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Featured Article

Anti-ICAM-2 Monoclonal Antibody Synergizes with Intratumor Gene Transfer of Interleukin-12 Inhibiting Activation-induced T-Cell Death[1]

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Abstract

Purpose: **Systemic treatment with an anti-ICAM-2 monoclonal antibody (mAb; EOL4G8) eradicates certain established mouse tumors through a mechanism dependent on the potentiation of a CTL-mediated response. However, well-established tumors derived from the MC38 colon carcinoma cell line were largely refractory to this treatment as well as to intratumor injection of a recombinant adenovirus encoding interleukin-12 (IL-12; AdCMVIL-12). We sought to design combined therapy strategies with AdCMVIL-12 plus anti-ICAM-2 mAbs and to identify their mechanism of action.**

Experimental Design: **Analysis of antitumor and toxic effects were performed with C57BL/6 mice bearing established MC38 tumors. Anti-ovalbumin T-cell receptor transgenic mice and tumors transfected with this antigen were used for *in vitro* and *in vivo* studies on activation-induced cell death (AICD) of CD8$^+$ T cells.**

Results: **Combined treatment with various systemic doses of EOL4G8 mAb plus intratumor injection of AdCMVIL-12 induced complete regression of MC38 tumors treated 7 days after implantation. Unfortunately, most of such mice succumbed to a systemic inflammatory syndrome that could be prevented if IFN-γ activity were neutralized once tumors had been rejected. Importantly, dose reduction of EOL4G8 mAb opened a therapeutic window (complete cure of 9 of 18 cases without toxicity). We also show that ICAM-2 ligation by EOL4G8 mAb on activated CTLs prevents AICD, thus extending IFN-γ production.**

Conclusions: **Combination of intratumor gene transfer of *IL-12* and systemic anti-ICAM-2 mAb display synergistic therapeutic and toxic effects. CTL life extension resulting from AICD inhibition by anti-ICAM-2 mAbs is the plausible mechanism of action.**

Introduction

ICAM-2 (CD102) is a glycosylated surface adhesion protein (1–3) expressed on endothelial cells and activated lymphocytes (2). Two ligands have been identified for this molecule: the leukocyte integrin LFA-1 (4) and the dendritic-cell-restricted C-type lectin DC-SIGN (CD209; Ref. 5).

In a recent study, we found that systemic administration of a mAb[3] specific for ICAM-2 (EOL4G8) was able to induce complete regression of tumors derived from the CT26 colon carcinoma cell line (6). Such tumor regression is dependent on a functional immune system and correlates with the induction of tumor-specific cytotoxicity mediated by CD8$^+$ T cells (6). The intrinsic mechanism underlying this antitumor activity is unknown, although it was found that EOL4G8 mAb was able to increase ICAM-2 binding to DC-SIGN (6). However, the relationship of this adhesion phenomenon to tumor immunity remains elusive. Recent evidence showed that cross-linking of human ICAM-2 was able to prevent the apoptosis of ICAM-2-transfected cells as well as in T cell lines (7). In those cellular systems, ICAM-2 binds ezrin in such a fashion that it becomes phosphorylated and recruits PI3K to the inner leaf of the plasma membrane, resulting in the elicitation of an antiapoptotic signaling cascade mediated by AKT (protein kinase B) phosphorylation and subsequent regulation of a number of substrates that result in the inhibition of BAD (7). Apoptosis is known to control clonal expansion in T cells to keep the immune response under control (8). AICD is a frequent outcome of T-lymphocyte activation through TCRs (8). Prominent roles played by FAS/FAS-L interactions (9), TNFR-II (10), IL-2 (11), IFN-γ (12) and the intracellular BCL-homologue BIM (13) have been described in this complex mechanism of immune regulation. Interference with AICD could be therapeutically useful to enhance desired immune responses.

IL-12 (14) is a potent antitumor agent (15) with a multilayered mechanism of action that includes potentiation of CTL

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[3] The abbreviations used are: mAb, monoclonal antibody; PI3K, phosphatidylinositol 3′kinase; AICD, activation-induced cell death; TCR, T-cell receptor; TNFR, tumor necrosis factor receptor; IL, interleukin; NK, natural killer; FACS, fluorescence-activated cell sorting; pfu, plaque-forming unit(s); AdCMVIL-12, (suppress) adenovirus encoding IL-12.

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responses, increases in NK activity (14), proinflammatory changes in tumor tissue (16, 17) and antiangiogenic effects (18). These actions of IL-12 are mainly mediated by its property of inducing IFN-γ release from T cells in an antigen-independent fashion (14). IFN-γ is also the main mediator of the severe systemic inflammatory shock that results from IL-12 overdose (19–22). Tumor gene transfer of *IL-12* genes has been used to treat tumors with the idea of confining production of the cytokine to malignant tissue, thus limiting collateral damage inflicted by IFN-γ. Intratumor gene transfer of IL-12 with adenovirus is a safe and efficacious approach against rodent transplanted tumors (23–25). Many strategies have been explored to increase efficacy including combinations with other transgenes (25–28) and with agonistic anti-CD137 (4–1BB) mAbs (29, 30). Those anti-CD137 mAbs mediate antitumor effects (31) by enhancing a weak CTL response as a result of mAb interactions with CTLs (32), NK cells (33), and maturing dendritic cells (34).

In this study, we have found that (*a*) EOL4G8 anti-ICAM-2 mAb synergistically enhances the antitumor effects of intratumor gene transfer of IL-12, (*b*) this combination may result in lethal IFN-γ-related toxicity, and (*c*) ICAM-2 ligation by specific anti-ICAM-2 mAbs on activated CTLs can inhibit AICD both *in vitro* and *in vivo*, thus extending the viable period of IFN-γ production. These findings provide a rationale for the efficacy and toxicity of this treatment combination.

Materials and Methods

Cell Lines, mAbs, and Peptides. MC38 colon carcinoma cells (35) were cultured in DMEM+10% FCS (Life technologies SA., Barcelona. Spain), and CTLL-2 immortalized T cells (American Type Culture Collection, Manassas, VA), which are absolutely dependent for growth on IL-2, were cultured in RPMI 1640 supplemented with 10 IU/ml human recombinant IL-2 (Chiron, Emeryville, CA) and 10% (v/v) FCS (Life Technologies, Inc.). EG.7 is a EL-4 lymphoma variant that has been transfected with an expression cassette to express full-length ovalbumin, which was obtained as a kind gift from Dr. M. Bevan (University of Washington. Seattle. WA; Ref. 36). EG.7 cells were cultured in DMEM+10%FCS.

EOL4G8 mAb (rat IgG2b) was obtained and purified by affinity chromatography on Sepharose-protein G columns (Amersham-Pharmacia Biotech, Uppsala. Sweden) as described previously (6). The anti-CD137 mAb (rat IgG2a) called 2A was kindly provided by Dr. Lieping Chen (Mayo Clinic, Rochester, MN; Ref. 37). Purified or fluorochrome-tagged anti-CD3, anti-CD4, anti-CD8, anti-CD69, and 3C4 anti-ICAM-2 mAbs were from PharMingen (San Diego, CA). Polyclonal rat IgG was purchased from Sigma (Alcobendas, Madrid, Spain).

Peptide SIINFEKL (OVA 257–264) was synthesized manually in a multiple peptide synthesizer using FMOC [*N*-(9-fluorenyl)methoxycarbonyl] chemistry. Ninhydrin test of Kaiser was used to monitor every step. At the end of the synthesis they were cleaved and deprotected with trifluoroacetic acid and washed with diethyl ether. Purity of the peptides was always above 90% as assessed by high-performance liquid chromatography.

Recombinant Adenoviruses. Recombinant adenoviruses carrying the *p40* and *p35* genes of murine *IL-12* (AdCMVIL-12) or β-*galactosidase* (AdCMVlacZ) under the control of the cytomegalovirus (CMV) promoter have been described previously (38, 39). Adenoviruses were purified by double cesium chloride ultracentrifugation and extensively dialyzed against 10 mM Tris/1 mM $MgCl_2$ before being stored at −80°C in the presence of 10% (v/v) glycerol. Titration was made by plaque assay (40).

Mice. Six-to-8-week-old C57BL/6 female mice were purchased from Harlan (Barcelona, Spain) and housed under specific-pathogen-free conditions in the University of Navarra. A breeding pair of OT-I transgenic mice in C57BL/6 background were obtained from Dr. Balbino Alarcón (Centro de Biología Molecular Severo Ochoa, Madrid, Spain) with permission from Dr. Francis R. Carbone (WEHI, Parkville, Victoria, Australia; 41) and were bred in our animal facility. All of the animal handling and *in vivo* experiments were performed following institutional guidelines and have been approved by the animal facility ethical committee.

***In Vivo* Tumor Experiments.** Five $\times 10^5$ MC38 cells were injected s.c. in the shaved right flank of syngeneic mice with a 280-G syringe (6). Tumor sizes were followed with a precision caliper and recorded at least weekly (most often every 3 days). The general well-being of the mice was recorded in comparison with control groups by observation, weighing, estimation of the amount of water drunk per cage, and watching the escaping response to stimulation by gentle poking with a blunt pencil.

To assess AICD *in vivo*, we injected 5×10^5 EG.7 cells in HBSS (100 μl/injection) s.c. in both flanks of OT-I transgenic mice and treated them i.p. with 100 μg of EOL4G8 or control antibody 4 days later. On day 7, draining lymph nodes were harvested from euthanized mice, and a cell suspension was obtained for flow cytometry studies.

FACS Staining and ELISA. Immunostaining with antibodies has been described previously (42). FITC-annexin V staining was performed with a commercial kit (PharMingen) in accordance with the manufacturer's instructions. Samples were processed with a FACScalibur instrument (Becton-Dickinson, Madrid, Spain), and results were analyzed with CellQuest software.

Mytomicin-C was commercially purchased from Sigma and was used at 50 μg/ml during 2 h at 37°C to arrest the proliferation of MC38 cells that were washed three times in DMEM before setting 4-day cocultures with splenocytes.

IFN-γ ELISAs were from PharMingen and were performed according to the manufacturer's instructions.

Results

Anti-ICAM-2 mAb + AdCMVIL-12 Combined Effects. Our published experiments (6) showed that i.p. treatment with two doses of 100 μg of EOL4G8 anti-ICAM-2 mAb could cure a fraction of tumors derived from s.c. inoculation of MC38 colon cancer cells in syngeneic mice, if given when tumor nodules were still unpalpable (as early as day 0 to day 4 after tumor cell inoculation). By contrast, if treatment onset was postponed to day 7 after tumor cell injection, no curative effects

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Table 1 Combined treatment with EOL4G8 mAb i.p. and intratumoral AdCMVIL-12 (IL-12-encoding adenovirus) synergyze both for therapeutic effects and toxicity

Results from two experiments in which C57BL/6 mice received injections s.c. of 5×10^5 MC38 cells and were monitored for tumor growth or regression after receiving three doses of 150 μg of EOL4G8 or a control antibody that were given i.p. every other day starting on day 7 after tumor inoculation combined or not with intratumor injection of a single dose of 10^8 pfu of AdCMVIL-12 on day 7.

	Experiment 1		Experiment 2	
Treatment	Tumor complete regressions[a]	Toxic deaths[b]	Tumor complete regressions	Toxic deaths
4G8	0/5	0/5	ND[c]	ND
Rat IgG	0/5	0/5	ND	ND
4G8 + Ad IL-12 i.t.	4/5	4/5	6/7	5/7
Rat IgG + Ad IL-12 i.t.	1/5	0/5	1/7	0/7

[a] Fraction of mice whose tumor nodules became undetectable.
[b] Fraction of mice that became severely ill between 2 and 3 weeks after treatment and that eventually died from tumor-unrelated causes.
[c] ND, not done; i.t., intratumoral/intratumorally.

were seen, and tumors progressed to kill the mouse, albeit with some delay if compared with control antibody-treated mice (6). In experiments shown in Table 1, we confirmed those treatment failures, because in a group of five mice that were treated with EOL4G8 mAb i.p on days 7, 9, and 11 after s.c. injection of MC38 cells, none of the tumors was rejected.

Our group has demonstrated the effectiveness of gene therapy strategies based on intratumor injections with an adenovirus encoding IL-12 (AdCMVIL-12) against a number of rodent tumors. However, MC38-derived tumors are highly resistant to treatment with doses of 10^8 to 10^9 pfu of AdCMVIL-12 (Table 1). Our hypothesis was that combined treatment with EOL4G8 mAb and AdCMVIL-12 could result in therapeutic activity against well-established MC38 tumors. As can be seen in Table 1, one dose of AdCMVIL-12 (10^8 pfu) given on day 7 (mean tumor diameter ranging from 4 to 7 mm) plus three i.p. doses of EOL4G8 mAb of 150 μg i.p. given every other day after day 7 resulted in complete regression in 10 of 12 cases, whereas AdCMVIL-12 plus an identical course of control antibody resulted in only 2 complete regressions in 12 cases ($P <$ 0.01 by Fisher′s exact test).

However, starting on day 24–25 after tumor inoculation (18 days after treatment onset), mice began to look dehydrated, lost activity, drank and ate poorly, showed altered fur, and, one day later, became clearly hypothermic. As a result of this condition, 9 of 12 mice died (Table 1) within 1 week after symptoms began. Such a syndrome was not seen in any animal of the control groups. Three moribund mice treated in the same way as those in the combined group of the second experiment in Table 1 were euthanized and subjected to postmortem examinations that disclosed congestive lungs and microscopic signs of edema consistent with acute heart failure. No macroscopic hemorrhages or inflamed areas were found, whereas the area of rejected tumor cells appeared normal without residual malignant tissue.

IFN-γ Neutralization Abrogates Toxicity of AdCMVIL-12+EOL4G8 mAb Treatment. Most of the toxicity reactions induced by IL-12 are known to be mediated by IFN-γ (22). Accordingly, we reasoned that mice given the toxic AdCMVIL-12+EOL4G8 regime but treated with 300 μl of high-titer ascitic fluid of neutralizing anti-IFN-γ mAb would be protected from the toxic shock syndrome (22). We chose day 21 after tumor inoculation for IFN-γ treatment, when mice were asymptomatic and most tumors were already macroscopically rejected or clearly shrinking in size. No toxic deaths took place in this group of seven mice (Fig. 1*a*), whereas three mice that did not receive anti-IFN-γ died on days 23, 25, and 30 from a toxic syndrome (data not shown). In the group that had been treated with EOL4G8+AdCMVIL-12 and that received anti-IFN-γ treatment, tumors were eradicated in five of seven mice (Fig. 1*a*), whereas AdCMVIL-12 + control antibody gave rise to only one tumor cure in seven cases (Fig. 1*a*). Interestingly, in the animals that had been treated with AdCMVIL-12 and three doses of EOL4G8 mAb and that were rescued with neutralizing anti-IFN-γ mAb on day 21, the two progressing tumors resulted from regrowth of an already shrinking tumor and from the relapse of a tumor nodule that had remained undetectable for 2 weeks after anti-IFN-γ mAb administration. These results highlight the activities of IFN-γ in the toxic reaction and in the therapeutic activity. Concentrations of IFN-γ in sera of mice ($n = 7$) that have received 10^8 pfu AdCMVIL-12 into MC38 tumors, plus a 100-μg dose of EOL4G8 i.p., were analyzed 6 days after treatment. Their IFN-γ concentrations were slightly higher than those of mice that had received AdCMVIL-12 and control antibody, although the difference did not reach statistical significance (Fig. 1*b*).

Dose Reduction of Anti-ICAM-2 Permits Significant Efficacy and Eliminates Toxicity. To find a therapeutic window for the combined treatment with AdCMVIL-12+EOL4G8 mAb, we treated a group of 18 mice bearing s.c. MC38 tumors implanted 7 days earlier. These mice received 10^8 pfu of AdCMVIL-12 intratumorally and a single dose of 150 μg of EOL4G8 i.p. immediately after. Nine of those mice underwent complete tumor regression (Fig. 2*a*), whereas another mouse of the group experienced a transient disappearance of the tumor nodule. All of the mice in this group were carefully inspected every day, and no signs of serious illness were detected (they remained active and steady to stimulation, showing general well-being). As a control, one group received AdCMVIL-12 intratumorally without antibody and only 1 of 6 rejected its tumor (Fig. 2*b*; differences between the AdCMVIL-12 and the AdCMVIL-12+EOL4G8 groups were significant with $P < 0.01$ according to Fisher′s exact test). In another control group, mice received an intratumoral injection of the saline vehicle (Fig. 2*c*).

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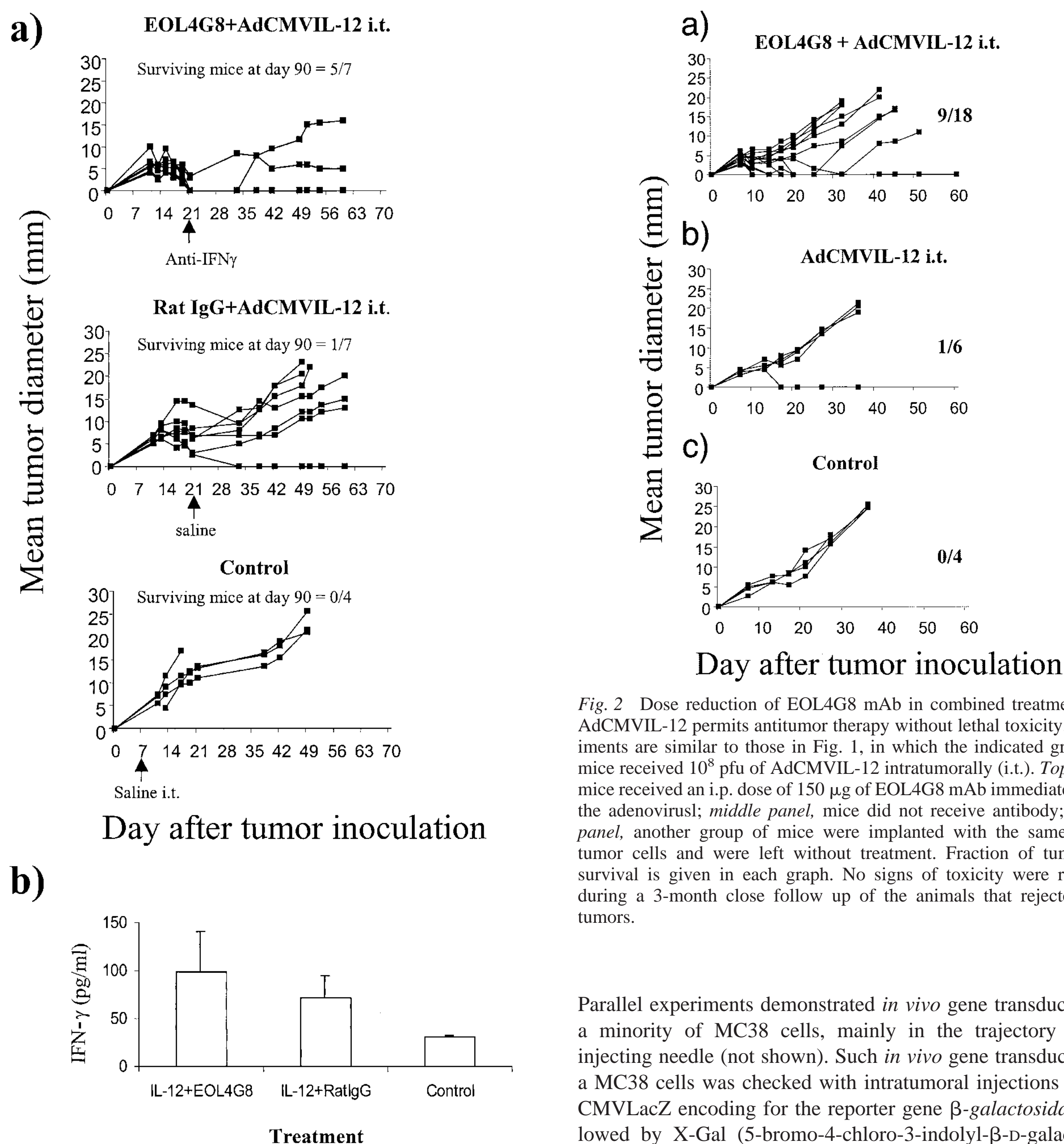

Fig. 1 *a*, Late *in vivo* neutralization of IFN-γ during treatment with AdCMVIL-2 + EOL4G8 anti-ICAM-2 mAb avoids toxicity and permits therapeutic effects. Diameter follow-up of tumor nodules derived from the s.c. injection of MC38 cells on day 0 that were treated as indicated on day 7 with 10^8 pfu of AdCMVIL-12 inside the tumor nodule. *Top graph*, mice received three i.p. doses of 150 μg of EOL4G8 mAb on days 7, 9, and 11, and one i.p. dose of ascitis fluid containing a neutralizing anti-IFN-γ mAb on day 21; *middle graph,* mice received a similar course, but with control polyclonal rat antibody and saline; *bottom graph,* control group, received an intratumoral (*i.t.*) volume of 100 μl saline buffer on day 7. *Not shown,* another group of three mice received a course similar to that in the top panel but with an injection of polyclonal rat IgG (instead of anti-IFN-γ treatment) on day 21; all of the mice died from toxicity between days 24 and 30 after tumor inoculation. The fraction of tumor-free surviving mice is given in each panel. In *b,* IFN-γ serum concentrations (mean ± SE) of seven mice per group 6 days after receiving 10^8 pfu of AdCMVIL-12 *i.t.* + 100 μg of EOL4G8 mAb or control antibody i.p.

Fig. 2 Dose reduction of EOL4G8 mAb in combined treatment with AdCMVIL-12 permits antitumor therapy without lethal toxicity. Experiments are similar to those in Fig. 1, in which the indicated groups of mice received 10^8 pfu of AdCMVIL-12 intratumorally (i.t.). *Top graph,* mice received an i.p. dose of 150 μg of EOL4G8 mAb immediately after the adenovirusl; *middle panel,* mice did not receive antibody; *bottom panel,* another group of mice were implanted with the same MC38 tumor cells and were left without treatment. Fraction of tumor-free survival is given in each graph. No signs of toxicity were recorded during a 3-month close follow up of the animals that rejected their tumors.

Parallel experiments demonstrated *in vivo* gene transduction of a minority of MC38 cells, mainly in the trajectory of the injecting needle (not shown). Such *in vivo* gene transduction of a MC38 cells was checked with intratumoral injections of Ad-CMVLacZ encoding for the reporter gene β-*galactosidase* followed by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining of frozen sections.

ICAM-2 Is Expressed on Activated CTLs. We have previously shown that ICAM-2 expression dramatically increases in mouse T cells on activation with concanavalin A+IL-2 from almost undetectable levels (6). When splenocytes from OT-I TCR-transgenic mice, which express a transgenic TCR specific for the ovalbumin peptide SIINFEKL ($OVA_{257–264}$) bound to the MHC class I molecule $H2\text{-}K^b$, were stimulated with the synthetic peptide at 10 ng/ml, high levels of ICAM-2 (CD102) expression were detected with the mAbs EOL4G8 and 3C4 (Fig. 3*a*) on $CD8^+$ cells. Interestingly, freshly isolated lymphocytes from such mice were virtually negative for EOL4G8 mAb staining both in the $CD4^+$ and $CD8^+$ subpopulations (Fig. 3*b*), indicating that ICAM-2 expression on T cells can be controlled by antigen stimulation. ICAM-2 is also expressed on immortalized murine CTL lines like CTLL-2, which

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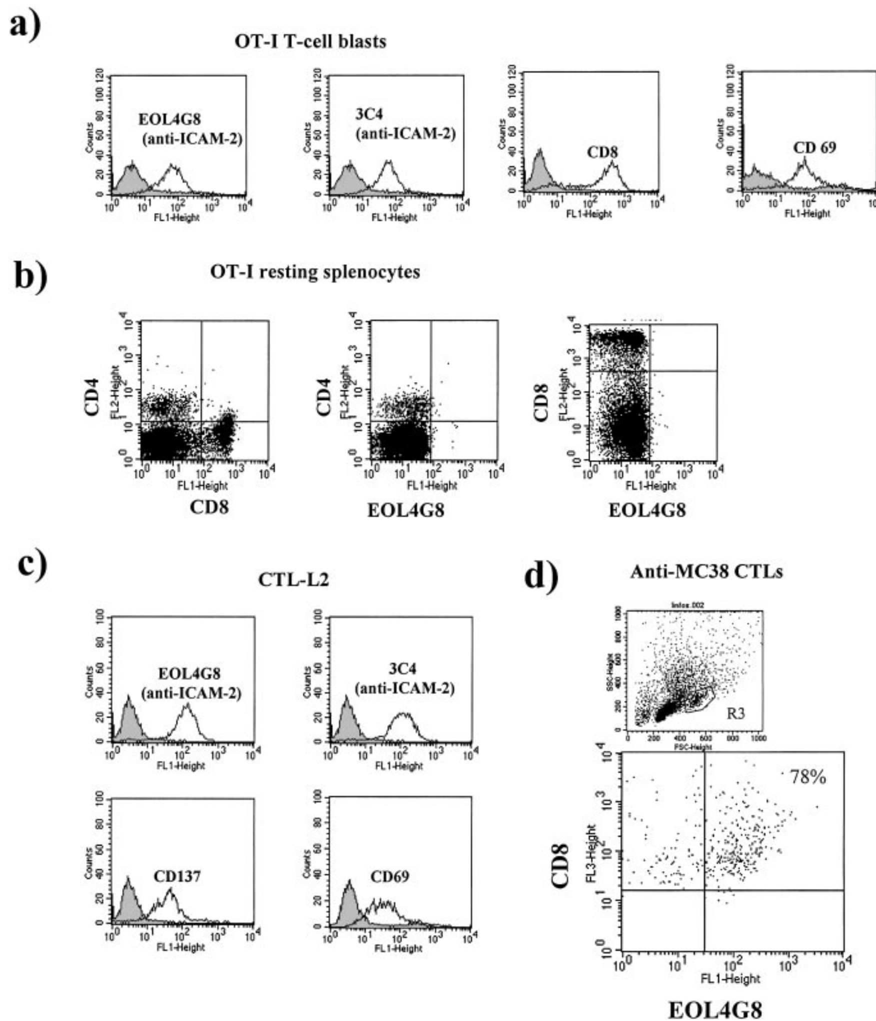

Fig. 3 ICAM-2 is expressed on activated $CD8^+$ CTLs. FACS analysis of immunofluorescence staining with the indicated mAbs of OT-I TCR transgenic splenocytes either after being stimulated for 72 h with 10 ng/ml of OVA257–264 (*a*) or freshly isolated (*b*). In *c,* a similar experiment on the CTLL-2 immortalized T-cell clone is shown. In *d,* a similar experiment displaying CD8/ICAM-2 double staining on gated lymphoblasts (*R3*) derived from spleens of mice that had rejected an established MC38 tumor. Splenocytes were restimulated *in vitro* for 4 days with mitomycin-C-pretreated MC38 cells plus 10 IU/ml of recombinant IL-2.

also expressed activation antigens such as CD137 (4–1BB) and CD69 (Fig. 3*c*). In addition, ICAM-2 can be detected on $CD8^+$ T-cell blasts from the spleen of mice cured from MC38 tumors restimulated for 4 days with mytomicin-C-treated MC38 cells plus 10 IU/ml of IL-2, thus indicating that activated CTLs with specificity for tumor antigens can interact with the anti-ICAM-2 therapeutic mAb (Fig. 3*d*).

ICAM-2 Cross-Linking Inhibits AICD in $CD8^+$ T Cells Both *in Vitro* and *in Vivo*. Human ICAM-2 cross-linking has been reported to transmit apoptosis-inhibitory signals in a number of cellular systems (7). Apoptosis, a natural outcome of TCR stimulation, thought to play a major role in the regulation of the immune response (8). FAS or TNFR ligation on cell surface (9, 10), as well as activation of the proapoptotic molecule BIM, have been involved in this activity, which is counteracted by BCL-2 (13). Accordingly, stimulation of OT-I splenocytes with 1 ng/ml $OVA_{257–264}$ peptide + 5 IU/ml IL-2 for 6 days resulted in 60–70% apoptosis of $CD8^+$ transgenic T cells, which were observed by FACS as cells that were stained both by annexin V coupled to FITC and by phycoerythrin-tagged anti-CD8α (Fig. 4, *a* and *b*). However, if EOL4G8 or 3C4 anti-ICAM-2 mAbs were applied to the cultured cells at 20 μg/ml, then the induction of apoptosis (measured as annexin V^+-$CD8^+$ cells) was partially inhibited with corresponding higher percentages of viable $CD8^+$ cells that did not bind annexin-V (Fig. 4, *a* and *b*). This effect can also be clearly seen with the number of cellular events satisfying the electronic gating for living lymphocytes (Fig. 4*b*). However, no difference was seen in the rate of proliferation during the first 3 days of culture as measured with carboxyfluorescein succinimidyl ester-labeled lymphocytes or by [^{3}H]-thymidine uptake into DNA (data not shown; confirming our previous data in Ref. 6). EOL4G8 mAb also failed to change the output of IFN-γ into the tissue culture medium of $OVA_{257–264}$-stimulated OT-I splenocytes within the first 48 h after stimulation again confirming our previous data (data not shown and Ref. 6). By contrast, if IFN-γ concentrations released into the culture medium were assessed after 6 days of culture, there was a 4- to 5-fold increase of the concentration of IFN-γ in those cultures containing 10 μg/ml of EOL4G8 mAb (Fig. 4*c*), which can be interpreted as the result of the extension of the life span of stimulated $CD8^+$ T cells.

To assess whether anti-ICAM-2 mAb were inhibiting

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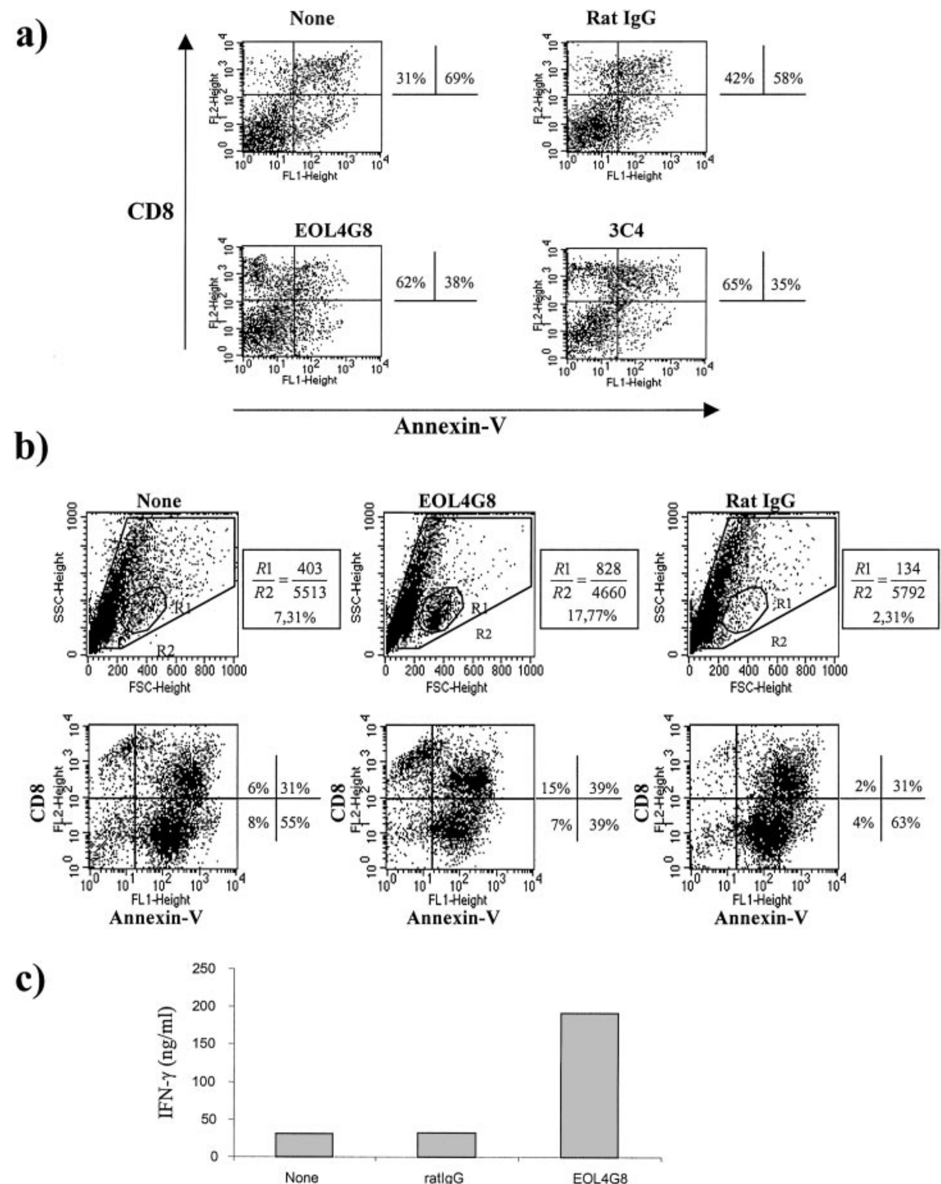

Fig. 4 Anti-ICAM-2 mAbs inhibit activation-induced T-cell death of CTLs. *a* and *b,* FACS analysis of splenocytes obtained from OT-I TCR-transgenic mice stimulated in culture for 6 days with 10 ng/ml $OVA_{257-264}$ in the presence of 20 μg/ml of the indicated monoclonal or control antibodies. FACS results are shown as percentage of double-stained $CD8^+$ lymphocytes with annexin V-FITC as a marker of apoptotic cells. In *a,* percentage of $CD8^+$ viable and nonviable cells is provided. In *b,* both annexinV staining and percentage of cell events within the forward scatter (*FSC*) and side scatter (SSC) features of viable cells are provided (% calculated from a ratio of viable events: total events that is also provided in the indicated figure). *c,* IFN-γ concentrations assessed by ELISA in the supernatant of cultures as those in *a* and *b*. Data are representative of four experiments similarly performed. R1, region 1; R2, region 2.

$CD8^+$ T-cell apoptosis *in vivo*, EG.7 tumor cells that express ovalbumin were injected s.c. into both flanks of OT-I mice. Four days later, mice were systemically treated with EOL4G8 or with polyclonal rat antibody as a control. As can be seen in Fig. 5, 3 days after antibody treatment, the draining lymph nodes of EOL4G8 mAb-treated mice contained less double-stained ($CD8^+$-annexin V^+) lymphocytes than in those treated with control antibody (6.6 *versus* 12.4%). At this time point, the total number of lymphocytes (mean ± SE) in draining lymph nodes was not significantly modified (900,000 ± 280,623 in control antibody group *versus* 940,000 ± 179,315 in EOL4G8-treated OT-I mice). These data as a whole strongly indicate that immunotherapy with anti-ICAM-2 antibody inhibits the apoptosis of CTLs that have been activated by a surrogate tumor antigen.

Discussion

This study shows the synergistic effects of systemic anti-ICAM-2 mAb and *IL-12* gene transfer into tumor tissue. Such synergy was associated not only with a potent therapeutic activity but also with a potentially lethal systemic inflammatory shock. In addition, a plausible mechanistic explanation of the synergy is provided because cross-linking of ICAM-2 on the plasma membrane of activated $CD8^+$ T cells was able to prevent AICD, thereby intensifying the total output of IFN-γ during the response.

Intratumor *IL-12* gene transfer with recombinant adenovirus is efficacious in several rodent tumors (23–25) but could be toxic above certain doses because of the overproduction of IFN-γ (22). Overdosing could also dampen the immune respon-

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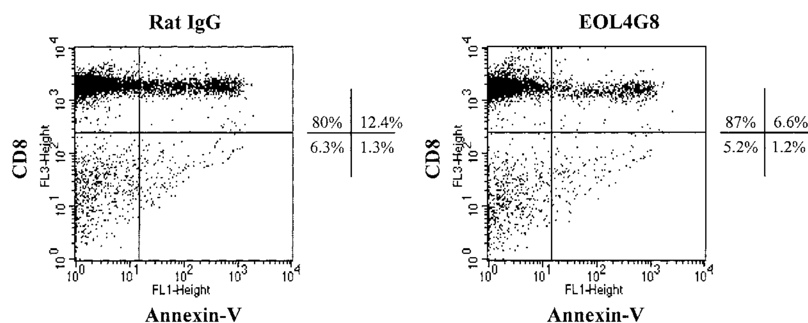

Fig. 5 *In vivo* inhibition of activation-induced T-cell death by treatment with anti-ICAM-2 mAbs. Flow cytometry analysis of $CD8^+$ lymphocytes stained with annexin-V obtained from the draining lymph nodes of TCR transgenic OT-I mice harvested 7 days after bilateral s.c. injection of EG.7 cells (OVA transfected EL-4). Groups of four mice each were treated on day 4 with 100 μg of either control polyclonal antibody (*left*) or EOL4G8 mAb (*right*). Each plot graph represents 10^4 events from eight pooled inguinal lymph nodes and provides the percentage of double-stained $CD8^+$ annexin-V^+ cells. Results are representative of two experiments similarly performed.

siveness of the host (43). Certain mouse strains, such as C57BL/6 are more prone to suffer a lethal syndrome when treated with AdCMVIL-12 (22). With 10^8 pfu of AdCMVIL-12 given intratumorally, none of the treated C57BL/6 animals showed any signs of toxicity, but a very limited tumor efficacy was observed with this particular tumor model. It should be noted that a similar dose, if given i.v. instead of intratumorally to this strain, would be lethal in 6–10 days because of toxic shock mediated by IFN-γ (22).

The combination of 10^8 pfu of AdCMVIL-12 intratumorally and three every-other-day doses of 150 μg of EOL4G8 mAb displayed a very dramatic and abrupt antitumor activity because most MC38-derived tumors were rapidly rejected, and the mice appeared otherwise healthy. The true synergistic nature of the therapeutic combination is shown because neither agent on its own would cause such an effect. However, during the 3rd week after treatment initiation, a high proportion of mice died from a condition with symptoms similar to those seen after overdosing AdCMVIL-12 i.v. (22), but with a much later onset. The involvement of IFN-γ in the toxicity was proved by showing that *in vivo* IFN-γ neutralization by specific mAbs 2 weeks after starting this treatment course, when most tumors were already rejected, prevented lethal toxicity. We observed that two tumors progressed on IFN-γ neutralization, one after being macroscopically undetectable and the other after being already shrinking its size. Both observations underscore the role of IFN-γ in the antitumor activity of IL-12, as has been confirmed in other immunotherapeutic regimes (15).

A clear therapeutic synergy could be observed also in the absence of lethal toxicity if only one dose (instead of three doses) of anti-ICAM-2 antibody were administered i.p. concomitantly with AdCMVIL-12 intratumor injection, thus allowing a dose-dependent therapeutic window. Anti-CD137 agonistic mAbs (37), which also have potent antitumor properties on their own (31, 37), have demonstrated synergistic effects with *IL-12* intratumoral gene transfer against a colon cancer and in a metastatic breast cancer model, with no systemic toxicity reported, at least at the doses tested (29, 30).

The idea of combining immunopotentiating mAbs and *IL-12* gene transfer to malignant cells will be important in the clinic. In support of this point, our results from a recently finished clinical trial, using escalating intratumoral doses of a first generation recombinant adenovirus encoding human IL-12 against advanced gastrointestinal tumors, have taught us that the effect of this gene therapy approach is not efficacious enough by itself.[4] Therefore, combination strategies are needed, and, among them, those showing truly synergistic complementary mechanisms of action should be preferred (25). Anti-CD40 antibodies (44) also could be considered an alternative that is worth testing in combination with AdCMVIL-12, although, in this case, even the antibody as a single agent caused a lethal systemic inflammatory syndrome in mice (45). Thus, comparative studies of anti-ICAM-2, anti-CD137, and anti-CD40 in combination with *IL-12* intratumor gene transfer have become one of our experimental priorities.

AdCMVIL-12 antitumor activity is attributed to CTL generation (39) and to increases in NK and NK T-cells activity (38), as well as to antiangiogenic actions (38). The mechanism or mechanisms of action of the EOL4G8 mAb are less well defined. Involvement of CTLs has been proved, but the intrinsic mechanism leading to their potentiation is not worked out (6). A recent publication showed that human ICAM-2, either as naturally expressed or as a transfected molecule in ICAM-2-negative cells, inhibited apoptosis on overexpression or cross-linking (7). The effect was studied in detail and was found to involve a chain of signaling events including ICAM-2-associated ezrin, PI3K, and AKT (PKB) and the inhibition of BAD (7). The prosurvival effect was very intense against apoptosis caused by growth factor deprivation and staurosporin treatment, as well as by FAS cross-linking.

In our hands, ICAM-2 cross-linking *in vitro* was capable of preventing induction of T-cell death by antigen in TCR-transgenic T cells stimulated with agonistic peptide and IL-2. Moreover, treatment with anti-ICAM-2 mAb *in vivo* prevented the induction of apoptosis of TCR-transgenic $CD8^+$ lymphocytes located at lymph nodes that drain the area in which tumor cells expressing the relevant antigen had been injected. This antipoptotic effect extended the life span of some of the responding

[4] B. Sangro *et al.,* manuscript in preparation.

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$CD8^+$ T cells with the overall result of a higher output of IFN-γ into the culture supernatant. Such an antiapoptotic effect was clearly dissociated from proliferation or early cytokine secretion. Anti-CD137 mAbs have also been described to be primarily antiapoptotic on activated CTLs (46). Activation-induced T-cell death is conceived as an important homeostatic mechanism to regulate immune responses. A role in AICD has been described for ligation of the T-cell surface proteins FAS and TNFR p75 as well as for the cytokines IFN-γ and IL-2 (9–12). The molecule BIM seems to be the mayor intracellular player in the connection of the apoptotic pathway to the mitochondria (13). Our hypothesis is that ICAM-2, when cross-linked with the antibody, sets in motion a signaling pathway that interferes with these cascades. Anti-CD137 mAbs may exert a similar type of effect leading also to effective tumor immunity (46, 47).

Alternatively or complementarily, EOL4G8 mAb has been shown to enhance the avidity of the ICAM-2/DC-SIGN interaction (6). This would provide the therapeutic antibody with the opportunity to enhance the immune response by acting on the synapse formed by preactivated T cells and dendritic cells. In this regard, we have preliminary evidence showing that ICAM-2 is located in the T-cell/APC contact area in an early and antigen-independent fashion.[5]

In conclusion, we herein describe an efficacious combined strategy for cancer treatment encompassing *IL-12* intratumoral gene transfer and systemic anti-ICAM-2 antibody treatment that requires caution because it can result in lethal toxicity. Both the efficacy and the late-onset toxicity are probably related to the ability of the anti-ICAM-2 mAbs to prevent the AICD of T lymphocytes, thus extending the effector time of IFN-γ-producing CTLs.

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