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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 antitumor 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: 4954–4966.
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 carried out 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 antitumor metabolic 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 CTL 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% FCS and penicillin-streptomycin (Life Technologies). 7A7 mAb specific for the extracellular domain of murine EGFR (12), was obtained at the Center of Molecular Immunology (Havana, Cuba). 7A7 F(ab’)2 was obtained by papain 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-erykkrinogenicity initiator factor 2a (eIF2a) and total eIF2a were used for immunoblot experiments and obtained from Cell Signaling Technology (Beverly, MA). Abs specific for mouse molecules, CD11c, I-A/II-E, CD40, CD80, CD86, CD3, CD8, CD4, 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 calreticulin (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 to 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.) or 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 lungs metastases was counted or lungs were subjected to histological examination as described below. The anti-EGFR effect of one dose (day 6) or two doses (days 6 and 13) of 7A7 mAb or AG1478 was measured. For spontaneous metastasis assays, C57BL/6 mice were challenged with D122 cells (5 × 10^3) s.c. into dorsal submolar regions. The evolution of primary tumors was monitored.

Immunogenic apoptosis assays

A total of 1.5 × 10^6 D122 cells treated with 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-2K 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; SYFFEITH1 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.

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-2K 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; SYFFEITH1 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 as described previously using a collagenase/DNase enzymatic digestion (21). Mononuclear cells were collected, and surface expression of CD3, CD8, CD69, and CD44 molecules was determined 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 × 10^5) 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 phosphorylation-serine exposure (24 h), an 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 Apo-specific to cleaved form of caspase 3. To analyze DNA fragmentation (72 h), cells were fixed with ice-cold methanol/acetone (4:1) and stained with an antibody to DNA fragmentation (30 μg/ml, Cell Death Detection ELISA, Roche Diagnostic, Mannheim, Germany). The number of apoptotic cells and total cell number per high-power microscopic field (original magnification ×10) in metastatic foci were counted, and eight high-powered fields were analyzed. Metastasis areas were defined by H&E routine staining. Results are expressed as percentages of TdT-positive cells of the total tumor cells counted.

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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. Isootype-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 NaN3, 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 NEBT buffer (0.15 M NaCl, 5 mM EDTA, 500 mM Tris-HCl [pH 7.5], 0.02% Tween 20, and 0.04% gelatin) and incubated with the primary Abs described above. Protein content was visualized using HRP-conjugated secondary Abs (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 ng/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 stimulation was 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 antimetasta-
between days 12 and 17 after tumor inoculation (13). As expected, administration of AG1478 did not stimulate measurable lysis against IFN-\(\gamma\)-treated D122 cells. In contrast, 7A7 treatment stimulated a potent CTL activity that eliminated a high percentage of IFN-\(\gamma\)-treated D122 target cells (\(\sim 75\%\) using an E:T ratio of 100:1). This response was not present when the IFN-\(\gamma\)-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 OV A 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. Two representative experiments are shown together. Bars represent mean ± SD of number of lung metastases counted for mice in each group (n = 8–16). For A and B, median values for each experimental group are included. Statistical analyses were performed using Mann–Whitney U test (B) or Kruskal–Wallis test and Dunn posttest (A, C) (groups with statistical differences are indicated): *p < 0.05, ***p < 0.001.
in vitro activity was also achieved in Western blot analyses of differential activity (p < 0.01). Median values for each experimental group are included.

7A7 F(ab')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(ab')2. First, we demonstrated in vitro biological activity of this molecule. We tested the capacity of 7A7 F(ab')2 to recognize the murine EGFR extracellular domain recombinant protein by ELISA. A similar recognition pattern was detected for 7A7 F(ab')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(ab')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 in vitro study of pharmacokinetic and biodistribution for 7A7 and 7A7 F(ab')2 (our unpublished data). When equimolar concentrations of both agents were injected i.v. into C57BL/6 mice, we found that F(ab')2 were cleared 10-fold more rapidly than the whole molecule. The dose used for in vivo treatment was then adjusted based on the t1/2 achieved for 7A7 mAb and 7A7 F(ab')2, similar to strategies followed by several groups (17, 25). Ten-fold larger amounts of the F(ab')2 were administered (56 μg of 7A7 and 37.33 μg × 10 of 7A7 F(ab')2).

Results from this experimental setup demonstrated the capacity of 7A7 F(ab')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(ab')2 appeared to produce less complete metastasis inhibition than 7A7 mAb, suggesting the involvement of Fc-mediated mechanisms in the anti-metastatic activity of the complete Ab. As multiple factors other than t1/2 could impact on the biological efficacy of F(ab')2 fragment, further experiments testing different doses should be conducted to define the relative contribution of the anti-tumor mechanisms induced by F(ab')2 versus Fc region to 7A7-mediated anti-metastatic effect.

Next, we determined whether CD8+ cells were involved in the in vivo effect of 7A7 F(ab')2 (Fig. 4B). Mice treated with the bivalent fragment were inoculated with a CD8-specific depleting Ab. CD8+ cell depletion completely abrogated the anti-metastatic effect of 7A7 F(ab')2. Finally, the induction of CD8+ T cells after 7A7 F(ab')2 treatment was evaluated (Fig. 4C). These experiments revealed that 7A7 F(ab')2 was able to stimulate a CTL activity. However, the percentages of specific lysis after 7A7 F(ab')2 administration were slightly lower than in 7A7-treated mice (29.6 ± 8.0% versus 38.6 ± 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 anchracyclines and UVC irradiation (26). In response to 7A7 or AG1478, D122 cells underwent apoptosis, as indicated by staining with...
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 the control group (12.5 ± 2.7% versus 1.0 ± 0.7%), whereas AG1478 treatment induced only a moderate increase in apoptotic cells (4.0 ± 1.2%) (Fig. 5D). It is noteworthy that differences in apoptotic index were detected using 7A7 and AG1478 regimes that induced similar antimetastatic activity, suggesting that apoptosis could be a mechanism particularly relevant for 7A7 in vivo effect.

FIGURE 3. Phenotypical and functional characterization of 7A7 mAb-induced CD8+ T cells in D122 experimental metastasis-bearing mice. A, 7A7 treatment induced a potent antitumor CD8+ T cell response. Mice (n = 10) treated with 7A7 or AG1478 as described in Fig. 1A were sacrificed at day 16, and axillary lymph node cells were grown as a pool with irradiated IFN-γ–treated D122 cells. After restimulation for 7 d, lymphocytes were isolated and tested for their capacity to lyse IFN-γ–treated D122 cells in a CTL assay using 100:1 and 50:1 E:T ratios. IFN-γ–treated B16F10 cells were used as negative control for EGFR expression. Percentage of specific lysis was calculated as: ([experimental release − effector cell release − spontaneous release] / [maximum release − spontaneous release]) × 100. Each point represents mean of triplicate measurements ± SD. B, CTL generated by 7A7 treatment recognized an EGFR-derived peptide in MHC class I context. Restimulated axillary lymph node cells from mice (n = 10) treated with 7A7 or PBS were tested for their capacity to lyse IFN-γ–treated D122 cells pulsed with DLHAFENL (EGFR-derived peptide) or mut 1 (D122 immunodominant peptide) using 50:1 and 25:1 E:T ratios. OVA-derived peptide SIINFEKL was used as irrelevant. CTL response versus nonpulsed IFN-γ–treated D122 was used as maximum percentage of specific lysis. One representative experiment out of three performed is shown in each case. Bars represent mean ± SD of triplicate measurements. Analyses in A and B were performed according to two-way ANOVA test and Bonferroni posttest (groups with statistical differences are indicated): *p < 0.05, **p < 0.01, ***p < 0.001. C, 7A7 administration increased lung infiltration of CD8+ T cells when compared with AG1478 treatment. After 16 d, lung mononuclear cells were isolated and analyzed by flow cytometry. Percentage of CD8+ cells (gated from CD3+ cells) migrating to metastatic site in mice treated with 7A7 or AG1478 is shown in the left panels. The expression of CD69 and CD44 in CD3+CD8+ cells are shown in the right panels. Numbers represent mean percentage of positive cells ± SD corresponding to three mice. D, Granzyme B and IFN-γ–producing lung mononuclear cells from mice treated with 7A7 gated from CD3+CD8+CD69+ cells. Representative flow cytometry pictograms are shown, and values represent mean ± SD of positive cells (n = 3).
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. 6A). 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 7A7 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 UV 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 posses 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, C). These results suggested that 7A7-treated D122 cells are particularly effective in stimulating 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 apoptosis assays. Further experiments must be conducted to clarify the relative contribution of cell death induced by EGFR inhibition to DC maturation.
responses to 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 1/2 values for both molecules, 23.94 h for 7A7 (our unpublished data) and 30 min for AG1478 (14), these data suggest that, in contrast to AG1478, the antimetastatic effect of 7A7 mAb occurs through target-indirect mechanisms.

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 metastatic 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-FcγR system in adaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system in adaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system in adaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system inadaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system inadaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system inadaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system inadaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system inadaptive immunity. Ag uptake in form of opsonized tumor cells has been associated with enhanced Ag presentation by DC system inadaptive immunity.

To understand why EGFR signaling inhibition by 7A7 but not by AG1478 induced a CTL response, we focused on the apoptosis inducible potential of 7A7 F(ab’)2, which 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.
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 ERp57 from the endoplasmic reticulum to the cell surface, whereas AG1478 failed to do so. These data underscore the notion from immunogenic chemotherapy that immunogenic apoptosis correlates with the early CRT/ERp57 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 exposure. 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 expression 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). They have also been associated with DC maturation (30). We need to study the impact of DC on the immune response induced in vivo by injection with 7A7-treated cells. However, we found evidence suggesting that apoptosis induced by 7A7, but not AG1478, was effective to induce DC maturation.

Recent data reported by Weiwa et al. (60) could contribute to understanding the mechanisms involved in the differential capability of Abs and T1K1s specific for EGFR to induce immunogenic cell death. This group demonstrated that EGFR, independently of its kinase activity, maintains basal intracellular glucose levels through interaction and stabilization of the sodium/glucose cotransporter 1, thereby preventing cells from autophagy (60). Interestingly, it has been demonstrated that autophagy might also be involved in Ag cross-presentation within the Ag donor cells (61). Moreover, several reports have revealed that autophagic features could in fact be essential for exposing the eat-me signals on apoptotic cells (62, 63). Thus, is it possible that the induction of cell death by anti-EGFR Abs, and not by T1K1s, follows a biochemically distinct subroutine characterized by apoptosis accompanied by autophagy.

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 immunogenic apoptosis. 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.


