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Genetic modification of a carcinoma with the IL-4 gene increases the influx of dendritic cells relative to other cytokines

Tumor cells genetically modified with certain cytokine genes gain immunogenic properties that allow the development of systemic anti-tumor immunity. Whether different cytokines may influence infiltration of transduced tumors by dendritic cells (DC) has not been investigated. Therefore, we analyzed the C26 murine colon carcinoma genetically modified to release interleukin (IL)-2, IL-4, IL-12, granulocyte colony-stimulating-factor (CSF) or granulocyte-macrophage (GM)-CSF for immunostaining with the monoclonal antibody NDLC145 recognizing the DEC205 determinant which, on tumor sections, is virtually restricted to DC. Infiltrating leukocytes were also characterized for expression of co-stimulatory molecules like CD54, CD86 and major histocompatibility complex class II. The intratumoral DC content was dependent on the type of transduced cytokines with C26/IL-4 being the most abundant in DEC205$^+$ cells. The effect of IL-4 in recruiting DC did not depend on the type of tumor since it was confirmed in the TSA mammary carcinoma. In comparison with C26/GM-CSF, C26/IL-4 had more B7.2$^+$ cells but less Ia$^+$ cells. Furthermore, the hypertrophic skin overlaying tumors producing GM-CSF showed numerous Langerhans cells stained by NDLC145 and the draining lymph nodes showed abundance and paucity of DC in C26/GM-CSF and C26/IL-4, respectively. When injected into the ear pinna, C26/GM-CSF stimulated, whereas C26/IL-4 inhibited DC-mediated priming of delayed-type hypersensitivity reaction by 2,4-dinitro-1-fluoro-benzene. These findings prove that transduced cytokines differently influence DC recruitment at the tumor site and DC function in nearby tissues. Along with the other leukocytes and their secondary produced cytokines, DC create an environment in which T cells can be differently modulated. Such a phenomenon may have implications on genetic modification of tumor cells to be used as cancer vaccine.

1 Introduction

Dendritic cells (DC) originate in the bone marrow, localize to epithelia and interstitial spaces, and migrate to secondary lymphoid organs where they signal the nature of the antigens encountered in the periphery and initiate the T cell-dependent immune response [1, 2].

DC infiltration of solid tumors has been correlated with a better prognosis [3], but which signal modulates their number has not been investigated. Inflammatory cytokines promote DC maturation and migration toward lymphoid organs [4]. Tumors transduced to express a variety of cytokines have been extensively characterized for the type of infiltrating leukocytes although infiltration by DC has not been investigated. However, cytokine-transduced

tumors offer the opportunity of studying which cytokines promote DC infiltration as well as their localization within the tumor stroma, their stage of maturation and, although indirectly, their number in the tumor-draining lymph nodes.

The immune response which follows the injection of cytokine-transduced tumors is generally capable of tumor destruction. The events caused by the injection of cytokine-transduced tumors include both the early infiltration of granulocytes and then of macrophages and lymphocytes, and the release of secondary cytokines. Tumor cell debris derived from tumor destruction may represent the source of tumor antigens that antigen-presenting cells (APC) process and bring to draining lymph nodes which appear often enlarged with an expanded cortical and paracortical area and which are rich in tingible-body macrophages [5]. Although cellular debris captured by phagocytic cells contains antigens that are presented through the MHC class II pathway, CD8$^+$ CTL are often induced in this setting since systemic immunity and generation of cytotoxic T lymphocytes generally follow tumor destruction [5]. The finding of granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent DC class I presentation of soluble proteins for CTL induction [6, 7] as well as the role of bone marrow-derived APC [8] in mediating cross-priming in vivo [9] indicates that intratumoral DC can induce protective immunity by taking up and processing antigen for presentation within their own MHC [8, 10].

Thus, following injection of cytokine-transduced tumor cells, DC participate in the initial process that leads to host immunization; however, it has not been investigated whether different cytokines influence the extent of DC tumor infiltration.

Here we present a characterization of DC infiltration into a C-26 colon carcinoma transduced to release IL-2, IL-4, IL-12, G- or GM-CSF. These results may contribute to understanding the way of how DC can be modulated and loaded directly *in vivo* by an engineered cell vaccine.

2 Materials and methods

2.1 Tumors and mice

C-26 is a colon adenocarcinoma line derived from BALB/c mice treated with N-nitroso-N-methylurethane [11]. TSA is a tumor line established from the first transplant *in vivo* of a moderately differentiated mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse [12]. Tumor cells were cultured in Dulbecco's modified Eagle's medium (Gibco, Paisley, GB) supplemented with 10% FCS (Gibco). BALB/cnAnCr mice (Charles River, Calco, Italy) were maintained at the Istituto Nazionale Tumori under standard conditions according to Institutional and EU guidelines.

2.2 Cytokine gene transduction

LXSN-based retroviral vectors carrying IL-2 [13], IL-4 [14], IL-12 [15], G-CSF [16] or GM-CSF [13] have been described. Target cells (10^6) were infected by exposure to undiluted supernatant for 3 h in the presence of 8 µg/ml polybrene, then selected in 0.5 mg/ml of G418. Single G418-resistant colonies were isolated and expanded.

2.3 Morphological analysis

Mice were killed at different times after injection of 10^6 cells s.c. into the right flank; where possible two tumors were analyzed at each time point. The flanks were gently dissected and the tumor mass was measured. Tumor fragments and draining lymph nodes were embedded in OCT compound (Miles Lab., Elkert, IN), snap-frozen in liquid nitrogen and stored at − 80°C. For EM, tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate (pH 7.2) for 3 h at 4°C, post-fixed with 1% OsO_4 in 0.1 M sodium phosphate (pH 6) for 1 h at 4°C, dehydrated with ethanol and embedded with Epon 812.

2.4 Immunocytochemistry

Five-millimeter croystat sections were fixed in acetone and immnostained with rat anti-mouse mAb against CD45 (M1/9.3.4.HL2 clone), CD8 (53.6.72 clone), CD4 (GK1.5 clone), Mac-3 (M3/84,6,34 clone), MHC-II (B21-2 clone) all from ATCC; CD86 (GL-1clone, Pharmingen, San Diego, CA), GR1 (RB6-8C5 kindly provided by Bob Coffman), DEC205 (NDLC-145, kindly provided by Ralph Steinman) and with hamster anti-mouse Ab against CD3ε

(Boehringer, Mannheim, Germany), CD54 (3C2 clone), and CD28 (37.51 clone) all from Pharmingen. Sections were preincubated with rabbit or hamster serum, and sequentially incubated with optimal dilutions of primary antibodies, biotinylated rabbit anti-rat IgG or rat anti-hamster IgG and streptavidin-peroxidase (Pharmingen). Each incubation step lasted 30 min and was followed by a 10 min wash in PBS. Sections were then incubated with 0.03% H_2O_2 and 0.06% 3.3' diaminobenzidine (BDH Chemicals, Poole, GB) for 2–5 min, washed in tap water, and counterstained with hematoxylin as described [17]. The number of immunostained cells was determined by light microscopy at 400 × magnification in five fields on a 1 mm^2 grid and is given as cells/mm^2 (mean ± SD).

2.5 Isolation of tumor-infiltrating dendritic cells (TIDC)

Tumor cells were implanted s.c. in the right flank at 5 × 10^6 cells/mouse and the tumors were surgically removed 7–10 days after implantation when they reached a mean diameter of 1.6–2 mm. Tumor masses were perfused with collagenase D solution (100 U/ml), gently minced in to small pieces and incubated in collagenase solution (400 U/ml) for 30–60 min at 37°C. After gentle pipetting, the fine suspension was washed several times with RPMI, cells were plated on 10-mm petri dishes at 10^6 cells/ml and incubated for 2 h at 37°C to let tumor cells adhere to the plastic. Nonadherent cells were collected and replated for additional 30 min, finally cells in suspension were loaded onto a 14.5% metrizamide gradient and the low-density fraction collected as TIDC-rich fraction. Contaminating lymphocytes were removed by C-mediated mAb lysis as described [18].

2.6 Contact hypersensitivity

Contact hypersensitivity was induced by injection of 2,4-dinitrobenzene sulfonic acid (DNBS; Eastman Kodak, Rochester, NY)-modified cells as described [18]. Briefly, TIDC or tumor cells or spleen-derived DC were derivatized with 1 mg/ml DNBS for 30 min at 37°C and inoculated s.c. into the dorsal skin of BALB/c mice. Each mouse received 5 × 10^4 cells resuspended in 250 µl final volume HBSS. Recipient mice and non-sensitized controls were challenged with a 0.2% solution of 2,4-dinitro-1-fluorobenzene (DNFB, Sigma) on both sides of right ear. Ear swelling was measured 24 h later.

To induce contact hypersensitivity in tumor-bearing mice, tumor cells (5 × 10^4) were injected into the pinna of the right ear of BALB/c mice. Mice were DNFB-sensitized 5 or 10 days after tumor injection by painting both sides of the ear into which the tumor had been injected, with 0.5% solution in 4:1 acetone/olive oil (20 µl) and challenged 5 days after sensitization by applying 0.2% DNFB solution to controlateral ears.

Mice sensitized epicutaneously on the shaved abdomen with 150 µg DNFB were included as positive controls for hapten sensitization; mice only painted on day 5 served as controls for nonspecific inflammatory edema caused by chemical painting. Ear thickness was measured immediately before and 24 h after DNFB challenge using an engi-
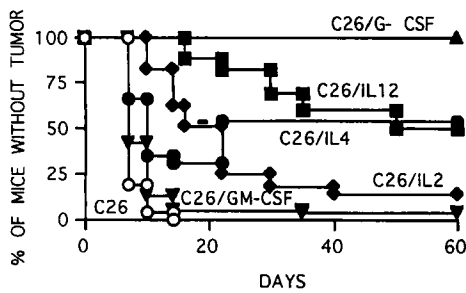
Figure 1. Outgrowth of C26 (open symbol) and its variants genetically modified to release IL-2, IL-4, IL-12, G-CSF and GM-CSF (black symbols) *in vivo*. Cells (5 × 10⁴) were injected s.c. into the right flank.

neer's micrometer. All the experiments were performed at least twice with five mice per group. Differences in ear swelling responses were statistically evaluated by the Student's *t*-test.

3 Results

3.1 DC content and distribution in cytokine-transduced C-26 colon carcinoma

C-26 cells transduced with IL-2, IL-4, IL-12, G or GM-CSF genes exhibited a different tumorigenicity varying from complete inhibition of take (with G-CSF-transduced cells), to unimpaired tumor growth (with GM-CSF-transduced cells) (Fig. 1). The host immune response against cytokine-transduced C-26 tumors cells has been

extensively characterized [13, 15–17]. A kinetics study describing the leukocytes infiltrating the site of tumor injection at day 3 as well as infiltrating the incipient tumors, excised at day 7 and 15, is shown in Table 1. Among the different mAb utilized for this characterization, NDLC145 [19] recognizes DEC205 [20] primarily on DC, epithelial cells of the thymic cortex and intestinal epithelia. DEC205 can be visualized on other tissues such as the stroma of bone marrow, pulmonary airways, and mature B cells, albeit at low levels compared to DC [21]. In tumor sections of C26 colon carcinoma grown subcutaneously, the only cells which can actually be detected by immunostaining with the mAb to DEC205 are DC. The density of intratumoral DC depended on the type of transduced cytokine and for each cytokine a higher number of DEC205⁺ cells was found at day 7. Whereas the number of DC was different among the engineered tumors analyzed, with IL-4 the richest (see Sect. 3.2), their distribution within and around the tumor was quite similar. DC were localized preferentially in the perinecrotic areas, and were frequently seen in clusters at the edge of the tumor and interdigitated with endothelial cells around the blood vessels (Fig. 2). Although B7.2 is expressed on other cell types, there was a correlation between the number and distribution of DC stained by antibodies to DEC205 and to B7.2 (Fig. 2C and Tables 1 and 2). Tumor transduced with G-CSF was so efficient in inducing a granulocyte-mediated tumor destruction that DC infiltration was prevented. Accordingly, mice which remained tumor-free did not develop immune memory and were susceptible to a challenge with parental tumor cells [16]. Leukocyte infiltration of C26/IL-2 was characterized by a predominant presence of GR-1⁺ cells as already reported for the TSA carcinoma
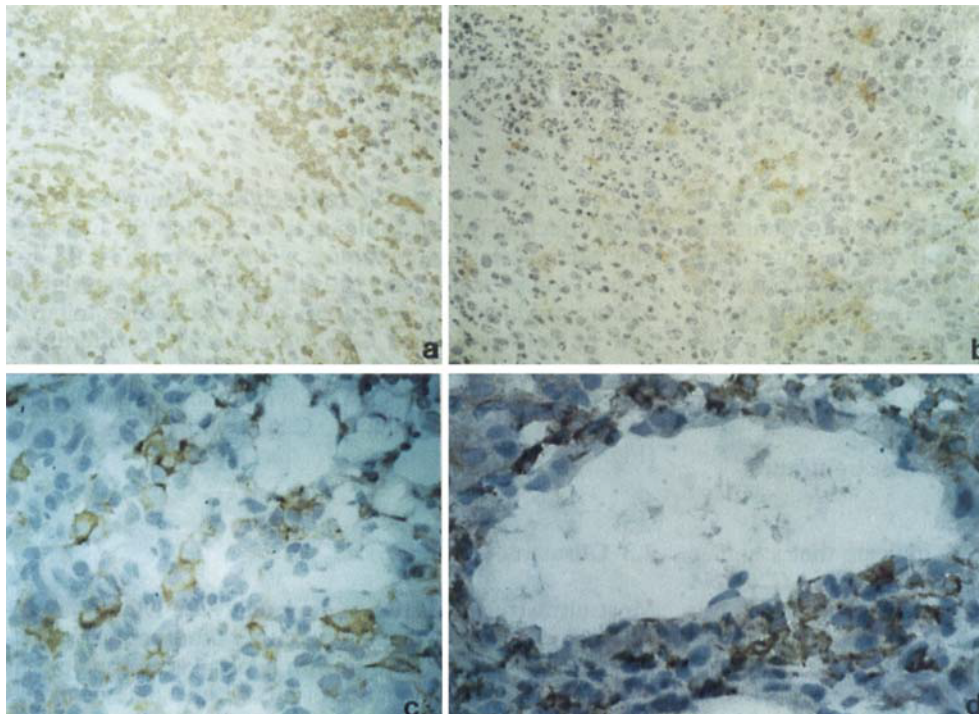

Figure 2. Intra- and peritumoral localization of DC. a) and b), sections from C26/IL-2 stained with mAb to CD45 (a) and to DEC205 (b): DC cells were outside the necrotic area where most of infiltrating leukocytes were localized, both × 350. c) Peritumoral localization of B7.2⁺ cells in C26/IL-4, × 600 and d), immunostaining with NDLC145 showing perivascular localization of DC in C26/GM-CSF, × 600.

Table 1. Immunocytochemical characterization of leukocytes infiltrating C26 colon carcinoma and its variants engineered to release cytokines by retroviral gene transfer

Tumor	Days	Tumor size	CD45	CD8	CD4	GR-1	Mac-3	DEC205
C26	3	3 × 4	213 ± 63[a]	5 ± 2	8 ± 6	115 ± 38	96 ± 21	3 ± 3 (1)[b]
	7	9 × 7	207 ± 32	15 ± 7	24 ± 6	55 ± 13	113 ± 46	3 ± 2 (1)
	15	11 × 8	189 ± 26	11 ± 3	29 ± 9	14 ± 8	118 ± 26	6 ± 4 (3)
C26//IL-2	3	1 × 3	nd	46 ± 15	nd	736 ± 12	nd	119 ± 58
	7	3 × 4	1715 ± 414	89 ± 12	107 ± 21	966 ± 215	238 ± 96	41 ± 41 (2)
	15	5 × 4	736 ± 103	97 ± 17	149 ± 37	193 ± 61	216 ± 51	41 ± 29 (6)
C26/IL-4	3	2 × 3	1318 ± 241	16 ± 9	39 ± 11	415 ± 71	143 ± 26	244 ± 28 (19)
	7	3 × 4	889 ± 173	23 ± 6	79 ± 15	394 ± 86	117 ± 45	261 ± 47 (29)
	15	9 × 8	514 ± 67	27 ± 11	96 ± 21	115 ± 22	133 ± 41	133 ± 12 (26)
C26/IL-12	3	3 × 3	907 ± 163	312 ± 47	107 ± 21	278 ± 26	81 ± 11	139 ± 61 (15)
	7	3 × 4	1137 ± 186	591 ± 98	164 ± 79	330 ± 99	78 ± 24	73 ± 37 (6)
	15	3 × 4	834 ± 123	450 ± 57	181 ± 37	114 ± 27	103 ± 13	43 ± 29 (5)
C26/G-CSF	3	2 × 3	958 ± 112	0	0	792 ± 98	115 ± 23	3 ± 2 (<1)
C26/GM-CSF	7	7 × 5	938 ± 89	26 ± 18	62 ± 18	407 ± 89	347 ± 96	60 ± 38 (6)
	15	10 × 9	942 ± 102	46 ± 11	67 ± 32	293 ± 61	405 ± 87	39 ± 31 (4)
TSA/IL-4	7	4 × 5	2115 ± 317	63 ± 34	115 ± 47	715 ± 105	431 ± 73	318 ± 44 (15)
	15	7 × 6	2311 ± 516	46 ± 22	146 ± 64	799 ± 126	467 ± 85	289 ± 47 (13)

a) Mean number of cells/mm^2 ± SD positive for immunostaining.
b) Percentage of positive cells among total number of infiltrating CD45$^+$ leukocytes.

Table 2. Immunocytochemical characterization of costimulatory molecules expressed by leukocytes infiltrating C26 and TSA tumors transduced with cytokine genes.

Tumor	Tumor size (mm)	MHC class II	CD54	CD86
C26	3 × 4	56 ± 18[a]	19 ± 11	3 ± 1
C26/IL-2	3 × 4	218 ± 41	398 ± 72	278 ± 93
C26/IL-4	3 × 4	109 ± 16	422 ± 41	622 ± 41
C26/IL-12	3 × 4	103 ± 14	905 ± 131	207 ± 89
C26/G-CSF	2 × 3	22 ± 13	nd	3 ± 2
C26/GM-CSF	7 × 5	87 ± 15	89 ± 27	89 ± 28
TSA/IL-4	3 × 5	189 ± 18	832 ± 76	949 ± 211

a) Mean number of cells/mm^2 ± SD positive for immunostaining.

[22]. Among the tumors analyzed, C26 engineered to release IL-12 was the most potent inducer of host immune response and the most active as cell vaccine for curing mice bearing lung metastasis [23]. Histological sections of these tumors were characterized by the early infiltration of DEC205$^+$ cells and by the higher number of CD8$^+$ cells (Table 1) as well as of cells expressing CD86 (Table 2).

3.2 Effect of IL-4 and GM-CSF on tumor infiltration by DC

The results shown in Table 1 clearly indicate that a higher number of DEC205$^+$ cells were localized within C26/IL-4 than within C26/GM-CSF. We further compared these tumors for expression of B7.2 and Ia molecules by immunostaining (Fig. 3 and Table 2). The number of cells expressing B7.2 was again higher in IL-4- than in GM-CSF-transduced C26 tumors whereas the number of Ia$^+$ cells was roughly similar. Therefore, the percentage of Ia$^+$ DC was higher in GM-CSF- than in IL-4-transduced tumors. These data were confirmed by double staining of C26/IL-4 and C26/GM-CSF with mAb to MHC class II and DEC205

(not shown). The GM-CSF released by the tumor also had some effect on the surrounding tissue such as the hypertrophic skin overlaying the tumors where the mAb NDLC145 stained more Langerhans cells than in the skin overlaying tumors producing IL-4 (Fig. 3E, F), which was similar to that of non-transduced C26 (not shown). Furthermore, staining with NDLC145 showed abundance and paucity (like normal node) of DC in the lymph-node draining GM-CSF- and IL-4-transduced C26 tumors, respectively (Fig. 3G, H).

3.3 Ultrastructural analysis

Most ultrastructural studies have analyzed DC differentiated *in vitro* [24] or, in the skin either after contact sensitization [25] or in association with hyperplasia or neoplasm [26]. The relative abundance of DC within some cytokine-transduced tumors allowed their morphological characterization by EM. We did not find morphological differences between IL-4- and GM-CSF-transduced tumors for cells recognized as DC. They showed typical DC features like enlarged Golgi apparatus, coated pits, large smooth and
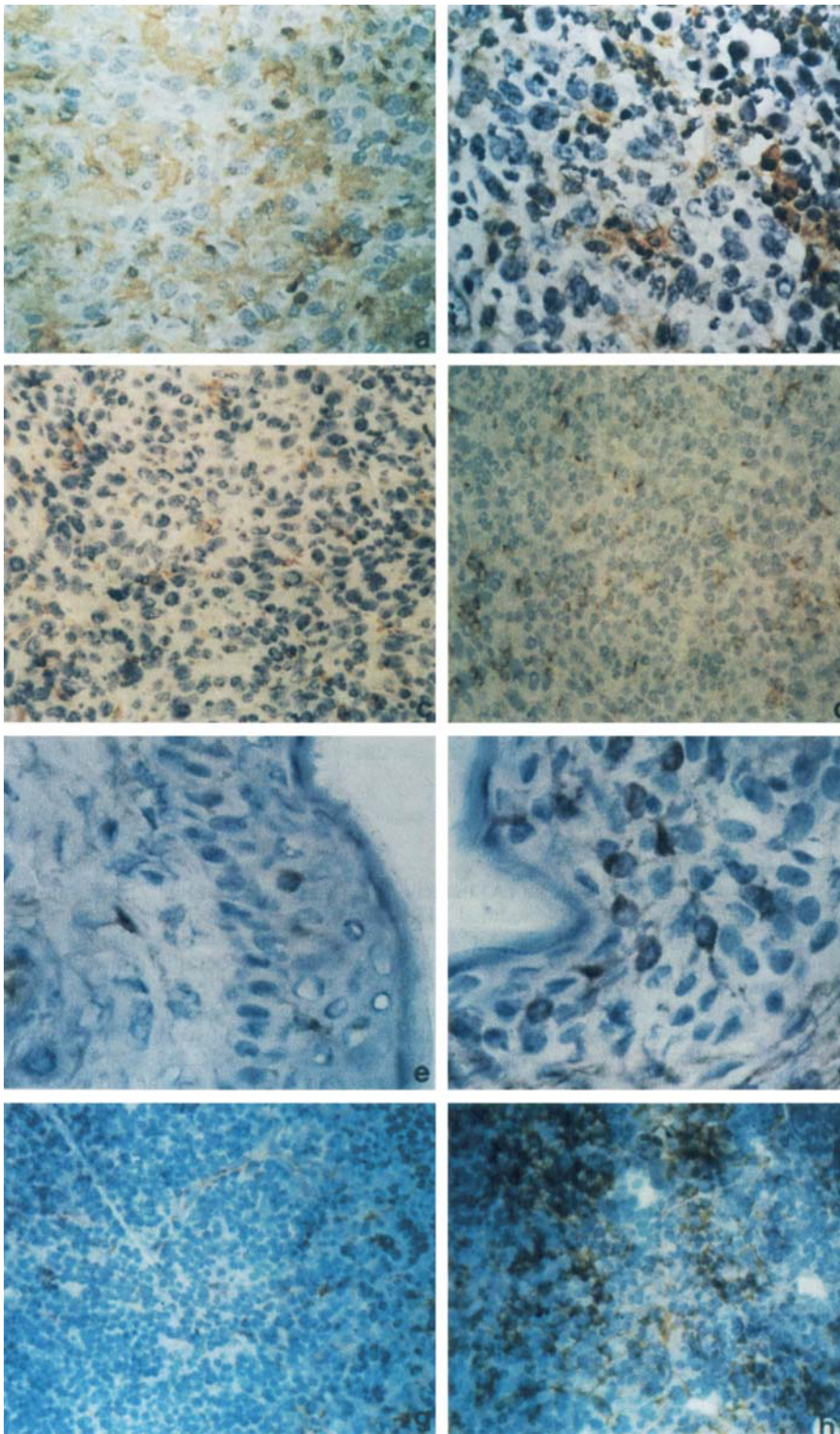
Figure 3. Comparison between C26/IL-4 (a, c, e, g) and C26/GM-CSF (b, d, f, h) for immunostaining with mAb to CD86 (a, b), MHC class II (c, d) and DEC205 (e–h). Sections from tumors (a–d), skin (e, f) and draining lymph nodes (g, h) were immunostained. a, × 650; b, × 750, c and h, × 400; d and g, × 350; e, × 800; f, × 1000.
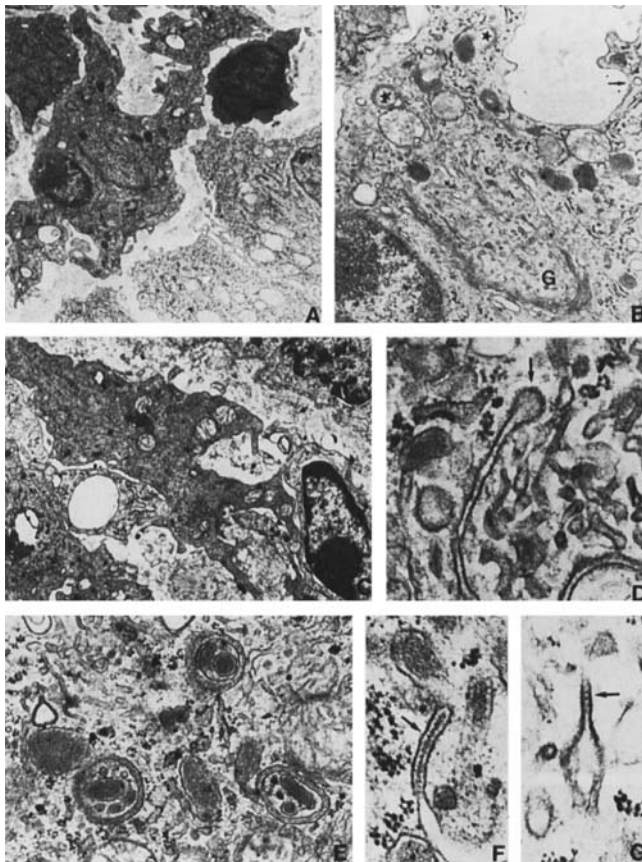
Figure 4. Ultrastructural analysis of infiltrating DC. A) Typical dendritic reticular cell with star shape, × 8000. B) Enlargement of A showing a large Golti apparatus (G), pentalaminar tubular structures (asterisks) and a coated pit (arrow), × 20000. C) Portion of a reticular cell with cytoplasmic projections, coated pits and vesicles; this cell has a darker cytoplasmic matrix than the cell shown in A, × 11500. D) A narrow tubule continuous with a coated vesicle (arrow), × 71000. E) Pentalaminar tubular structures surrounding dense core granules, × 35000. F) A rod-shaped Birbeck granule (arrow) showing central linear striated density which appears to be continuous with a narrow tubule, × 85000. G) A racket-shaped Birbeck granule (arrow), × 85000.

Figure 5. (A) Induction of contact hypersensitivity by injection of DNBS-modified TIDC. TIDC or tumor cells were derivatized with DNBS for 30 min at 37 °C, and inoculated s. c. into the dorsal skin of syngeneic mice. Recpient and nonsensitized mice (control sham) were ear challenged 5 d later with DNFB. As positive controls mice sensitized epicutaneously with DNFB were included as well as mice immunized with DNBS-modified DC. Ear thickness was measured with an engineer's micrometer immediately before and 24 h after the challenge. The x-axis shows the differences in ear thickness. (B) Induction of contact hypersensitivity in tumor-bearing mice. Cytokine-transduced tumor cells were injected into the ear at day 0; mice were DNFB-sensitized by painting both sides of tumor-treated ears (right) 5 days following tumor injection. Mice were challenged 5 days after sensitization by applying DNFB to controlateral ears (left). Ear swelling was measured 24 h later. Mice only painted on day 5 served as controls for nonspecific inflammatory edema caused by chemical painting. The x-axis shows differences in ear thickness before and after challenge. Each group included five mice; statistical analysis was performed using Student's t test; * $p < 0,05$; ** $p < 0,01$.

rough endoplasmic reticulum, numerous core tubules, often pentalaminar, filled with electron-dense materials, narrow tubules continuing with coated vesicles and sometimes rod- or racket-shaped Birbeck-like granules (Fig. 4). A few cells probably related to the spectrum of differentiation of dendritic reticular cells were also observed (Fig. 4C). They showed numerous dark cytoplasmic projections contacting surrounding cells as well as coated pits and vesicles but did not show other morphological markers typical of DC.

3.4 Abundance of DC within TSA tumors transduced with IL-4

Since IL-4 was the cytokine more efficient in recruiting DC, we tested whether IL-4 transduced into a different tumor produced the same effect. Therefore, the TSA mammary carcinoma was transduced with the same viral supernatant used for transducing the IL-4 gene into C-26
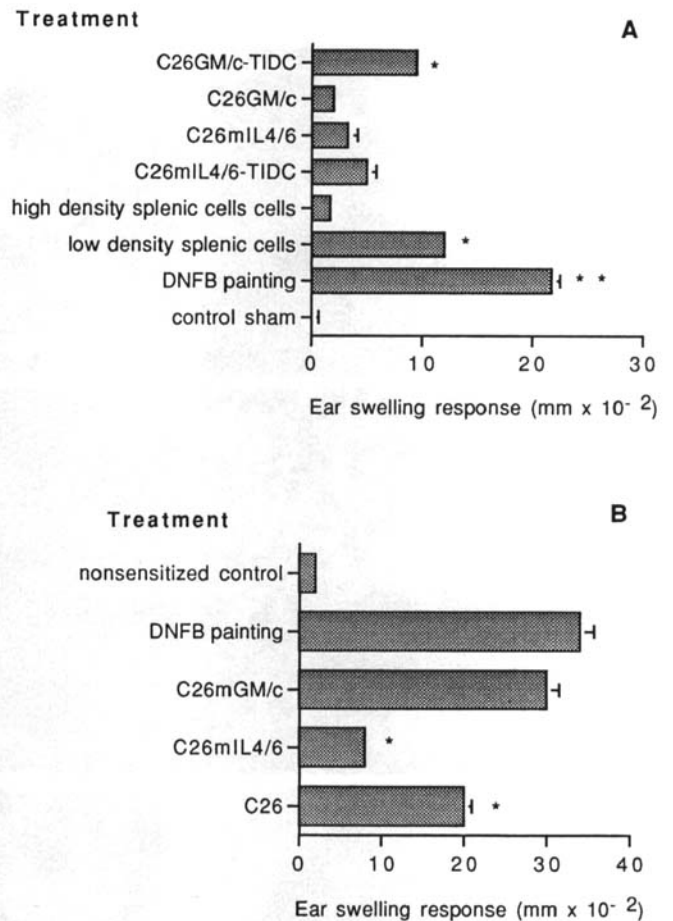
cells. The amount of IL-4 released by TSA/IL-4 and C-26/IL-4 ranged between 20–26 and 18–22 ng/ml/10^6 cells/48 h, respectively, and although the total number of CD45$^+$ infiltrating leukocytes was higher in TSA/IL-4, the different leukocyte populations (as % of CD45$^+$ cells), including DEC205$^+$ cells, were similarly represented (Table 1). Intra- and peritumoral distribution of cells immunostained with mAb to DEC205, B7.2 and Ia was very similar in the two carcinomas (Table 2 and data not shown).

3.5 C26/IL-4 impairs DTH reaction

We investigated whether the differences between C26/IL-4 and C26/GM-CSF in terms of DC number, expression of MHC class II molecules as well as size and NDLC-145 staining of draining lymph nodes have any functional implication on DC function. DC were isolated by C26/IL-4 and C26/GM-CSF tumors, derivatized *in vitro* with DNBS and injected into the skin of naive mice to prime them against an ear challenge with DNFB. Fig. 5A shows that DC from C26/GM-CSF, but not from C26/IL-4, were able to induce a swelling response. This type of experiment required isolation and therefore manipulation of DC *in vitro*, and did not allow to test whether parental C26 may have any effect on DC function because the parental tumor is virtually devoid of DC. Since tumor-derived factors may impair DC function [27], we repeated DTH experiments with the aim of testing whether cytokines at the tumor site or tumor-derived factors influence the function of DC seeded into nearby tissues. C26, C26/IL-4 or C26/GM-CSF were injected into the ear pinna and DNFB sensitization was performed 5 or 10 days later around the tumor areas. DNFB challenge was performed 5 days after sensitization into the controlateral ear. The results show that C26 reduces the ear swelling measured in DNFB-sensitized ears of naive mice. Such inhibition was corrected by C26/GM-CSF but worsened by C26/IL-4 (Fig. 5B).

4 Discussion

Several *in vitro* studies have described the role of different cytokines on DC proliferation, maturation, antigen uptake and presentation [1, 28]; *in vivo*, cytokines were injected intradermally either to recruit DC or to favor migration of Langerhans cells from skin to the draining lymph nodes [4]. Among the studies reporting immunohistological characterization of DC into human tumors [3, 29], one described a correlation between the amount of GM-CSF produced by lung carcinomas and distribution/differentiation of tumor-associated DC [30]. GM-CSF is certainly an important cytokine for DC maturation, proliferation and function [31]. Some tumors transduced with GM-CSF have been described as the most immunogenic [32] whereas this finding was not always confirmed when other tumor cell types were tested [33, 34]. This does not infringe the immunostimulatory property of GM-CSF but simply underlines that the inherent immunogenicity of different tumors, the amount of GM-CSF, as well as other factors, probably of tumor origin, may concur in determining the final outcome.

Knowledge of which cytokine causes the sequential events of DC recruiting, antigen uptake, migration and presentation may improve vaccine activity without need of additional adjuvant. The proposal of using GM-CSF as adjuvant is in line with this view [35]. A direct instruction of DC *in vivo* avoids any manipulation *in vitro* that may change DC physiology; in fact, treatments that increase antigen presentation may impair antigen uptake [36, 37].

Our results clearly show that the cytokines produced by transduced tumor cells drastically influence the number of DC which infiltrate the tumor. However, their absolute number is meaningless since maturation, lineage and func-

tion likely depend on the microenvironment to which other infiltrating leukocytes and their produced cytokines concur. This is strictly a phenomenon *in vivo* that can not be reproduced *in vitro*. Even s.c. or i.d. injection of recombinant cytokines gives partial or different information because of the short half-life of the injected cytokine and of the short time window in which the injected tissues can be analyzed.

Neither DC number nor their function appears to correlate with take inhibition of primary injected tumor (Fig. 1). In fact, C26/GM-CSF is fully tumorigenic although tumor-produced GM-CSF is able to restore DC function for DTH priming. Furthermore, C26/IL-4 which is the richest in DC number, shows reduced tumor take while abrogating DTH reaction. Taken together, these data show that DC do not participate in the early effector phase that is responsible of tumor rejection *in situ*. It is likely that DC present tumor antigen for the forthcoming systemic T cell-mediated immune response. Since irradiated tumor cells are used for vaccination, the early effector phase is not required, whereas a prompt DC recruitment for antigen uptake and presentation is necessary.

Among the cytokines analyzed in this study, GM-CSF and IL-4 have been shown to directly interact with DC [28] but, *in vivo*, the final effect on DC results from the concomitant action of secondary cytokines, in particular IL-1 and TNF, IFN-γ and IL-10, and of cell-cell contacts, in particular with CD40L present on activated Th lymphocytes. TNF-α, IL-1 and CD40L down-regulate macropinocytosis [37] and up-regulate adhesion co-stimulatory molecules [36, 37]. Ligation of CD40 on DC make them produce high levels of IL-12 [38, 39] which boost proliferative response and IFN-γ production by T cells.

An unsolved question is whether the environment created at the tumor site by the transduced cytokine offers a milieu sufficient for T cell priming [40]. Pulaski and colleagues [41] reported that CTL can be generated directly at the tumor site in the presence of IL-3-recruited APC of macrophage morphology. Staining for CD40L⁺ T cells might indicate that primed T cells are either generated or recruited at the tumor site. Activated T cells may be boosted for IFN-γ production via CD40 on DC [38] but also turned down if T cells are CTLA4⁺, via B7 on DC [42]. Cytokine-transduced tumor environment may not only be an inflammatory site but also a site where immunoregulatory interactions take place.

The mechanism by which DC influx was more pronounced in IL-4-producing tumors (in both C26 and TSA carcinomas) remains unknown. It is likely that chemokines activated at the tumor site by infiltrating leukocytes are the final effectors. DC express an array of chemokine receptors [43] and, *in vitro*, C-C chemokines show chemotactic property for human DC [44]. No matter what the final effector(s), are it is clear that cytokines transduced into tumor cells do influence the type and the number of infiltrating leukocytes including DC.

We focused on cytokines like IL-2, IL-4, GM-CSF and IL-12 which have been transduced into human tumor cells or fibroblast to treat cancer patients. The efficacy of such vaccines largely depends on their ability to provide all the

repertoire of relevant antigens and the cytokines and co-signals that favor DC recruitment and function for both T cell priming and T-T help. In light of the results presented here, we can now test, as cell vaccine, tumor cells equipped with CD40L and releasing GM-CSF or IL-4 rather than a tumor cell transduced with both B7 and IL-12 [45]. The former combination is likely to favor the interaction with DC while the latter would transform tumor cells into APC-like cells which should directly interact with T cells, thus bypassing the need of DC. Comparison of these formulations in the therapeutic setting with different tumor models is expected to provide clues for improving the initial success obtained by using the IL-12 gene alone [23, 46, 47].

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