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_J Immunol_ 2005; 174:7194-7201; doi: 10.4049/jimmunol.174.11.7194

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IL-4-Transfected Tumor Cell Vaccines Activate Tumor-Infiltrating Dendritic Cells and Promote Type-1 Immunity

Junichi Eguchi,2* Naruo Kuwashima,2* Manabu Hatano,* Fumihiko Nishimura,*, Jill E. Dusak,† Walter J. Storkus,‡ and Hideho Okada3*‡§

We previously demonstrated that IL-4 gene-transfected glioma cell vaccines induce effective therapeutic immunity in preclinical glioma models, and have initiated phase I trials of these vaccines in patients with malignant gliomas. To gain additional mechanistic insight into the efficacy of this approach, we have treated mice bearing the MCA205 (H-2b) or CMS-4 (H-2k) sarcomas. IL-12/23 p40−/− and IFN-γ−/− mice, which were able to reject the initial inoculation of IL-4 expressing tumors, failed to mount a sustained systemic response against parental (nontransfected) tumor cells. Paracrine production of IL-4 in vaccine sites promoted the accumulation and maturation of IL-12p70-secreting tumor-infiltrating dendritic cells (TIDCs). Adoptive transfer of TIDCs isolated from vaccinated wild-type, but not IL-12/23 p40−/−, mice were capable of promoting tumor-specific CTL responses in syngeneic recipient animals. Interestingly, both STAT4−/− and STAT6−/− mice failed to reject IL-4-transfected tumors in concert with the reduced capacity of TIDCs to produce IL-12p70 and to promote specific antitumor CTL reactivity. These results suggest that vaccines consisting of tumor cells engineered to produce the type 2 cytokine, IL-4, critically depend on type 1 immunity for their observed therapeutic efficacy. The Journal of Immunology, 2005, 174: 7194–7201.

As a result of their profile of secreted cytokines, T cell responses have been frequently subdivided into two types, type 1 and 2, with the type 1 response being represented by predominant expression of IL-2 and IFN-γ and the type 2 response preferentially producing IL-4, IL-5, and IL-10 (1). Although IL-4 is generally regarded as an inducer of type 2 immunity for their observed therapeutic efficacy.

In comparing various cytokine gene therapies in the rat 9L gliosarcoma model, we observed that peripheral vaccination with IL-4-transfected 9L cells yielded the most effective therapeutic effects among the cytokines examined (i.e., IL-4, IL-12, GM-CSF, or IFN-α) (10). These results prompted our initiation of phase I clinical trials of vaccinations with autologous glioma cells engineered to produce IL-4 (10–12). Our preliminary analyses of underlying mechanisms critical to antitumor immunity induced by these vaccines suggested that remarkably high levels of IFN-γ were produced by splenocytes (SPCs), suggesting that this modality induced type 1 T cell responses (13).

In the present study, we tested our hypothesis that IL-4 GT promotes IL-12p70 expression from tumor-infiltrating APCs, which may play a critical role in the development of durable antitumor immunity. Because STAT4 and 6 are required for intracellular signaling following IL-12 and IL-4 stimulation, respectively (reviewed in Refs. 14 and 15), we examined and confirmed roles for both STAT4 and 6 in the effectiveness of IL-4 GT in our tumor model.

Materials and Methods

Animals

Female BALB/c, C57BL/6, and C57BL/6-background nude mice were purchased from Taconic Farms. BALB/c backcrossed STAT4 deficient (STAT4−/−) or STAT6−/− mice and C57BL/6 backcrossed IFN-γ−/−, IL-12/23 p40−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Vα14 NKT−/− mice with specific deletion of the Jα281 gene (16) were originally obtained from Dr. M. Taniguchi (Chiba University, Chiba, Japan), and maintained at University of Pittsburgh (Pittsburgh, PA). (17) Animals were maintained under aseptic conditions in microisolator cages in the Central Animal Facility at the University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol, and in accordance with recommendations for the proper care and use of laboratory animals.
Cell lines and culture
The MCA205 (H-2d) and CMS4 (H-2d) fibrosarcoma cell lines and YAC-1 lymphoma cells were cultured in complete medium (RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM t-glutamine (all reagents from Invitrogen Life Technologies) in a humidified incubator in 5% CO₂ at 37°C. The MCA205 and CMS4 tumor cells were transfected with retroviral vector DFG-mIL-4-neo, TFG-mIL-12-neo- or MFG-Neo, and stable transfectants were selected with G418 as previously described (10). Based on specific ELISA, the MCA205-IL-4 and CMS4-IL-4 cell lines secreted between 60 and 80 ng of IL-4 per 10⁶ cells per 48 h, whereas neither the MCA205-neo nor CMS4-neo control cell lines produced detectable levels of IL-4. NK cell-sensitive YAC-1 cells were used as target cells for assessing nonspecific killing in CTL assays.

Tumor establishment and protection models
To investigate the growth of tumors that were engineered to stably express transgene-derived cytokines (establishment model), C57BL/6, IFN-γ⁻/⁻, p40⁻/⁻, C57BL/6 mice received s.c. injections in the right flank with 2 × 10⁵ syngeneic MCA205-Neo, -IL-4, or -IL-12 cells. BALB/c, STAT4⁻/⁻, or STAT6⁻/⁻ mice received s.c. injections with 2 × 10⁶ syngeneic CMS4-Neo or CMS4-IL-4 cells. Tumor size was measured at least twice weekly using vernier calipers in a blinded fashion. Mice that were tumor-free in the establishment model on day 35 were rechallenged in the left (contralateral) flank with 2 × 10³ wild-type MCA205 tumor cells. Mice with ulcerated tumors or tumors larger than 30 mm in diameter were sacrificed. Results are displayed as mean tumor area (square millimeters) ± SD.

Tumor-infiltrating dendritic cells (TIDC) and CTL preparation
C57BL/6 mice were injected s.c. with 2 × 10⁵ MCA205-Neo or a mixture of 2 × 10⁵ MCA205-IL-4 cells and 2 × 10⁵ MCA205-Neo (MCA205-IL-4/Neo). On days 7 and 10 after the initial inoculation, MCA205-IL-4/Neo and MCA205-Neo tumors received intratumoral injections with 1 × 10⁶ MCA205-IL-4 or MCA205-Neo, respectively. On day 12, tumors were harvested and minced, and TIDCs were isolated using anti-CD11c-Microbeads, following the manufacturer’s instructions (MiniMacs; Miltenyi Biotec). This method was developed to obtain sufficient numbers of TIDCs, as syngeneic mice rejected s.c. inoculation of up to 2 × 10⁶ MCA205-IL-4 cells (our unpublished data). Greater than 90% of isolated cells expressed CD11c based on flow cytometry (data not shown). The TIDCs (2 × 10⁶ cells/ml) were incubated in the presence of 1000 U/ml GM-CSF and 1000 U/ml IL-4 for 48 h (Cell Sciences) and the concentration of IL-12 secreted between 30 and 40 ng IL-12p70 per 10⁶ cells per 48 h, whereas neither the MCA205-neo nor CMS4-neo control cell lines produced detectable levels of IL-4 or IL-12p70. NK cell-sensitive YAC-1 cells were used as target cells for assessing nonspecific killing in CTL assays.

Adoptive transfer of TIDCs
For adoptive transfer experiments, C57BL/6 mice or p40⁻/⁻ mice received s.c. injections with a mixture of 2 × 10⁵ MCA205-IL-4 and 2 × 10⁵ MCA205-Neo cells. When tumors reached average size of 5–8 mm in diameter, TIDCs were purified using anti-CD11c-Microbeads. Each of recipient C57BL/6 mice received 2 × 10⁵ TIDCs i.p. twice with a 1-wk interval. SCID mice were harvested from the recipient mice or control mock-injected mice on day 10 following the second adoptive transfer, and then stimulated in vitro with irradiated MCA205 tumor cells for 6 days in the presence of 30 IU/ml human IL-2. Standard 4-h ⁵¹Cr-release assays were performed using MCA205 or YAC-1 cells as target cells.

Statistical analyses
Comparative growth of s.c. tumors and T cell responses were assessed using a Student’s t test for two samples with unequal variances. Statistical significance was determined at the <0.05 level.

Results
Syngeneic fibrosarcoma cells stably transfected with IL-4 or IL-12 are rejected in immunocompetent mice
As an initial evaluation of the effects of paracrine cytokine production within the tumor microenvironment, fibrosarcoma cells were stably transfected with cDNAs for IL-4 or IL-12 by recombinant retroviral infection and were inoculated s.c. into syngeneic mice (Fig. 1). Because the overall goal of the study was to determine the role of type 1 and type 2 cytokines in IL-4 GT, we also included IL-12 GT as a prototypical type 1 cytokine model to compare with IL-4 GT in the initial sets of experiments. Although cytokine-gene transfection did not alter the growth rate of parental MCA205 or CMS4 cell lines in vitro (data not shown), in both C57BL/6 and BALB/c mice, IL-4 or IL-12 cDNA-transfected tumors were rejected, whereas mock-transfected tumors grew aggressively in all mice tested. These findings indicate that local production of IL-4 or IL-12 within the tumor microenvironment dramatically reduces the tumorigenicity of the parental sarcoma cells in vivo.

Rejection of IL-4 cDNA-transfected tumors is independent of endogenous IL-12/23p40 and IFN-γ
To assess the roles of host type 1 cytokines in the rejection of cytokine gene engineered tumors, C57BL/6-background (H-2d) IL-12/23 p40⁻/⁻ or IFN-γ⁻/⁻ mice received s.c. injections with 2 × 10⁵ MCA205 stably transfected to secrete IL-4 or IL-12. As shown in Fig. 2A, p40⁻/⁻ mice rejected both MCA205-IL-4 and MCA205-IL-12, suggesting that rejection was independent of endogenous IL-12 or IL-23 production. In contrast, as shown in Fig.

FIGURE 1. IL-4 or IL-12 gene transduction leads to the rejection of MCA205 and CMS4 tumor cells in syngeneic mice. A. C57BL/6 mice received s.c. injections of 2 × 10⁵ syngeneic MCA205-Neo (○), -IL-4 (●), or -IL-12 (△) cells (n = 5 per group). B. BALB/c mice received s.c. injections of 2 × 10⁵ syngeneic CMS4-Neo (□), CMS4-IL-4 (●), or CMS4-IL-12 cells (△) (n = 5/group). Tumor size was measured every 3 days in a blinded fashion. Mice with tumors exceeding 30 mm in diameter were sacrificed. Results are displayed as mean tumor area (square millimeters) ± SD for each group. Fractions of mice with palpable tumors are indicated by different symbols. These data are representative of three independent experiments performed.

A

B

C57BL/6

BALB/c

MCA205-Neo

MCA205-IL-4

MCA205-IL-12

CMS4-Neo

CMS4-IL-4

CMS4-IL-12
FIGURE 2. Neither IL-12/23 p40, IFN-γ, nor NKT cells are required for the efficacy of IL-4 GT; however, long-term, antitumor protection requires intact host T cell immunity. C57BL/6-background IL-12/23p40−/− (A) or IFN-γ−/− (B) mice received s.c. injections with 2 × 10⁵ syngeneic MCA205-Neo (□), MCA205-IL-4 (●), or MCA205-IL-12 cells (△) (n = 5 per group). C. Athymic (○) or NKT−/− (●) mice were challenged s.c. with 2 × 10⁵ MCA205-IL-4 (n = 5 per group). Tumor growth was monitored as indicated in Materials and Methods. Results are displayed as mean tumor area (square millimeters) ± SD for each group. Significance at 95% confidence limits is indicated. Fractions of mice with palpable tumors are indicated for each group. These data are representative of three independent experiments performed.

2B, IFN-γ−/− mice allowed the growth of MCA205-IL-12 tumors, suggesting that the antitumor effects associated with locally secreted IL-12p70 were at least partially dependent on the capacity of the host to produce IFN-γ. Interestingly, the growth of MCA205-IL-4, but not control MCA205-Neo tumors was suppressed in IFN-γ−/− mice (p = 0.0007 vs Neo), as only one of five mice with MCA205-IL-4 challenge had palpable tumors, suggesting that effective host immunity against MCA205-IL-4 was largely independent of endogenous IL-12 and IFN-γ production.

T cells, but not NKT cells, are required for rejection of IL-4 expressing tumors

To examine the immune cells responsible for the rejection of MCA205-IL-4 tumors, wild-type C57BL/6, NKT−/−, and C57BL/6-background athymic mice were injected with 2 × 10⁵ MCA205-IL-4 s.c. As shown in Fig. 2C, long-term observation of up to 60 days following tumor-challenge indicated that MCA205-IL-4 tumors failed to progress in NKT−/− mice, but eventually grew out by day 35 in athymic mice. This indicates that NKT cells were not responsible for the acute rejection of IL-4 expressing tumors. In contrast, in the nu/nu recipients, T cells appeared to be responsible for sustained, long-term antitumor immunity, although the delayed tumor growth in these mice suggested the potentially important role of alternate innate immune effectors, such as eosinophils, for the early phase of antitumor response (19, 20).

Endogenous IL-12/23p40 and IFN-γ production are required for long-term systemic immunity

To address the roles of endogenous IL-12/23p40 and IFN-γ in the IL-4 GT-induced generation and maintenance of long-term memory T cell-mediated immunity against parental MCA205, we re-challenged each of the animals that had rejected an initial inoculation of MCA205-IL-4 cells with a s.c. injection of 2 × 10⁵ parental MCA205 cells. Fig. 3A depicts the growth of parental MCA205 tumors in the flank of C57BL/6 wild-type or p40−/− mice that had previously rejected MCA205-IL-4 tumors. All wild-type mice rejected the rechallenged tumors, whereas parental MCA205 cells grew progressively in all of the primed IL-12/23 p40−/− (or IFN-γ−/−; data not shown) mice. To evaluate the profile of cytokine-responses in SPCs isolated from mice vaccinated with MCA205-IL-4 cells, SPCs were stimulated with irradiated MCA205 cells in vitro and assessed for their ability to produce IFN-γ and IL-4 as measured by specific ELISA (Fig. 3B). SPCs from IL-12/23p40−/− mice produced a severely reduced level of IFN-γ in comparison to wild-type SPCs, consistent with the inability of these animals to produce biologically active IL-12p70, a potent inducer of IFN-γ (21, 22). In contrast, IL-4 production levels by SPCs were comparable between wild-type, p40−/−, and IFN-γ−/− mice. Hence, although IL-12/23p40 and IFN-γ did not appear to have major roles in the initial rejection of IL-4-secreting tumor cells, these two cytokines are clearly important in supporting memory responses against the parental tumor.

IL-4 GT enhances the accumulation of TIDCs and their expression of IL-12p70

To investigate the role of IL-4 GT on APCs in the vaccine site, we analyzed TIDCs. In comparison to nonengineered tumors, the secretion of transgene-derived IL-4 within the vaccine site attracted enhanced numbers of CD11c+ TIDCs (Fig. 4A), that were competent to produce IL-12p70 (Fig. 4B). We also evaluated surface markers on freshly isolated CD11c+ TIDCs to assess their maturation status (Fig. 4C). Although the CD11c+ TIDCs demonstrated heterogeneous subpopulations with regard to the I-Aβ and CD86 markers in both groups, IL-4-transfected tumors contained a higher frequency of I-Aβhigh and CD86high cells (i.e., matured DCs) than the control tumors. These data suggest that IL-4 GT enhances the recruitment and maturation of IL-12p70 producing TIDCs in association with enhanced therapeutic efficacy.
Adoptive transfer of TIDCs promotes tumor-specific CTL response in recipient mice in an IL-12/23p40-dependent manner.

To further determine the functional roles of TIDCs within IL-4 GT treatment sites, TIDCs were isolated from IL-4-transfected tumors in wild-type mice or IL-12/23p40/H11002/H11002 mice and adoptively transferred into recipient C57BL/6 mice as a vaccine. The size of tumors and the density of TIDC obtained from wild-type or IL-12/23p40/H11002/H11002 mice were comparable, and the lack of IL-12p70 production in IL-12/23p40/H11002/H11002 mice-derived DC was confirmed (data not shown). Control mice received a mock i.p. injection with PBS. SPCs from the recipient mice were in vitro restimulated with MCA205 cells for 6 days and then subjected to standard 4-h 51Cr-release assays to evaluate the levels of specific CTL responses (Fig. 5C). TIDCs isolated from wild-type mice induced tumor-specific CTL responses against parental MCA205 (p < 0.05 at all E:T ratios vs p40/H11002/H11002 mice and mock-injected mice), whereas TIDCs isolated from MCA205-IL-4 tumor grown in IL-12/23p40/H11002/H11002 TIDC failed to expand any tumor-specific T cell responses. The lytic activities against MCA205 were considered to be tumor-specific as control target YAC-1 cells demonstrated only background levels of susceptibility to the killing effects of the SPCs. These data suggest that TIDCs and their ability to produce IL-12/23 are important for developing adaptive immunity against the parental tumor cells.

Host STAT4 and STAT6 are required for the antitumor effectiveness of IL-4 GT

Subsequently, we examined the role of STAT pathway using BALB/c-background STAT4/H11002/H11002 and STAT6/H11002/H11002 mice as recipients. Syngeneic CMS4 tumors grew aggressively and comparably in BALB/c, STAT4/H11002/H11002, and STAT6/H11002/H11002 mice (data not shown). To
evaluate the effect of local IL-4 expression on TIDC status in these mice. IL-4-transfected and nontransfected CMS4 fibrosarcoma cells were admixed and injected s.c., thereby allowing for the harvest of sufficient TIDC even in wild-type mice. As shown in Fig. 6A, tumors grew progressively in both STAT4+/−/− and STAT6+/−/− mice in comparison to wild-type mice (p < 0.022 for BALB/c vs STAT4+/−/−, p < 0.046 for BALB/c vs STAT6+/−/−), suggesting that both STAT4 and STAT6 are required for the induction of antitumor immunity by IL-4 GT. As depicted in Fig. 6B, TIDC secretion of IL-12p70 was moderately reduced in STAT4+/−/− mice, and severely diminished in STAT6+/−/− mice when compared with TIDC isolated from wild-type mice. We also evaluated the maturation status of TIDCs by flow cytometry (Fig. 6C). As was also the case in Fig. 4, the CD11c+ TIDC populations were heterogeneous in their composition. Nevertheless, there appeared to be a tendency for TIDCs isolated from STAT6+/−/− mice to display lower MHC class II (I-Ad) and CD86 levels than TIDCs harvested from wild-type mice or STAT4+/−/− mice.

FIGURE 5. Tumor-specific adaptive immunity requires TIDC-derived IL-12/23 p40. CD11c+ TIDCs from wild-type (□) or IL-12/23 p40−/− (■) mice were adoptively transferred into recipient C57BL/6 mice i.p. (2 × 10^5 TIDCs per mouse) twice on days 0 and 7. SPCs were then harvested from the recipient animals 10 days after the last TIDC injection (i.e., day 17) and were restimulated in vitro with irradiated MCA205 tumor cells for an additional 6 days in the presence of 30 IU/ml human IL-2. SPCs from mock-PBS injected mice (△) were used as negative control. Standard 4-h ^51Cr-release assays were performed using MCA205, or YAC-1 cells as target cells. Results are displayed as mean percent specific lysis + SD. A value of p < 0.05 at all E:T ratios for C57BL/6-TIDC vs other groups on MCA205 target. These data are representative of two independent experiments performed.

FIGURE 6. Both host-STAT4 and STAT6 are required for IL-4 GT-induced antitumor responses, with STAT6 playing a critical role in the activation of TIDC. A. BALB/c (□), STAT4+/− (○), or STAT6+/− (△) mice received s.c. injections with a mixture of 2 × 10^5 CMS4-Neo and 2 × 10^4 CMS4-IL-4 cells (n = 5/group). On days 7, 10, 14, and 21 after the initial inoculation, tumors were injected with 1 × 10^5 CMS4-IL-4 to provide a continuous level of local IL-4 production. Tumor size was measured every 3 days in a blinded fashion. Results are displayed as mean tumor area (square millimeters) ± SD for each group. Significance at 95% confidence limits is indicated. B. TIDCs were isolated from s.c. tumors using anti-CD11c MACS beads. Purified TIDCs were incubated for 48 h in the presence of GM-CSF and IL-4. Production of IL-12p70 in the supernatant was assessed by ELISA (p < 0.001 at BALB/c vs STAT4+/−, BALB/c vs STAT6+/−, and STAT4+/− vs STAT6+/−). C. TIDC surface expression levels of I-A^d and CD86 were determined on freshly isolated cells by flow cytometry. Numbers indicate mean fluorescence intensity levels. Broken lines indicate the same cells stained with isotype control Abs. These data are representative of three independent experiments performed.
Both type 1 and type 2 immune responses are required for the optimal efficacy of IL-4 GT

To further delineate the roles of type 1 and 2 cytokine responses in the effectiveness of IL-4 GT, we examined the CTL responses and cytokine production profiles from SPCs obtained from STAT4−/−, STAT6−/−, or wild-type mice, all of which had been previously inoculated s.c. with CMS4-IL-4 fibrosarcoma cells. As shown in Fig. 7A, and as expected, SPCs from STAT4−/− mice produced a reduced level of IFN-γ in comparison to the wild-type mice, while SPCs from STAT6−/− mice failed to produce detectable levels of IL-4. CTL assays using these SPCs demonstrated that both STAT4−/− and STAT6−/− mice mounted CTL responses of a reduced magnitude against CMS4 fibrosarcoma cells in comparison to wild-type mice (Fig. 7B). All these SPCs demonstrated only a background level of killing activity against YAC-1 cells, suggesting a CMS4-specific killing activity of the SPCs. These results suggest that the abrogation of antitumor efficacy in STAT4−/− and STAT6−/− mice (Fig. 6) may be due, at least partially, to the impaired development of tumor-specific CTL in these animals; and that host type 1 cytokine responses as well as type 2 responses play critical roles in the efficacy of IL-4 GT.

Discussion

The most significant findings in the present study are that local IL-4 production within the site of a tumor vaccine leads to the activation of TIDCs through a STAT6-dependent pathway, and that these activated TIDCs induce antitumor T cell responses in an IL-12p40/IFN-γ-dependent manner. The critical role of host-derived type 1 cytokines was further supported by the abrogation of IL-4 GT-induced antitumor effects in STAT4−/− mice.

Although long-term antitumor protection of animals required host type 1 and type 2 cytokines, our data in establishment models indicated that the initial rejection of IL-4 gene-transfected tumors was independent of host type 1 cytokines and NKT cells. We hypothesize that this early event was most likely mediated by eosinophils and macrophages as demonstrated by experiments using Abs that specifically blocked the accumulation of eosinophils at the site of IL-4-expressing tumors, as we (20) and others (19) have previously demonstrated. The role of the innate immunity against IL-4-expressing tumors is also suggested in our current study by the delayed growth of MCA205-IL-4 tumors in athymic mice.

In contrast to IL-4 GT, IL-12 GT was dependent on the ability of the host to produce IFN-γ. This result is expected given the known role for IL-12 as a major inducer of IFN-γ and IFN-inducible protein 10, both of which play major roles in IL-12 mediated antitumor responses (21, 22). Although NKT cells did not appear to be critical mediators or effectors in our IL-4 GT establishment model, antitumor immunity stimulated by vaccination with GM-CSF-secreting tumor cells has been reported to be abrogated in CD1d−/− and NKT cell−/− mice (23). In this referenced study, the lack of antitumor immunity was accompanied by impaired tumor-induced type 2 cytokine production, although IFN-γ secretion and cytotoxicity appeared to be preserved. We are currently evaluating whether IL-4 GT requires NKT cells for systemic, long-term antitumor responses.

Our results demonstrate the critical role of TIDCs that express IL-4 GT-induced antitumor effects in STAT4−/− mice.

**FIGURE 7.** Induction of optimal antitumor efficacy by IL-4 GT requires both STAT4 and STAT6. SPCs were harvested from BALB/c ( ), STAT4−/− ( ), or STAT6−/− ( ) mice immunized with IL-4-transfected CMS4 on day 25. A, SPCs were stimulated with irradiated CMS4 fibrosarcoma cells for 48 h in the absence of IL-2, and concentrations of IFN-γ and IL-4 in the culture supernatant were measured by ELISA. A value of p = 0.018 for BALB/c vs STAT4−/−, p = 0.022 for BALB/c vs STAT6−/−, and p = 0.012 for STAT4−/− vs STAT6−/− on IFN-γ ELISA. B, SPCs were further cocultured with irradiated CMS4 fibrosarcoma cells in the presence of 30 IU/ml human IL-2 from days 2 to 6. Standard 4-h 51Cr-release assays were performed using CMS4 or YAC-1 cells as target cells. Results are displayed as mean percent specific lysis ± SD. A value of p < 0.04 at all E:T ratio for BALB/c vs STAT4−/−, p < 0.05 at E:T ratio of 80 and 40 for BALB/c vs STAT6−/−, and p < 0.05 at all E:T ratios for STAT4−/− vs STAT6−/− on CMS4 target cells. These data are representative of three independent experiments performed. N/D, not detectable.
transfer experiments. It has been demonstrated that GM-CSF and CD40L-cotransfected C-26 tumors attract DCs that are able to stimulate specific CTL clones and to prime naive mice for a CTL response against the parental tumor (24). Our results also support the notion that tumor cell-based vaccines engineered to express DC-stimulating cytokines (i.e., GM-CSF or IL-4) are able to enhance the accumulation and activation of TIDCs that are critical for cross-priming tumor-specific T cell responses.

Our data also demonstrate the critical role of host-derived IL-12/23, particularly that expressed by TIDCs, in supporting the ability of IL-4GT to induce tumor-specific T cell-mediated immunity in vivo. Using bone marrow-derived DCs, it has been previously reported that the absence of IL-12/23 p40 does not affect the ability of vaccination with Ag-loaded DCs to elicit OVA-specific CTL cytotoxicity (25), which contrasts with our current results. Although this referenced study demonstrated a requirement for CD4+ Th cells and CD40L in vaccine effectiveness, immunizations with IL-12/23 p40−/− DC were performed using an H-2Kb-restricted peptide epitope in the absence of Th epitopes. Therefore, it is possible that this study may have underestimated the central importance of CD40-CD40L interactions in triggering DC of IL-12p70 (26). In contrast, in our present study, we presume that TIDCs have both engulfed tumor Ags and cross-presented both MHC class I and II epitopes to responder CD8+ and CD4+ T cells, respectively.

These data suggest that IL-4 GT relies upon host type 1 cytokine responses in mediating its antitumor effects. Indeed, there appears to be accumulating evidence that type 1 responses are promoted by IL-4 GT. IL-4−/− mice are severely impaired in their development of antitumor immunity to tumor lines that are typically rejected by control, wild-type animals (27). The lack of antitumor immunity in IL-4−/− mice was associated with reduced IFN-γ production and undetectable CTL activity, indicating that defective type 1 responses occur in the absence of endogenous IL-4 production. When IL-4 is provided in the vicinity of tumor cells, tumor immunity was induced in IL-4−/− mice, supporting a role for IL-4 in the priming phase of effector T cells (27). In a colon 26 tumor model, IL-4 GT has been reported to enhance IFN-γ, IL-12 p35, and p40 mRNA expression in the draining lymph nodes (28). Furthermore, mice deficient in the IFN-γ gene did not reject colon 26/IL-4 cells, suggesting that IL-4-induced generation of memory CTL requires IFN-γ production in the draining lymph nodes to generate protective immunity.

Based on our current study using both STAT4−/− and STAT6−/− mice, we propose the following mechanisms related to how IL-4 GT induces type 1 dependent antitumor immunity. First, local production of IL-4 in the tumor vaccine site activates TIDCs through STAT6-dependent pathways. Activated TIDCs become polarized to induce type 1 effector responses as demonstrated by their enhanced IL-12p70 production. In the induction of adaptive antitumor response, IL-12 and STAT4-mediated signals play pivotal roles as observed in our TIDC adoptive transfer experiments and the failure of IL-4 GT to induce antitumor immune responses in STAT4−/− mice.

Although STATs 1, 3, 4, and 5 have all been reported to be activated by IL-12, STAT4 appears to be the major player in IL-12-mediated responses (29) and STAT4−/− mice exhibit defective type 1 T cell proliferation, IFN-γ production, and NK cell activity in response to IL-12 stimulation (reviewed in Refs. 14 and 15).

STAT6 is required for IL-4R signaling and for the activation and differentiation of Ag-specific type 2 T cells (30). In our current study, TIDCs isolated from STAT6−/− mice demonstrated an immature phenotype and a diminished capacity for IL-12p70 production, which is in accordance with a previous study by Lutz et al. (31) demonstrating that STAT6−/− DC did not become matured or produce enhanced levels of IL-12p70 in response to IL-4 when they were cultured in low concentrations of GM-CSF.

Based on our results, we believe that STAT6−/− mice fail to reject the IL-4-transfected tumors for at least two reasons. First, as discussed above, at the level of Ag-presentation, STAT6−/− TIDCs fail to respond to local IL-4 stimulation, appear to remain functionally immature, and produce suboptimal levels of IL-12p70 required for the support of specific CTLs. Secondarily, STAT6−/− mice-derived SPCs do not secrete detectable levels of IL-4. Although Kachra et al. (32) demonstrated accelerated tumor rejection and augmented IFN-γ producing tumor-specific CTL activity in STAT6−/− mice, in our IL-4 GT model, we hypothesize that IL-4 production from T effector cells may be important for optimal antitumor effectiveness. Indeed, as has been demonstrated by others, tumor eradication resulting from IL-4 GT is not restricted to a type 1 response, but can also be mediated by a type 2-biased T cell response. Indeed, IL-4-transfected C26/FRx tumor cell vaccines induced antitumor type 2 effector T cells that released IL-4 upon specific stimulation, and these T cells were capable of mediating the rejection of lung metastasis (33). Interestingly, CD8+ T cells isolated from IL-4-vaccinated IFN-γ−/−, but not from IL-4−/−, mice were able to cure established lung metastases, supporting the conclusion that IL-4 produced by Tc2 cells was critical for tumor rejection in this model system (33). Anti-TS/A tumor memory immune responses induced by IL-4-transfected TS/A tumor cells were more potent than those elicited by IL-2 gene-transduced TS/A cells, with the former responses characterized as type 2 (34). Our previous studies also suggested that both type 1 and type 2 responses play coordinated roles in host protection against the rat 9L glioma (13, 35). Lastly, it has been demonstrated that STAT6-mediated type 2 responses attract eosinophils that possess potent cytolytic activity against tumor cells (36) and that the effective generation of antitumor CTLs requires IL-4 production from CD8+ T cells (37).

Overall, our study strongly suggests that the efficacy of IL-4 GT relies upon intact expression of both type 1 and type 2 cytokines by host immune cells. We believe our observations are clinically relevant because it has been well-documented that cancer patients, including glioma (38), display suppressed type 1 immune-reactivity (39, 40), but prevalent type 2 and/or regulatory type T cell responses against tumor Ags (41, 42). In addition, expression of IL-12 from PBMC is decreased in cancer patients (43), and patients with advanced metastatic cancers often present with significantly lower frequencies of circulating DCs and serum concentrations of IL-12 (44, 45). In this light, analyses of both type 1 and type 2 cytokine response status in patients undergoing IL-4 GT trials (10–12) are clearly warranted to determine those factors that are critically linked to immunological and clinical responses to this treatment modality.

Disclosures
The authors have no financial conflict of interest.

References


