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IL-4-Transduced Tumor Cell Vaccine Induces Immunoregulatory Type 2 CD8 T Lymphocytes That Cure Lung Metastases Upon Adoptive Transfer

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Vaccinations with tumor cells engineered to produce IL-4 prolonged survival and cured 30% of mice bearing pulmonary metastases, an effect abrogated by in vivo depletion of T cells. Vaccination induced type 2 T cell polarization in both CD4 and CD8 T lymphocyte subsets. We focused on the antitumor activity exerted by type 2 CD8\(^+\) T cells (Tc2) activated by IL-4 tumor cell vaccination. Tc2 lymphocytes lacked in vitro tumor cytotoxicity, but released IL-4 upon stimulation with tumor cells, as shown by limiting dilution analysis of the frequencies of tumor-specific pCTL and of CD8 cells producing the cytokine. In vivo fresh purified CD8\(^+\) T lymphocytes from IL-4-vaccinated mice eliminated 80–100% of lung metastases when transferred into tumor-bearing mice. CD8\(^+\) lymphocytes from IL-4-vaccinated IFN-\(\gamma\) knockout (KO), but not from IL-4 KO, mice cured lung metastases, thus indicating that IL-4 produced by Tc2 cells was instrumental for tumor rejection. The antitumor effect of adoptively transferred Tc2 lymphocytes needed host CD8 T cells and AsGM1 leukocyte populations, and partially granulocytes. These data indicate that Tc2 CD8\(^+\) T cells exert immunoregulatory functions and induce tumor rejection through the cooperation of bystander lymphoid effector cells. Tumor eradication is thus not restricted to a type 1 response, but can also be mediated by a type 2 biased T cell response. The Journal of Immunology, 1999, 163: 1923–1928.

In contrast to the current view indicating that a type 1 immune response is required for tumor regression, type 2 cytokines have been successfully used in murine models for augmenting the immunogenicity of tumor cells such to determine in vivo tumor rejection (1).

IL-4, initially known as B cell stimulatory factor (2), has a pivotal role in conditioning type 2 T cell differentiation (3) leading to IgG1 and IgE isotype switching. In several studies, IL-4 showed in vivo tumor growth inhibition through a strong inflammatory effect associated with local recruitment of granulocytes, macrophages, T lymphocytes, and dendritic cells (4–7). Tumor rejection required polymorphonuclear cells, either eosinophils (8) or neutrophils (9), whereas the role of CD8 T cells remains controversial, since they have been found necessary in one study (6) but not in others (5, 10). IL-4–releasing tumor cell vaccines showed efficacy when used in preclinical therapeutic setting (5, 11), and pilot studies in cancer patients have been proposed (12, 13) and conducted (51).

The aim of this study was to test whether type 2 antitumor CD8 T cells are activated by IL-4–producing tumor cell vaccines and thus studying the mechanism by which they produce tumor eradication. The colon carcinoma C26/IL-4/FR\(\alpha\) cells, engineered to produce IL-4 and to express the human folate receptor \(\alpha\) (FR\(\alpha\)) as a model Ag, were irradiated and used to treat mice bearing C26/FR\(\alpha\) pulmonary metastases. We have recently shown that vaccination with C26/IL-4/FR\(\alpha\) elicits a strong anti-FR\(\alpha\) IgG1 response that is not correlated with the therapeutic outcome, thus suggesting that mechanisms other than the Ab response should be implicated (14). We show here that treatment with IL-4 vaccine induces noncytotoxic CD8\(^+\) T cells producing type 2 cytokines able to induce tumor rejection through interaction with host CD8\(^+\), AsGM1\(^+\), and GR-1\(^+\) cells.

Materials and Methods

Mice

Eight- to ten-week-old female BALB/c mice were purchased from Charles River (Calco, Italy); BALB/c IL-4 knockout (KO) and BALB/c IFN-\(\gamma\) KO mice were from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained and treated in compliance with institutional guidelines.

Immunotherapy by vaccinations with IL-4-producing C26/FR\(\alpha\) cells

C26/FR\(\alpha\), C26/IL-4/FR\(\alpha\), and C26/IL-12/FR\(\alpha\) cells producing murine IL-4 or IL-12 and expressing human FR\(\alpha\) were obtained by retroviral transduction, as described (14). For immunotherapy experiments, lung metastases were induced by the i.v. injection of \(10^6\) C26/FR\(\alpha\) cells, and mice were vaccinated on days 3, 6, 9, and 13, by injecting s.c. \(3 \times 10^6\) irradiated C26/IL-4/FR\(\alpha\) cells, producing 20–30 ng/ml IL-4 (10\(^6\) cells/ml/48 h). CD4 and CD8 T cell depletion was conducted by treating mice, on day 1 and every 2 wk thereafter, with mAbs (0.5 mg, i.p.) obtained from the hybridomas GK 1.5 and 2.43, respectively. For survival, follow-up mice were euthanized when showing dyspnea; survivors were shown to be tumor-free. Lymphocytes were collected from vaccinated mice 5–7 days after last vaccination.

1 Abbreviations used in this paper: FR\(\alpha\), folate receptor \(\alpha\); KO, knockout; MLTC, mixed lymphocytes tumor cell culture; LDA, limiting dilution analysis; Tc2, type 2 CD8\(^+\) T cells.

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Adoptive transfer assay

Recipient mice (5–10 mice/group) were i.v. injected with 7.5 × 10⁶ C26/FRα cells to induce lung metastases and treated on the following day by i.v. injection of fresh spleen cells (3.5 × 10⁹) obtained from donors vaccinated with C26/IL-4/FRα tumor cells, as described above, and treated with anti-CD4 mAb 3–4 days previously. CD8+ lymphocytes were enriched by elution on nylon wool columns (Wako Chemicals, Dusseldorf, Germany), and 5 × 10⁶ lymphocytes were transfected. The transfected population was shown to be 85–95% CD8+ by flow cytometric analysis. Recipient mice were sacrificed 2 wk after lymphocyte transfer, and the number of superficial lung metastases was counted after lung insufflation, as previously described (14). Recipient mice were depleted of lymphoid subpopulations by treatment with specific mAbs. T cell depletion was obtained by surgical thymectomy at 1 mo of age, followed by treatment with anti-CD4 and/or anti-CD8 i.v. 1 wk before the beginning of the experiment. Treatment with anti-AsialoGM1 rabbit antiserum (Wako Chemicals) and with anti-granulocyte mAb (RB6-8C5 hybridoma) was given 3 days before and on the day of lymphocyte transfer, at the dose of 10 µl and 0.5 mg, respectively. Ablating treatments were checked by FACS analysis and functional assays to verify target leukocyte depletion. Hybridomas were obtained from (American Type Culture Collection, Manassas, VA); mAbs were purified by using a commercial kit (E-Z-SEP; Amersham Pharmacia Biotech., Uppsala, Sweden).

Cytokine production

Lymphocytes obtained from mice vaccinated with C26/IL-4/FRα or with C26/IL-12/FRα cells were restimulated in mixed lymphocyte tumor cell cultures (MLTC) with C26/FRα irradiated cells for 6 days, as described (15). CD4+ and CD8+ cells were then positively selected by paramagnetic beads conjugated with anti-CD8a mAb (Miltenyi Biotec, Bergish Gladbach, Germany) and tested for cytokine production by culturing lymphocytes (10⁶/well) in 96-well flat-bottom plates precoated or not with 0.5 µg/well of anti-CD3 mAb (145-2C11 hybridoma) at 37°C for 20 h. Supernatants were assayed for cytokine content by specific ELISAs (PharMingen, San Diego, CA).

Cell-mediated cytotoxicity and precursor (p) CTL determination in limiting dilution analyses (LDA) assay

CTL response was assessed in a standard 4-h ⁵¹Cr-release assay after 6 days MLTC, as described (15). For LDA, lymphocytes were serially 1:2 diluted (from 8 × 10⁶/well to 500/well) and cultured with 15,000 rad irradiated C26/FRα cells (5 × 10⁵/well), 2000 rad irradiated syngeneic splenocytes as feeder cells (5 × 10⁵/well), and 50 U/ml rhIL-2 (Chiron-Italy, Milan, Italy), in 32 identical replicates, in 96 U-well plates for 10 days. ⁵¹Cr-release assays were performed with 10⁴ target cells/well. Specificity for C26/FRα was evaluated by testing replica plates with unrelated target cells. Values of lysis exceeding three times the SD of the mean spontaneous release were used as a threshold for scoring positive cytolyis.

Calculation of the frequencies from limiting dilution data was done as previously described (16). The same method was used for calculation of frequencies of IL-4 and IFN-γ-producing precursors; in this case, after 18 h of incubation with unlabeled target cells, supernatantans were tested for cytokine content by specific ELISA. The experiments were conducted by directly comparing lymphocytes from mice treated with IL-4 vaccine or with IL-12 vaccine.

Flow cytometry for cytokines

Lymphocytes from MLTC were cultured for 18 h with immobilized anti-CD3 mAb and 10 µg/ml brefeldin A added for the last 2 h (Sigma, Milan, Italy). Cells were suspended in PBS containing 1% FCS, fixed with paraformaldehyde 4% for 20 min at room temperature, and permeabilized with saponin buffer containing saponin 0.5% and 0.1% NaCl (Sigma) for 10 min. For triple staining, FITC-labeled anti-IFN-γ, PE anti-IL-4, and Cy-Chrome anti-CD4 mAbs were used; for double staining, purified CD8+ cells were labeled with FITC anti-CD8, PE anti-IL-4, and PE anti-IFN-γ. Iso-type-matched conjugated mAbs were used for background determination. mAbs were purchased from PharMingen. Cytofluorometric analysis was performed with a FACScan (Becton Dickinson, Mountain View, CA). mAbs dilutions were established by preliminary dose/response experiments, and 50,000 events were acquired.

Results

Immunotherapy with IL-4-producing C26 tumor cell vaccines prolongs survival of mice bearing lung micrometastases, and the therapeutic effect is T cell-dependent

Active immunotherapy with C26/FRα cells engineered to produce IL-4 increased survival and cured 33% of mice bearing C26/FRα lung metastases (Fig. 1, and Ref. 14). The therapeutic effect was dependent on host T cells, since it was abrogated in mice depleted of CD4 or CD8 T cells by in vivo treatment with specific mAbs (Fig. 1).

C26/IL-4/FRα vaccination induces type 2 T cell polarization

We analyzed the type of T cell response activated by vaccinations with C26/IL-4/FRα cells by assessing cytokine production by ELISA and by flow cytometry. Splenic lymphocytes obtained 5–7 days after the course of vaccinations were restimulated in MLTC with C26/FRα cells for 6 days; CD4+ and CD8+ T cells were then purified and cytokine production examined after stimulation with anti-CD3 mAb for 18 h. The pattern of cytokines produced by either CD4+ (Fig. 2, upper panel) or CD8+ lymphocyte subsets (Fig. 2, lower panel) indicated that treatment with an IL-4-releasing cell vaccine induced polarization toward the type 2 phenotype in both CD4+ and CD8+ T cells. In fact, IL-4, IL-5, and IL-10 were induced in both CD4+ and CD8+ T cells
were measured at higher levels and IFN-γ at lower quantity, compared with T cells from mice vaccinated with C26/FRα cells engineered to produce IL-12, included in this analysis as a control of type 1 T cell activation (15–17). The analysis of cytokines produced by T cells from lymph nodes draining the vaccination site showed similar results (data not shown).

Intracellular staining of purified CD4+ T cells showed a higher number of cells producing IL-4 rather than IFN-γ in IL-4-vaccinated mice (Fig. 3A), whereas an opposite pattern of expression was found in mice treated with IL-12 vaccine (Fig. 3B). In addition, these data indicated that IL-4 and IFN-γ were expressed by distinct cells in IL-4-vaccinated mice. Similarly, in CD8+ T lymphocytes, the frequency of IL-4-producing cells was higher than that of IFN-γ-producing cells (Fig. 3, C and D).

**CD8 lymphocytes activated by IL-4 vaccine lack cytotoxic activity and produce IL-4 upon interaction with tumor target cells**

Type 2 CD8+ T cells activated by C26/IL-4/FRα were tested for CTL activity against the tumor. Absence or low levels of C26/FRα tumor cell lysis were detected in short-term cytotoxicity assays (Fig. 4), a result confirmed by testing the frequencies of anti-C26/FRα pCTL in LDA assays. In fact, the frequencies of pCTL against C26/FRα in IL-4-vaccinated mice were shown to be 5- to 10-fold lower than in lymphocytes from IL-12-vaccinated mice in three different assays (Table I).

The same LDA was used to determine the frequencies of CD8+ lymphocytes producing cytokines upon recognition of tumor target cells (16). In this assay, to avoid any bias due to CD4-secreted cytokines, we used lymphocytes depleted of CD4+ T cells. In lymphocytes from IL-4-vaccinated mice, the frequency of CD8+ T cell precursors producing IL-4 and specific for C26/FRα was 1/1700, whereas the frequency of those producing IFN-γ was 1/20,345; in the same experiment, lymphocytes from IL-12-vaccinated mice showed a frequency of 1/207,597 and 1/6006 cells producing IL-4 and IFN-γ, respectively (Table I).

**Noncytotoxic type 2 CD8 are endowed with in vivo antitumor capacity**

To test whether type 2 CD8+ T cells (Tc2) may induce tumor rejection, splenic lymphocytes from C26/IL-4/FRα-vaccinated mice were depleted of CD4 cells, and CD8+ lymphocytes were purified or not before being adoptively transferred into syngeneic recipients bearing C26/FRα lung metastases. Two weeks after lymphocyte transfer, recipient mice were sacrificed to count superficial lung metastatic nodules. Splenocytes depleted of the CD4+ T cell population from IL-4-vaccinated mice completely eliminated lung metastases (mean number of metastases, 1.6 ± 0.7), whereas lymphocytes from C26/FRα tumor-bearing or from naive donors did not affect the number of metastatic nodules compared with untreated controls (169 ± 38, 139 ± 39, and 147 ± 46). The transfer of CD8+ cells, purified from the CD4-depleted splenocytes, determined >85% reduction of metastases (24 ± 7), while lymphocytes from vaccinated donors depleted of both CD4 and CD8 cells induced 60% reduction of lung metastases (52 ± 13) (Fig. 5A). These data indicated that adoptively transferred Tc2 cells can eliminate lung metastases, and that their therapeutic activity is enhanced by coadministration of uncharacterized splenocyte populations not belonging to CD4+ cells.

To assess the role of IL-4 production in the antitumor effect of the transferred lymphocytes, syngeneic IL-4 KO mice were used as lymphocyte donors. C26/IL-4/FRα vaccination of IL-4 KO mice induced CD8+ T cells producing the same level of IFN-γ as the wild-type vaccinated counterparts, but no IL-4 in response to stimulation with C26/FRα tumor cells (data not shown). The transfer of IL-4 KO-purified CD8+ T cells was ineffective (Fig. 5B), indicating that IL-4 production by the infused lymphocytes is needed for mediating the antitumor effect. In contrast, the transfer of lymphocytes from IFN-γ KO-vaccinated donors significantly reduced the number of metastases, thus ruling out an effect of IFN-γ-producing lymphocytes.
CD8+ cells within the Tc2 population in the rejection of metastases (Fig. 5C).

**Tc2 lymphocytes eliminate metastases by interacting with host CD8 T cells, NK cells, and granulocytes**

To investigate the host lymphoid effectors enrolled in the mechanism of tumor rejection, recipient mice were depleted of different leukocyte populations by treatment with specific mAbs before lymphocyte transfer. When recipients were depleted of CD8 T cells or of AsGM1-positive cells, the eradication of metastases was abolished, supporting a fundamental role of host CD8 and NK cells that are likely activated by transferred Tc2 cells, to eliminate lung metastases. Also, granulocyte depletion reduced the antimitastatic effect, whereas depletion of CD4 cells did not change the therapeutic effect of adoptively transferred Tc2 lymphocytes (Fig. 6). Thus, Tc2 lymphocytes eradicated lung metastases through the cooperation of bystander host CD8 T cells, NK cells, and granulocytes, but not CD4 lymphocytes.

**Discussion**

Initially shown for CD4 T cells, it is now clear that both CD4 and CD8 T cell subsets differentiate into Ag-specific type 1 and type 2 cells characterized by the production of IFN-γ or of IL-4 (18, 19). The IFN-γ or IL-4 conditioned cytokine milieu in which primary Ag stimulation takes place has a major role in directing the differentiation of CD8 T cells from naive T lymphocytes into either type 1 or type 2 effectors (20–23). Tc2 have also been isolated from PBL and mucosal tissues (24, 25) in healthy individuals and during infectious diseases (26, 27). Tc2 are cytotoxic in some systems (20, 22) but not in others (28, 29), can provide B cell help (29), induce delayed-type hypersensitivity reactions (30), and their type 2 phenotype is maintained in memory responses (22, 23). Although one of the first reports showing IL-4 production by CD8 lymphocytes dealt with tumor immune mice (31), no data are available about the presence and function of tumor-specific Tc2 cells. For this reason, after Tc2 cells were detected in mice treated with IL-4 vaccine, the present study was focused on the assessment of their antitumor potential.

Tc2 cells activated in mice treated with IL-4-vaccine were poorly cytolytic. When purified from MLTC and stimulated by anti-CD3 mAb, CD8+ cells from mice treated with IL-4 vaccine were shown to produce IL-4, IL-5, and IL-10, while IFN-γ was measured at lower quantities as compared with lymphocytes obtained from mice treated with the IL-12-producing vaccine. In addition, TNF-α and GM-CSF were released at levels similar between the two groups (data not shown). Intracellular immunostaining directly demonstrated the presence of IL-4-producing CD8+ cells. However, the general poor staining of intracellular IL-4 (32), did not allow to obtain a trustworthy quantitative data about the frequency of IL-4-producing CD8+ cells in lymphocytes from mice treated with IL-4 vaccine. To solve this difficulty, we set up LDA experiments that showed frequencies of IL-4-producing CD8+ cells were 20-fold higher than that of IFN-γ-producing cells and 20-fold higher than that of cytotoxic pCTL.

The adoptive transfer of enriched CD8+ cells from mice vaccinated with C26/IL-4/FRα cells in mice bearing C26/FRα lung metastases resulted in an 80–100% reduction in the number of tumor metastases.
nodules in four different experiments. Noncytolytic CD8 lymphocytes obtained from tumor infiltrate (TIL), as well as CD4 Th cells, have been reported to eradicate tumors upon adoptive transfer through their release of cytokines in different murine models (33–36) and in a recent pilot trial (37). Although the production of type 2 cytokines has not been evaluated in TILs (38, 39), the ability of a Th2-type CD4 T cell clone to initiate tumor eradication has been reported (40).

Our data underline a direct relationship between IL-4 production and the rejection of metastases. Tc2-secreted IL-4 may act through the enhancement of cytotoxicity of host CD8 T cells (41) and by activating eosinophils and basophils, in concert with IL-5 (42). In addition, the released TNF-α may synergize with IL-4 in activating endothelial cells, thus increasing vascular permeability and facilitating leukocyte infiltration, and by induction of NK cell activation (43). Moreover, IL-10 has a chemoattractant effect on CD8 T cells (44).

Depletion experiments clearly showed the participation of host CD8 T cells, asialoGM1-positive lymphocytes, and granulocytes in the process of metastases rejection. Although asialoGM1 is not expressed only on NK cells but also on a subset of CD8 T cells, these data indicate that all the mentioned leukocyte populations participate in the reduction of metastases.

The antitumor activity of Tc2 cells in adoptive immunotherapy can be potentiated by other donor leukocyte populations. In fact, injection of 35 × 10⁶ spleen cells depleted of CD4 lymphocytes reduced the number of metastases to a greater extent compared with injection of 5 × 10⁶ purified CD8⁰ cells, the estimated CD8⁰ content of such splenocytes (Fig. 6A). Whether antitumor Ab-producing B cells, activated macrophages, or granulocytes play the major role in this phenomenon remains to be determined. Whether Tc2 are endowed with antitumor activity similar to that of Tc1 cells was not evaluated in the present study. When tested in parallel, CD8⁰ lymphocytes obtained from mice treated with IL-4 vaccine or with IL-12 vaccine reduced metastases to the same extent upon adoptive transfer (data not shown). However, in the studied tumor model, IL-12-releasing vaccine showed a superior therapeutic effect compared with IL-4 vaccine treatment, probably because of the activation of a stronger Ab response (14). Mice cured after either vaccine treatment were immune to a subsequent tumor challenge injection, and either Tc2 or Tc1 CD8⁰ cells were shown when the memory response was assayed in vitro (data not shown).

The data presented here have a number of important implications for tumor immunotherapy. First, they indicate Tc2 cells as a new lymphocyte population endowed with antitumor potential. Second, they highlight the independence from a strict type 1 response for immune tumor rejection. The T1/T2 paradigm implicated that cytokines produced by T1 cells being primarily associated with cell-mediated immune response would augment T cell response against tumors, whereas type 2 cytokines, like IL-4 and IL-10, were considered immunosuppressive and therefore inhibiting antitumor responses (45). Recent reports suggest that the T1/T2 paradigm is not predictive of whether a particular pathway is protective or not (46, 47). In addition, the requirement for both T1/T2 paradigm is not predictive of whether a particular pathway is protective or not (46, 47). In addition, the requirement for both T1/T2 paradigm is not predictive of whether a particular pathway is protective or not (46, 47).

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