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## Low Surface Expression of B7-1 (CD80) Is an Immunoescape Mechanism of Colon Carcinoma

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### Abstract

**Artificially enforced expression of CD80 (B7-1) and CD86 (B7-2) on tumor cells renders them more immunogenic by triggering the CD28 receptor on T cells. The enigma is that such B7s interact with much higher affinity with CTLA-4 (CD152), an inhibitory receptor expressed by activated T cells. We show that unmutated CD80 is spontaneously expressed at low levels by mouse colon carcinoma cell lines and other transplantable tumor cell lines of various tissue origins. Silencing of CD80 by interfering RNA led to loss of tumorigenicity of CT26 colon carcinoma in immunocompetent mice, but not in immunodeficient Rag<sup>-/-</sup> mice. CT26 tumor cells bind CTLA-4Ig, but much more faintly with a similar CD28Ig chimeric protein, thus providing an explanation for the dominant inhibitory effects on tumor immunity displayed by CD80 at that expression level. Interestingly, CD80-negative tumor cell lines such as MC38 colon carcinoma and B16 melanoma express CD80 at dim levels during *in vivo* growth in syngeneic mice. Therefore, low CD80 surface expression seems to give an advantage to cancer cells against the immune system. Our findings are similar with the inhibitory role described for the dim CD80 expression on immature dendritic cells, providing an explanation for the low levels of CD80 expression described in various human malignancies.** (Cancer Res 2006; 66(4): 2442-50)

### Introduction

CD80 (B7-1) is a surface glycoprotein shown to increase the immunogenicity of tumor cell lines when its gene is transfected into them (1, 2). As a consequence, in tumor grafting experiments, CD80 transfectants are rejected in syngeneic hosts causing protective and therapeutic immunity against untransfected tumor cells, provided that they bear antigen determinants available for CTL recognition (3). Tumor cell transfection with the CD80 close relative CD86 (B7-2) also conferred increased immunogenicity to transplanted tumors with only subtle differences with CD80 (4, 5).

CD80 and CD86 molecules share their ligands on T cells (6–8). The T-lymphocyte surface molecules CD28 and CTLA-4 (CD152) bind to them, although with a conspicuously higher affinity in the

case of CTLA-4 (100- to 1,000-fold higher; refs. 9, 10). CD28 is constitutively expressed on the membrane of resting T lymphocytes (6), whereas CTLA-4 expression is induced on stimulation (6) and retained in internal cell compartments (11). Upon T cell receptor engagement, CTLA-4 molecules are selectively directed to emerge at the immunologic synapse (11, 12). It has been also observed that when T cells meet a dendritic cell presenting cognate antigen, surface CD28 goes to the lipid raft-rich central synapse (13). After the engagement of ligands, CD28 induces signaling cascades that enhance proliferation, intensify cytokine secretion, up-regulate antiapoptotic genes (14), and fuel metabolism for lymphoblast transformation (15). In fact, CD28's key role as a costimulatory molecule has been shown in CD28<sup>-/-</sup> mice, in which both cellular and T cell-dependent humoral immunity are deficient in a certain degree (16).

On the contrary, CTLA-4 delivers a signal that decreases T cell activation by the recruitment of tyrosine (17, 18) and serine/threonine phosphatases (19). In fact, the function of CTLA-4 is inhibitory for T cell activation as illustrated *in vivo* by the uncontrolled lymphoproliferative/autoimmune syndrome observed in CTLA-4<sup>-/-</sup> mice (20, 21). Recent genetic evidence using CD80<sup>-/-</sup> dendritic cells strongly converge to suggest that the low level of surface CD80 expressed by immature (steady state) dendritic cells is involved in down-regulating the immune response (22, 23), by means of its interaction with CTLA-4 (24, 25). In contrast, some published observations have suggested that CD80 engagement on tumor cells by CTLA-4 would lead to a better T cell-mediated destruction of malignant cells in certain mouse models (26, 27), whereas other authors sustain that B7-CTLA-4 interactions may shield target tumor cells against CTL-mediated destruction (28). The reason(s) for this set of discrepant results are unclear.

Nonetheless, the inhibitory function of CTLA-4 against tumor immunity is best illustrated by the potent immunotherapeutic effect of monoclonal antibodies (mAb) that interfere with the function of CTLA-4 (29) in such a way that they induce tumor rejection in a number rodent tumors (30) with the potential to induce autoimmunity (31). Interestingly, anti-CTLA-4 antibodies have been tested in early trials with patients suffering from melanoma and ovarian cancer, showing evidence of certain clinical efficacy and unwanted autoimmunity as a side effect (32, 33).

Other members of the CD28/CTLA-4 family, such as PD-1 and BTLA have also been described to mediate inhibitory effects for the activation of the lymphocytes on which they are expressed, suggesting a common theme in the regulation of immune responses (34, 35). Furthermore, other members of the B7 family such as B7-H1 and B7-H4 have been shown to inhibit T-cell

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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activation (34, 36, 37). In the case of B7-H1, various mouse and human tumors express the molecule as a tumor escape mechanism (38), that if interfered with using blocking antibodies, fosters immunotherapy (39).

The expression of low levels of CD80 and CD86 has been detected on an important fraction of human melanomas (40, 41), myelomas (42), and acute myeloid leukemias (43). Moreover, low levels of expression of CD80 and CD86 detected by RT-PCR and surface staining has been reported in a series of cell lines derived from human carcinomas including some of colorectal origin (44). Interestingly, expression of CD86 was found to be associated with poor prognosis of leukemia and myeloma (42, 43). However, the mechanism underlying these clinical observations remains unknown.

In this study, we found CD80 surface expression at relatively low levels in various colon carcinoma cells that are widely used as cancer therapy models upon grafting onto immunocompetent syngeneic mice, as well as in other mouse malignant cell lines. We carried out experiments in immunocompetent versus immunodeficient mice to assess the relative immunogenicity displayed by carcinoma cells that express CD80 spontaneously, or the same cell lines transfected either to specifically silence or to overexpress CD80. Our results suggest that a low level of CD80 expression confers an advantage for tumor growth, thus helping to avoid tumor rejection, whereas high-level CD80 induces immune-mediated tumor regression.

## Materials and Methods

**Mice and cells.** BALB/c, athymic nude, and C57BL/6 mice were obtained from Harlan (Barcelona, Spain) and were used between 7 and 14 weeks of age. A breeding pair of Rag2<sup>-/-</sup> in BALB/c background mice was purchased from Harlan and bred in our animal facility under pathogen-free conditions. All animal handling and laboratory procedures were approved by the institutional animal facility ethical committee and are in accordance with Spanish regulations.

Five murine colon adenocarcinoma cell lines were used. Three of BALB/c origin (CT26, C26, and C51) and two of C57BL/6 origin (MC38 and C38). C51 and MC38 cells were transfected with a previously described recombinant retrovirus expressing murine *b7-1* gene (3, 45). Transfections were done by incubating supernatant of the packaging  $\psi$ -2 lines in the presence of polybrene as previously described (46). B7<sup>+</sup>-transfected cells were positively-selected by immunofluorescence-activated cell sorting (using an EPICS-C, Coulter, Fullerton, CA) and drug selection as previously described (3). Transfectants were routinely cultured in the presence of selecting drug. Cell lines were cultured at 37°C in 5% CO<sub>2</sub> in DMEM with 2 mmol/L L-glutamine, 100 units/mL streptomycin, 100 µg/mL penicillin and supplemented with 10% heat-inactivated fetal bovine serum. All cell culture reagents were from Life Technologies (Basel, Switzerland). For transfectant selection, hygromycin and puromycin were from Sigma-Aldrich (Madrid, Spain) and geneticin from Life Technologies. RENCA (renal cell carcinoma) and B16OVA melanoma were a kind gift from Dr. Allan Melcher (Leeds, United Kingdom). Lewis lung carcinoma was obtained through Dr. Lea Eisenbach (Rehovot, Israel), BNL (hepatocellular carcinoma) was obtained from Dr. Cheng Qian (Pamplona, Spain), and HOPC (myeloma) was obtained through the American Type Culture Collection (Manassas, VA). Dr. Paola Ricciardi-Castagnoli kindly provided us with the D1 dendritic cell line cultured as previously described (47). A cell line from a spontaneous T cell lymphoma arising from a peripheral lymph node of an elderly C57BL/6 mouse has been derived in our laboratory.<sup>5</sup>

CD11c<sup>+</sup> splenic cells were purified (>98%) with immunomagnetic beads from Miltenyi Biotec (Gladbach, Germany) according to manufacturer-recommended procedures in an Automacs instrument. Dendritic cell maturation was induced with 24 hours of culture in the presence of 10 µg/mL of lipopolysaccharide (Sigma-Aldrich).

**Tumor model and *in vivo* experiments.** For assessment of the tumorigenicity of the CT26 cell line, 5 × 10<sup>5</sup> cells were injected s.c. in the right flank of BALB/c, Rag2<sup>-/-</sup>, and athymic nude mice. Similarly, 5 × 10<sup>5</sup> MC38 and MC38-B7 cells were injected into C57BL/6 mice. Tumor growth was monitored weekly by measuring two perpendicular diameters using a Vernier caliper. Tumor explants were obtained after animal sacrifice by grinding a minced fragment of solid tumor and plating it in 24-well plates.

**Immunofluorescence and flow cytometry.** Cells were washed and labeled with FITC rat anti-mouse CD80 (BD PharMingen, San Diego, CA) for 30 minutes at 4°C. Unbound mAb was removed by washing twice with ice-cold PBS and immunostaining was determined by flow-cytometry (FACScalibur, Becton Dickinson, San Jose, CA). An isotype-matched FITC-tagged mAb was used as a negative control. CD28Ig and CTLA-4Ig were purchased from R&D (Abingdon, United Kingdom) and used in indirect immunofluorescence staining with the appropriate FITC-tagged secondary antibody purchased from Caltag (Burlingame, CA). Anti-CD45 mAb (BD PharMingen) was used to gate out myeloid-derived cells in cell suspensions of explanted tumors.

**Northern blot and probe preparation.** Total cellular RNA was extracted by the guanidineisothiocyanate technique, run in 20 µg aliquots on 1.0% agarose-formaldehyde gel, transferred onto nylon membrane (Hybond-N; Amersham, United Kingdom) by Northern blot. Blots were hybridized with the *HpaI-XhoI* fragment of pLmB7-ISH plasmid, containing the murine B7-1 cDNA and labeled with <sup>32</sup>P-dCTP by means of Multiprime kit from Amersham.

**Cloning and sequencing of murine CD80.** Tumor RNA was extracted by ULTRASPEC-II RNA isolation system (Biotecx, Houston, TX) and cDNA obtained by reverse transcription using random primers. A DNA fragment encoding the open reading frame of murine B7-1 was amplified by PCR using the primers: 5'CCCCATCATGTTCTCCAAAGC3', 5'ACTAAA-GGAAGACGGTCTGTTC3'. Another pair of primers was used for B7-1 detection as described (48). In this series of PCRs, the antisense primer was located in exon 3, which is spliced off to generate a B7-1a molecule. Therefore, these primers only amplified mCD80 cDNA but not B7-1a cDNA. PCR products were analyzed by 1% agarose gel electrophoresis and DNA bands were isolated using Concert Rapid Gel Extraction System (Life Technologies, Eggenstein-Leopoldshafen, Germany) and TA-cloned into pcDNA3.1/V5-His-TOPO (Invitrogen, Groningen, the Netherlands). Ten clones were selected and plasmids were isolated using QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) and fully sequenced (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Foster City, CA).

**Gene silencing of CD80.** pMSCVpuro (Clontech, Mountain View, CA) was modified to accommodate the small interfering RNA (siRNA) expression cassette by sequential digestion and religation of *BglII* and *HindIII* sites (all restriction enzymes were from New England Biolabs, Ipswich, MA). The expression cassette of pSUPER (49) was extracted by *EcoRI-XhoI* digestion and directionally cloned into the modified retroviral plasmid to yield pMSCV3SUPER. The target sites for CD80 siRNA were selected using the criteria proposed by Tuschl et al. (50). Three target sites were selected, and the following oligonucleotides (ordered from Sigma-Genosys, Cambridge, United Kingdom) were phosphorylated and cloned into the *HindIII-BglII* sites of pMSCV3SUPER. Sequences of the primers synthesized were (from 5' to 3'): 273<sup>+</sup>, GAT CCC CAC ATG ACA AAG TGG TGC TGT TCA AGA GAC AGC ACC ACT TTG TCA TGT TTT TTG GAA A; 273<sup>-</sup>, AGC TTT TCC AAA AAA CAT GAC AAA GTG GTG CTG TCT CTT GAA CAG CAC CAC TTT GTC ATG TGG G; 424<sup>+</sup>, GAT CCC CAA GAA GGA AAG AGG AAC GTT TCA AGA GAA CGT TCC TCT TTC CTT CTT TTT TTG GAA A; 424<sup>-</sup>, AGC TTT TCC AAA AAA AGG AAA GAG GAA CGT TCT CTT GAA ACG TTC CTC TTT CCT TCT TGG G; scr<sup>+</sup>, GAT CCC CCT ACA GTA ACT CCG TCA CTT TCA AGA GAA GTG ACG GAG TTA CTG TAG TTT TTG GAA A; scr<sup>-</sup>, AGC TTT TCC AAA AAC TAC AGT AAC TCC GTC ACT TCT CTT GAA AGT GAC GGA GTT ACT GTA GGG G.

<sup>5</sup> Arina et al., unpublished data.

Plasmids were transfected into CT26 cells by using a 22-kDa linear polyethylenimine from Polyplus Transfection (Illkirch, France) as described (51). Twenty-four hours posttransfection, the medium was removed, cells were washed and fed with fresh medium containing 8  $\mu\text{g}/\text{mL}$  of Puromycin (Sigma-Aldrich). Stable transfectants were picked 10 days later and subcultured for analysis.

## Results

### Constitutive expression of CD80 on murine tumor cell lines.

We assessed CD80 expression in CT26, C26, C51, and MC38 murine colon carcinoma cell lines by immunofluorescence and flow-cytometry analysis. To this end, we used a FITC-conjugated monoclonal antibody specific for murine CD80 (Fig. 1). CT26 and C26 expressed mouse CD80 (mCD80) at similar dim levels, whereas mCD80 was almost undetectable on C51 cells and was consistently undetectable on MC38 cells, thus indicating that CD80 expression is not a constant feature in murine colon cancer. As shown in Fig. 1, anti-CD80 mAb also stained the surface of Lewis lung carcinoma, RENCA (renal cell carcinoma), BNL (hepatocellular carcinoma), HOPC (multiple myeloma), and a spontaneous T cell lymphoma cell line derived from C57BL/6 mice that has been recently derived in our laboratory from a peripheral lymph node.<sup>5</sup> These data indicate that the low level of expression of CD80 is not an exclusive property of colon cancer but is a feature shared by many types of transplantable mouse malignancies, including MB49 bladder carcinoma and PANC02 pancreatic carcinoma (data not shown). However, there exist clear exceptions because cultured B16 melanoma and MC38 colon carcinoma cells do not express surface CD80 (Fig. 1).

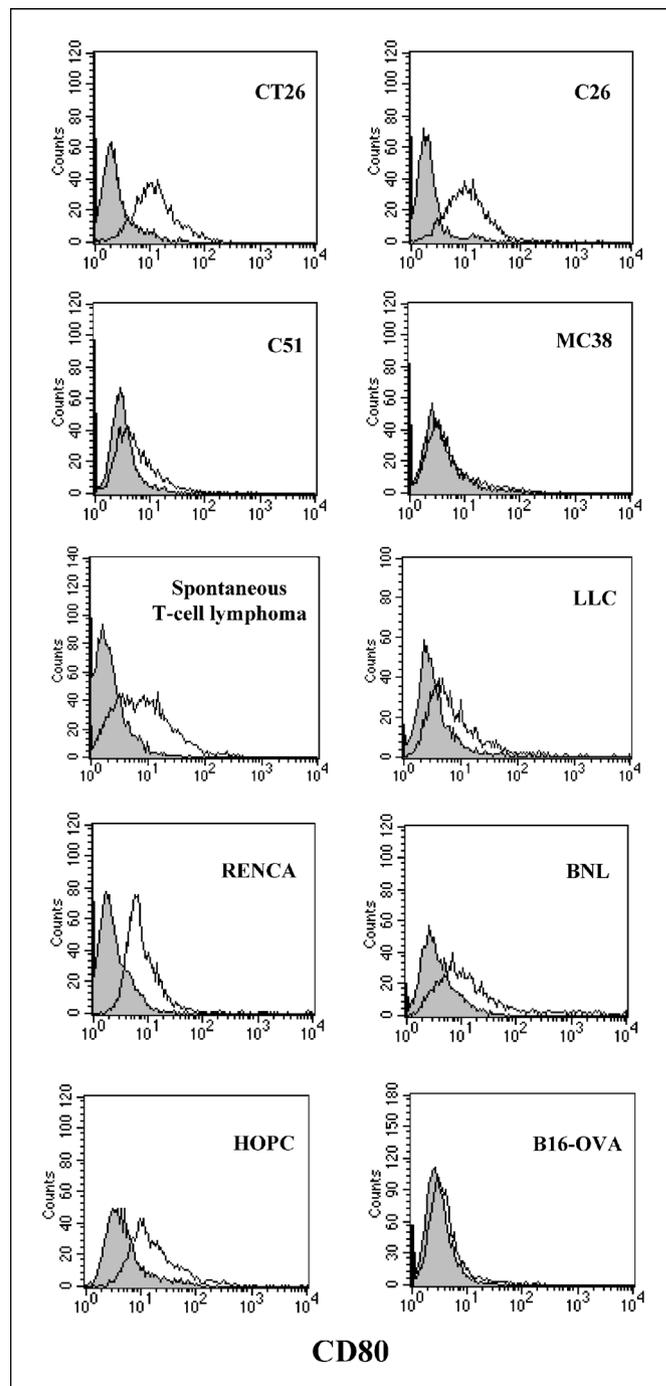
Northern blot analysis of RNA isolated from these colon cancer cell lines readily showed the presence of two bands (3.9 and 2.2 kb) when using a mCD80-specific probe. These two bands correspond with the previously reported splicing alternatives of the *cd80* gene and were also detected on RNA from lipopolysaccharide-stimulated splenocytes or IFN- $\gamma$ -stimulated macrophages (Fig. 2A).

cDNA was synthesized from colon carcinoma cell lines using random primers revealed that CD80 has two variants arising by alternative splicing that are expressed on the plasma membrane, one with IgV (membrane distal) + IgC (membrane proximal) domains, whereas the other contains only the IgV domain (B7-1a; ref. 52). Both isoforms have similar ligand-binding properties that map to the IgV domain. Therefore, two different PCRs were done. One was used for mCD80 mRNA detection with a sense primer located in the IgV-like domain whereas the antisense primer is located in the IgC-like domain. The IgC-like fragment of the CD80 protein is encoded by exon 3 which is spliced off to generate B7-1a molecule (IgV-only isoform). Accordingly, these primers only amplified mCD80 cDNA but not B7-1a cDNA. As a control, these primers amplified a similar band from RNA isolated from murine bone marrow-derived dendritic cells (Fig. 2B). A second series of PCRs were done in which another pair of primers were used to amplify cDNA encoding the whole mCD80 open reading frame. In this PCR, the two alternative splicing variants were found to coexist in CT26 (Fig. 2B).

Full-length CD80 cDNA was TA-cloned in pCDNA3.1 in order to verify its sequence. The sequence of at least two independent clones from CT26 total cDNA was identical to the one published. The shorter alternative splicing isoform (B7-1a; ref. 53) was also cloned and sequenced without finding any change when compared with the published sequence (data not shown). These experiments

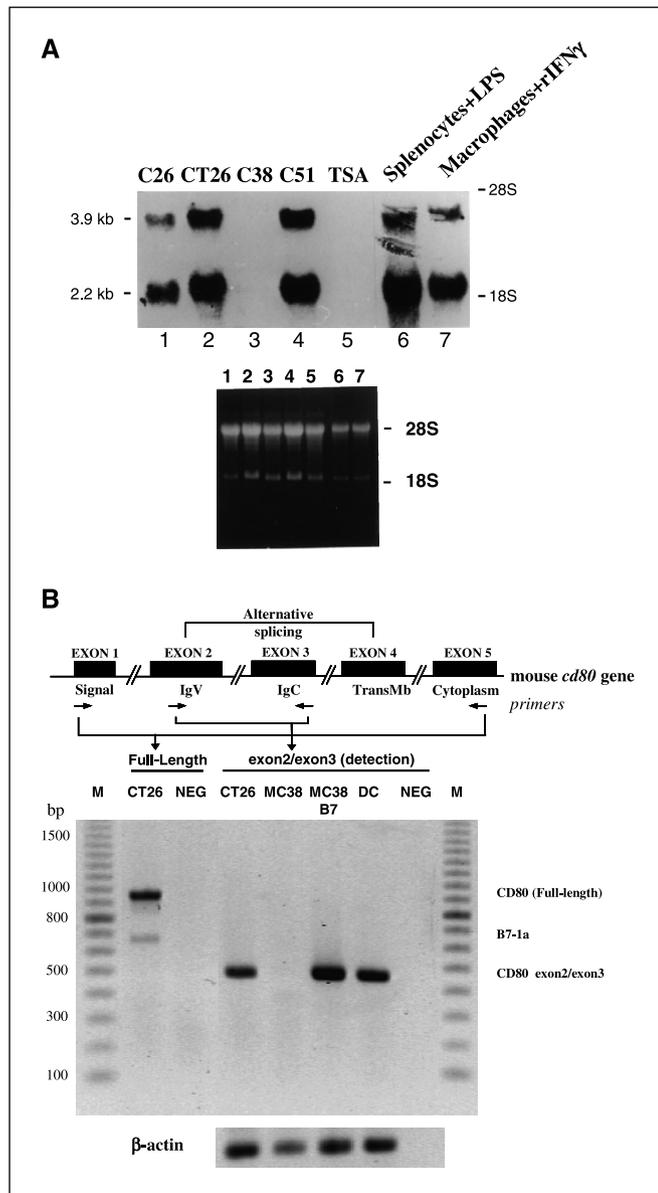
conclude that the unmutated, wild-type, and alternative splicing forms of the *cd80* gene are expressed on three murine colon carcinoma cell lines widely used in tumor immunology experiments on transplantation to syngeneic mice.

**Selective binding of CTLA-4 by spontaneously expressed CD80 on CT26 colon cancer cells.** CT26 cells were brightly

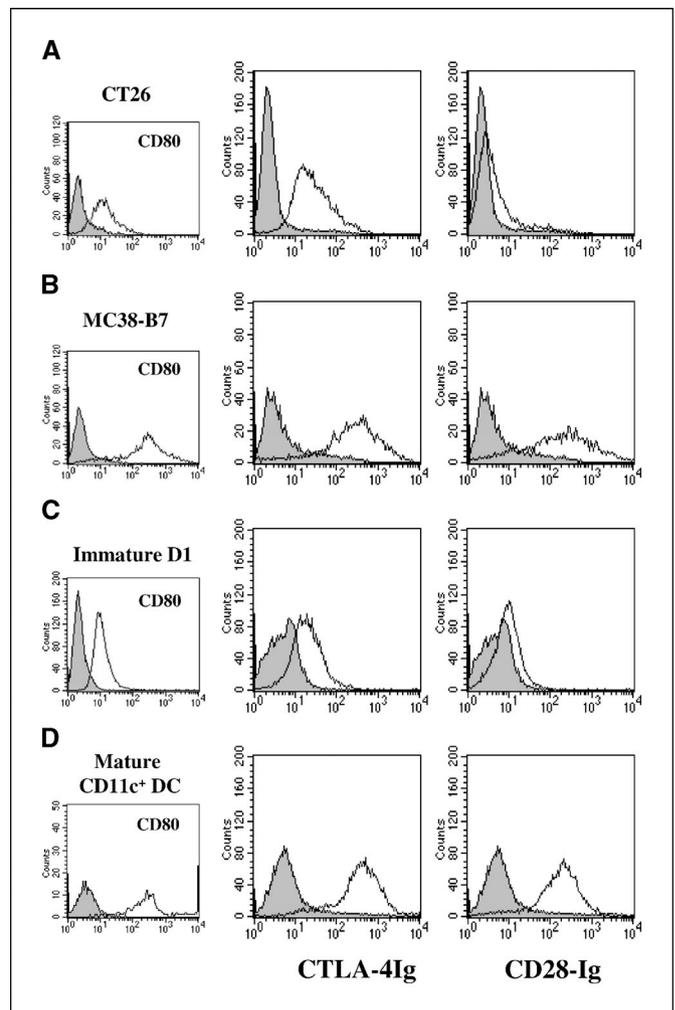


**Figure 1.** Surface expression of CD80 on transplantable tumor cell lines. CD80 surface levels assessed by direct immunofluorescence and FACS analysis on CT26, C26, and C51 murine colon carcinomas cell lines, on a spontaneous T-cell lymphoma cell line recently established, on Lewis lung carcinoma (LLC), RENCA (renal cell carcinoma), BNL (hepatocellular carcinoma), and HOPC (myeloma). Expression was not detected on the surface of MC38 (colon carcinoma) and B16-OVA cells (melanoma).

stained at the cell surface by chimeric proteins containing the extracellular portion of CTLA-4 and an immunoglobulin tail, indicating that the tumor molecule was functional at least for binding this inhibitory ligand (Fig. 3A). However, a similar chimeric protein containing the extracellular domains of CD28 barely bind CT26 cells even at 100  $\mu$ g/mL, whereas readily



**Figure 2.** Expression of CD80-encoding RNA in murine cultured colon carcinoma cell lines. **A**, Northern blot analysis of CD80 mRNA expression on RNA samples isolated from CT26, C26, C51, and MC38 cell lines, as well as from splenocytes activated with lipopolysaccharide and from macrophages treated with rIFN- $\gamma$ . RNAs were transferred onto nylon membranes and hybridized with a  $^{32}$ P-labeled probe specific for CD80 mRNA. The gel stained with 18S rRNAs is provided as a control of total RNA load per lane (**B**) RT-PCR analysis of CD80 expression. cDNAs were obtained by reverse transcription and a series of two PCRs were done. In one series, we used a pair of primers which amplified only full-length CD80 because one of the primers was located in exon 3, which is spliced off when generating B7-1a mRNA. In these PCRs, CD80 cDNA was found in CT26 and in a B7-transfected cell line (MC38-B7), whereas no amplification was obtained from MC38 cDNA. We used RNA obtained from dendritic cells as a positive control. The second series of PCRs with the indicated oligos amplified both splicing alternative isoforms of CD80.



**Figure 3.** Selective binding of CTLA-4 to CD80 as spontaneously expressed by CT26 colon cancer cells resembles selective binding of CTLA-4 to immature dendritic cells. FACS analyses of CT26 cells (**A**), MC38 cells transfected with recombinant retrovirus to express high levels of CD80 (**B**), the immature D1 dendritic cell line (**C**), and lipopolysaccharide-matured CD11c<sup>+</sup> dendritic cells (**D**). Cells were stained with FITC-tagged anti-CD80 mAb or with CTLA-4Ig or CD28Ig followed by antihuman Ig-FITC by indirect immunofluorescence. Analysis of CD11c<sup>+</sup> immature spleen cells rendered comparable results to those in D1 cells (data not shown).

stained MC38 cells that had been retrovirally transfected to stably express high levels of CD80 (Fig. 3B). These data indicate that CD80 at the levels displayed by CT26 cell shows selective binding for the inhibitory receptor CTLA-4 if compared with CD28. The CD80 levels on CT26 resemble those detected on immature dendritic cells, represented in Fig. 3C by the D1 immortalized dendritic cell line that also preferentially binds CTLA-4Ig. By contrast, splenic mature dendritic cells express high levels of CD80 that bind both CTLA-4Ig and CD28Ig (Fig. 3D). Our observations on tumor cells echo those by other authors that strongly suggest a role in immune down-regulation for low, but not completely negative, levels of CD80 and CD86 expression on immature dendritic cells (23–25).

**Transfection of *cd80* gene into colon cancer cells results in increased immunogenicity and tumor rejection.** MC38 is another mouse colon carcinoma cell line that is negative for CD80 surface expression by fluorescence-activated cell sorting

(FACS) analysis (Fig. 1 and corresponding histograms in Fig. 3B). These cells were retrovirally modified to express high levels of CD80 protein on their surface. After pharmacologic selection of stable transfectants, CD80<sup>high</sup> cells were selected and cloned by FACS-assisted sorting. Mock-transfected MC38 and mCD80-transfected cells (MC38-B7) were s.c. injected into the right flank of C57BL/6 mice. MC38 cells grafted as lethal tumors in all injected mice. In contrast, MC38-B7 displayed only transient growth in syngeneic mice that was constantly followed by complete tumor regression (Fig. 4A). This is in agreement with an extensive series of published experiments showing that artificial B7-1 expression, at least at high levels, increases tumor immunogenicity (1) and also causes tumor rejection in experimental colon carcinomas (2).

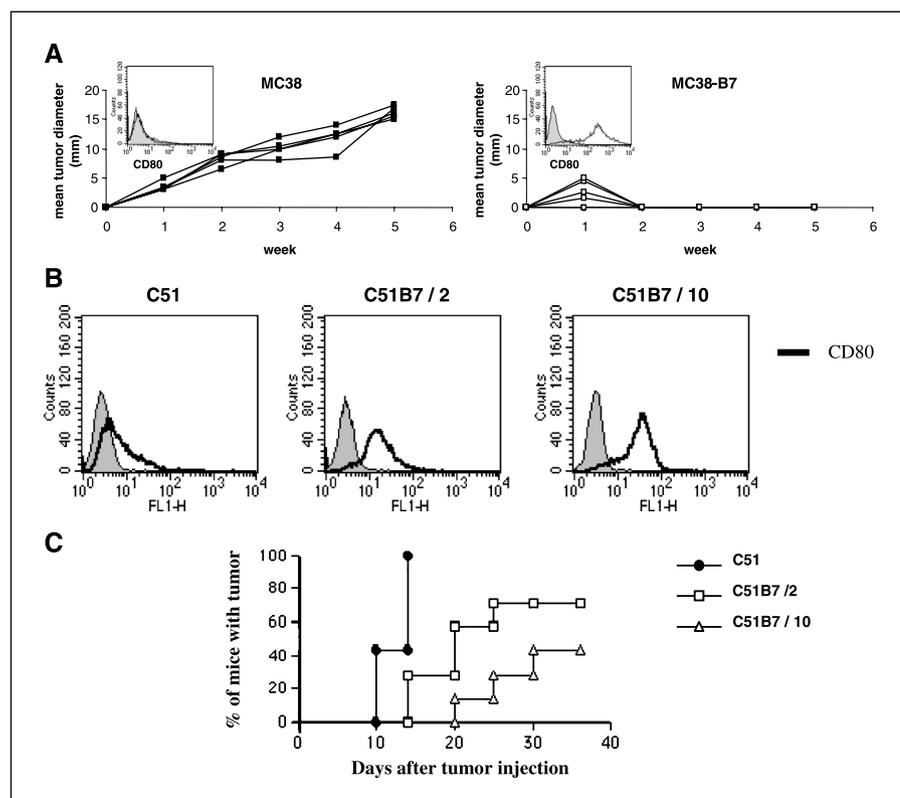
We also transfected the *mcd80* gene into the C51 cell line that expresses very low but detectable levels of endogenous CD80 (Fig. 1). Two cloned transfectants were generated, which are clearly different in gradual levels of CD80 expression (Fig. 4B). S.c. injection of such transfected cells into BALB/c mice showed that the transfected tumors had longer latency than the mock-transfected C51 in accordance with the levels of CD80 expression on plasma membrane (Fig. 4C). Indeed, in 50% of the cases, the brightest expression of CD80 avoided tumor grafting. These experiments indicated that the intensity of CD80 expression on transfected cells inversely correlated with tumor progression, but did not tell whether the low spontaneous level of CD80 had any biological function.

**Silencing of CD80 expression in CT26 results in lack of tumorigenicity.** To study the role of CD80 endogenous expression in the ability of CT26 colon carcinoma to graft as progressive tumors, stable transfectants were generated to express siRNAs targeted to different regions of the CD80's mRNA in order to silence its expression. For this purpose, we cloned the pSUPER expression

cassette (49) into retroviral plasmid pMSCV3puro, and in this new vector, we cloned a hairpin encoding oligonucleotides that would yield siRNA directed to CD80 mRNA (Fig. 5A). As a control, we used a scrambled sequence of roughly the same GC content as the other siRNAs. Puromycin-selected stable transfectants from two different RNAi constructions were cultured and cloned under limiting dilutions. Expression of CD80 in two different clones and in bulk culture cells transfected with an irrelevant scrambled sequence as a control is shown in Fig. 5B.

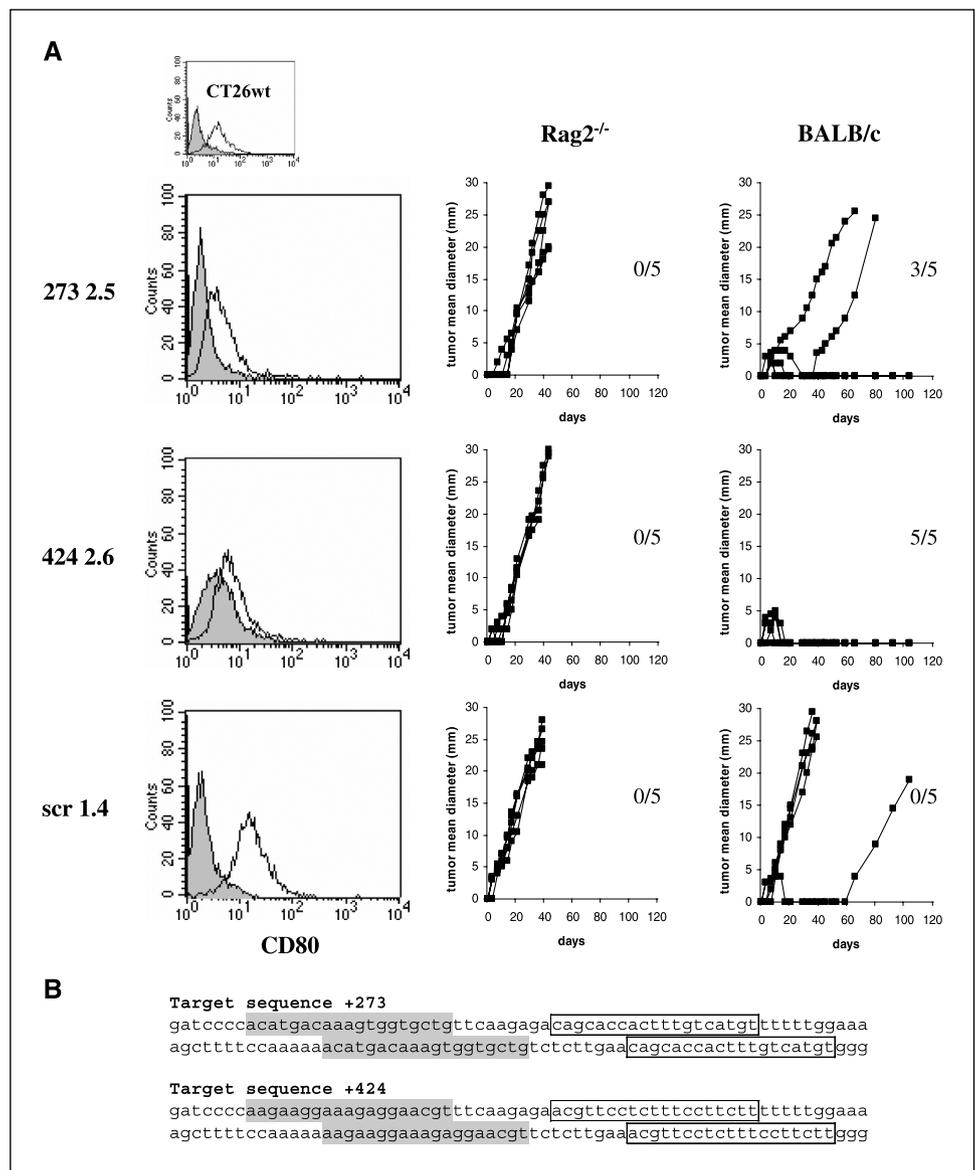
The experiments on *in vivo* growth of each silenced or control transfectant in normal BALB/c and Rag-2<sup>-/-</sup> are shown in Fig. 5B. Transfectants in which CD80 expression at the protein level were highly decreased, lost their capacity to graft as terminal tumors in immunocompetent mice, but preserved tumorigenicity in immunodeficient hosts of identical genetic background. Differences were not attributable to changes in MHC class I levels of expression because CD80-silenced cells express almost identical levels of H2-K<sup>d</sup> when compared with CT26 wild-type (data not shown). In addition, when those BALB/c mice who had rejected CD80-silenced CT26 tumors were rechallenged 3 months later with unmodified CT26 cells, those tumors were rejected in all mice, indicating that the mice had been immunized by exposure to CD80-silenced tumor cells (data not shown). As a whole, these data indicate that CT26 tumor cells silenced for CD80 expression elicit stronger antitumor immune responses.

Moreover, one of the repeated culture passages of the clone 424 2.6 spontaneously gave rise to a variant that homogeneously regained CD80 expression in spite of keeping resistance to puromycin (Supplementary Fig. S1A). This cell line progressed in immunocompetent mice indicating that the revertant regaining CD80 expression has an advantage against the antitumor immune response. In addition, if these revertant cells were preincubated



**Figure 4.** Increased immunogenicity of cells expressing high levels of CD80. *A*, comparative study of s.c. tumor development of CD80-negative MC38 cells and a retrovirally transfected MC38 clone selected for high stable expression of mCD80. Cells ( $5 \times 10^5$ ) from each one of these two cell lines were injected s.c. in the right flank of two groups of syngeneic C57BL/6 mice. Levels of expression of CD80 in transfected or untransfected cells are provided in accompanying histograms. *B*, gradual levels of expression in stable transfectants of the *cd80* gene generated with recombinant retrovirus in the C51 colon carcinoma cell line as detected by immunofluorescence with anti-CD80 mAb (*C*). Sequential analysis of the fraction of BALB/c mice ( $n = 7$  per group) developing lethal tumors after being injected s.c. with C51 wild-type colon cancer cells expressing dim levels of surface CD80 or with cloned variants that had been transfected with a retrovirus encoding an expression cassette of CD80. These variants were selected for bearing different levels of stable and gradually brighter expression of CD80 (shown in *B*).

**Figure 5.** CD80 silencing in CT26 cells abrogates tumorigenicity in immunocompetent hosts. **A**, siRNA sequences cloned in pSUPER to inhibit CD80 expression. A scrambled sequence with the same base composition in different order was used as a control. **B**, comparative analysis of tumor progression (size follow-up and fraction of mice completely rejecting their tumors) in BALB/c and Rag-2<sup>-/-</sup> syngenic mice after a s.c. injection of  $5 \times 10^5$  cells from stable transfectants with a plasmid encoding the siRNAs whose sequence is specified in (A) or a randomly scrambled sequence of similar length and base composition as a control. Levels of CD80 expression in the indicated stably transfected clones are provided in the accompanying histograms as well as the level of CD80 in untransfected CT26 (CT26-WT). Data pooled from three independent experiments with a total of 17 mice per group permitted a Fisher's exact test that showed a two-sided  $P < 0.0001$  when comparing rejection rates of the silenced versus control (nonsilenced) CT26 variants.



and coinjected with 100  $\mu\text{g}/\text{mL}$  of an anti-CD80 blocking antibody, those tumors were completely rejected in three out of six cases, whereas all tumors injected with control antibody progressed in another group of immunocompetent BALB/c mice (Supplementary Fig. S1B). These data further reinforce the notion that CD80 is the molecule involved in the escape mechanism and helps to rule out the possibility that the effects of the silencing could be explained by clonally variable immunogenicity among CT26 cells. In this regard, an independently generated polyclonal silenced variant of CT26 cells transfected with the 424 siRNA construction was also rejected in four out of six cases in immunocompetent mice, whereas it progressed in every case in T cell-deficient nude mice (Supplementary Fig. S1C).

**Preservation of CD80 expression on *in vivo* passage.** If low CD80 expression is considered as an advantageous feature in immunocompetent hosts, its expression will likely be evolutionarily preserved in tumor cells explanted from CT26 tumors growing in immunocompetent mice. This was confirmed in experiments shown in Supplementary Fig. S2, in which the relative intensity of

CD80-specific immunofluorescence in explanted tumor cells from immunodeficient or immunocompetent mice is plotted referred to CD80 level of expression on cultured CT26 (taken as 100%). It can be seen that CD80 expression is preserved both in immunocompetent and immunodeficient Rag<sup>-/-</sup> hosts even if they had been depleted of natural killer cells. Again, this observation suggests that low surface CD80 might have been selected for tumor escape from immunity.

These findings are unlikely explained by clonal heterogeneous expression of CD80 because in 18 randomly chosen limiting dilution-derived CT26 clones, the means of CD80 fluorescence intensity were almost identical, as illustrated by a coefficient of variation (CV = SD/mean) inferior to 3% of the mean (data not shown).

**CD80-negative tumor cell lines are induced to express low levels of CD80 on *in vivo* grafting, whereas successfully grafted CD80<sup>high</sup> transfected tumor cells reduce, but do not lose, CD80 expression.** MC38 and B16OVA melanoma are completely negative for CD80 immunostaining in tissue culture

but become positive in cell suspensions obtained from grafted tumors in syngeneic mice (Fig. 6A and B). Electronic gating and exclusion of CD45<sup>+</sup> hematopoietic cells in the FACS analyses ensured that the CD45-negative malignant cells were the only ones analyzed in Fig. 6. Interestingly when these cells were plated in culture for 7 days, a complete loss of surface CD80 took place.

As shown in Fig. 4C, the C51B7/10 CD80<sup>bright</sup> transfectant grafted as a terminal tumor in only 50% of the cases. Explanted cell suspensions of such tumors showed lower, but importantly not negative, CD80 expression levels than the original transfectant cell line in culture (Fig. 6C). As a whole, these data provide evolutionary evidence for a selective advantage of low but not negative expression of CD80 on cancer cells.

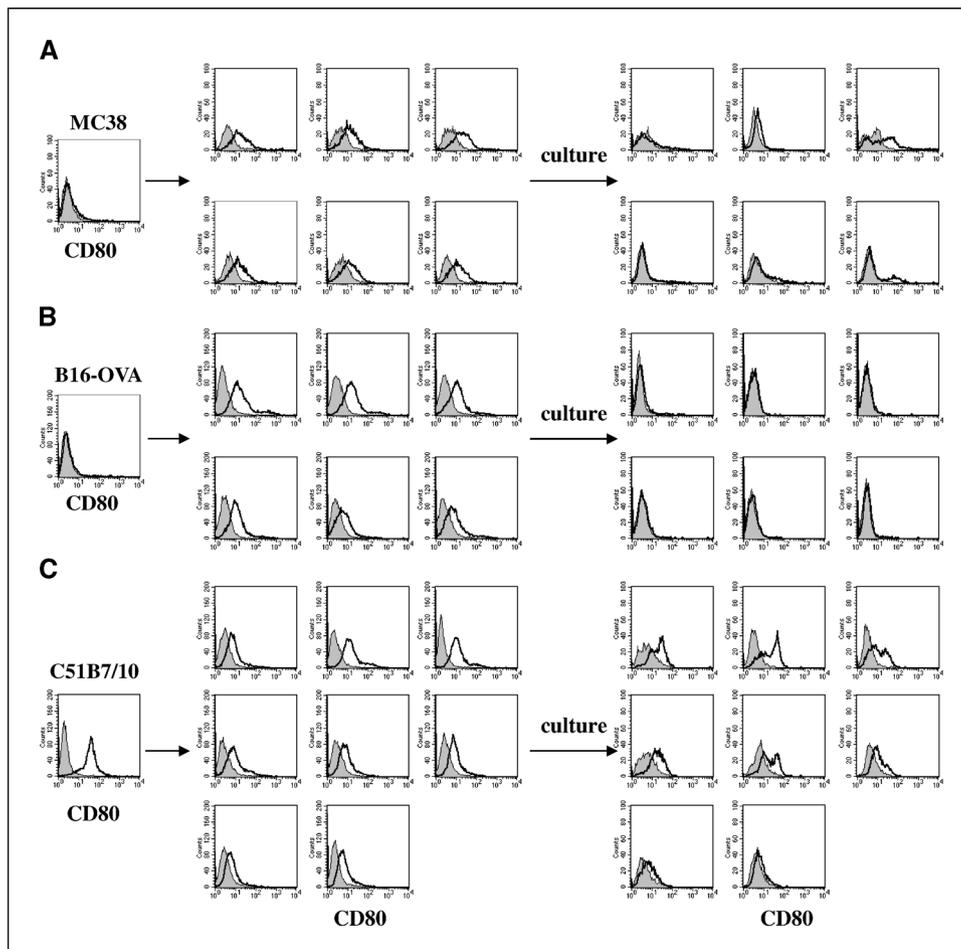
## Discussion

The main findings in this study are the unexpected basal and spontaneous expression of CD80 in transplantable tumor cell lines that are commonly used for experimental cancer immunotherapy experiments, in addition to observations on the role of low CD80 expression as an immune evasion mechanism.

To the best of our knowledge, this is the first report showing spontaneous CD80 expression in mouse tumor cells of epithelial origin, but it should be considered that CD80 up-regulation has been detected in mouse tumors treated with chemotherapy or radiotherapy (54, 55). These reports suggest the inducibility of

CD80 under stress conditions. The costimulatory molecule 4-1BBL has also been found to be spontaneously expressed in some tumor cell lines (56), a finding that is also in contrast with the fact that 4-1BBL transfection to high levels of expression augments the immunogenicity of various tumors (57, 58).

It is not possible to tell whether the original tumors were CD80<sup>+</sup> or if it was an acquired event that took place during *in vitro* or *in vivo* passage. Sequencing of the cDNA disclosed no mutation, suggesting that the membrane glycoproteins were fully functional, as confirmed by CT26 staining with CTLA-4Ig. Interestingly, we found by Northern blot, RT-PCR, and sequencing, the coexistence of two alternative splicing variants of *cd80* mRNA, as occurring in splenic cells stimulated with lipopolysaccharide or IFN- $\gamma$  and in cultured dendritic cells used as positive controls. Accordingly, it should not be expected that CD80 would function differently on the colon cancer cells compared with professional antigen-presenting cells. Indeed, the function of CD80 on antigen-presenting cells seems to be dual and related to the level of expression because CD80<sup>dim</sup> immature dendritic cells suppress T cell immunity in a CD80-dependent fashion, whereas CD80<sup>bright</sup> mature dendritic cells promote immunity under proper conditions (22, 23, 25). Our results with dendritic cells are in agreement with the view that the low levels of CD80 expression on immature dendritic cells would bind inhibitory CTLA-4 with competitive advantage to stimulatory CD28, as suggested by other authors with functional data using bone marrow chimeras with defects in CD80 expression on dendritic cells (24, 25).



**Figure 6.** Dim CD80 expression is induced in MC38 and B16-OVA during *in vivo* passage, whereas low levels of CD80 expression are selected on *in vivo* passage of CD80-high transfectants. MC38 (A), B16-OVA (B), and C51B7/10 (C) were inoculated in the flank of syngeneic mice. Tumors were explanted when they reached an average diameter of 15 mm and cell suspensions obtained by grinding minced tumors. For FACS analysis, CD45-positive cells were electronically gated and excluded so histograms only reflect CD80 levels of CD45-negative cells satisfying the FSC/SSC features of malignant cells. Cell suspensions were cultured for 1 week and CD80 expression reassessed. Each histogram represents an independent explanted tumor cell suspension.

B7-1 (CD80) and B7-2 (CD86) expression on tumor cells has been found to strongly raise the immunogenicity of transplantable cell lines (3, 4). The transfection of B7-1 generates cells that can even work as prophylactic or therapeutic vaccines against untransfected tumors by means of eliciting a strong CTL response (1). Most of these experiments were carried out with stable transfectants that had been sorted and selected for expression of very high levels of CD80 on every cell. Moreover, no detailed study has been published on the dose dependency of CD80 levels of expression and tumor immunogenicity, a factor that might prove crucial when considering that CD80 has two counter-receptors with dramatically opposite effects on the immune response. CD28 enhances T cell receptor-induced proliferation and activation of effector functions (14, 15), whereas CTLA-4 ligation arrests T cell cycle progression (59). Expression of CTLA-4 is only induced on activated cells with low levels of membrane expression but exquisitely directed to the area of T-cell engagement (11). Importantly, the inhibitory CTLA-4 receptor displays >100-fold higher avidity for CD80 than CD28 (9). It is tantalizing to speculate that low levels of CD80 might confer, by selective binding affinity for CTLA-4, some advantage to tumor progression in immunocompetent mice. In fact, lymphocytes that infiltrate tumors have an activated membrane phenotype and therefore are susceptible to CTLA-4-mediated inhibition (data not shown). The possibility that CD80 could be shielding CT26 tumor cells as targets for the CTL effector phase has been explored in light of the effects reported by Saudemont et al. (28) and the effects also shown by Hirano et al. for B7-H1 (39). Although we did our cytotoxicity experiments from 4 to 20 hours with anti-CT26-specific CTL, no increase of specific lysis upon CD80 blockade, neither by mAbs nor CTLA-4Ig, was observed (Supplementary Fig. S3). However, the *in vivo* situation could be different and therefore we cannot completely disregard such a mechanism in our tumor model.

Alternatively, tumor CD80 might enhance the function of regulatory T cells (60). We have done an extensive series of experiments aimed at costimulating CD4<sup>+</sup>CD25<sup>+</sup> Treg suppressor function with CD80<sup>+</sup> CT26 cells rendered negative results in our investigation. However, this mechanism is not definitively ruled out because Treg cells are known to express relatively high levels of membrane CTLA-4 that paradoxically costimulates this population (61, 62), and CT26 grafting is prevented by depleting CD25<sup>+</sup> lymphocytes (63).

Another mechanistic possibility is that CD80 ligation by CTLA-4 on CT26 could provide advantageous signals to the tumor cell. Although unlikely in epithelial cells, this possibility has been observed in mouse dendritic cells in which CD80 engagement by CTLA-4 promotes IFN- $\gamma$  secretion that in turn induces the immune inhibitory enzyme indoleamine 2,3-dioxygenase (IDO; ref. 64). We have explored this possibility in detail measuring IDO expression at the RNA and protein level as well as the production of IDO products without finding any involvement of CD80 cross-linking in CT26 cells. Up-regulation of IDO expression and function occurred following IFN- $\gamma$  stimulation, but was not further costimulated by CD80 cross-linking in these cells (data not shown). Therefore, the exact mechanism(s) behind the anti-immune advantage of low CD80 expression on the malignant cell surface remains elusive as is the case for the low CD80 expression on immature dendritic cells (25).

Gene silencing with siRNA is a powerful tool used to examine the role of a cellular protein. By these means, controlled selective decrease of expression of a specific gene is achieved with minimal

residual expression (49). To generate stable transfectants, we used two different constructs targeting distinct sequences of CD80 to assure specificity. Selection of cloned CT26 variants that clearly express much lower levels of CD80 showed that CD80 was not absolutely required for grafting in animals devoid of a functional immune system, but was necessary to avoid immune rejection. On the other hand, CD80 levels on CT26 were only minimally reduced after *in vivo* passage in BALB/c mice, even if compared with both B cell- and T cell-deficient mice and to B cell-, T cell-, and natural killer cell-deficient mice (Rag-2<sup>-/-</sup> mice depleted of asialoGM-1<sup>+</sup> cells; Supplementary Fig. S2). It can be concluded that, *in vivo*, the immune system exerts little or no pressure against low surface CD80 whereas reducing, but not abolishing, CD80 expression in successfully grafted CD80<sup>bright</sup> transfectants. Moreover, we clearly show that CD80 is induced *in vivo* on the surface of malignant cell lines that are negative during *in vitro* culture. We are currently exploring the stimuli that could be involved in such *in vivo* regulation.

Importantly, CD80 and CD86 expression has been found in a series of human melanoma in which low levels of RNA expression have been frequently documented (40, 41). These findings, precisely in a human malignancy characterized by a certain degree of intrinsic immunogenicity, suggest that B7 family molecules could be involved in subverting routes of immune destruction of tumors. In fact, B7 molecules are more frequently detected in those cases of melanoma with a higher number of metastatic nodules (41). In addition, the expression of B7 molecules in leukemia and myeloma cells is correlated with faster disease progression (42, 43). The presence of CD80 in other human cell lines or in tissue sections from human colorectal carcinomas is currently under investigation.

Another molecule of the family, B7-H1, has been described to be expressed on human and mouse tumor cells of various tissue origins as observed both in cultured cell lines and in tissue sections (38). By expression of B7-H1, human cancers may evade adaptive immune responses by promoting the apoptosis of activated T cells or by stimulating IL-10 production to deactivate T cells (65). B7-H1 does not bind CD28 or CTLA-4, but it binds PD-1, a T-cell surface molecule also involved in the down-regulation of immune responses (36, 38). It has been recently described that B7-H1 expression renders tumors resistant to immunotherapy and that this effect could be reverted by blocking B7-H1 or PD-1 with specific antibodies (39). The overall emerging picture is that B7 family members can be broadly exploited by tumor cells as a way to escape immune destruction. In the particular case of CD80, tumors cunningly exploit the dual function of this molecule by expressing low surface levels, which preferentially engage its high-avidity inhibitory T-cell ligand CTLA-4.

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