Antigen-specific regression of established tumors induced by active immunization with irradiated IL-12- but not B7-1-transfected tumor cells

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Abstract

Transfection of modestly immunogenic tumors to express B7 family co-stimulator molecules results in their rejection by syngeneic mice, suggesting a possible clinical application in cancer patients. Immunization of naive mice with irradiated B7-1-transfected P1.HTR cells is sufficient to induce specific cytolytic T lymphocytes (CTL) and to protect against tumor challenge. However, patients to be treated will have an existing tumor burden; thus, preclinical models should examine therapeutic efficacy in an established tumor setting. Contrary to expectations, immunization of mice with irradiated B7-1-transfected P1.HTR cells had no impact on the growth of pre-established control-transfected tumors. Mice bearing control-transfected P1.HTR tumors successfully rejected living B7-1 transfectants on the contralateral flank, demonstrating the ability of tumor-bearing mice to respond to B7 co-stimulation. Inasmuch as IL-12 is another important factor for CTL maturation, P1.HTR transfectants expressing B7-1 and/or IL-12 were then constructed. Remarkably, regression of pre-established tumors was achieved following immunization with irradiated IL-12 transfectants, even without co-expression of B7-1. Rejection required a shared antigen with the tumor used for immunization, could not be reproduced with rIL-12 alone, depended on host T lymphocytes and correlated with a high IFN-\(\gamma\) producing T cell phenotype. In addition, IL-12-facilitated tumor rejection required co-operation with a CTLa-4 ligand provided by the host, and correlated with up-regulation of B7-1 and B7-2 on host antigen-presenting cells. Thus, active immunization in the established tumor setting is benefitted greatly by the provision of IL-12, which may recruit participation of sufficient B7 co-stimulation from the host that it need not be provided exogenously.

Introduction

The cloning and characterization of several human genes encoding antigens specifically expressed on tumor cells but not most normal cells has opened the way for the antigen-specific immunization of cancer patients (1–4). Immunization approaches will depend on the activation of class I MHC-restricted CD8\(^+\) T lymphocytes. Because of the complexities of T cell regulation, provision of cofactors and co-stimulator molecules necessary for differentiation into highly lytic effector cells may be a required component of tumor antigen immunization protocols. Because of many parallels with human tumors expressing defined tumor antigens, the well-characterized P815 tumor model is an ideal murine system for the study of anti-tumor immunization schemes (1,5–10). One advantage of this system is the availability of a highly transfectable variant of P815, P1.HTR (11).

Despite expression of recognizable antigens, tumors grow progressively in immunocompetent hosts, implying the existence of mechanisms for tumor escape from immune destruction. Several progressor tumor cell lines have been transfected to express the T cell co-stimulator molecule B7-1, resulting in their rejection by immunocompetent syngeneic mice (12–16). It therefore has been postulated that failure of tumor
rejection may be a consequence of the absence of B7 expression by tumor cells, which in turn results in the induction of tumor-specific T cell anergy and systemic tolerance. However, other mechanisms of tumor escape certainly exist. Numerous studies have suggested that transfection of tumors to express other immunomodulatory molecules also results in their rejection in vivo, leading to protection against tumor challenge (17–22).

We recently have focused on analyzing the immune response generated against immunogenic tumors that are naturally rejected, in order to identify cofactors and cytokines that participate in a successful anti-tumor response when it does occur, which in turn may indicate factors that are deficient in the case of progressive tumors. Natural rejection of tumor variants of P815 was blocked by a murine CTLA-4–murine IgG3 fusion protein (mCTLA-4/3), indicating that host B7 family co-stimulation was indeed necessary for generation of tumor-specific cytotoxic T lymphocytes (CTL) (23). Tumor rejection also was prevented by an anti-IL-12 antiserum, indicating the importance of host IL-12 for generating an anti-tumor response (24). In addition, rejection appeared to correlate with a high IFN-γ-low IL-4-producing T cell phenotype in draining lymph nodes, the generation of which also was prevented by IL-12 blockade, suggesting that a Th1/Th1- like cytokine profile was predictive of, and necessary for, successful tumor rejection (24). In addition, primary generation of anti-tumor CTL in vitro was optimized by provision of both B7-1 and IL-12 (25), the combination resulting in increased expression of cytotoxic granule-related serine esterase and a high IFN-γ-producing phenotype. Collectively, these results have implicated synergistic co-operation between B7-1 and IL-12 in the induction of a Th1/Th1-like T cell phenotype, which may be optimal for mediating tumor rejection. Ensuring appropriate activation towards this phenotype might therefore induce rejection of progressively growing established tumors, which otherwise might fail to elicit a Th1-like response.

In order to move toward a clinically applicable system, we have employed an immunization scheme employing irradiated rather than living tumor cell transfectants. We have observed previously that multiple immunizations with irradiated B7-1- but not B7-2-transfected P1.HTR cells was sufficient to induce tumor-specific CTL and protection against living tumor challenge in the majority of naive mice (23). However, in the present study, the same immunization approach had no effect on the growth rate of pre-established tumors. On the contrary, immunization with irradiated IL-12 transfectants did successfully induce regression of pre-established tumors, by a mechanism dependent upon B7 family co-stimulation from the host. Thus, provision of exogenous IL-12 may be more critical than supplying exogenous B7 during active immunization in the pre-established tumor setting. Our results are consistent with a model of incomplete differentiation toward a Th1/Th1 phenotype as a cause for failure to reject a progressor tumor.

Methods

Mice

DBA/2 mice were bred and housed in a specific pathogen-free animal facility at the Catholic University of Louvain or at the University of Chicago. Female mice 8–10 weeks of age were used for experiments.

Cells and transfectants

The P1 subclone of the DBA/2-derived mastocytoma P815, its highly transfectable variant P1.HTR and the multiple antigen loss variant P0.HTR were derived and cultured as described (7,11). P1 cells were used as targets for cytolytic assays and P1.HTR cells as recipients for transfection. The irrelevant syngeneic tumor L1210 was grown as described (25) and used as a negative control target in cytolytic assays. The NK-sensitive target YAC-1 was cultured in DMEM containing 10% FCS, 2-mercaptoethanol, and additional glucose and amino acids (25).

The cDNAs encoding murine B7-1, IL-12 p35 and IL-12 p40 were cloned by PCR as described (25). The IL-12 inserts were subcloned into pEF.BOS.puro for supertransfection into P1.HTR cells that had been transfected previously with empty pcDSSra (generating HTR.C) or pcDSSra.B7-1 (generating HTR.B7-1). HTR.C cells were used as control transfectants for in vivo experiments. Transfection using both IL-12 subunits or an empty vector control was performed by calcium phosphate precipitation as described (11) and selection was performed with puromycin (1 µg/ml). Drug-resistant cells were cloned by limiting dilution, screened for IL-12 secretion and matched for expression of class I MHC molecules (yielding HTR.C/C, HTR.B7-1/C, HTR.C/IL-12 and HTR.B7-1/IL-12). The chosen clones secreted ~1000 U/ml IL-12 activity per 10^6 cells in 24 h as measured by proliferation of murine concanavalin A blasts (26); control transfectants produced no detectable IL-12 activity. rIL-12 was produced as a histidine-tagged fusion protein in P1.HTR cells and purified by nickel chelate chromatography as described (24).

Immunization with irradiated tumor cell transfectants

Either naive DBA/2 mice or mice bearing a s.c. HTR.C tumor (5×10^5 cells on the left flank) were immunized. Cultured P1.HTR transfectants were washed three times with PBS, adjusted to 1×10^6 cells/ml and irradiated (10,000 rad). Mice received 50 µl per hind footpad (5×10^5 cells) via a 26-gauge needle each week for three consecutive weeks. Two weeks after the last injection, either splenocytes were stimulated in a mixed lymphocyte tumor culture (MLTC) or mice were challenged with a s.c. inoculum of 5×10^5 living HTR.C cells in 50 µl PBS. In the latter case, mice were scored for incidence of macroscopic tumor 4 weeks after the challenge, positive tumors were measured in two dimensions, and a mean and SEM for each experimental group were calculated.

Rejection of B7 transfectants in tumor-bearing mice

HTR.C cells (5×10^5) were implanted s.c. on the right flank of multiple mice. At various times after (day 0, day 4, day 7 or day 14), HTR.B7-1 cells were implanted s.c. on the left flank. Both tumors were measured in two dimensions at least twice weekly, and a mean and SEM for each group of mice were calculated.

MLTC and cytolytic assays

Splenocytes (6×10^6) from treated DBA/2 mice were stimulated with irradiated (5000 rad) HTR.B7-1 cells (2×10^5) in a volume
Immunization to induce regression of pre-established tumors

After 6 days, effector cells from three replicate wells were collected and washed, adjusted to $2 \times 10^6$/ml, and titrated in duplicate in V-bottom microtiter plates to give the indicated E:T ratios, along with 2000 $^{51}$Cr-labeled target cells (either P1, L1210 or YAC-1). Supernatants were collected after 4 h and radioactivity was measured using a 96-well plate $\gamma$-counter (Wallac Microbeta, Turku, Finland). Percent specific lysis was calculated as described (27).

IFN-γ assay

After a 6 day MLTC, effector cells were harvested and re-stimulated with P1 cells. After 24 h, supernatants were collected and levels of IFN-γ were assessed using a specific ELISA (the reagents having been kindly provided by Dr R. Schreiber, Washington University, St Louis, MO). Serial dilutions of test supernatants were compared to serial dilutions of a defined standard, and concentrations were calculated and expressed as U/ml.

Production of and treatment of mice with mCTLA-4γ3

A fusion protein between the extracellular domain of murine CTLA-4, and the hinge, CH2 and CH3 domain of a murine IgG3 isotype mAb (C3110) was constructed (generating mCTLA-4γ3) as described (23). The IgG3 isotype was chosen to minimize binding to FcγRs, thus eliminating potential artifacts derived from cross-linking of B7 family molecules on the cells which express them. For preparation of large quantities of mCTLA-4γ3, P1.HTR cells expressing this protein were cultured in 10 l batches. Supernatant was harvested, cells were cleared by centrifugation and then ultracentrifugation, and purification was performed by binding to Protein A-Sepharose, elution at pH 3.0 and dialysis against PBS. Yield was generally 1–2 mg/l of supernatant. Purification of control IgG3 was similarly performed from C3110 supernatant.

For treatment of mice, purified mCTLA-4γ3 and control IgG3 were adjusted to 100 µg/ml in PBS. Mice received 1 ml i.p. of either of these reagents on days 0, 1, 2, 4, 7, 10, 14, 17, 21 and 24. Tumor cells were implanted in the flank a few hours after the first injection on day 0. No apparent toxicities were observed. In other experiments, HTR.C cells were implanted s.c. first and administration of mCTLA-4γ3 or control IgG3 was initiated 1 h prior to immunization with irradiated HTR.IL-12 cells, according to the same schedule. In some experiments, T cells were depleted by administration of anti-Thy-1 mAb (A6-703-A8) in the form of 1 ml of ascites on day –2 followed by 0.5 ml weekly.

Flow cytometry (FCM)

Cells were prepared from the right popliteal lymph node of mice that had received HTR.C/C or HTR.C/IL-12 cells ($1 \times 10^9$) 4 days earlier. Staining was performed with directly coupled mAb specific for B7-1 or B7-2 (PharMingen, San Diego, CA) and biotinylated anti-MAC-1 produced in our laboratory, followed by streptavidin–phycoerythrin or streptavidin–FITC. Gates were set using second-step reagents alone. Data were collected on 20,000 viable cells based on forward light scatter and side scatter using a FACSScan, and processed via CellQuest software (Becton Dickinson, Mountain View, CA).

Results

Immunization of mice with irradiated B7-1 transfectants has no effect on the growth of pre-established tumors

In order to more closely approximate the clinical scenario with cancer patients, an immunization model of P1.HTR tumor-bearing rather than naive mice was employed. Given the prior successes of immunization with irradiated B7-1-transfected P1.HTR cells in naive mice (23), these transfectants were the first to be examined in a pre-established tumor setting. Control-transfected P1.HTR cells (HTR.C) were implanted s.c. on the flank, and beginning on day 7, mice were immunized with irradiated B7-1 or control transfectants in both hind footpads on a weekly schedule. As shown in Fig. 1, none of these immunization approaches had any impact on the progressive growth of pre-established tumors.

Mice bearing large P1.HTR tumors can reject living B7-1 transfectants

It was conceivable that immunization in the pre-established tumor setting failed because of an inability of T cells from tumor-bearing mice to respond to B7 co-stimulation, perhaps because of clonal anergy having been induced. To this end, living HTR.C cells were implanted s.c. on the right flank of several groups of seven mice each. At various times later, living HTR.B7-1 cells were implanted s.c. on the left flank. As shown in Fig. 2(A), the growth rate of the original HTR.C implant was comparable between groups. Interestingly, even in mice in which an HTR.C tumor had been pre-established for 2 weeks (thus generating a tumor size of ~1 cm), the B7-1 transfectant was readily rejected...
Immunization to induce regression of pre-established tumors

Experiment 1: A: Original implant

![Graph A: Original implant](image)

B: B7+ challenge

![Graph B: B7+ challenge](image)

Experiment 2: C: Tumor-bearing mice

![Graph C: Tumor-bearing mice](image)

D: Normal mice

![Graph D: Normal mice](image)

Fig. 2. Experiment 1. Tumor-bearing mice can reject living B7-1-transfectants. (A) Five groups of seven DBA/2 mice each received $5 \times 10^5$ living HTR.C cells s.c. on the right flank. Tumor measurements were performed twice per week as described in Methods. At each time indicated by the arrows, all mice of one group were challenged with living HTR.B7-1 cells ($5 \times 10^5$) on the left flank or with nothing (Control). (B) Bidimensional measurements were performed of HTR.B7-1 tumors that had been implanted at various times after implantation of HTR.C tumors from panel (A). These are compared to the growth curve of HTR.B7-1 tumors in naive mice (Control). Experiment 2. Dependence of rejection of HTR.B7-1 in tumor-bearing mice on T cells. (C) Two groups of seven mice each received living HTR.C cells ($5 \times 10^5$) s.c. on the right flank. Beginning on day 12, mice were treated either with anti-Thy-1 mAb (circles) or PBS (squares); on day 14, all were challenged with HTR.B7-1 cells ($5 \times 10^5$) on the left flank. Measurements of both tumors (original implant, open symbols; HTR.B7-1 challenge, solid symbols) were performed as above. (D) Two groups of seven naive mice were treated either with anti-Thy-1 mAb (circles) or PBS (squares). Two days after the first injection, each mouse was challenged with HTR.B7-1 cells ($5 \times 10^5$) s.c. on the left flank and tumor measurements were performed twice per week.

Immunization of naive mice with irradiated P1.HTR transfectants expressing B7-1 and/or IL-12

It was conceivable that an IL-12-dependent, Th1-like phenotype might be better equipped to penetrate and eliminate an established tumor. Given the known co-operation between B7-1 and IL-12 in CTL maturation, it seemed logical to provide both B7-1 and IL-12 during active immunization in vivo. P1.HTR cells were co-transfected to express B7-1 and/or IL-12; control cells and single expressors were super-transfected with the appropriate empty vector controls (thus generating HTR.C/C, HTR.B7-1/C, HTR.C/IL-12 and HTR.B7-1/IL-12). Living transfectants expressing either B7-1 or IL-12 were rejected when implanted s.c. in naive DBA/2 mice (data not shown). All rejecting mice developed P815-
Immunization to induce regression of pre-established tumors

**Table 1. Protection after immunization with irradiated transfectants expressing B7-1 and/or IL-12**

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Fraction without tumor</th>
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<tbody>
<tr>
<td>PBS</td>
<td>1/10</td>
</tr>
<tr>
<td>HTR/C</td>
<td>2/10</td>
</tr>
<tr>
<td>HTR.B7-1/C</td>
<td>9/10</td>
</tr>
<tr>
<td>HTR.C/IL-12</td>
<td>9/10</td>
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<tr>
<td>HTR.B7-1/IL-12</td>
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Mice were immunized by three weekly injections of $5 \times 10^6$ irradiated P1.HTR transfectants or PBS in each hind footpad and challenged 2 weeks after the last injection with $5 \times 10^5$ living P1.HTR cells s.c. The fraction of tumor-free mice is indicated 4 weeks after the challenge. Normal mice develop a measurable tumor in $<1$ week. Values considered to be significantly different from controls are in italic. Similar results were obtained in two independent experiments.

**Immunization of tumor-bearing mice with irradiated P1.HTR transfectants**

Immunization with either irradiated B7-1-expressing or IL-12-secreting P1.HTR cells was next attempted in mice bearing pre-established tumors. Control-transfected P1.HTR cells were implanted s.c. in the left flank and 6 days later weekly immunization in the hind footpads with irradiated P1.HTR transfectants was initiated. As observed in previous experiments, immunization with irradiated control transfectants or B7-1 transfectants had no impact on the growth rate of pre-established HTR.C tumors. In contrast, immunization with irradiated IL-12 transfectants resulted in a marked slowing of tumor growth in all mice and complete rejection in 80%. Immunization with irradiated B7-1/IL-12 double transfectants also led to regression of established tumors (data not shown), but expression of IL-12 alone was clearly sufficient. When mice that rejected pre-established tumors were rested for 2 weeks and re-challenged again with HTR.C cells, all mice demonstrated protective immunity (Fig. 3B).

We next examined the effect of immunization with IL-12 transfectants on larger pre-established tumors. As shown in Fig. 4, even when HTR.C tumors were pre-established for 2 weeks (thus generating a large tumor of approximately 1 cm in diameter), weekly immunization in the hind footpads with irradiated IL-12 transfectants markedly slowed tumor growth. The fraction of mice completely rejecting a pre-established tumor decreased as the delay before the first immunization was prolonged. Immunization schedules with injections more frequent than once per week were not attempted.

Because the pre-established tumors were implanted s.c. on the flank, it was conceivable that immunization via the hind footpads was having a potent effect because of being in continuity with a draining lymph node site of the flank region. We therefore compared i.p. injection to footpad injection. As shown in Fig. 5 (Experiment 1), systemic immunization with irradiated IL-12 transfectants via i.p. injection was at least as effective as immunization via the footpad route.

**Specific CTL activity detected from splenocytes after a 6-day MLTC**

Naive syngeneic mice were then immunized by three weekly injections of irradiated transfectants in the hind footpads and challenged with living HTR.C cells. As shown in Table 1, immunization with irradiated transfectants expressing either B7-1 or IL-12 was sufficient to prevent growth of control-transfected tumors in the majority of mice, demonstrating protective immunity.
Immunization to induce regression of pre-established tumors

**Fig. 4.** Effect of immunization with irradiated IL-12 transfectants on mice bearing larger established tumors. Groups of seven mice each were inoculated s.c. with HTR.C cells and either 7 days (A), 10 days (B) or 14 days (C) later they were immunized weekly with irradiated HTR.C/C cells (open squares) or HTR.C/IL-12 cells (solid circles) in the hind footpads. Tumor sizes were measured as above and the fraction of mice completely rejecting is indicated at the end of each curve.

**Fig. 5.** Experiment 1. Efficacy of i.p. immunization and lack of effect of rIL-12 on growth of established HTR.C tumors. Groups of 10 mice each were inoculated s.c. with living HTR.C cells and beginning on day 7 they were immunized weekly (arrows) as indicated: irradiated HTR.C cells i.p. (open squares), irradiated HTR.C/IL-12 cells i.p. (solid circles) or rIL-12 (1 µg each footpad) for the first 4 days of each week. Other regimens for rIL-12 injection gave similar results, including 2 µg i.p. on day 1 only or on days 1–4. Experiment 2. Tumor specificity of immunization with irradiated IL-12 transfectants. Groups of 10 mice each were inoculated s.c. with living HTR.C cells (A) or L1210 cells (B). At the indicated times (arrows), mice were immunized with irradiated HTR.C/C cells (open squares) or irradiated HTR.C/IL-12 cells (closed circles) and tumors were measured as above. Similar results were obtained in two experiments.

any of the five defined P815 tumor antigens. As depicted in Fig. 5 (Experiment 2), although the growth rate of pre-established L1210 tumors was slightly delayed by immunization with irradiated HTR.C/IL-12 cells (Fig. 5B), clearly no mice completely rejected the tumor and the effect was marginal compared to the marked growth retardation and rejection seen with HTR.C cells (Fig. 5A).

The characterization of specific tumor antigens expressed by P1.HTR cells allowed a further investigation into the specificity of the rejection response. P0.HTR cells, which were selected to lack expression of the four major P815 tumor antigens (S), were implanted s.c. and the mice were immunized with irradiated HTR.C/IL-12 cells. As shown in Fig. 6(A), immunization had no effect on the growth rate of P0.HTR tumors, whereas HTR.C tumors were rejected by the majority of treated mice. To pursue this issue further, L1210 cells were transfected to express the major P815 tumor antigen gene, P1A, or with a control vector. These cells failed to form measurable solid tumors when implanted s.c., but rather metastasized widely and killed recipient mice. Nonetheless, immunization with irradiated HTR.C/IL-12 cells was capable of preventing death in the majority of mice bearing L1210.P1A but not L1210.C tumors (Fig. 6B). Collectively, these results argue for an antigen-specific component to successful immunization with irradiated IL-12-transfected P1.HTR cells.
Immunization to induce regression of pre-established tumors

**Fig. 6.** Effect of immunization with IL-12-transfected P1.HTR cells on established P0.HTR and L1210.P1A tumors. (A) Groups of 10 mice each were inoculated s.c. with living HTR.C cells (squares) or P0.HTR cells (circles). At the indicated times (arrows), mice were immunized with PBS (open symbols) or irradiated HTR.C/IL-12 cells (closed symbols) and tumors were measured as above. The fraction of mice completely rejecting is indicated at the end of each curve. (B) Groups of 10 mice each were inoculated s.c. with living L1210.C or L1210.P1A cells. Mice were immunized weekly with PBS or irradiated HTR.C/IL-12 cells and tumor-free survival was assessed 4 weeks after tumor implantation. Similar results were obtained in two experiments.

**Fig. 7.** Effector cell phenotype induced by immunization of naive mice with irradiated P1.HTR transfectants expressing B7-1 or IL-12. Naive DBA/2 mice were immunized with irradiated HTR.B7-1/C cells (solid lines) or irradiated HTR.C/IL-12 cells (dotted lines) as described in Methods and splenocytes were re-stimulated in a 6 day MLTC. Lysis from individual mice was assessed against P815 cells (A) or YAC-1 cells (B) or re-stimulation with P815 cells was performed to assess for IFN-γ release (C). Specific lysis of control L1210 cells was ~15% under all conditions. Production of IFN-γ in the absence of P815 cells was ~20 U/ml. Shown are results from four representative mice from each group; similar results were obtained with another three mice per group.

Immune phenotype induced by immunization with irradiated B7-1 versus IL-12-transfected P1.HTR cells

Naive DBA/2 mice were immunized in the hind footpads with three weekly injections of irradiated P1.HTR transfectants, and specific CTL activity was assessed from splenocytes following a 6 day MLTC. As shown in Fig. 7(A), immunization with irradiated transfectants expressing either B7-1 or IL-12 was sufficient to generate anti-P815 CTL activity in 100% of mice. Lysis against L1210 control cells was ~15% at all effector:target ratios, demonstrating specificity (data not shown). Immunization with irradiated control transfectants resulted in specific CTL in only ~20% of mice (data not shown and 23). Since IL-12 is also known to facilitate activation of NK cells (28), lysis against the NK-sensitive target YAC-1 also was assessed. Not surprisingly, immunization with irradiated IL-12 transfectants resulted in substantially greater NK activity than immunization with irradiated B7-1 transfectants (Fig. 7B). P815 and L1210 are known to be relatively NK resistant. When effector cells were harvested after MLTC and re-stimulated with P815 cells, nearly two orders of magnitude greater IFN-γ was produced with IL-12-facilitated immunization compared to B7-1-facilitated immunization (Fig. 7C). In parallel experiments, similar high levels of IFN-γ were observed following stimulation with anti-CD3 mAb, consistent with T cells being the source of IFN-γ production (data not shown and 24). Immunization with combined B7-1/IL-12
transfectants gave results similar to those obtained with the IL-12-only transfectants (data not shown). Thus, the improved efficacy of immunization with irradiated IL-12-transfected P1-HTR cells may be related to the co-operative participation of NK cells, the generation of a high IFN-γ-producing T<sub>H</sub>1/T<sub>C</sub>1 phenotype or both.

IL-12-mediated tumor rejection depends on synergy with a host CTLA-4 ligand

Because of the dependence of natural rejection of immunogenic tumors on an endogenous CTLA-4 ligand (23), it was surprising in the current model that immunization with transfectants expressing IL-12 alone was sufficient to result in such potent anti-tumor immunity in vivo. It was reasoned that the immune response facilitated by IL-12-only-transfectants might indeed require co-operation with a B7 family co-stimulator molecule, but that it may have been provided endogenously by host accessory cells. To test this hypothesis, mice inoculated s.c. with living HTR.C/IL-12 cells were treated from day 0 with mCTLA-4γ3 to block host B7 family molecules and tumor size was monitored over time. As shown in Fig. 8A, the IL-12 transfectants were readily rejected s.c.; control transfectants grew progressively (data not shown). Administration of mCTLA-4γ3 but not of control IgG3 prevented this rejection and resulted in progressive tumor growth in all mice. In addition, depletion of T cells with anti-Thy-1 mAb also prevented rejection (Fig. 8A). The dependence of rejection on T cells makes it unlikely that the reported anti-angiogenic property of IL-12 is solely responsible for the anti-tumor effect of IL-12 (29).

Because a host CTLA-4 ligand was required for rejection of IL-12-transfected P1-HTR cells, it was conceivable that the presence of IL-12 may have resulted in increased expression of B7 molecules on host accessory cells. To address this possibility, living HTR.C/IL-12 cells were implanted in the right hind footpad of DBA/2 mice, and the draining popliteal lymph node cells were analyzed 4 days later for expression of B7-1 and B7-2. Preliminary one-color FCM analysis revealed a subtle increase in the percentage of B7-1<sup>+</sup> and B7-2<sup>+</sup> cells, and also of MAC-1<sup>+</sup> cells, in lymph node cells from mice receiving IL-12 transfectants (data not shown). Two-color analyses were then performed to examine co-expression of both MAC-1 and B7-1 or B7-2. As shown in Fig. 9, normal lymph node cells contained a small number (0.2%) of MAC-1<sup>+</sup>/B7-1 double-positive cells, which was increased 7-fold (1.4%) in the presence of IL-12-transfected but not of control-transfected P1-HTR cells. B7-1 expression on MAC-1<sup>+</sup> cells was less significantly altered (2.7 to 3.3%). In contrast, an increase in the number of B7-2<sup>+</sup> cells was seen in both the MAC-1<sup>+</sup> (0.8 to 3%) and MAC-1<sup>−</sup> (17 to 42%) subpopulations. These changes were seen reproducibly in three experiments, with two or three independently analyzed mice per group per each experiment. Inasmuch as the absolute number of lymph node cells increased ~4-fold during the 4 days of tumor presence (data not shown), the increase in B7<sup>−</sup> cells could be secondary to influx of new cells, proliferation of B7<sup>+</sup> cells already present or induction of increased expression of B7-1 and B7-2 on those cells.

Finally, it was of interest to determine if, like in naive mice, rejection induced by immunization of tumor-bearing mice with irradiated IL-12 transfectants also depended upon a host CTLA-4 ligand and T cells. As can be seen in Fig. 8B), this was indeed the case, as rejection of pre-established tumors was prevented by treatment of mice with mCTLA-4γ3 or pretreatment with anti-Thy-1 mAb.
Obtained with two additional individual mice in this experiment and sary both for generation of the high IFN-

The percentage of cells in the key regions are as follows: (A) MAC-33 was observed previously that ~30% of DBA/2 mice1 axis, upper panels) or anti-B7-2 mAb (horizontal axis, lower panels). antigen at that time as well.

Fig. 9. Expression of B7-1 and B7-2 on lymph node cells draining tumor sites. Mice were inoculated in the right hind footpad with either nothing (left panels), 1×10^6 living HTR/C cells (middle panels) or 1×10^6 living HTR/C-IL-12 cells (right panels). Popliteal lymph node cells were prepared 4 days later and double stained with labeled anti-MAC-1 mAb (vertical axis) and either anti-B7-1 mAb (horizontal axis, upper panels) or anti-B7-2 mAb (horizontal axis, lower panels). The percentage of cells in the key regions are as follows: (A) MAC-1^+/B7-1^+: normal, 0.2%; HTR.C, 0.1%; HTR.IL-12, 1.4%. MAC-1^+/B7-

10^6 living HTR.C/C cells (middle panels) or differentiation. In the setting of a pre-established tumor, a fresh set of naive lymphocytes must be accessed for successful immunization to occur, requiring provision of a source of antigen at that time as well.

We have observed previously that ~30% of DBA/2 mice could reject P1.HTR cells implanted in the hind footpad, that this proportion correlated with the fraction of mice generating a high IFN-γ-producing phenotype, and that IL-12 was necessary both for generation of the high IFN-γ phenotype and prevention of tumor growth (24). Since all mice did acquire activated T cells in that system, failure to reject did not appear to be a result of the lack of an inflammatory response nor a consequence of immune ignorance. These results suggest that incomplete T cell differentiation may be responsible for the failure of rejection of P1.HTR cells in vivo. Provision of IL-12, but not B7-1, during active tumor immunization thus ensures differentiation into a T_h1-like phenotype and is therefore able to overcome the deficit by generating a fresh supply of effector cells.

It was surprising at first to find that expression of IL-12 alone, without B7-1, was sufficient to facilitate differentiation of tumor-specific CTL in vivo. However, participation of a B7 family ligand still appeared to be necessary for IL-12-mediated tumor rejection, but was provided by host immune cells. Recent published work has indicated the importance of host antigen-presenting cells (APC), perhaps via indirect antigen presentation and co-stimulation, in the immune response against tumors (33,34). Whether host APC were involved in direct or bystander co-stimulation during the immune response directed by IL-12 transfectants has not yet been addressed. B7-1 expression was up-regulated on MAC-1^+ lymph node cells, and B7-2 expression on MAC-1^+ and MAC-1^-populations, following exposure to IL-12 transfectants. It is not yet clear if the increase in B7^+ cells is a direct effect of IL-12 or an indirect consequence of the type of immune response that evolves in the presence of IL-12. Preliminary attempts to neutralize IFN-γ activity have failed to prevent this effect. It is possible that another mediator is involved and that the successful immune response facilitated by IL-12 induces B7 expression as a secondary, positive reinforcement of that response. The possibility that IL-12 itself can induce
expression of B7 on accessory cells has not yet been examined.

Co-operation between B7 and IL-12 in the induction of proliferation and IFN-γ production by human T cells has been reported previously (35). In that study, induction of IFN-γ secretion by IL-12 alone was inhibited by hCTLA-4–Ig, suggesting that B7 family molecules expressed on accessory cells were required for an optimal IL-12 effect even when B7+ cells were not provided exogenously. Similarly, B7-1 and IL-12 act synergistically to induce IFN-γ production during a primary MLTC in vitro, and co-operate to induce helper-independent, anti-tumor CTL with a Tn1/Tn1-like phenotype upon re-stimulation (25). Hence, synergy between these two cofactors may generally be important for successful immune-mediated tumor rejection.

Collectively, our results support a model in which optimal tumor rejection depends on an immune response driven by co-stimulation by a B7 family co-stimulator molecule and IL-12. Participation of both of these signals may be necessary to optimize the phenotype of CTL induced during tumor antigen immunization schemes. Indeed, preliminary results immunizing with tumor antigen peptide-pulsed APC have transfectable variant from mouse mastocytoma P815.

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