 F(\(\text{ab}^\prime\))\(_2\) to recognize the murine EGFR extracellular domain recombinant protein by ELISA. A similar recognition pattern was detected for 7A7 F(\(\text{ab}^\prime\))\(_2\) and 7A7 at equimolar concentrations (Supplemental Fig. 3A). Similar in vitro activity was also achieved in Western blot analyses of EGFR phosphorylation status and in cell viability experiments using 7A7 F(\(\text{ab}^\prime\))\(_2\) and 7A7-treated D122 cells (Supplemental Fig. 3B, 3C).

Due to differences in the m.w. of both molecules and the described differences in clearance by the reticuloendothelial system and excretion by the kidneys (24), we previously had conducted an study of pharmacokinetic and biodistribution for 7A7 and 7A7 F(\(\text{ab}^\prime\))\(_2\) (our unpublished data). When equimolar concentrations of both agents were injected i.v. into C57BL/6 mice, we found that F(\(\text{ab}^\prime\))\(_2\) were cleared 10-fold more rapidly than the whole molecule. The dose used for in vivo treatment was then adjusted based on the \(t_{1/2}\) achieved for 7A7 mAb and 7A7 F(\(\text{ab}^\prime\))\(_2\), similar to strategies followed by several groups (17, 25). Ten-fold larger amounts of the F(\(\text{ab}^\prime\))\(_2\) were administered [56 \(\mu\)g of 7A7 and 37.33 \(\mu\)g \(\times\) 10 of 7A7 F(\(\text{ab}^\prime\))\(_2\)].

Results from this experimental setup demonstrated the capacity of 7A7 F(\(\text{ab}^\prime\))\(_2\) to reduce metastases number when compared with PBS group, suggesting that EGFR inhibition is sufficient to mediate an in vivo effect (Fig. 4A). This response pattern was observed in three independent experiments. With this schedule of Ab and bivalent fragment administration, 7A7 F(\(\text{ab}^\prime\))\(_2\) appeared to produce less complete metastasis inhibition than 7A7 mAb, suggesting the involvement of Fc-mediated mechanisms in the antimitastic activity of the complete Ab. As multiple factors other than \(t_{1/2}\) could impact on the biological efficacy of F(\(\text{ab}^\prime\))\(_2\) fragment, further experiments testing different doses should be conducted to define the relative contribution of the antitumor mechanisms induced by F(\(\text{ab}^\prime\))\(_2\) versus Fc region to 7A7-mediated antimitastatic effect.

Next, we determined whether CD8\(^+\) cells were involved in the in vivo effect of 7A7 F(\(\text{ab}^\prime\))\(_2\) (Fig. 4B). Mice treated with the bivalent fragment were inoculated with a CD8-specific depleting Ab. CD8\(^+\) cell depletion completely abrogated the antimitastic effect of 7A7 F(\(\text{ab}^\prime\))\(_2\). Finally, the induction of CD8\(^+\) T cells after 7A7 F(\(\text{ab}^\prime\))\(_2\) treatment was evaluated (Fig. 4C). These experiments revealed that 7A7 F(\(\text{ab}^\prime\))\(_2\) was able to stimulate a CTL activity. However, the percentages of specific lysis after 7A7 F(\(\text{ab}^\prime\))\(_2\) administration were slightly lower than in 7A7-treated mice (29.6 \(\pm\) 8.0% versus 38.6 \(\pm\) 4.4%), suggesting a possible contribution of 7A7 Fc region to CTL response generation.

EGFR inhibition by 7A7 mAb induces a potent proapoptotic effect on D122 lung metastases

In an attempt to understand the mechanisms involved in the differential activation of T cells for 7A7 versus AG1478, we examined the contribution of apoptosis induction. The capacity of cells dying by apoptosis to elicit an effective antitumor immune response has been recently demonstrated for anthracyclines and UVC irradiation (26). In response to 7A7 or AG1478, D122 cells underwent apoptosis, as indicated by staining with

FIGURE 2. Dependence on CD8\(^+\) cells to 7A7 mAb effect on D122 primary tumor and spontaneous metastasis. A, Schematic representation of D122 spontaneous metastasis model. Mice were challenged s.c. with D122 (5 \(\times\) 10\(^5\)) cells. Administration of 7A7 (56 \(\mu\)g i.v.), AG1478 (1 mg orally), or PBS (i.v.) began on day 14 when tumors reached \(~0.3\) cm\(^3\) and continued as shown. Each experimental group was separated into two subgroups that received i.p. injections of anti-CD8–depleting Abs or PBS at days 14, 18, 22, 26, and 30. Primary tumor growth was monitored, and lung metastases were counted on day 35. One representative experiment out of two performed is shown (\(n = 7\)). B, 7A7 effect on primary tumor, in contrast to AG1478, was partially affected by CD8\(^+\) cell depletion. Each point represents mean \(\pm\) SD of tumor volume for mice in each group. Analyses were performed according to two-way ANOVA test and Bonferroni posttest. Statistical differences between groups are indicated in the graph. C, Depletion of CD8\(^+\) cells completely abrogated 7A7 antimitastatic effect but not AG1478 activity. Statistical analyses were performed using Kruskal–Wallis test and Dunn posttest (different letters indicate statistical differences): a versus b, \(p < 0.01\). Median values for each experimental group are included.
Annexin V-PI. A 4-fold increase of phosphatidyl serine-positive D122 cells was detected for both agents (Fig. 5A). In 7A7- and AG1478-treated D122 cells, we also found similar levels of caspase-3 activation (Fig. 5B) and DNA fragmentation (Fig. 5C).

However, differences in the capacity of both therapeutic agents to induce apoptosis in vivo were detected. Histological analyses of lung samples revealed that 7A7 treatment, in contrast to AG1478 administration, induced a potent proapoptotic effect on metastatic areas (Fig. 5D). Percentages of apoptotic (TdT-positive) cells in 7A7-treated mice were significantly higher with respect to AG1478-treated and control groups. However, the percentages of apoptotic cells in 7A7-treated mice were slightly lower than in 7A7-treated mice (Fig. 5D). It is noteworthy that differences in apoptotic index were detected using 7A7 and AG1478 regimes for the in vivo apoptosis induction by 7A7, but, in addition, suggested that Ab Fc region could also be involved. As previously suggested by Kono and Rock (27), quantitative differences in apoptosis induction by 7A7 and AG1478 could contribute to the exclusive capacity of the Ab to generate an effective antitumor CTL response. However, differential release of endogenous danger signals could also be involved.
7A7 mAb induces immunogenic apoptosis on D122 tumor model with independence on its Fc region

To explore the putative intrinsic immunogenicity of cells dying by treatment with anti-EGFR agents, D122 cells were treated in vitro to program them for cell death and s.c. injected into the right flank of mice. Inoculation of equivalent percentages of apoptotic cells for the groups of anti-EGFR agents was verified by Annexin V/PI staining (Fig. 6d). D122 cells treated with DX or MC were used as positive and negative controls of immunogenic apoptosis induction, respectively (28). Seven days later, these animals were challenged with live D122 cells into the opposite flank, and tumor growth was monitored (Fig. 6b). The absence or delay of tumor growth was then scored as an indication of antitumor immune response elicited by dying cells (29).

Similar to animals that received DX-treated D122, inoculation of 7A7-treated D122 cells reduced the frequency of tumor developing from live cells (∼60% of tumor-free mice for DX group and ∼50% for 7A7 group on day 39). In contrast, all mice injected with AG1478-treated D122 developed tumors on day 15. An identical tumor incidence was found in mice injected with MC-treated and untreated cells (Fig. 6b, left panel). Furthermore, in mice injected with 7A7-treated D122 cells that developed tumors, a significant delay in tumor growth was achieved. This effect was not detected in tumor-bearing mice from the AG1478 group (Fig. 6b, left panel). Antitumor immune response induced by injection with 7A7-treated D122 was specific because mice were not protected against a challenge with an unrelated tumor (Fig. 6b, right panel). Taken together, these results suggested that 7A7, in contrast to AG1478, was able to induce apoptosis with molecular determinants of immunogenicity.

Next, we wondered whether 7A7 F(ab’)_2 induced apoptosis with immunogenic features. As shown in Fig. 6b, an identical degree of protection was obtained in mice inoculated with 7A7 F(ab’)_2 or AG1478 mAb-treated D122 cells, suggesting that Fc-independent mechanisms are associated to the immunogenic apoptosis induced by 7A7. In accordance with these experimental results, we also found that 7A7 or 7A7 F(ab’)_2-treated D122 cells elicited a similar cytotoxic T cell response after in vivo inoculation (Fig. 6c). This response was not detected in mice treated with cells dying by AG1478 treatment. In addition, depletion experiments revealed that CD4+ and CD8+ cells were required for the 7A7 F(ab’)_2 and 7A7 mAb-elicited immunogenic effect (Fig. 6d).

We therefore studied the expression of molecular determinants for immunogenic apoptosis in D122 cells treated with anti-EGFR agents (30). Similar to DX, we found that 7A7 and 7A7 F(ab’)_2 induced an early (12 h) preapoptotic exposure of CRT and ERp57 on the plasma membrane (Fig. 7a). Using identical conditions, AG1478 failed to elicit early CRT and ERp57 exposure. A wide range of AG1478 concentrations was proved with similar results (data not shown). Our experimental data suggested that mechanisms behind CRT/ERp57 translocation induced by 7A7 mAb are similar to those reported by Kroemer’s group for anthracyclines and UVC irradiation (29, 31). In 7A7-treated D122 cells, the CRT/ERp57 translocation to the cell surface is a rapid process that occurs within hours, well before phosphatidyl-serine exposure, which manifests in ∼24 h (Fig. 7b). Similar to DX, the treatment of D122 cells with 7A7 in presence of brefeldin A, an inhibitor of anterograde endoplasmic reticulum–Golgi traffic, did not induce CRT/ERp57 exposition (Fig. 7c). We also found that 7A7 provoked an early (6 h) phosphorylation of eIF2α (Fig. 7d), a key step for anthracycline-induced immunogenic apoptosis (31).

In addition, D122 exposed to 7A7 or 7A7 F(ab’)_2 exhibited an increase in the expression of HSP 70 and HSP 90 in plasma membrane, whereas AG1478 failed to provoke this effect, as measured 48 h after stimulation (Fig. 7e). In accordance with in vivo immunogenic apoptosis experiments, these results suggested that EGFR inhibition by 7A7 is sufficient to induce the translocation of molecules with immunogenic properties to the cell surface. Differential capacity of 7A7 versus AG1478 to elicit these danger signals could contribute to the divergence in the in vivo induction of immunogenic apoptosis by these anti-EGFR passive agents.

D122 cells dying by 7A7 mAb treatment possess the capacity of inducing DC maturation

To explore the capacity of 7A7-treated dying tumor cells to stimulate DC maturation, we challenged DC with dying D122 cells and measured their maturation. DC maturation has been commonly associated with an increase in cell size; to detect this morphological change, we measured in CD11c+ cells the forward-scattered (FSC)
light intensity (Fig. 8A). DC cocultured with 7A7 mAb or DX-treated D122 cells showed an increased FSC signal when compared with DC cocultured with untreated D122. In contrast, neither in DC cocultured with AG1478-treated D122 nor in DC cocultured with MC-treated D122 was the intensity of this parameter increased. The exclusive capacity of 7A7 to induce DC maturation was confirmed when the expression of costimulatory molecules CD80, CD86, and CD40, as well as MHC class II (MHC II), was studied in CD11c+ cells cocultured with 7A7-treated or AG1478-treated D122 cells (Fig. 8B, 8C). These results suggested that 7A7-treated D122 cells are particularly effective in stimulating DC maturation.

As expected, dying D122 cells by treatment with 7A7 F(ab’)2 also induced DC maturation (Fig. 8). However, in DC cocultured with 7A7 F(ab’)2-treated cells, we detected a slight decrease in MHC II mean fluorescence intensity (MFI) values when compared with 7A7-treated D122 cells. We did not find these differences between 7A7 and 7A7 F(ab’)2 in in vitro and in vivo immunogenic apoptosis assays. Further experiments must be conducted to clarify the relative contribution of cell death induced by EGFR inhibition to DC maturation.

Discussion
Development of anti-EGFR agents might represent a major breakthrough in cancer therapy, but several issues still need to be clarified to achieve better clinical results. These include understanding of the mechanisms involved in the antitumor effect of anti-EGFR passive therapeutic agents. In this regard, main investigations have been focused in the capacity of Abs and TKI to interfere with EGFR signaling. Additionally, recent preclinical and clinical data have suggested the relevance of Ab-dependent cellular cytotoxicity as an immunological mechanism of anti-EGFR Abs (32–34). However, there are clinical observations suggesting that short-term mechanisms such as EGFR signaling inhibition and Ab-dependent cellular cytotoxicity are not the only ones involved. Some studies have indicated that maximal clinical and molecular

**FIGURE 5.** Significant proapoptotic effect in D122 metastatic niches by 7A7 mAb and 7A7 F(ab’)2 treatment. A–C, 7A7, 7A7 F(ab’)2, and AG1478 induced similar levels of apoptosis on D122 cells. Tumor cells were treated as indicated. A, Twenty-four hours later, D122 were double-stained with Annexin V-FITC and PI and analyzed by flow cytometry. Percentages of Annexin V-positive cells from three independent experiments are shown as mean ± SD. B, Forty-eight hours later, the expression of cleaved caspase-3 was detected by flow cytometry. Dot plot graphs show the results of one representative experiment indicating the percentages of cleaved caspase-3-positive cells for each group. A quantitative analysis of four independent experiments is included. C, Seventy-two hours later, cells were fixed with ice-cold methanol/acetone and stained with PI. Percentages of cells with DNA fragmentation (M1: sub G0-G1 phase) from three independent experiments are shown as mean ± SD. D, 7A7 or 7A7 F(ab’)2 treatment induced a higher apoptotic index than AG1478 administration in D122 lung metastases. An experiment as described in Fig. 1A was carried out (n = 10). After 21 d, lung sections were immunostained with TUNEL system. Bars represent mean ± SD of deoxinucleotidyl transferase enzyme (TdT)-positive cells from eight fields randomly counted. Microscope images show the results of one representative field (original magnification ×10) for each experimental group. Black arrows show representative apoptotic cells. Statistical analyses were performed using Kruskal–Wallis test and Dunn posttest (different letters indicate statistical differences). For B: a versus b, p < 0.001. For D: a versus b, p < 0.01; a versus c, p < 0.001; c versus b, p < 0.05.
anti-EGFR Abs may take several months (35–37). Mechanisms underlying the induction of long-time disease stabilization by EGFR Ab treatment have not been formally investigated. To address this idea, using an animal model, we previously generated an Ab that recognizes murine EGFR named 7A7 (12). Our previous results indicated the involvement of T cells in the 7A7 effect on D122 experimental metastasis (13). In the present report, we confirmed that 7A7 antimetastatic activity was dependent on T cells in D122 experimental and spontaneous metastasis assays, whereas the activity of AG1478 was not affected in CD4+ and CD8+ cell-depleted mice. Consistent with these findings, one or two doses of 7A7 Ab were able to induce an effective elimination of D122 experimental metastasis. On the contrary, the reduction of AG1478 inoculation frequency provoked a significant decrease on its antimetastatic potential, as expected for passive drugs. Taking into consideration the reliance on CD8+ and CD4+ cells to the antimetastatic activity of 7A7, we evaluated whether mAb therapy could lead to tumor-specific CTL and humoral responses. We found that 7A7 treatment of D122 experimental metastasis-bearing mice, in contrast to AG1478 administration, was an effective strategy to stimulate a cytotoxic CD8+ T cell response against D122 tumor Ags, including EGFR. Moreover, 7A7 administration markedly increased CD8+ T cell infiltration into lung metastases. When we examined the phenotype of lung-infiltrating CD8+ T cells, an upregulation of CD69 molecule, an activation marker, was verified in 7A7-treated mice when compared with the group that received AG1478. Importantly, 7A7-stimulated CD8+ T cells were able to produce IFN-γ and granzyme B in the absence of ex vivo stimulation, suggesting that lung-infiltrating T cells are functional in the metastasis microenvironment. Given that T cell dysfunction is an event frequently induced by tumors (38), further experiments must be conducted to confirm the lytic function of lung-infiltrating CD8+ T cells. In contrast, we did not detect a tumor-specific humoral response in 7A7-treated mice. A relevant issue that needs to be explored to complete the characterization of
the adaptive immune response induced by this Ab is the capacity of 7A7 to induce a long-lasting antitumor response. In this regard, the involvement of CD4+ T cells in the antitumor effect of 7A7 could suggest the generation of a memory CD8+ T cell response in this model (39). In line with this idea, we found in Ab-treated mice a high percentage of lung-infiltrating CD8+ T cells that expressed the CD44 molecules (>75%), which is a marker associated to memory phenotype. However, in-depth experiments should be performed to formally demonstrate the induction of a memory T cell response by 7A7 treatment.

An obvious scenario could be envisaged in which the differential capacity to stimulate an effective CTL response for both anti-EGFR agents is due to the effector functions mediated by Ab Fc region. Several studies have demonstrated the relevance of the Fc-γRI system in adaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC and the generation of Ag-specific CD4+ and CD8+ T cell responses (40). It has been described that some Abs approved for cancer treatment, including those specific to EGFR, promote trogocytosis in vitro upon binding to their target cells (41). In this process, FcγRI on acceptor cells (e.g., DC) remove and internalize cognate ligands and cell membrane fragments from tumor cells (42). In addition, it has been proposed recently that the Fc region of cetuximab, an EGFR-directed Ab used in the clinic, could contribute to adaptive immune activation by the induction of an NK/DC cross talk (43).

Therefore, we decided to test whether not only Fc region but also the EGFR signaling inhibition by Abs could contribute to the generation of a tumor-specific T cell immunity. To address this issue, we used the bivalent fragment of 7A7. Experiments to evaluate the effect of 7A7 F(ab')2 on D122 experimental metastases demonstrated its capacity to reduce the metastases number, although the fragment activity was less complete (~50%) than that of the native Ab. This result suggested the relevance of the Fc region for the in vivo effect of 7A7 mAb. The possibility that dual activities involving both pharmacological receptor blockade and Fc-mediating mechanisms may contribute to antitumor activity of anti-EGFR Abs has been suggested previously by Fan and colleagues (17). We used a similar schedule for bivalent fragment administration to that reported by these authors. Our next step was to determine the involvement of CD8+ T cells in the 7A7 F(ab')2 in vivo activity. The antitumor effect of 7A7 F(ab')2 was completely reliant on the presence of this cell population. In vitro CTL experiments showed that 7A7 F(ab')2 was able to induce a tumor-specific CTL response. However, a slight decrease in the cytotoxic activity was achieved in 7A7 F(ab')2-treated mice when compared with mice that received the whole molecule, suggesting that 7A7 Fc region contribute to CTL generation. These data demonstrated that EGFR-mediated signaling inhibition by 7A7, and not by AG1478, plays an important role in establishing a link with adaptive immunity.

To understand why EGFR signaling inhibition by 7A7 but not by AG1478 induced a CTL response, we focused on the apoptosis induction. It has been shown in recent reports that therapy with Abs provoking apoptosis either by direct damage to tumor cells (44) or...
through antiangiogenic effects (7) can activate CTL responses. Apoptotic cells are an especially attractive source of Ag for cross-presentation (45–47). The immunological consequence of the ingestion of apoptotic cellular material by DC has been controversial. In general, apoptosis is thought to be intrinsically tolerogenic, whereas necrosis is inherently immunogenic and elicits inflammatory reactions (48, 49). However, in-depth investigations have shown that cells dying by apoptosis can be highly immunogenic (50). For example, the apoptosis of tumor cells induced by some chemotherapeutic agents (28, 51) or radiotherapy (52) has been shown to prime a tumor-specific immune response.

Why, then, is apoptosis sometimes silent and other times proinflammatory? A likely key factor is how rapidly the apoptotic cells are cleared by phagocytes. This is because, over time, apoptotic cells undergo a process known as secondary necrosis in which their membranes become permeable to proinflammatory components (27). Another important element is the nature of the molecules exposed and/or released by dying cells even at early stages. It is now assumed that apoptosis can elicit an immune response only if dying cells emit specific danger signals that mediate their efficient phagocytosis by DC and the consequent DC maturation (26).

Previous reports have described apoptosis induction on tumor cells using anti-EGFR passive agents. Apoptosis associated with EGFR blockade has been characterized by an increase of molecules with proapoptotic functions (53, 54) and the activation of the caspase cascade (55, 56). Importantly, the capacity of anti-EGFR therapies to promote immunogenic apoptosis has not yet been explored. In the current study, we found that both agents, 7A7 and AG1478, were able to induce apoptosis in lung metastatic lesions, but quantitative differences were detected. The percentage of apoptotic cells in remaining D122 metastases was markedly increased in 7A7-treated mice when compared with the AG1478-treated group. In addition, our experimental data showed an important contribution of EGFR interference to the apoptosis induced by 7A7 in metastatic niches. Interestingly, apoptosis levels detected with 7A7 and 7A7 F(ab')2 treatment were similar to those reported for murine carcinomas treated in vivo with chemotherapeutic agents (57). Based on the considerations about factors contributing to immunogenic apoptosis, 7A7's capacity to generate higher apoptosis rates than AG1478 could contribute to the exclusive ability of the Ab to induce a vaccine effect.

However, results from our experiments demonstrated that not only quantitative differences can be found between 7A7 and AG1478-induced apoptosis. Despite their similar capacity to induce the apoptosis routine (phosphatidyl-serine exposure, caspase-3 activation, and detectable DNA fragmentation), 7A7 and AG1478 differed in their ability to elicit apoptosis with immunogenic
features. When mice were challenged s.c. with 7A7- or AG1478-treated D122 cells having similar percentages of apoptosis, in the absence of an adjuvant, only 7A7 was successful to mediate a specific protection against a second challenge with live tumor cells. This effect was dependent on CD8+ and CD4+ cells. Also, we confirmed in this scenario the exclusive capacity of 7A7-treated cells to prime a tumor-specific CTL response. At the biochemical level, we found differences between 7A7- and AG1478-triggered apoptosis that could explain their differential capacity to induce immunogenic apoptosis in vivo. 7A7 stimulated the translocation of CRT and ERPs from the endoplasmic reticulum to the cell surface, whereas AG1478 failed to do so. These data underscore the notion from immunogenetic chemotherapy that immunogenic apoptosis correlates with the early CRT/ERPs57 exposure. In fact, the only biochemical difference between classical nonimmunogenic and immunogenic apoptosis inducers is the early wave of CRT exposure (29). We also identified for 7A7- and AG1478-induced apoptosis differences in HSP expression. This event has also been associated with immunogenic chemotherapy (58). Importantly, our experiments demonstrated that the Fc region of 7A7 was not involved in the immunogenic apoptosis induction measured by in vivo injection of 7A7 F(ab’)2-treated cells and in vitro characterization of endogenous danger signals in F(ab’)2-treated cells.

Induction of chaperone exposure by 7A7 could suggest an involvement of DC in the Ab capacity to induce an effective T cell response. In vitro and in vivo studies have demonstrated that CRT is an eat-me signal allowing engulfment of tumor cells by DC (29). It has been suggested that HSP 70 and HSP 90 can capture tumor Ags and facilitate their presentation following uptake by DC (59). It has been suggested that HSP 70 and HSP 90 can capture tumor Ags and facilitate their presentation following uptake by DC (59).

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In summary, using 7A7 as a model treatment of EGFR-positive tumor cells, we described a novel antitumor mechanism for anti-EGFR Abs based on the induction of immunogenic apoptosis. We provide evidence regarding how EGFR inhibition by 7A7, in an Fc-independent manner, leads to molecular events defined as hallmarks of immunogenic apoptosis. Our data suggested that the induction of apoptosis with immunogenic features by 7A7 could have an important contribution to its capacity to generate a tumor-specific response. Thus, the next step will be to define the capacity of anti-EGFR Abs approved for cancer treatment to trigger an immunogenic apoptosis, and to evaluate if CTL responses are critically involved in the clinical response after anti-EGFR Ab administration. These data could be relevant in the design of new combination-based approaches for patients with EGFR-positive tumors.

Disclosures

The authors have no financial conflicts of interest.

References
