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BRIEF COMMUNICATION

Intratumoral injection of bone-marrow derived dendritic cells engineered to produce interleukin-12 induces complete regression of established murine transplantable colon adenocarcinomas

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Stimulation of the antitumor immune response by dendritic cells (DC) is critically dependent on their tightly regulated ability to produce interleukin-12 (IL-12). To enhance this effect artificially, bone marrow (BM)-derived DC were genetically engineered to produce high levels of functional IL-12 by ex vivo infection with a recombinant defective adenovirus (AdCMVIL-12). DC-expressing IL-12 injected into the malignant tissue eradicated 50–100% well established malignant nodules derived from the injection of two murine colon adenocarcinoma cell lines. Successful therapy was dependent on IL-12 transfection and was mediated only by syngeneic, but not allogeneic BM-derived DC, indicating that compatible antigen-presenting molecules were required. The antitumor effect was inhibited by in vivo depletion of CD8+ T cells and completely abrogated by simultaneous depletion with anti-CD4 and anti-CD8 mAbs. Mice which had undergone tumor regression remained immune to a rechallenge with tumor cells, showing the achievement of long-lasting systemic immunity that also was able to reject simultaneously induced concomitant untreated tumors. Tumor regression was associated with a detectable CTL response directed against tumor-specific antigens probably captured by DC artificially released inside tumor nodules. Our results open the possibility of similarly treating the corresponding human malignancies.

Keywords: *dendritic cell; interleukin-12; colon cancer; adenovirus; CTLs*

DC are specialized APC for T-lymphocytes that initiate CD4- and CD8-mediated immune responses.[1–3] Fuctional DC can be differentiated *in vitro* from myeloid precursors or peripheral blood monocytes in the presence of GM-CSF and IL-4.[4–6] Effective protection and therapy has been achieved by adoptive transfer of DC cultures in which tumor antigens have been artificially introduced into their antigen-presenting pathways by a number of procedures.[5–11] Various groups have shown that *ex vivo* infection of DC with recombinant adenovirus encoding for tumor rejection antigens is one of the most efficient systems for delivering tumor antigens and other transgenes into DC.[12,13]

Typical DC differentiated in culture with GM-CSF and IL-4 are not yet fully matured and need further stimulation to upregulate their immunostimulatory and antigen-presenting capabilities,[1–3] by increasing MHC antigen-presenting molecules, membrane-bound costimulatory molecules, and cytokine secretion. Maturation is triggered by various bacterial products such as LPS and DNA,[14–16] cytokines such as TNFα and IL-1[17] and importantly, by activated T cells through CD40L–CD40 interactions.[18] Among the changes that occur in DC during maturation, the induction of IL-12 synthesis is considered an absolute requirement for the activation of the anti-tumor cellular immune response.[19]

Artificial gene transfer of IL-12 into DC or their precursors has been used to enhance their ability to stimulate T cells both *in vivo* and *in vitro*.[20,21] An important limitation in those studies was the low frequency of DC actually transfected with the IL-12 genes. We reasoned that *ex vivo* infection of DC with defective recombinant adenovirus would overcome such an obstacle by genetically modifying most DC.

Accordingly, we derived DC cultures from lymphocyte-depleted bone marrow cell suspensions by culturing them for 7 days with GM-CSF and IL-4 as described[5,10] obtaining 70–90% purity according to the combined immunostaining with mAb anti-MHC class II, CD11c, CD80 and CD86 (data not shown). Cells in our cultures of bone marrow-derived DC displayed a relatively immature phenotype which can be driven to full maturation by LPS and CD40 ligation increasing the expression of CD80 and CD86 whereas losing phagocytic activity.

To assess frequency of productive gene transfer into a variety of cell types, we have described a recombinant defective adenovirus encoding for β-galactosidase (AdCMVLacZ),[22] that was used to study the infectivity and gene transfer of DC cultures with defective recombinant adenoviruses. We infected the cells with AdCM-

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VLacZ at different MOIs and studied the percentage of cells showing enzyme expression. As shown in Figure 1a, a significant proportion of DC expressing β-galactosidase activity were detected 48 h after infection at MOIs ranging from 10^4 to 10^3. However very small percentage of β-galactosidase$^+$ cells were observed at lower MOIs, in contrast to reports in which MOIs of 100 were effective.[11,12] Repetition of the experiments with independently titrated batches of AdCMVLacZ confirmed our results. We also found that preincubation of the recombinant adenovirus with lipofectamine decreased the MOI needed to obtain similar percentage of transduction of BM derived DC by approximately one log (data not shown) as recently reported.[23] Since sensitivity of the assay to detect β-gal activity has been proposed to underestimate the actual number of transfected cells,[24] subtle differences in the detection threshold can explain the quantitative differences among laboratories to estimate the optimal MOI. In this regard, in accordance with our transduction data, recent evidence shows that production of adenovirally transduced IL-2 and IL-6 in DC greatly increases in the MOI range between 10^3 and 10^4.[25] Consistently we got up to 90–100% transduction with AdCMVLacZ at MOI of 100 in various cell lines (data not shown) indicating that DC are somehow more difficult to infect with recombinant adenovirus. In our hands infection of BM-derived DC at MOI of 3000 routinely gave 60–80% transfection with AdCMVLacZ with less than 10% loss of viability after 48 h in culture. Those were therefore selected as our working conditions of infection. Lipofectamine was not introduced in the protocols to avoid additional complexity but it should be considered an alternative to downsize adenovirus production needs.[23]

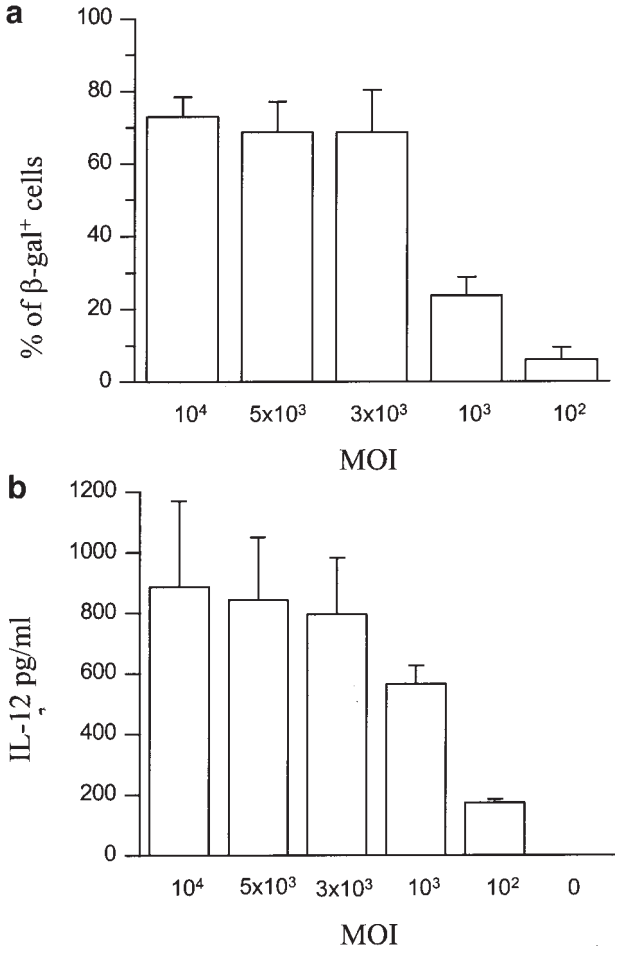

Figure 1 *Recombinant adenovirus productively transduce gene expression into BM-derived DC. Seven-day cultures of BM-derived DC were infected with AdCMVLacZ (a) or AdCMVIL-12 (b) at the indicated MOIs. Forty-eight hours after AdCMVLacZ infection percentage of β-galactosidase$^+$ cells was assessed by X-gal staining (a) and p70 IL-12 concentration in the supernatants of the DC infected with AdCMVIL-12 was measured by ELISA (b). Results are the mean ± s.e.m. of five (a) and three (b) different experiments. BM-derived DC were obtained essentially as described.[5] Briefly, erythrocyte-free cell suspensions flushed out from the long bones of the rear limbs of Balb/c mice were depleted of granulocytes, T and B lymphocytes by treatment with a mAb cocktail containing anti-CD4, anti-CD8, anti-Gr-1, anti-B220 (Pharmingen, San Diego, CA, USA) and rabbit complement (Sigma, Madrid, Spain). Cells were subsequently cultured in RPMI 1640 5% FCS containing recombinant GM-CSF (1000 IU/ml) (Peprotech, London, UK) and 20 ng/ml of recombinant mouse IL-4 (Peprotech) for 7 days. Purity of DC cultures was assessed by FACS analysis upon staining with FITC conjugated anti-I-A^d, anti-CD11c, anti-CD80, anti-CD86 (Pharmingen). X-gal staining was performed as described[22] and p70 IL-12 determinations in the supernatant were carried out with a commercial ELISA (Endogen, Woburn, MA, USA) according to the manufacturer's instructions. Defective recombinant adenovirus AdCMVIL-12 and AdCMVLacZ were prepared and titered as described.*[26]

We had generated a recombinant defective adenovirus encoding both chains of murine IL-12 (p35 and p40) separated by a ribosomal entry site (IRES) under the transcriptional control of the CMV promoter (AdCMVIL-12).[26] Intratumoral injections of AdCMVIL-12 have been shown to induce a powerful, often curative CD8-mediated immune response.[26] As shown in Figure 1b, infection with AdCMVIL-12 modified *in vitro* cultured DC to express high levels (1362 pg/ml/10^6 DC) of the IL-12 p70 heterodimer as detected in the supernatant of infected DC cultured for 48 h. It was concluded that AdCMVIL-12 could be a good tool to engineer BM-derived DC to produce IL-12 in order to enhance their immunostimulatory properties. We asked as to whether IL-12 gene transduction made DC capable of unleashing effective cellular immune responses against tumor antigens when tranferred into the tumor nodules. In this scenario, DC are not artificially loaded *ex vivo* with tumor antigens and they need to capture and present tumor antigens after being taken up from the neighboring malignant tissue. Experiments were set up in two murine models of colon adenocarcinoma: CT26 (Balb/c origin) and MC38 (C57Bl/6 origin) which grow aggressively and are considered poorly immunogeneic. As summarized in Table 1, a single injection of 2.5×10^5 syngeneic BM-derived DC, which had been infected *ex vivo* with AdCMVIL-12, induced complete regressions in 84% of the CT26-derived subcutaneous nodules and in 50% of the MC38-derived s.c. tumor nodules when treated 7 days after innoculation of the malignant cells (mean diameter of the nodules between 3 and 6 mm). When similarly raised allogeneic, rather than syngeneic DC, also infected with AdCMVIL-12 and secreting comparable quantities of IL-12 were used, no significant antitumor effect was observed, indicating that compatible antigen presentation was required. Intratumoral injection with transformed fibroblasts similarly infected with AdCMVIL-12 did not show antitumor effect neither in a syngeneic (Balb/c) nor allogeneic (C57Bl/6) setting. Therefore properties of DC absent in transformed fibro-

Table 1 Intratumoral injection of BM-derived DC infected with AdCMVIL-12 induces tumor regression[a]

In vitro infection[b] / *Intratumoral treatment*[c]		*AdCMVIL-12*			*AdCMVLacZ*	*None*	*HBSS*
Tumor	*Exp*	*Syngeneic DC*	*Allogeneic DC*	*Fibroblasts*[d]	*Syngeneic DC*	*Syngeneic DC*	
CT26	1	4/5	ND	ND	0/5	1/5	0/4
	2	4/6	0/5	0/5	1/5	0/5	0/5
	3	8/8	0/4	0/5	1/5	0/5	0/8
MC38	1	3/6	0/5	0/5	0/5	0/5	0/5
Total (%)		19/25 (76)[e]	0/14 (0)	0/15 (0)	2/20 (10)	1/20 (5)	0/22 (0)

[a]Fraction of mice surviving tumor-free after 10 weeks post s.c. tumor cell injection. 5×10^5 CT26 or 2×10^5 MC38 tumor cells were injected in 50 μl of HBSS in the right flank. Resulting tumor nodules were followed by weekly inspection and diameters recorded.
[b]Cultured BM-derived DC or fibroblasts were infected with the indicated recombinant adenovirus at a MOI of 3000, 15 h before their intratumoral injection. *In vitro* infection was carried out for 1 h in serum-free RPMI 1640 at room temperature and followed by a 14-h incubation in RPMI 1640 5% FCS at 37°C.
[c]Tumor nodules were slowly injected at day 8 after tumor cell inoculation with the indicated cells in 50 μl of HBSS using a 29 G needle.
[d]The fibroblast cell line IZA2.1 was derived from adherent cells of healthy Balb/C livers by transfection with a plasmid encoding for SV40 large T Ag (Melero I *et al*, manuscript in preparation).
[e]χ^2 test found this group different from the others with a $P < 0.01$.
ND, not done.

blasts were required for the antitumor effect beyond the mere transduction with IL-12 genes. It has been reported that fibroblasts retrovirally engineered to secrete IL-12 were efficacious upon peritumoral injection in other tumor models, but at doses at least 10 times higher than the ones used in this study.[27] Autologous DC transfected with control AdCMVlacZ, or untransfected, displayed a marginal antitumor effect within the range of doses used in these experiments. In the case of tumor nodules treated with AdCMVIL-12 transduced syngeneic DC, the malignant masses started to regress several days (5 to 10) after the procedure (data not shown), as if the antitumor effector mechanisms required this period of time to generate a curative response.

Because CT26 tumors were successfully treated in most instances by a single dose of 2×10^5 IL-12-producing DC, it was not considered an adequate model to optimize the therapeutic regimen although injections of larger doses of 10^6 IL-12-engineered DC seem to achieve better antitumor efficacy according to our preliminary data (I Melero *et al*, unpublished observations). With MC38, which is only cured in half of the cases, we are currently exploring potential improvements in the overall antitumor effect using higher and/or repeated intratumoral doses of IL-12-transduced BM-derived DC. It is noteworthy that the level of IL-12 secreted seems to play an important role since in the case of a batch of DC secreting 10 times less IL-12 due to a mistake in the MOI of AdCMVIL-12 used for gene transfer only a very marginal antitumor effect was observed (data not shown).

Cellular requirements for the induction of CT26 tumor regression were studied by selective *in vivo* depletion with specific anti-CD4 and anti-CD8 mAbs (Figure 2). Both $CD4^+$ and $CD8^+$ T cells seemed to play a role since only simultaneous depletion completely abrogated the antitumor effect of IL-12-engineered DC injected into the tumor, with eight of eight tumors progressing under these conditions, while only two of eight progressed in the non-depleted control group. Single CD8 depletion partially impaired the antitumor effect (six of eight tumors progressed), whereas CD4 depletion by itself did not impair or even improve the antitumor effect (none of eight tumors progressed). Such results in this tumor model are consistent with observations obtained in studies using immunotherapy with tumor antigen-pulsed on DC,[19] which showed a crucial role for both CD8 and CD4 subsets during induction of immunity and only for $CD8^+$ T cells during the effector phase. In our case CD4 depletion by itself did not affect tumor regressions indicating that IL-12 gene transduction into DC might be partially bypassing the need for CD4 help in CTL generation. Nevertheless the data obtained upon simultaneous CD4 and CD8 depletion may point to a beneficial involvement of CD4 cells in the overall elicited antitumor effect, which could explain the elimination of the observed residual antitumor activity after single CD8 depletion.

Paraffin-embedded CT26 tumor sections obtained on days 4 and 7 after intratumoral injection of IL-12-engineered DC were examined upon staining with hematoxylin and eosin and compared with untreated tumors. Treated tumors show a marked mononuclear infiltrate and abundant areas of intense necrosis. Granulocytes are also seen in much smaller numbers surrounding necrotic areas. Frozen samples of the same tumors were analyzed by immunostaining showing that the majority of tumor infiltrating mononuclear cells were $CD8^+$ $CD4^-$ but a small contribution of $CD4^+$ cells to the infiltrate could be detected (data not shown). These data are in agreement with the *in vivo* depletion experiments.

Memory in the immune mechanisms was found when mice who had rejected a tumor nodule 10 weeks earlier upon treatment with AdCMVIL-12-infected DC, remained tumor-free after a second lethal challenge in the opposite flank with tumor cells which aggressively progressed in naive, sex- and age-matched mice (Figure 3). These data also indicated that the procedure has induced systemic immunity against tumor cells which can be therapeutically useful to prevent the outgrowth of micrometastasis. In this regard, mice who carried s.c. CT26 tumors bilaterally were able to reject both tumor

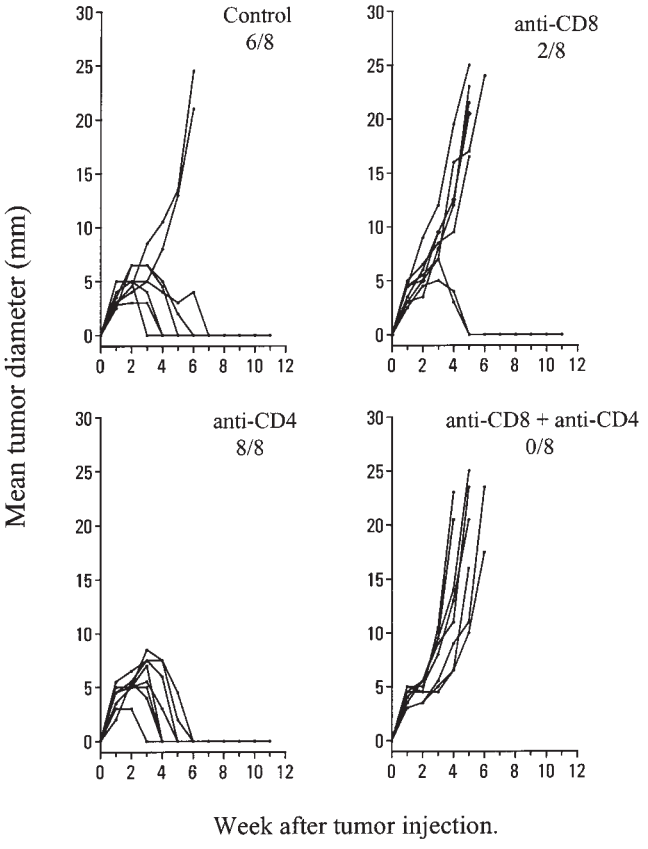

Figure 2 *T-lymphocytes are required for the antitumor effect of intratumoral injections of BM-derived DC infected with AdCMVIL-12. Individual size evolution of the tumor nodules in Balb/c mice injected subcutaneously with 5×10^5 CT26 cells. Seven days later, and weekly thereafter, mice were injected i.p. with 100 μl of HBSS (control) or ascites fluid of the anti-CD4 (GK15), anti-CD8 (53.6.72), or both hybridomas as indicated. Tumor nodules in every group were injected with 2.5×10^5 BM-derived DC infected with AdCMVIL12 (MOI, 3000) at day 9 after tumor inoculation. High titer ascites fluid was produced by i.p. injection of the hybridomas in Balb/c nude mice. Four control mice were injected with 100 μl of each ascites and depletion of the corresponding lymphocyte subsets was found to be complete 1 day later upon immunostaining and FACS analysis of the relevant populations in the spleen of these mice as described.[26] 5–9-week-old male mice Balb/c, C57Bl/6, and Balb/c$^{nude/nude}$ were purchased from Charles River (Barcelona, Spain) and housed in our animal facility according to institutional guidelines. The hybridomas GK15 and 53.6.72 were obtained through ATCC; CT26 was obtained from Dr K Brand (Max Plank-Institut fur Biochemie, Germany) and MC38 from Dr Lieping Chen (Bristol Myers Squibb, Seattle, WA, USA). Cell lines grown in DMEM 10% FCS from GIBCO (Basel, Switzerland). For tumor follow-up, opposite maximal tumor nodule diameters were measured weekly with a precision calliper until mice spontaneously died or were killed when nodules surpassed 25 mm or when they developed severe ulceration.*

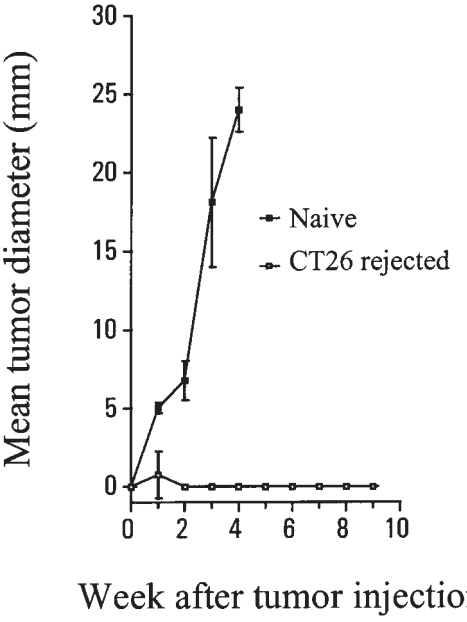

Figure 3 *Rejection of CT26 tumors after intratumoral injection with AdCMVIL-12 infected DC induces long-lasting systemic antitumor immunity. Mice (n = 4) who have rejected a primary CT26 tumor after intratumoral injection of 2.5×10^5 BM-derived DC infected with AdCMVIL-12 were rechallenged s.c. 10 weeks later with a dose of 5×10^5 CT26 viable cells on the opposite flank. As a control, naive littermates (n = 4) were also injected with a similar dose of CT26. Tumor development and size ± s.d. were monitored weekly in the area of the tumor rechallenge. Similar mice which had rejected CT26 tumors after intratumoral injection of BM-derived DC infected with AdCMVIL-12 developed lethal malignancies when s.c. rechallenged with A20 lymphoma cells indicating tumor-specificity of the protection (data not shown).*

nodules in six of eight cases when only one of the nodules received intratumoral injection of 10^6 IL-12-engineered DC, whereas all tumors progressed bilaterally in a control group (n = 8) identically intratumorally injected in one of the nodules but with non-transduced DC (data not shown).

It was observed that intratumoral injection of IL-12-transduced DC induced CTL specific for CT26 without cytolytic activity against P815 and YAC-1 cells (Figure 4). In addition, we took advantage of the fact that CT26 expresses a tumor rejection antigen named AH1.[28] This epitope has been identified as a 8-mer peptide presented by H-2L^d, derived from the *env* gene of a retroviral sequence silent in the genome of cells from normal Balb/c mice, but expressed in CT26 cells. CTL cultured from mice whose tumor nodules had been injected with IL-12-transduced DC showed specificity for this epitope pulsed on P815 cells (H-2^d) (Figure 4). Bearing in mind these data and the current view on the physiology of DC,[1–3] we postulate a model in which artificially injected IL-12-transduced DC pick up tumor antigens during a transient residence in the malignant tissue and become T cell stimulators after migration into lymphoid tissues.[29] This would be consistent with the fact that BM-derived DC have been shown to acquire tumor antigens upon *in vitro* coculture with a tumor cell line without further manipulation.[30] In this regard, it is of interest that DC are very efficient at internalizing apoptotic bodies[31,32] and maybe they take up antigen from compartments of the tumor nodule undergoing programmed cell death. We are in the process of investigating whether pretreatment of the tumor with agents increasing apoptosis enhance antigen transfer and presentation in our model. It is also conceivable that mechanical effects of injection of fluid into the experimental tumor facilitate release and capture of intracellular antigens. However direct demonstration in this system of tumor antigen capture and transport into T cell areas of lymphoid tissue by the injected DC is the aim of our current and future research. It is noteworthy that our defective recombinant adenovirus have

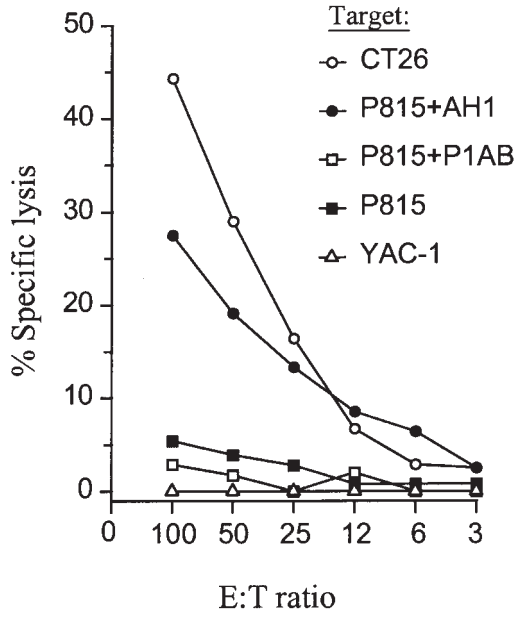

Figure 4 *Intratumoral injection with BM-derived DC infected with AdCMVIL-12 induces tumor-specific CTLs. Cytolytic activity in 4 h ^{51}Cr-release assays of CTL cultures derived from three pooled spleens of CT26 tumor-bearing Balb/c treated on day 7 after tumor inoculation with BM-derived DC infected with AdCMVIL-12 and harvested 7 days later was measured against ^{51}Cr-labelled CT26, P815 and YAC-1 cells at different effector:target ratios. When indicated P815 targets were pulsed with 10 μm of AH1 (SPSYVYHQF) or control P1AB (LPYLGWLVF) peptides during the assay. Results are representative of two independent experiments. ^{51}Cr-release assays were performed as described.[42] CTL cultures were raised by a 5-day coculture of spleen cell suspensions harvested 7 days after intratumoral injection of IL-12 producing DC with non-viable CT26 cells in RPMI 1640 10% FCS.[42] YAC-1 and P815 were from ATCC. Peptide synthesis was performed as described.[28,43]*

a deletion in the E3 region so infection does not interfere with the MHC class I antigen presenting pathway.[22,26]

If the scenario of local antigen uptake and following migration through lymphatic vessels holds true, IL-12 secreted by DC will be a potent stimulator of Th1 cells and CTL precursors[33,34] upon arrival in T cell areas of draining lymph nodes. In addition, IL-12 secreted at the tumor site may downregulate angiogenesis[35] or mediate other antitumor effects, although these mechanisms must be secondary under our conditions because neither IL-12-transfected allogeneic DC nor fibroblasts mediate any measurable antitumor effect. More importantly, IL-12 has been shown to act in an autocrine fashion on DC promoting maturation and migration of DC through the activation of NFκB transcription factors.[36] In fact, culture of DC with exogenous recombinant IL-12 induces the secretion of endogenous IL-12 conferring DC with the ability to prime CTL responses.[37] According to these reports, part of the IL-12 detected in our transfected DC could result from the activation of endogenous IL-12 genes in a positive autocrine feed-back. In addition, it has also been reported that spleen mononulear cells or purified B cells pulsed with a defined murine tumor antigen can induce protective immunity only if recombinant IL-12 is coinjected into the footpad.[38] Although only protective but not therapeutic immunity is seen in that model, it would be interesting to understand the role of rIL-12 in enhancing the immunostimulatory properties of DC or other APCs. Moreover, our preliminary data suggest that at least part of the effects seen after transduction of DC by AdCMVIL-12 are mediated by IL-12 in an autocrine fashion (Galofre JC *et al*, manuscript in preparation).

In conclusion, the reported therapeutic effect of intratumoral injections with AdCMVIL-12 infected DC might have clinical application for a number of reasons: (1) it does not require the identification of tumor antigens; (2) it can be done in every patient provided that DC can be cultured from peripheral blood; (3) avoids the toxicity of systemic doses of IL-12,[39] which have been used to enhance immunotherapy with DC.[38] The advantage in our approach is that IL-12 is presumably secreted at the precise site of antigen presentation and lymphocyte activation. Although clinical feasibility of these kinds of procedures awaits further safety experimentation, we have not observed any noticeable side-effect in animals rejecting their tumors. In this regard, the potential appearance of immunity against normal components of the injected tissue should be carefully evaluated.[40] It will also be important to study comparatively the efficacy of this new approach with other means to use DC in tumor immunotherapy in different tumor models, including those which propose the use of DC transfected with other cytokines.[41]

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