IL-12 and IL-10 Expression Synergize to Induce the Immune-Mediated Eradication of Established Colon and Mammary Tumors and Lung Metastasis[1]

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Preclinical studies demonstrated that certain cytokines are potentially useful for the induction of antitumor immune responses. However, their administration in clinical settings was only marginally useful and evoked serious toxicity. In this study, we demonstrate that the combination of autologous inactivated tumor cells expressing IL-12 and IL-10 induced tumor remission in 50–70% of mice harboring large established colon or mammary tumors and spontaneous lung metastases, with the consequent establishment of an antitumor immune memory. Mice treatment with tumor cells expressing IL-12 was only marginally effective, while expression of IL-10 was not effective at all. Administration of the combined immunotherapy stimulated the recruitment of a strong inflammatory infiltrate that correlated with local, increased expression levels of the chemokines MIP-2, MCP-1, IFN-γ-inducible protein-10, and TCA-3 and the overexpression of IFN-γ, but not IL-4. The combined immunotherapy was also therapeutically effective on established lung metastases from both colon and mammary tumors. The antitumor effect of the combined immunotherapy was mainly dependent on $CD8^+$ cells although $CD4^+$ T cells also played a role. The production of IFN-γ and IL-4 by spleen cells and the development of tumor-specific IgG1 and IgG2a Abs indicate that each cytokine stimulated its own Th pathway and that both arms were actively engaged in the antitumor effect. This study provides the first evidence of a synergistic antitumor effect of IL-12 and IL-10 suggesting that a Th1 and a Th2 cytokine can be effectively combined as a novel rational approach for cancer immunotherapy. *The Journal of Immunology,* **2005, 175: 5885–5894.**

Since the initial reports using bacillus Calmette-Guérin to stimulate the influx of inflammatory cells, much work has been done to establish the role that cytokines play in the immune-mediated recognition and rejection of tumor cells (Ref. 1 and references therein). An important number of preclinical studies have shown that cytokine administration was beneficial for cancer treatment. However, the systemic administration of several potentially useful recombinant cytokines in clinical settings had only marginal effects and evoked serious toxicity, hampering their use but stimulating the development of delivery systems providing relatively high levels, locally (2–4).

IL-12 is considered the most potent antitumor and antimetastatic cytokine (5, 6). It stimulates NK cells, promotes maturation of CTL, and induces IFN-γ production stressing its role as an efficient molecule for the initiation of a Th1 response (5, 6). IL-12 also inhibits Th2 cells and their cytokine production (7). The potential usefulness of IL-12 gene expression for cancer treatment in preclinical models was clearly demonstrated (6, 8, 9), although its excessive toxicity and modest clinical effects reduced the initial expectations (10). Previous studies have demonstrated that ectopic expression of IL-12 by CT26 colon cancer cells induced a $CD8^+$ T cell-mediated tumor rejection (8).

IL-10 was originally described as a B cell growth factor (11) and has attracted attention for therapeutic purposes for its inhibition of Th1 lymphocyte proliferation and cytokine production (12). IL-10 is not a general inhibitor of immune responses. IL-10 potentiates IL-2-induced proliferation and differentiation of $CD8^+$ cells (13) and stimulates monocyte mediated-Ab-dependent cell cytotoxicity (14). Moreover, immunization of mice with tumor cells expressing IL-10 promoted the loss of tumorigenicity and induced a protective antitumor immune memory response mediated either by NK cells or $CD8^+$ T cells (15–19). Interestingly, IL-10 expression by CT26 colon cancer cells induced a T cell-mediated tumor rejection in the context of a systemic Th2 response (18).

The Th1/Th2 paradigm plays a pivotal role in the understanding of the resolution of microbial infections as well as other diseases (20). Qualitative analysis of the immune response in tumors has been also facilitated by the Th1/Th2 model of immune response (21). Similar to what has been observed in infectious diseases, it was proposed that the eradication of human cancer would be favored by the effective induction of a Th1 response (22). It was reasoned that abnormally elevated levels of IL-10 skews a Th2 response that favors tumor growth. Current paradigms in immunology suggest that the combination of potent and antagonistic Th1 and Th2 cytokines such as IL-12 and IL-10 must lead to counterinhibition of their respective activities (5, 6, 12, 23). However, in view of the reported antitumor effects of each single cytokine gene and their different mechanisms of action, we decided

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to evaluate their concurrent production via autologous tumor cells on established colon or mammary primary tumors and spontaneous lung metastasis.

Materials and Methods

Vector construction, transfection of packaging cells, and transduction of tumor cells

The construction of retroviral vectors, transfection of packaging cells, screening of IL-10 and IL-12-producing clones, transduction of target cells, and assessment of IL-10 and IL-12 levels have been previously described (8, 18). All the cell lines were routinely tested for the absence of mycoplasma (MYCOTECT; Invitrogen Life Technologies). CT26-IL-12 and LM3-IL-12 cells produced 0.21 and 0.37 ng/ml per 10^5 cells per 24 h of murine IL-12 (mIL-12),[4] respectively. CT26-IL-10 and LM3-IL-10 produced 5.8 and 6.3 ng/ml per 10^5 cells per 24 h of mIL-10, respectively. CT26-neo, LM3-neo, CT26-puro, and LM3-puro correspond to the tumor cell lines transduced with a retroviral vector without insert and carrying the gene that confers resistance to neomycin or puromycin, respectively.

In vivo studies

All the experiments were performed with 8- to 11-wk-old male BALB/c mice obtained from the animal facility of the National Institute of Drugs and Clinical Trials (INAME). Mice were housed in the animal facility at the Leloir Institute for 2 wk before their use. In preimmunization assays, mice were s.c. injected with different types and amounts of cells into the left flank in a total volume of 0.1 ml of PBS. Tumor size was monitored every 2 days. Three to 5 wk after the initial administration, mice were challenged in the opposite flank with 3×10^5 parental cells.

Mice were s.c. injected in the left flank with tumorigenic inocula of 3×10^5 tumor cells in a total volume of 0.1 ml. Mice bearing 20-day-old tumors of 200–300 mm^3 size were injected weekly for 4 wk with live (L-TC), mitomycin C-treated (MitC-TC) or gamma-irradiated (γI-TC) autologous tumor cells producing IL-10, IL-12, or the combination of both cell types. Tumor cells carrying the transduced cytokine gene were injected close to the established tumor in four different places equidistant from the tumor area or as specified in the experiments. Inactivation of cells by mitomycin C was performed as previously described (8), while irradiated cells received 5000 rad from a ^{137}Cs source (Cerbisa). Inactivation of tumor cells did not abrogate secretion of cytokines in vitro over the course of 7 days (data not shown). In some experiments mice bearing s.c. CT26 tumors were treated weekly for 4 wk with 3×10^6 NIH3T3 or amphotropic retroviral vector producer cells (RVPC) expressing IL-10 and IL-12 mixed or not with irradiated CT26 cells. NIH3T3 cells remained viable in BALB/c mice at least 11 days as assessed with cells expressing the enhanced GFP (data not shown).

The antimetastatic effect of administering tumor cells producing the cytokines was assessed in a model of spontaneous metastases. LM3 cells were injected in the flank and after 22 days of primary tumor development large lung metastatic nodules are visible in 100% of mice. At this time point, we started injecting mice with Mit C-treated autologous tumor cells. Mice were sacrificed for evaluating metastatic nodules as described in *Results*. Experimental metastases were induced by injecting 1×10^6 CT26 cells in 100 μl of PBS, pH 7.4, via the tail vein. Mice were sacrificed for evaluation of metastatic nodules 7 days after the administration of the last treatment. The lungs were fixed in 10% formaldehyde and the metastatic nodules were counted under $\times$10 magnification.

For evaluating the establishment of an antitumor memory immune response, mice that rejected established tumors were contralaterally challenged with tumorigenic inocula of parental cells. Tumor cell growth was evaluated up to the end of the different experiments.

Histological studies

Mice carrying 20-day-old tumors were injected once with MitC-treated autologous tumor cells as described above. One week later, three to four mice were sacrificed and all the area was removed for histological analyses. Tissues were fixed, embedded in paraffin, sectioned at 5 μm, and stained with H&E. Immunohistochemical analysis of macrophages was performed with a rat anti-mouse Ab (Anti-F4/80; Serotec; 1/50 final dilution) followed by biotin-labeled goat anti-rat antisera (Jackson ImmunoResearch Laboratories). After washing, sections were incubated with ABC Vectastain Elite reagent (Vector Laboratories). Staining was developed with diaminobenzidine and sections were counterstained with hematoxylin. NK cells, $CD4^+$ and $CD8^+$ lymphocytes were detected in frozen sections embedded in OCT, using anti-Ly49G2 (NK) hybridoma (4D11 from American Type Culture Collection, code HB240, kindly provided by M. Guthman, Hospital de Clinicas, Buenos Aires, Argentina), anti-$CD4^+$ and anti-Ly2.1 and Ly2.2 ($CD8^+$) Abs, both from Serotec, followed by a Cy2-conjugated donkey anti-rat Ab (Jackson ImmunoResearch Laboratories). For histological analyses, the lungs were removed, fixed, and embedded in paraffin. Frozen spleen sections were used as a positive control for $CD4^+$ and $CD8^+$ T cells (data not shown).

RNase protection assays

For analysis of chemokine expression, mice bearing established tumors were injected once with cytokines expressing tumor cells. At different time points, all the area was excised under a stereoscopic microscope, and the tumor put aside. The rest of the tissue was quick-frozen in liquid nitrogen and stored at -80°C until total RNA extraction using TRIzol (Invitrogen Life Technologies). Expression of chemokines was assessed with the RiboQuant RNase Protection System and template set mCK-5c (BD Pharmingen). The probe was labeled with [α-^{32}P]UTP (3,000 Ci/mmol; 10 μCi/μl; Amersham Biosciences) using T7 RNA polymerase. After overnight hybridization with 20 μg of total RNA, samples were treated with proteinase K-SDS mixture, extracted with phenol-chloroform, and precipitated in the presence of ammonium acetate. The protected RNA fragments were separated on a 4.75% denaturing polyacrylamide gel. Dried gels were exposed for 1–2 h and scanned with a phosphorimager (Storm 820 PhosphoImager; Molecular Dynamics) or alternatively exposed to an X-OMAT film (Eastman Kodak) at -70°C. The signals were quantified by suitable software (ImageQuant; Molecular Dynamics). The chemokine mRNA levels were corrected for RNA loaded by dividing the chemokine hybridization signal by the L32 signal for the same sample.

Cell depletion

Immune cells were selectively depleted by in vivo treatment with specific Abs for different lymphocytes subpopulations as reported (18). The Abs used were mAb YTS 191.1 for $CD4^+$ cells, mAb YTS 169.4 for $CD8^+$ cells, and 4D11 hybridoma for NK cells. Mice received the Ab dose 1 day before administration of autologous tumor cells carrying the cytokines.

Assessment of IL-4, IFN-γ, and IgG levels

The isolation of spleen cells and assessment of IL-4 and IFN-γ was performed by ELISA essentially as described (18). Total levels of circulating and anti-CT26-specific IgG2a and IgG1 were evaluated as described previously (8, 18).

Assessment of IL-4 and IFN-γ mRNA levels at the tumor site

Total RNA was used to assess IL-4 and IFN-γ mRNA levels by competitive RT-PCR (24). Single-stranded cDNA was synthesized using a First Strand cDNA Synthesis kit (Invitrogen Life Technologies). The cDNA and cytokine competitor (cf; kindly provided by F. Pitossi, Instituto Leloir, Buenos Aires, Argentina) were then PCR amplified for IFN-γ (forward, 5′-GCTCTGAGACAATGAACGCT-3′; reverse, 5′-AAAGAGATAATC TGGCTCTGC-3′), yielding 400- and 227-bp fragments, respectively. The cDNA and cytokine competitor were also PCR-amplified for IL-4 (forward, 5′-TCGGCATTTTGAACGAGGTC-3′; reverse, 5′-GAA AAGC CCGAAAGAGTCTC-3′), yielding 400- and 216-bp fragments, respectively, and finally amplified for the β_2-microtubulin gene (housekeeping: forward, 5′-TGACCGGCTTGTATGCTATC-3′; reverse, 5′-CAGTGTG AGCCAGGATATAG-3′), yielding 400- and 222-bp fragments, respectively. PCR was performed in a PTC-200 thermocycler (MJ Research) as follows: 94°C for 2.5 min, then 41 cycles at 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s, followed by a final extension at 72°C for 3 min. The bands were quantified with the software Gelworks1D Intermediate version 3.01 (UVP 2.5; Ultra Violet Products). For each cytokine a standard curve was established. The relative amounts of IL-4 and IFN-γ mRNA to β_2-microtubulin was calculated by the following formula: Relative amounts = (cytokine product of standard curve)/(housekeeping product of standard curve).

Statistical analysis

Survival analyses were performed using Kaplan-Meier's method. Statistical comparisons between the different groups were made using the log-rank test. We used a one-way ANOVA to analyze the experimental data in Figs. 1, 3, 4, and 5. A p value < 0.05 was considered significant. Data corresponding to Table II were analyzed by Fisher's exact test.

[4] Abbreviations used in this paper: m, murine; TC, tumor cell; L-TC, live TC; MitC-TC, mitomycin C-treated TC; γI-TC, gamma-irradiated TC; IP-10, IFN-γ-inducible protein 10.

Results

Local administration of autologous tumor cells expressing the combination of IL-12 and IL-10 reproducibly led to the rejection of large, established s.c. tumors

In previous studies we have shown that 28/28 and 12/19 of mice challenged with 3×10^6 CT26-IL-12 or with 5×10^5 CT26-IL-10 cells, respectively, rejected tumor growth and developed an antitumor immune memory able to reject a challenge with parental cells (8, 18). Here, we established that 100% of mice challenged with the combination of 3×10^6 CT26-IL-12 and 5×10^5 CT26-IL-10 cells rejected tumor growth and also developed an antitumor memory immune response ($n = 14$; data not shown). We extended these preliminary studies and observed that 25 of 26, 9 of 11, and 16 of 16 mice injected with 3×10^6 LM3-IL-12 cells, 5×10^5 LM3-IL-10 cells, and their combination, rejected tumor growth and developed an antitumor immune memory. All the controls developed rapidly growing tumors (data not shown). Overall, these data indicated the effectiveness of IL-12 and IL-10 expression by tumor cells, alone or in combination, in eliciting an antitumor response in preimmunization protocols.

Our next attempt was to establish whether this cytokine combination expressed by autologous tumor cells could be also effective in the rejection of established tumors. For this purpose, mice were injected s.c. with tumorigenic inocula of either CT26 or LM3 cells. Twenty days later, when average tumor size reached 200–300 mm^3, mice were treated with four consecutive administrations of L-TC locally as described in *Materials and Methods*. None of the mice harboring established CT26 tumors benefited from the administration of control CT26-neo L-TC or CT26-IL-10 L-TC as all the tumors reached 2.5 cm^3 in $<$ 35 days and mice were considered not survivors (Fig. 1*A*). Administration of CT26-IL-12 L-TC had a minimal but statistically significant effect on tumor growth and 8.3% of mice (2 of 24) survived at the end of the experiments (Fig. 1*A*). However, the therapeutic effect was greatly improved by the coadministration of CT26-IL-10 cells because 100% of mice survived longer than control mice when treated with CT26-(IL-10 + IL-12) and 69.2% of them (18 of 26) remained alive up to end of the experiments (Fig. 1*A*). The effectiveness of the combined immunotherapy was confirmed with the LM3 mammary tumor model. Although the administration of LM3-IL-12 L-TC in tumor-bearing mice had some effectiveness because 12.5% (2 of 16) of mice survived at the end of the experiments, mice treatment with LM3-(IL-10 + IL-12) L-TC raised this percentage to 58.9% (10 of 17) (Fig. 1*B*). Again, the administration of LM3-neo L-TC and LM3-IL-10 L-TC had no effect at all (Fig. 1*B*).

To establish the therapeutic potential of this cytokine combination on established tumors, we injected mice also with mitomycin C-inactivated tumor cells (MitC-TC) expressing the different cytokines. In line with the results observed with L-TC, the administration of CT26-IL-12 MitC-TC induced a statistically significant improvement in mice survival ($p < 0.0001$) but none of the mice remained alive at the end of the experiments. Meanwhile, mice treatment with CT26-(IL-10 + IL-12) MitC-TC was highly effective and 57.1% of the mice (20 of 35) survived up to the end of the experiments (Fig. 1*C*). The therapeutic effect observed in the CT26 colon cancer model with the combined immunotherapy was confirmed in the LM3 model because only the administration of LM3-(IL-10 + IL-12) MitC-TC promoted the survival of 50% of mice carrying established tumors (12 of 24) while the administration of CT26-IL-12 MitC-TC had only a minor but statistically significant effect (Fig. 1*D*).

The therapeutic efficacy of the combined immunotherapy was further confirmed using γI-TCs. CT26-(IL-10 + IL-12) γI-TC delayed tumor growth in >70% of mice and 41.7% of total mice (10 of 24) showed tumor regression even after 6 mo (Fig. 1*E*). Similarly, LM3-(IL-10 + IL-12) γI-TC were effective in inducing the survival of 50% of mice carrying mammary tumors (8 of 16), while administration of γI-TC expressing a single cytokine were mostly ineffective (Fig. 1*F*). Fig. 1 shows also a typical experiment showing the in vivo tumor growth rate following the injection of mice carrying established CT26 colon carcinomas with the different MitC-TCs. Injection of mice with CT26-IL-12 MitC-TC induced only slight delays in primary tumor growth in several mice compared with the growth rate observed in control mice (Fig. 1*G*). On the contrary, injection with the combined CT26-(IL-10 + IL-12) MitC-TC cured 4 of 7 mice (Fig. 1*H*).

To understand the mechanism by which the combined expression of both cytokines induced rejection of established tumors, we injected CT26-tumor bearing mice with CT26-(IL-12 + IL-10) MitC-TC contralaterally to the tumor. In parallel experiments we administered tumor-bearing mice with CT26-IL-12 MitC-TC close to the tumor and CT26-IL-10 MitC-TC in the opposite flank. In both cases, there was no statistically significant therapeutic benefit compared with the control (Fig. 1*I*). This result indicated that the combined immunotherapy must be administered locally to obtain its full effect and that IL-10 synergized with IL-12 only when coexpressed close to the tumor area (Fig. 1*I*).

One-hundred percent of mice that rejected established tumors developed an antitumor memory and rejected a contralateral challenge with tumorigenic inocula of parental cells, at days 60–65 after the initiation of the injections regime. Interestingly, histological analysis of the tumor area following mice autopsies showed a central necrotic area surrounded by a ring of viable tumor cells heavily infiltrated by neutrophils, macrophages and lymphocytes indicating that the immune system was being actively recruited locally (data not shown). Mice autopsies showed no evidence of tumor cell dissemination to secondary organs even after 6 mo of follow-up.

Tumor cells were not merely acting as biological pumps but they appear to contribute to the antitumor effect exerted by the combined cytokine expression. Indeed, treatment of mice bearing s.c. CT26 tumors with weekly administration of NIH3T3 or RVPC producing IL-10 and IL-12, in combination or not with irradiated autologous tumor cells demonstrated that the cytokine combination was much more effective than the use of a single cytokine agent (not shown). However, all the animals treated with nontumor heterologous cells eventually died following the different approaches. Overall, these data confirmed the superior therapeutic benefit of using autologous tumor cells for the combined immunotherapy of primary colon and mammary tumors.

Combined immunotherapy with IL-10 and IL-12 promotes the rejection of established lung metastasis

To establish whether the combined immunotherapy might be effective also in the eradication of established metastatic foci we performed two different set of experiments. First, we took advantage of the fact that LM3 cells spontaneously develop large lung metastases in <20 days after s.c. injection of 3×10^5 cells in the flank (Table I and Ref. 25). Mice harboring 20-day-old LM3 tumors were injected four consecutive times locally with the different MitC-TCs without removing the primary tumor, and sacrificed when primary tumors in control mice reached an average of 2.5 cm^3 (days 40–42 after the first injection). None of the animals treated with the combined LM3 cells-based immunotherapy developed lung metastases at the end of the experiment, despite the fact that some (5 of 12) showed slow growing s.c. tumors (Table I). In comparison, 83% of mice injected with the MitC-treated LM3 cells

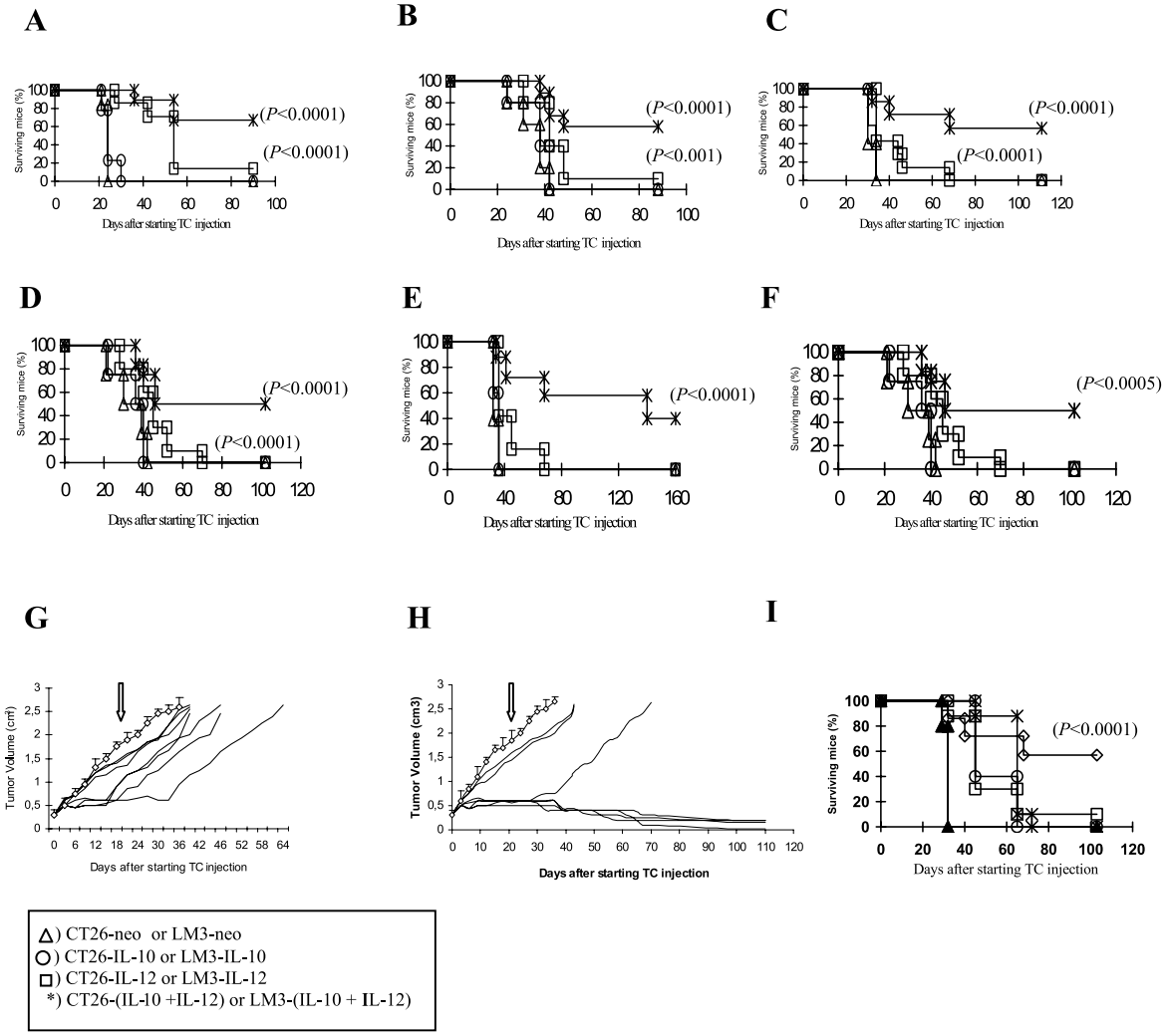

FIGURE 1. Treatment of mice bearing s.c. CT26 and LM3 tumors with autologous tumor cells expressing IL-10 and IL-12. Mice bearing 20-day-old s.c. tumors were injected weekly for 4 wk with live (L-TC) (*A* and *B*), mitomycin C-inactivated (MitC-TC) (*C* and *D*), and gamma-irradiated tumor cells (γI-TC) (*E* and *F*). Values of *p* correspond to groups showing statistically significant differences compared with their respective controls that correspond to mice injected with cells carrying empty retroviral vector. The figures represent the cumulative data obtained from the following number of experiments: *C*) four; *A*, *D,* and *E*) three; *B*, *F*, and *I*) two. Each experiment included 6–10 mice per group. *G* and *H*, Typical experiments extracted from *C* are shown. *G*, Mice carrying established CT26 tumors were injected with CT26-IL-12 MitC-TC ($n = 7$) and their growth in individual mice was followed. The average tumor growth in CT26-tumor bearing mice injected with CT26-IL-10 MitC-TC is shown as a comparison (◇). *H*, Corresponds to mice carrying established CT26 tumors and injected with CT26-(IL-10 + IL-12) MitC-TC ($n = 7$). Tumor growth is shown in individual mice. The average tumor growth in CT26-tumor bearing mice treated with CT26-neo MitC-TC is shown as a comparison (◇). Arrows show the end of injection protocol. *I*, Mice bearing CT26 tumors in the left flank were injected with Mit C cells as follows: ▲) CT26-IL-10 in the right flank; ○) CT26-IL-12 in the right flank; □) CT26-IL-12 in the left flank and CT26-IL-10 in the right flank; ∗) CT26-(IL-10 + IL-12) in the right flank; ◇) CT26-(IL-10 + IL-12) in the left flank. Each group corresponds to at least 10 mice.

producing only IL-12 and 100% of mice corresponding to the two other groups developed lung metastasis (Table I). Histological analysis showed that both the control group and those mice injected with autologous cells producing a single cytokine demonstrated a remarkable amount of neutrophils infiltrating the lung vessels and the alveoli (compare Fig. 2*A* with Fig. 2*C*). However, a dramatic shift to a lymphocyte infiltrate was observed in lungs of all the mice treated with the combined immunotherapy (Fig. 2*B*). Lymphocytes were located surrounding interalveolar blood vessels and attached to alveoli, and in some cases next to residual tumor cells, while intraluminal areas were mostly devoid of lymphocyte (compare Fig. 2*B* and Fig. 2*C*). These lymphocytes were identified as $CD8^+$ cells while no positive staining was observed for $CD4^+$ T cells and NK cells (Fig. 2*D* and data not shown).

In another model of experimental lung metastases, 1×10^6 CT26 cells were injected in the tail vein of syngeneic BALB/c mice. Two days later we started to administer MitC-TC weekly for 4 wk. Mice were sacrificed 7 days after the fourth administration, the lungs were removed and the metastatic nodules were evaluated. Eighty percent of control mice (4 of 5) developed lung metastases that ranged from 2 to >200 nodules. In contrast, only 12% of mice (1 of 8) that received CT26-IL-12 MitC-TC and 33% of mice (3 of 9) that received CT26-(IL-10 + IL-12) MitC-TC showed 1–10 nodules indicating that both treatments were equally effective (*p*, was not statistically significant when comparing the CT26-IL-12 MitC-TC with the CT26-(IL-10 + IL-12) MitC-TC groups).

Histological assessment of the potent inflammatory response following primary tumor treatment with combined immunotherapy

Our data indicated that both cytokines must be coexpressed locally to achieve their maximal therapeutic effect. Therefore, we decided

Table I. *Therapeutic effect of the different treatments on established lung metastasis originated from primary LM3 tumor cells*

Treatment[a]	Day of Mice Sacrifice	Mice with Lung Metastases	Nodules per Mice
None	13	4/5[b]	1–2
None	19	6/6	1–3
None	23	4/4	2–3
None	27	4/4	1–5
None	30	2/2	3–5
None	39	3/3	2–6
LM3-Neo MitC-TC	60–62	5/5	1–3
LM3-IL-10 MitC-TC	60–62	6/6	1–6
LM3-IL-12 MitC-TC	60–62	5/6	1–4
LM3-(IL-10 + IL-12) MitC-TC	60–62	0/12	0

[a] Mice were injected s.c. with 3×10^5 LM3 mammary tumor cells and treated or not with the corresponding protocol starting 20 days after injection of tumor cells.

[b] Mice were sacrificed at the indicated time and histologically analyzed for development of spontaneous lung metastases. The numerator indicates mice bearing metastases and the denominator, the total number of mice.

to histologically assess the area between the tumor and the MitC-TC cells. Control mice showed a slight neutrophil infiltrate all along the treatment with a small increase in macrophage recruitment starting from the second administration (data not shown). Injection of CT26-IL-10 MitC-TC induced a 3-fold increase in neutrophil recruitment that was followed by the recruitment of macrophages and lymphocytes after the third injection (data not shown). CT26-IL-12 MitC-TC induced an 8-fold increased recruitment of neutrophils after the first administration (Fig. 3*A*) followed by 2- and 2.2-fold increased recruitment of macrophages and lymphocytes, respectively, after the second administration (data not shown). Remarkably, the combined immunotherapy induced 13.2-, 4.4-, and 4.9-fold increased recruitment of neutrophils, lymphocytes, and macrophages, respectively, compared with the control, immediately after the first administration. By specific immunostaining, we could identify neutrophils, macrophages (Fig. 3*B*), NK cells, (Fig. 3*B*, *inset*) as well as $CD4^+$ and $CD8^+$ T cells (Fig. 3, *C* and *D*). This potent inflammatory infiltrate was located at the interface between the Mit C-treated cells and the tumor area (Fig. 3*E*). In addition, we also identified neutrophils and NK cells at day 2 after the administration of the combined immunotherapy or the IL-12-based monotherapy (data not shown). Seven days after the administration of the second combined injection, the infiltrate was deeply penetrating the tumor area and 70% of the tumor area was necrotic compared with only 30% of necrosis in the case of mice treated only with CT26-IL-12 MitC-TC (data not shown). These strong differences in the amount and heterogeneity of the inflammatory response were also observed in the LM3 model (data not shown).

The increased recruitment of inflammatory cells is associated with increased local chemokine production

The potent recruitment of inflammatory cells that followed the administration of the combined immunotherapy led us to assess whether this recruitment was associated with chemokine production. Mice harboring established CT26 tumors were injected once with each of the different types of MitC-treated tumor cells and sacrificed for evaluation of chemokines production using an RNase protection assay. MIP-2, a chemotactic factor for neutrophils and T lymphocytes (26), was overexpressed from day 2 up to day 7 with a maximal 5-fold increase ($p < 0.01$) at day 3 compared with control mice (Fig. 3*F*, *lane 7*). Dramatic increases in the expression levels of the macrophage chemotactic factor MCP-1 ($p < 0.05$) and in the T cell chemoattractants IFN-γ-inducible protein-10 (IP-10; $p < 0.05$) and TCA-3 ($p < 0.01$) (16-, 12-, and 19-fold increase, respectively, compared with control mice), were observed at day 7 after administration of the combined immunotherapy (Fig. 3*F*, *lane 11*). Thus, a close correlation was observed between the increased recruitment of an inflammatory response and increased chemokine production.

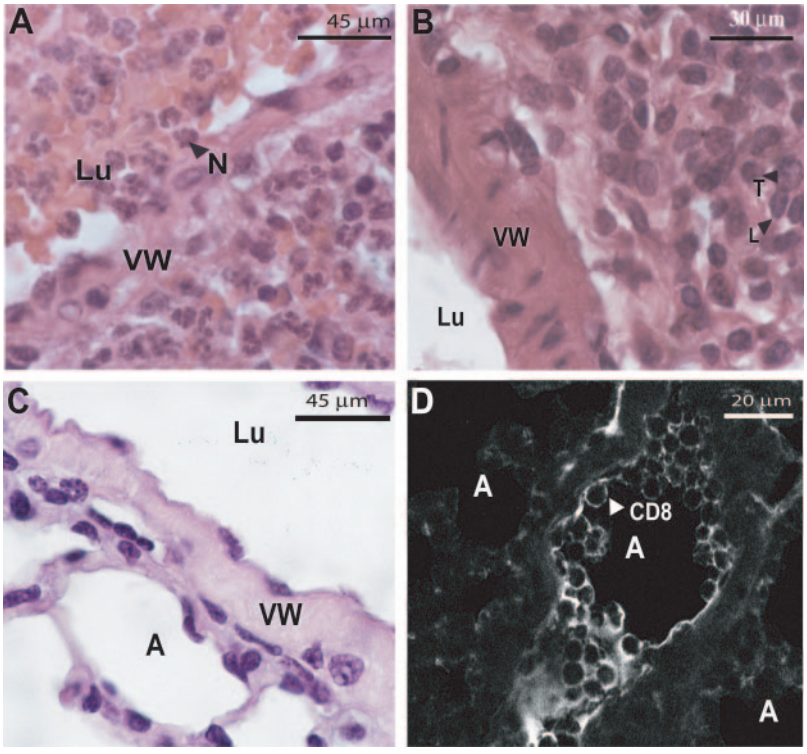

FIGURE 2. Histological analysis of lungs following treatment of mice carrying established lung metastasis. *A*, Microscopic section corresponding to LM3 tumor-bearing mice injected with LM3-IL-12 MitC-TC showing a high neutrophil infiltrate. *B*, Microscopic section corresponding to LM3-tumor bearing mice treated with LM3-(IL-10 + IL-12) MitC-TC showing a vast number of lymphocytes surrounding lung vessels, and next to tumor cells. *C*, Microscopic section corresponding to naive mice showing no immune infiltrate and normal intraluminal areas. *D*, Similar to *B*, stained for $CD8^+$ T cells (arrow). N, neutrophil; L, lymphocyte; T, tumor cell; A, alveoli; VW, vessel wall; Lu, intraluminal space.

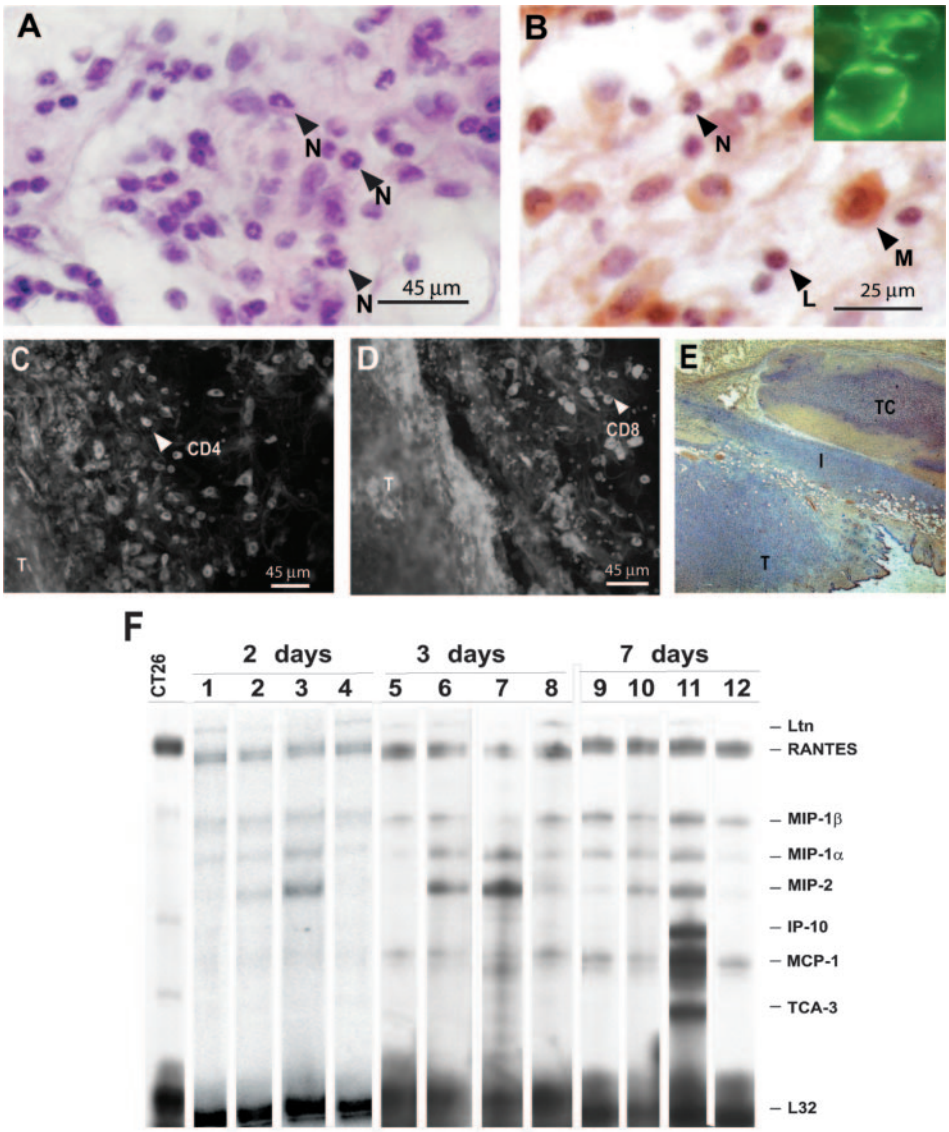

FIGURE 3. Histological evaluation and chemokine production at the injection areas. CT26-tumor-bearing mice were injected with Mit C cells and sacrificed at day 7 for histological evaluation. *A*, Corresponds to the area of administration of CT26-IL-12 MitC-TC and shows predominant neutrophil recruitment (arrows, N). *B*, Corresponds to the area of administration of CT26-(IL-10 + IL-12) and the inflammatory infiltrate includes macrophages (arrow, M) staining positive with an Ab anti-mouse F4/80 Ag, lymphocytes (arrow, L), and neutrophils (N); *inset*, NK-specific immunostaining. *C*, $CD4^+$ T cells at the area of administration of CT26-(IL-10 + IL-12)-Mit C TC. *D*, $CD8^+$ T cells at the area of administration of CT26-(IL-10 + IL-12)-Mit C TC. *E*, Tumor area showing the tumor nodule (T), the site of injection of Mit C cells expressing the cytokine combination (TC) and the inflammatory infiltrate (I) between the TC and the tumor. *F*, Chemokine mRNA expression pattern. Chemokine gene expression was determined as described in *Materials and Methods*. This is a representative result from three independent experiments and three to four animals per experiment. Expression of IL-10 or IL-12 induced no change in the chemokine expression pattern of CT26 cells (CT26 *lane* and data not shown). References: CT26-IL-10 MitC-TC (*lanes 1, 5, 9*), CT26-IL-12 MitC-TC (*lanes 2, 6, 10*), CT26-(IL-10 + IL-12) MitC-TC (*lanes 3, 7, 11*) and CT26 MitC-TC (*lanes 4, 8, 12*). Two days after injection (*lanes 1–4*), 3 days after injection (*lanes 5–8*), and 7 days after injection (*lanes 9–12*).

A Th1-related infiltrate prevails locally after combined immunotherapy

Because IL-12 promotes a Th1 response and IL-10 induces a Th2 response (6, 8, 18, 21), we sought to establish which Th response predominates when both cytokines are expressed in conjunction. First, we used quantitative PCR analysis to evaluate the levels of the Th1 and Th2 markers IFN-γ and IL-4 produced locally after administration of the combined immunotherapy. Mice harboring 20-day-old-CT26 tumors were injected once with each of the different MitC-treated cells. At different times, mice were sacrificed to assess mRNA levels of both cytokines. As expected, mice injected locally with CT26-IL-10 MitC-TC showed high expression levels of IL-4 (Fig. 4*A*), while IFN-γ was only slightly detectable (Fig. 4*B*). Unexpectedly, expression of IL-12 alone led to an initial peak of IFN-γ at day 2 but the levels diminished dramatically at day 7 (Fig. 4*B*), with the concomitant increase in IL-4 expression (Fig. 4*A*). Interestingly, the combined expression of IL-12 and IL-10 induced the highest levels of IFN-γ mRNA at days 2 and 7 (Fig. 4*B*), while IL-4 mRNA levels were slightly elevated at day 2 and undetectable at day 7 (Fig. 4*A*). These results suggest that a Th1-associated-inflammatory infiltrate prevails locally after the local administration of the combined immunotherapy.

Combined immunotherapy activates both the Th1 and the Th2 arms, systemically: absolute need of $CD4^+$ and $CD8^+$ T cells for complete tumor rejection

We sought to establish which Th arm was systemically activated by the combined immunotherapy. Spleen cells obtained from mice treated with CT26-IL-10 MitC-TC or LM3-IL-10 MitC-TC produced IL-4 (Fig. 5, *A* and *B*). Increased IFN-γ production was observed in spleen cells obtained from mice treated with CT26-IL-12 MitC-TC or LM3-IL-12 MitC-TC (Fig. 5, *C* and *D*). Interestingly, the combined expression of IL-12 and IL-10 stimulated the production of both IL-4 and IFN-γ by spleen cells, indicating the engagement of both the Th1 and Th2 responses (Fig. 5, *A–D*). In line with this finding, mice injection with CT26-IL-10 MitC-TC induced the increased production of total circulating and tumor-specific IgG1, a marker of a Th2 response (Fig. 5*E*). Whereas injection of CT26-IL-12 MitC-TC led to a clear increase in circulating and CT26-specific IgG2a levels, a marker of a Th1 response (Fig. 5*E*). Finally, the combined immunotherapy increased both total circulating and CT26-specific IgG2a and IgG1 (Fig. 5*E*). Thus, by using different approaches we confirmed that both cytokines were engaged in stimulating their respective Th responses systemically with no counterinhibition.

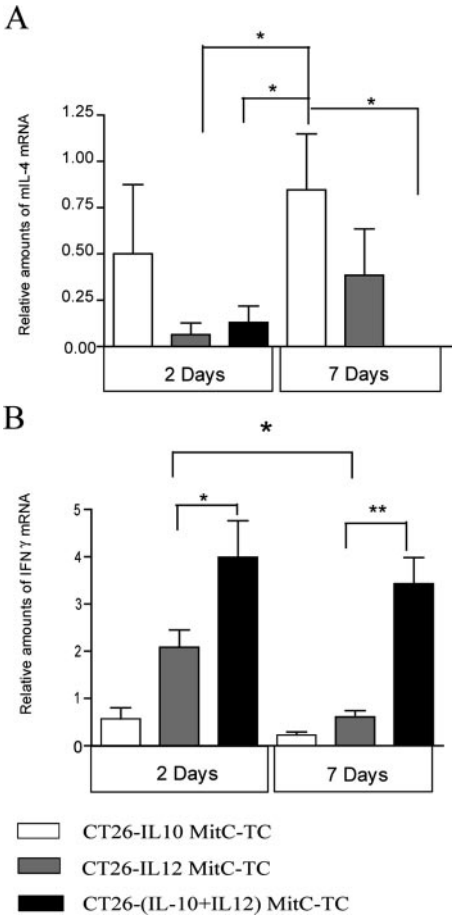

FIGURE 4. Local expression of IL-4 and IFN-γ after administration of cytokine-expressing autologous tumor cells. Mice bearing CT26 tumors were injected once with MitC-TC. Two and 7 days after injection, mice were sacrificed and mRNA was extracted from the injection area. IFN-γ and IL-4 mRNA levels were determined by a competitive quantitative RT-PCR assay. Data correspond to the mean ± SD of at least five mice. ** and * correspond to groups showing statistically significant differences ($p < 0.01$ and $p < 0.05$, respectively). *A,* IL-4 levels; *B*, IFN-γ levels.

Finally, we established which T cell subtype is involved in the rejection of established tumors. For this purpose mice harboring 20-day-old CT26 tumors were injected locally with the combined immunotherapy. A day before, mice received Abs to deplete from NK or T cell-specific subtype. We observed that depletion of either $CD4^+$ or $CD8^+$ T cells partially restored CT26 growth in syngeneic mice, although it was statistically significant only in the case of $CD8^+$ T cells ($p = 0.019$; Table II). However, the simultaneous depletion of $CD4^+$ and $CD8^+$ T cells completely abrogated the anti-tumor effect of the combined immunotherapy ($p = 0.001$) indicating that both T cell types were required to achieve a complete antitumor effect (Table II). Depletion of NK cells had no effect ($p > 0.05$, Table II).

Discussion

In this study, we show for the first time the eradication of large, established primary colon and mammary tumors, and lung metastases, through the synergism of IL-12 and IL-10 gene expression. Treatment of mice harboring established tumors with cells expressing a single cytokine have marginal or no effect. Several mechanistic advantages of the combined approach contributed to this outcome. First, the improved therapeutic efficacy was concurrent with the recruitment of a potent inflammatory response and increased chemokine production. IL-12 and IL-10 also did not impede each other's induction of their respective Th response. Concurrent IL-10 expression locally provided an additional benefit because it inhibited IL-4 expression that followed the initial IFN-γ expression caused by IL-12. The synergistic effect of the combined immunotherapy was demonstrated in the poorly immunogenic CT26-colon and LM3-mammary tumor models.

We observed that after the initial administration of the combined immunotherapy, the area was infiltrated by a heterogeneous pattern of inflammatory cells that was coincidental with the increased production of specific chemokines. At day 2 the area was infiltrated by neutrophils and NK cells. This initial infiltrate could result from the chemotactic activity induced by the concurrent production of IL-10 and IL-12. Indeed, IL-10 was shown to stimulate neutrophils recruitment when expressed by mammary tumor cells (27). IL-12 was found to be chemotactic for neutrophils and to induce the production of MIP-2 (28, 29) a chemokine produced by the neutrophils themselves and that we found to be the first chemokine to be overexpressed in the CT26 tumor model. In addition, both IL-10 and IL-12 were shown to recruit, and in the case of IL-12 to induce a direct chemotactic effect on, NK cells (15–17, 30). Infiltrating NK cells are likely to be responsible for the local production of IFN-γ at day 2.

We also observed an increased expression of IP-10, MCP-1 and TCA-3 at day 7 after administration of the combined immunotherapy. These chemokines are mainly produced by macrophages and T lymphocytes and are chemotactic for macrophages and T lymphocytes themselves (31–33). In addition, IP-10 was shown to mediate, at least in part, the antitumor effects of IL-12 (34, 35). MCP-1 induce monocyte differentiation to dendritic cell thus helping to promote maturation of secondary immune responses (36). In this sense, it is interesting to mention that IL-10 tends to inhibit APC maturation and migration to draining lymph nodes prolonging their capacity to Ag uptake (37). Thus, after an initial inhibition exerted by IL-10, further APC maturation might occur in the presence of increased expression levels of MCP-1. It is also interesting that the ectopic expression in malignant cells of MCP-1, TCA-3, and IP-10 induced tumor rejection and prevented lung metastases formation in certain animal models (34, 38–40). Thus, the two cytokines acting in concert with these specific chemokines might potentiate the intrinsic capacity of each other leading to an exacerbated local antitumor immune response.

An interesting result was also the persistent expression of IFN-γ locally and the abrogation of IL-4 increase following the combined immunotherapy, indicating that infiltrating cells were expressing a Th1 phenotype. Both infiltrating macrophages and T cells could be responsible for the production of IFN-γ locally at day 7 (6). On the contrary, the initial expression of IFN-γ that followed IL-12 expression was counterbalanced by the expression of IL-4 at day 7. Previous studies have shown that spleen cells from mice immunized with a soluble Ag and IL-12 developed both a Th1 and a Th2 response upon ex vivo stimulation with the Ag as a means to protect the organism from an exacerbated Th1 response (41). Moreover, tumor treatment with locally injected IL-12 was less effective than the systemically injected protein (28), consistent with an immunosuppressive effects of high doses of locally injected IL-12 (42). The question as to whether IL-10 may abrogate IL-4 expression and behave as a proinflammatory cytokine is an intriguing possibility. A recent review challenged the perception of IL-10 solely as a Th2 immunosuppressive cytokine speculating that IL-10 produced by certain tumors might precondition the tumor microenvironment to the antitumor effects of other cytokines

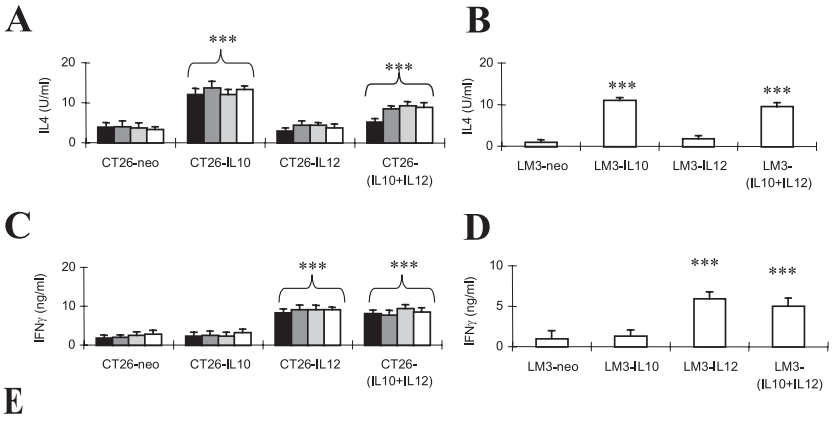

FIGURE 5. Evaluation of the Th response. *A* and *C,* Mice bearing CT26 tumors were injected with MitC-TCs and 1 wk after each injection spleen cells were obtained for cytokine quantification. *B* and *D*, Mice bearing LM3 tumors were injected with MitC-TC and 1 wk after the last injection spleen cells were obtained for cytokine quantification. ***, Corresponds to groups showing statistically significant differences ($p < 0.001$) compared with their respective control groups of animals injected with cells expressing no cytokine. Each point corresponds to the mean ± SD obtained from six mice. *E*, Total circulating and CT26-specific IgG2a and IgG1 subclass levels. Serum was obtained from each mice 1 wk after the last injection of autologous TC expressing the different cytokines.

Type of treatment	Total IgG2a	Total IgG1	IgG2a/ IgG1	Anti-CT26 IgG2a	Anti-CT26 IgG1	IgG2a/ IgG1
None	81 ± 10.8[a]	16 ± 3.2	5.5[b]	ND[c]	ND	
CT26-Neo	79.5 ± 5.6	13.2 ± 2.5	6.0	20.5 ± 2.8	4.2 ± 1.5[d]	4.9[b]
CT26-IL-10 MitC-TC	76.5 ± 4.8	23.5 ± 1.8	3.2	18.6 ± 2.3	18.8 ± 6.3	0.6
CT26-IL-12 MitC-TC	138 ± 2.5	10.5 ± 2.9	13.1	158 ± 15	3.4 ± 2.8	46.5
CT26-(IL10+IL12) MitC-TC	129 ± 3.1	21.3 ± 3.2	6	147 ± 18	29.5 ± 3.9	5

[a]Data are expressed as mg/ml (mean±SD)
[b]Ratio in arbitrary units. Each value was obtained as an average of 5-8 mice.
[c]ND: non detectable
[d]Data are expressed as ng of bound Ig/10^6 cells (mean±SD)

(43). This provocative view arose from the fact that IL-10 promoted T cell expansion and cytotoxic potential of anti-human papilloma virus CTLs when added in combination with IL-2 (44). In addition, intratumor expression of IL-10 correlated with clinical regression following treatment of melanoma patients with IL-2 (45). Moreover, the combination of IL-10 with IL-2 administration induced the differential expression of close to 200 genes, most of them related to proinflammatory processes (37). Based on the evidence presented here it appears that the concurrent production of IL-10 and IL-12 support the view that these cytokines are promoting a proinflammatory environment locally.

An important result from the present studies is that the combined immunotherapy activated simultaneously the Th1 and the Th2 arms. It was suggested that human neoplasia is associated with an imbalanced production of Th1 and Th2 type cytokines and that production of Th2 type cytokines favored cancer progression (reviewed in Ref. 22). However, changes in Th1 and Th2 cytokines described in cancer patients are the result of an advanced disease in which the tumor itself contributes to cytokine imbalance by secreting immunoregulatory factors. In a previous study in which mice were preimmunized with B16 melanoma cells expressing GM-CSF it was demonstrated that both Th arms can develop systemically and contribute to the rejection of a challenge with parental cells (46). Previous in vitro studies have demonstrated that murine Th cells will produce Th1 or Th2 cytokines in a mutually exclusive way (47–49). Moreover, Th1 cytokines such as IL-12 will promote Th1 differentiation and Th2 inhibition (50–52), while Th2 cytokines such as IL-10 will promote the opposite (12, 53, 54). The present findings provide evidence that the simultaneous expression of antagonistic cytokines such as IL-12 and IL-10 not only did not lead to antagonistic effects but promoted a synergistic antitumor and antimetastatic effect. Although a proinflammatory Th1 response appears to prevail locally, each cytokine promoted the expected Th response systemically, suggesting that both arms were important in achieving complete rejection.

Table II. *Depletion of subsets of immune cells using specific Abs*

Ab used	Treatment	Tumor Progression/Regression[a]
None	None	7/0
None	MitC-TC[b]	3/8
Anti-CD8	MitC-TC	7/1
Anti-CD4	MitC-TC	6/2
Anti-CD4 + anti-CD8	MitC-TC	11/0
Anti-NK	MitC-TC	2/5

[a] Mice in which tumors progressed/mice in which tumors regressed.
[b] CT26-(IL-10 + IL-12) MitC-TC.

The combined expression of IL-10 and IL-12 was effective in the induction of an immune mediated antitumor response when expressed by the tumor cells themselves and as a combined immunotherapy both on primary tumors and established metastasis. In the LM3 model the maximal therapeutic effect was observed when the expression of both cytokines occurred in the vicinity of the primary tumor mass. The decreased efficacy to induce primary tumor rejection when injected contralaterally is likely due to the large size of the growing tumors that render them not amenable for treatment. Consistent with this, is the fact that the combined immunotherapy was able to reject 2 days established lung metastasis in the CT26 model indicating that small size distant metastatic foci responded to treatment. In addition, the concurrent expression of IL-10 and IL-12 induced a shift toward a lymphocyte infiltrate associated with the complete elimination of the lung metastatic foci in the mammary tumor model. The complete elimination of established lung metastasis only when this shift occurred is consistent with previous evidence indicating that neutrophils seem to have no role in the destruction of lung metastases, whereas they might have an important role in the anti- primary tumor response (28).

In conclusion, the combined immunotherapy with IL-10 and IL-12 expressed by autologous tumor cells was superior to single

treatment in eliminating established primary tumors and metastasis and prolonging survival. The increase in therapeutic efficacy was due to effects that occurred locally (increased inflammation and chemokines expression, sustained production of IFN-γ) and systemically (simultaneous activation of the Th1 and the Th2 arms). Thus, the use of this combination might have implications in cancer treatment.

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Disclosures

The authors have no financial conflict of interest.

References

1. Davis, I. D., M. Jefford, P. Parente, and J. Cebon. 2003. Rational approaches to human cancer immunotherapy. *J. Leukocyte Biol.* 73: 3–29.
2. Parmiani, G., M. Rodolfo, and C. Melani. 2000. Immunological gene therapy with ex vivo gene-modified tumor cells: a critique and a reappraisal. *Hum. Gene Ther.* 11: 1269–1275.
3. Mach, N., and G. Dranoff. 2000. Cytokine-secreting tumor cell vaccines. *Curr. Opin. Immunol.* 12: 571–575.
4. Rosenberg, S. A., J. C. Yang, and N. P. Restifo. 2004. Cancer immunotherapy: moving beyond current vaccines. *Nat. Med.* 10: 909–915.
5. Trinchieri, G. 1994. Interleukin-12: a cytokine produced by antigen-presenting cells with immunoregulatory functions in the generation of T-helper cells type 1 and cytotoxic lymphocytes. *Blood* 84: 4008–4027.
6. Trinchieri, G. 2003. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nat. Rev. Immunol.* 3: 133–146.
7. Hofstra, C. L., I. Van Ark, G. Hofman, M. Kool, F. P. Nijkamp, and A. J. M. Van Oosterhout. 1998. Prevention of Th2-like cell responses by coadministration of IL-12 and IL-18 is associated with inhibition of antigen-induced airway hyperresponsiveness, eosinophilia, and serum IgE levels. *J. Immunol.* 161: 5054–5060.
8. Adris, S., E. Chuluyan, A. Bravo, M. Berenstein, S. Klein, M. Jasnis, C. Carbone, Y. Chernajovsky, and O. L. Podhajcer. 2000. Mice vaccination with interleukin 12-transduced colon cancer cells potentiates rejection of syngeneic non-organ-related tumor cells. *Cancer Res.* 60: 6696–6703.
9. Brunda, M. J., L. Luistro, R. R. Warrier, R. B. Wright, B. R. Hubbard, M. Murphy, S. F. Wolf, and M. K. Gately. 1993. Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med.* 178: 1223–1230.
10. Colombo, M. P., and G. Trinchieri. 2002. Interleukin-12 in anti-tumor immunity and immunotherapy. *Cytokine Growth Factor Rev.* 13: 155–168.
11. Itoh, K., and S. Hirohata. 1995. The role of IL-10 in human B cell activation, proliferation, and differentiation. *J. Immunol.* 154: 4341–4350.
12. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, and A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* 147: 3815–3822.
13. Chen, W. F., and A. Zlotnik. 1991. IL-10: a novel cytotoxic T cell differentiation factor. *J. Immunol.* 147: 528–534.
14. te Velde, A., R. de Waal Malefijt, R. Huijbens, J. de Vries, and C. Figdor. 1992. IL-10 stimulates monocyte FcγR surface expression and cytotoxic activity: distinct regulation of antibody-dependent cellular cytotoxicity by IFN-γ, IL-4, and IL-10. *J. Immunol.* 149: 4048–4052.
15. Zheng, L., D. Ojcius, F. Garaud, C. Roth, E. Maxwell, Z. Li, H. Rong, J. Chen, X. Wang, J. Catino, and I. King. 1996. Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. *J. Exp. Med.* 184: 579–584.
16. Kundu, N., and A. M. Fulton. 1997. Interleukin-10 inhibits tumor metastasis, downregulates MHC class I, and enhances NK lysis. *Cell. Immunol.* 180: 55–61.
17. Giovarelli, M., P. Musiani, A. Modesti, P. Dellabona, G. Casorati, A. Allione, M. Consalvo, F. Cavallo, F. di Pierro, C. De Giovanni, et al. 1995. Local release of IL-10 by transfected mouse mammary adenocarcinoma cells does not suppress but enhances antitumor reaction and elicits a strong cytotoxic lymphocyte and antibody-dependent immune memory. *J. Immunol.* 155: 3112–3123.
18. Adris, S., S. Klein, M. Jasnis, E. Chuluyan, M. Ledda, A. Bravo, C. Carbone, Y. Chernajovsky, and O. Podhajcer. 1999. IL-10 expression by CT26 colon carcinoma cells inhibits their malignant phenotype and induces a T cell-mediated tumor rejection in the context of a systemic Th2 response. *Gene Ther.* 6: 1705–1712.
19. Gerard, C. M., C. Bruyns, A. Delvaux, N. Baudson, J. L. Dargent, M. Goldman, and T. Velu. 1996. Loss of tumorigenicity and increased immunogenicity induced by interleukin-10 gene transfer in B16 melanoma cells. *Hum. Gene Ther.* 7: 23–31.
20. Yazdanbakhsh, M., P. G. Kremsner, and R. van Ree. 2002. Allergy, parasites, and the hygiene hypothesis. *Science* 296: 490–494.
21. Lucey, D. R., M. Clerici, and G. M. Shearer. 1996. Type 1 and type 2 cytokine dysregulation in human infectious, neoplastic, and inflammatory diseases. *Clin. Microbiol. Rev.* 9: 532–562.
22. Clerici, M., G. M. Shearer, and E. Clerici. 1998. Cytokine dysregulation in invasive cervical carcinoma and other human neoplasias: time to consider the TH1/TH2 paradigm. *J. Natl. Cancer Inst.* 90: 261–263.
23. Seder, R. A., and W. E. Paul. 1994. Acquisition of lymphokine-producing phenotype by $CD4^+$ T cells. *Annu. Rev. Immunol.* 12: 635–673.
24. Pitossi, F., A. del Rey, A. Kabiersch, and H. Besedovsky. 1997. Induction of cytokine transcripts in the central nervous system and pituitary following peripheral administration of endotoxin to mice. *J. Neurosci. Res.* 48: 287–298.
25. Aguirre Ghiso, J., M. Diament, I. D'Elia, E. Bal de Kier Joffe, and S. Klein. 1997. Effects of in vivo culture of murine mammary adenocarcinoma cells on tumor and metastatic growth. *Tumour Biol.* 18: 41–52.
26. Kishimoto, C., H. Kawamata, S. Sakai, H. Shinohara, and H. Ochiai. 2001. Enhanced production of macrophage inflammatory protein 2 (MIP-2) by in vitro and in vivo infections with encephalomyocarditis virus and modulation of myocarditis with an antibody against MIP-2. *J. Virol.* 75: 1294–1300.
27. Di Carlo, E., A. Coletti, A. Modesti, M. Giovarelli, G. Forni, and P. Musiani. 1998. Local release of interleukin-10 by transfected mouse adenocarcinoma cells exhibits pro- and anti-inflammatory activity and results in a delayed tumor rejection. *Eur. Cytokine Netw.* 9: 61–68.
28. Cavallo, F., E. Di Carlo, M. Butera, R. Verrua, M. P. Colombo, P. Musiani, and G. Forni. 1999. Immune events associated with the cure of established tumors and spontaneous metastases by local and systemic interleukin 12. *Cancer Res.* 59: 414–421.
29. Di Carlo, E., A. Comes, S. Basso, A. De Ambrosis, R. Meazza, P. Musiani, K. Moelling, A. Albini, and S. Ferrini. 2000. The combined action of IL-15 and IL-12 gene transfer can induce tumor cell rejection without T and NK cell involvement. *J. Immunol.* 165: 3111–3118.
30. Allavena, P., C. Paganin, D. Zhou, G. Bianchi, S. Sozzani, and A. Mantovani. 1994. Interleukin-12 is chemotactic for natural killer cells and stimulates their interaction with vascular endothelium. *Blood* 84: 2261–2268.
31. Rollins, B. J. 1997. Chemokines. *Blood* 90: 909–928.
32. Zingoni, A., H. Soto, J. A. Hedrick, A. Stoppacciaro, C. T. Storlazzi, F. Sinigaglia, D. D'Ambrosio, A. O'Garra, D. Robinson, M. Rocchi, et al. 1998. Cutting edge: the chemokine receptor CCR8 is preferentially expressed in Th2 but not Th1 cells. *J. Immunol.* 161: 547–551.
33. Luo, Y., J. Laning, S. Devi, J. Mak, T. Schall, and M. Dorf. 1994. Biologic activities of the murine β-chemokine TCA3. *J. Immunol.* 153: 4616–4624.
34. Narvaiza, I., G. Mazzolini, M. Barajas, M. Duarte, M. Zaratiegui, C. Qian, I. Melero, and J. Prieto. 2000. Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN-γ-inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. *J. Immunol.* 164: 3112–3122.
35. Tannenbaum, C. S., R. Tubbs, D. Armstrong, J. H. Finke, R. M. Bukowski, and T. A. Hamilton. 1998. The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J. Immunol.* 161: 927–932.
36. Omata, N., M. Yasutomi, A. Yamada, H. Iwasaki, M. Mayumi, and Y. Ohshima. 2002. Monocyte chemoattractant protein-1 selectively inhibits the acquisition of CD40 ligand-dependent IL-12-producing capacity of monocyte-derived dendritic cells and modulates Th1 immune response. *J. Immunol.* 169: 4861–4866.
37. Mocellin, S., M. C. Panelli, E. Wang, D. Nagorsen, and F. M. Marincola. 2003. The dual role of IL-10. *Trends Immunol.* 24: 36–43.
38. Manome, Y., P. Y. Wen, A. Hershowitz, T. Tanaka, B. J. Rollins, D. W. Kufe, and H. A. Fine. 1995. Monocyte chemoattractant protein-1 (MCP-1) gene transduction: an effective tumor vaccine strategy for non-intracranial tumors. *Cancer Immunol. Immunother.* 41: 227–235.
39. Laning, J., H. Kawasaki, E. Tanaka, Y. Luo, and M. E. Dorf. 1994. Inhibition of in vivo tumor growth by the β chemokine, TCA3. *J. Immunol.* 153: 4625–4635.
40. van Deventer, H. W., J. S. Serody, K. P. McKinnon, C. Clements, W. J. Brickey, and J. P. Ting. 2002. Transfection of macrophage inflammatory protein 1 α into B16 F10 melanoma cells inhibits growth of pulmonary metastases but not subcutaneous tumors. *J. Immunol.* 169: 1634–1639.
41. Bliss, J., V. Van Cleave, K. Murray, A. Wiencis, M. Ketchum, R. Maylor, T. Haire, C. Resmini, A. K. Abbas, and S. F. Wolf. 1996. IL-12, as an adjuvant, promotes a T helper 1 cell, but does not suppress a T helper 2 cell recall response. *J. Immunol.* 156: 887–894.
42. Kurzawa, H., M. Wysocka, E. Aruga, A. E. Chang, G. Trinchieri, and W. M. Lee. 1998. Recombinant interleukin 12 enhances cellular immune responses to vaccination only after a period of suppression. *Cancer Res.* 58: 491–499.
43. Mocellin, S., F. Marincola, C. Riccardo Rossi, D. Nitti, and M. Lise. 2004. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. *Cytokine Growth Factor Rev.* 15: 61–76.
44. Santin, A. D., P. L. Hermonat, A. Ravaggi, S. Bellone, S. Pecorelli, J. J. Roman, G. P. Parham, and M. J. Cannon. 2000. Interleukin-10 increases Th1 cytokine production and cytotoxic potential in human papillomavirus-specific $CD8^+$ cytotoxic T lymphocytes. *J. Virol.* 74: 4729–4737.
45. Mocellin, S., G. A. Ohnmacht, E. Wang, and F. M. Marincola. 2001. Kinetics of cytokine expression in melanoma metastases classifies immune responsiveness. *Int. J. Cancer* 93: 236–242.

46. Hung, K., R. Hayashi, A. Lafond-Walker, C. Lowenstein, D. Pardoll, and H. Levitsky. 1998. The central role of $CD4^+$ T cells in the antitumor immune response. *J. Exp. Med.* 188: 2357–2368.
47. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136: 2348–2357.
48. Cher, D. J., and T. R. Mosmann. 1987. Two types of murine helper T cell clone. II. Delayed-type hypersensitivity is mediated by TH1 clones. *J. Immunol.* 138: 3688–3694.
49. Cherwinski, H. M., J. H. Schumacher, K. D. Brown, and T. R. Mosmann. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. *J. Exp. Med.* 166: 1229–1244.
50. Gajewski, T. F., E. Goldwasser, and F. W. Fitch. 1988. Anti-proliferative effect of IFN-γ in immune regulation. II. IFN-γ inhibits the proliferation of murine bone marrow cells stimulated with IL-3, IL-4, or granulocyte-macrophage colony-stimulating factor. *J. Immunol.* 141: 2635–2642.
51. Gajewski, T. F., and F. W. Fitch. 1988. Anti-proliferative effect of IFN-γ in immune regulation. I. IFN-γ inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. *J. Immunol.* 140: 4245–4252.
52. Fernandez-Botran, R., V. M. Sanders, T. R. Mosmann, and E. S. Vitetta. 1988. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. *J. Exp. Med.* 168: 543–558.
53. Fiorentino, D. F., M. W. Bond, and T. R. Mosmann. 1989. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* 170: 2081–2095.
54. Fiorentino, D. F., A. Zlotnik, P. Vieira, T. R. Mosmann, M. Howard, K. W. Moore, and A. O'Garra. 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 146: 3444–3451.