Adoptive Transfer of Immature Dendritic Cells with Autologous or Allogeneic Tumor Cells Generates Systemic Antitumor Immunity

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Abstract

Dendritic cells (DCs) are potent antigen-presenting cells that are capable of priming systemic antitumor immune responses in animal tumor models. However, many of the model tumor systems tested need definition of the specific tumor antigens involved. To use DCs in situations that are more relevant to the majority of human cancers, where the antigens are unknown, we have tested the adoptive transfer of immature DCs in mouse colorectal and melanoma models of varying immunogenicity but with undefined antigens. When DCs admixed with a syngeneic primary tumor inoculum were seeded s.c., the growth of the primary tumor was unchanged; however, if the primary tumor was then surgically excised and the animal was rechallenged with the same tumor, significant protection (75%) was generated when DCs were present in the original primary inoculum of a moderately immunogenic colorectal model (CMT93tk). This effect was not observed when a nonimmunogenic melanoma (B16) was tested in an identical protocol. Next, DCs were injected directly into established tumors; again, protection (55%) was achieved against a secondary tumor challenge following excision of the primary, but only in the CMT93tk model of moderate immunogenicity. To increase the clinical relevance of this approach still further, we tested irradiated allogeneic K1735 melanoma cells mixed with syngeneic DCs as a vaccine against subsequent challenge with the poorly immunogenic syngeneic melanoma B16. The allogeneic vaccine alone was ineffective, but when admixed with DCs, a significant number of animals rejected a subsequent B16 challenge, suggesting that DCs are able to prime an immune response against melanoma antigens shared between K1735 and B16. The generation of systemic antitumor immunity by adoptive transfer of DCs has significant clinical potential because it is technically straightforward and does not require the definition of specific tumor antigens.

Introduction

DCs are highly potent antigen-presenting cells that are uniquely capable of priming naive T-cell responses (1, 2). DCs pulsed with tumor antigens in murine tumor models have been shown to generate effective immune responses in established disease as well as vaccine/challenge protocols (3–5). The maturation state of DCs in such experiments depends on culture conditions (6). In the mouse, GM-CSF is the critical cytokine in generating myeloid-derived DCs, and used alone, it results in an immature DC phenotype. Addition of lipopolysaccharide or CD40 ligand to DC cultures then matures DCs such that they express high levels of MHC class II and costimulatory molecules and are more potent at stimulating T cells in an allogeneic mixed lymphocyte reaction (7). However, there is evidence that, if DCs are to acquire antigen prior to immune presentation, immature rather than mature DCs are most potent (8, 9). Many of the protocols using DC thus far for immune priming against tumor antigens require definition and characterization of the epitopes involved. However, in the majority of common solid human adult malignancies, antigenicity is less clear; if DCs could be exposed to all potential epitopes within tumors in vivo, a more effective clinical response might follow. In addition, delivery of DC in vivo without manipulation of tumors derived from individual patients could further increase the broad application of DC-based immunotherapy. Therefore, we have designed protocols in murine models to test whether DCs are able to take up undefined tumor antigens in vivo, both from syngeneic and allogeneic tumors, and prime an effective immune response against them.

Materials and Methods

Cell Culture. The MHC class I-negative B16 F1 cell line used in this study has been described previously (10). CMT93tk is a clone derived from colorectal CMT93 cells by transfection with the HSVtk gene, driven by the cytomegalovirus promoter, and it is MHC class I positive. B16 and CMT93tk cells are syngeneic in H-2k C57BL mice, whereas K1735 is a melanoma cell line derived from H-2b C3H/He mice. X63 cells transfected with the mouse GM-CSF gene (X63 Ag8 12; a generous gift from D. Gray, RPMS, London, United Kingdom) were cultured, and supernatant was harvested for use in generation of bone marrow-derived DCs. All cell lines were grown in DMEM supplemented with 10% FCS and 4 mM l-glutamine. For culture of CMT93tk cells, puromycin (1.25 μg/ml) was added to the culture medium to ensure selection for the HSVtk transgene.

DC Culture. DCs were cultured from the bone marrow of C57BL mice according to a protocol modified from that of Inaba et al. (11). Briefly, bone marrow was flushed from femurs and tibias and cultured in 75-cm² flasks (25 mL per flask) in RPMI 1640 supplemented with 10% FCS, 5 × 10⁻³ M 2-mercaptoethanol, and 10% X63-GM-CSF supernatant. The medium was changed on day 3 of culture. On days 7–9, cells in suspension were collected together with those dislodged by vigorous pipetting from aggregates on the bottom of the flasks. In agreement with other studies, these cells, cultured in medium with GM-CSF being the only cytokine, were of a relatively immature phenotype, with low MHC class II expression, weak stimulation in an allogeneic mixed lymphocyte reaction, and high phagocytic activity (data not shown; Ref. 7). For animal experiments, day 7–9 DCs were spun down, washed thoroughly, and resuspended in PBS for injection.

Adoptive Transfer of DCs in Syngeneic Tumor Cell Vaccines. C57BL/6 mice were obtained from colonies bred at the Imperial Cancer Research Fund. Mice were age- and sex-matched for individual experiments. In all cases, 10 mice were used per group, and results shown are representative of at least two independent experiments. To establish s.c. tumors, we injected 2 × 10⁵ CMT93tk cells or 5 × 10⁵ B16 cells s.c. into the flank region. The diameters of the tumors, in two dimensions, were measured twice weekly using calipers. Animals had tumors surgically excised when tumor size was −1.0 × 1.0 cm in two perpendicular directions. For immediate adoptive transfer of DCs, 1 × 10⁹–2 × 10⁹ DCs per mouse were thoroughly admixed with the primary tumor cell challenge immediately before s.c. injection. For adoptive transfer of DCs into established tumors, 1 × 10⁹–2 × 10⁹ DCs were injected directly into palpable 6–9-mm-tumors using a 28-gauge needle. Following excision of the primary tumor at −1.0 × 1.0 cm (this was between 3 and 6 days following injection of DC in protocols involving intratumoral DC administration), mice were rechallenged 7–10 days later on the opposite flank with the same tumor
type as their primary challenge, at a dose of $2 \times 10^5$ B16 cells or $1 \times 10^6$ CMT93tk cells. In each experiment, a naive group of mice was also injected with the same preparation of cells at the same time. Growth of the secondary tumor was monitored in the same way as growth of the primary. Animals were killed when tumor size was $\sim 1.0 \times 1.0$ cm in two perpendicular directions.

**Allogeneic/DC Vaccines.** In allogeneic vaccine/challenge experiments, mice were immunized as follows: on day 1, $5 \times 10^6$ K1735 cells were irradiated to 100 Gy (250-kV HF320; Pantak, Reading, United Kingdom) and administered s.c. in the flank as a vaccine either alone or thoroughly admixed just prior to inoculation with $1 \times 10^3$–$2 \times 10^4$ unirradiated syngeneic DCs cultured from C57BL mouse bone marrow, as described above. This vaccine was repeated on day 8, and on day 15, $5 \times 10^4$ B16 cells were seeded on the opposite flank. A naive group of mice was also challenged, and B16 tumor growth was monitored as described previously; the animals were killed when tumor size was $\sim 1.0 \times 1.0$ cm in two perpendicular directions.

**Results and Discussion**

**Adoptive Transfer of DCs with a Primary Syngeneic Tumor Cell Inoculum.** When $2 \times 10^6$ CMT93tk colorectal tumor cells were seeded s.c. in C57BL mice, tumors grew to $1.0$ cm in diameter in $16$ days. When these tumors were surgically excised and the animals were rechallenged with CMT93tk $7$–$10$ days after excision, consistently, $25\%$ of mice were protected from the secondary challenge and remained tumor free for at least $60$ days (Fig. 1A). This demonstrates that this tumor model has moderate inherent immunogenicity. We take such protection generated against secondary challenge by growth and excision of a primary tumor to be a test of immunogenicity, with greater relevance to the natural history of most tumors in cancer patients having ablative therapies than, for example, the efficacy of a vaccine comprising irradiated tumor cells. It is not certain to what extent the presence of the HSVtk gene (or its puromycin selection marker) contributes to the immunogenicity of CMT93tk (although the parental cell line is less immunogenic; data not shown), and the precise nature of any antigens involved remains undefined. This makes CMT93tk a suitable model for testing the ability of DCs to enhance the immunogenicity of tumors with potential yet uncharacterized antigenic determinants.

When $1 \times 10^3$–$2 \times 10^5$ immature DCs cultured from bone marrow over $7$–$9$ days with GM-CSF were admixed with the same number of CMT93tk cells just prior to s.c. seeding, the growth rate of the primary tumor was unchanged (data not shown). This suggests that any immune response instigated by presence of DC within the primary tumor as it became established was insufficient to prevent its growth and continued progression. However, when these mice were rechallenged with CMT93tk $7$–$10$ days after excision of the primary tumor, the presence of DCs in the primary inoculum increased the percentage of animals rejecting the secondary challenge from $25$ to $75\%$ (Fig. 1A). This suggests that, in this model, DCs that were seeded and admixed with the initial tumor challenge were able to acquire relevant undefined tumor antigens in vivo and prime a systemic immune response against them. Hence, once the bulk primary tumor had been removed surgically, the systemic immune response generated was effective at increasing the percentage of animals protected against a second challenge with the same tumor cell type.

It was reasoned that the efficacy of DCs in enhancing the immunogenicity of a surgically excised primary CMT93tk tumor may be related to its inherent (moderate) immunogenicity. Therefore, B16 melanoma, which is, in contrast, a poorly immunogenic MHC class I-negative tumor model, was also tested. B16 tumors were more aggressive in vivo: following seeding of fewer ($5 \times 10^5$) cells, the tumors reached $1.0$ cm in diameter and required excision after, on average, $13$ days. In B16, as with CMT93tk, addition of DCs to the primary tumor challenge did not significantly affect their growth rate (data not shown). B16 was, as expected, poorly immunogenic, in that surgical excision of a primary tumor alone generated no significant long-term protection against a secondary challenge (Fig. 1B). In contrast to CMT93tk, however, in the B16 model, mice succumbed to the secondary challenge with a similar time course, whether the primary inoculum had contained DCs or not (Fig. 1B).

Hence, when DCs were admixed with a primary tumor challenge, the growth of the tumor was unaffected in both tumor models. However, when the primary tumor was excised and the animal was subjected to a second tumor challenge with the same cell type, the presence of DCs in the primary inoculum was able to increase the level of secondary protection against the inherently moderately immunogenic CMT93tk model but not against poorly immunogenic B16. This correlation between the efficacy of adoptive transfer of DCs with the inherent immunogenicity of the model may be explained by differences in the availability of antigens to DCs. More antigenic proteins may be cross-primed into adoptively transferred DCs from...
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Fig. 2. Immature DCs increase the immunogenicity of CMT93tk tumors (but not B16) on adoptive transfer into established tumors. C57BL mice were inoculated with 2 × 10^6 CMT93tk (A) or 5 × 10^5 B16 (B) cells and growth of the tumor monitored. When tumors became palpable at 6–9 mm, 1 × 10^6 immature DCs were injected directly into the tumor. Primary tumors were excised when they reached ~1.0 × 1.0 cm, and 7–10 days later, mice were rechallenged on the opposite flank with the same cell type (1 × 10^6 cells for CMT93tk or 2 × 10^5 cells for B16). In each case, a naive group of mice was also challenged with these same cells at the same time. The key indicates the nature and treatment of the primary inoculum, and the figure shows the growth of the secondary rechallenge.

Fig. 3. Adoptive transfer of immature DCs admixed with an irradiated allogeneic K1735 vaccine generates protection against a subsequent syngeneic B16 challenge. On days 1 and 8 of the experiment, H-2^d C57BL mice were injected s.c. with 5 × 10^5 irradiated (100Gy) K1735 H-2^d cells with or without 1 × 10^6 immature syngeneic DCs admixed in with the inoculum. On day 15, mice were challenged with 5 × 10^5 live syngeneic B16 tumor cells on the opposite flank. A naive group of mice was also challenged with these cells at the same time. The figure shows the growth of the syngeneic B16 challenge.

CMT93tk cells than B16 as the primary tumor becomes established. Alternatively, the effector arm of the T-cell response may be more effective in CMT93tk, which expresses MHC class I, than B16, which does not. Hence, these experiments support the hypothesis that DCs are able to process and present undefined tumor antigens in vivo and prime an effective systemic immune response, but only in tumors with demonstrable immunogenicity.

Adoptive Transfer of DCs into Established Syngeneic Tumors. The next set of experiments was designed to test adoptive transfer of DCs into established tumors in a protocol that could readily be translated into the clinical setting. CMT93tk and B16 tumors were seeded as above and allowed to grow to between 6 and 9 mm in diameter. DCs (1 × 10^5–2 × 10^5) were then injected directly into these palpable tumors. Injection of DC into tumors did not affect their continuing growth (data not shown), and the primary tumor was again excised at a diameter of 1.0 × 1.0 cm. As before, mice were subjected to a secondary rechallenge with the same tumor type, and as shown in Fig. 2, the levels of protection achieved by intratumoral injection of DCs were 55% for CMT93tk and 10% for B16. These data suggest that, in the environment of an established tumor as well as in a newly seeded inoculum, DCs are able to acquire relevant tumor antigens and prime an immune response against them. As in the protocol introducing DCs at the start of a primary tumor inoculum, the level of protection generated by intratumoral DCs was greater for the moderately immunogenic CMT93tk than for poorly immunogenic B16.

The adoptive transfer of DCs by direct injection into established tumors holds particular appeal for clinical application. DCs could readily be injected into a single primary tumor deposit before definitive surgical excision; by processing undefined antigens, DCs may be able to prime an immune response capable of eradicating micrometastatic disease that is undetectable at initial diagnosis. Alternatively, DCs injected into accessible disease, even when the cancer is advanced, may instigate an antitumor response that, although not curative, might offer some control of tumor growth with low toxicity.

With regard to the immunological mechanisms responsible for the antitumor effects of adoptively transferred DC with syngeneic tumor challenges, studies, thus far, have shown the effectiveness of these protocols is abolished in nude mice (data not shown), suggesting that a T cell-mediated mechanism is responsible.

Allogeneic Tumor Cell/Syngeneic DC Vaccines. Ex vivo manipulation of individual patients’ tumors is a major barrier to widespread immunotherapy with DCs. However, allogeneic tumor cell vaccines are available and have shown some promising results when used alone in clinical melanoma trials (12). Therefore, as a further potential use for adoptive transfer of DCs, we next tested syngeneic DCs transferred with an allogeneic vaccine to test whether DCs could potentiate the immunogenicity of putative antigens shared between an allogeneic vaccine and a syngeneic tumor. Incorporating allogeneic tumor cells into DC protocols may have further benefits because there is evidence that allogeneic MHC molecules can act as an adjuvant to enhance antitumor responses (13). The allogeneic vaccine used was K1735, a melanoma cell line derived from H-2^d C3H/He mice. An allogeneic parental K1735 vaccine (5 × 10^6 cells) was irradiated to 100 Gy (because irradiation would be required for even allogeneic vaccines in
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clinical trials) and administered s.c. to C57BL mice on days 1 and 8 of the schedule, with or without $1 \times 10^5 - 2 \times 10^5$ nonirradiated syngeneic immature DCs admixed just prior to inoculation. On day 15, mice were challenged with $5 \times 10^4$ cells of the poorly immunogenic syngeneic melanoma B16. In the experiment shown in Fig. 3, 2 of 10 naive mice did not succumb to the B16 challenge, probably because of the reduced cell challenge dose used in this protocol. Mice vaccinated with K1735 cells alone had no greater protection against the B16 challenge than the naive group, whereas in animals vaccinated with DCs in addition to K1735, 70% protection was achieved. In additional experiments, DCs alone, delivered as a vaccine, had no effect on B16 tumor growth (data not shown). Furthermore, DCs combined with irradiated B16 cells were ineffective at protecting against a subsequent live B16 challenge, suggesting that the allogeneic nature of K1735 does, indeed, contribute to its efficacy. This data suggests that in an allogeneic melanoma model K1735 can protect against B16, but only if adoptively transferred syngeneic DCs are present to take up, process, and present potential shared tumor antigens. As in the syngeneic setting, adoptive transfer of DCs with allogeneic tumor cells is ineffective in nude mice (data not shown).

In view of the success of the allogeneic approach, we wondered whether allogeneic vaccines expressing particular cytokines may be still more effective. K1735 cells expressing GM-CSF or IL-12 were tested because these cytokines have been implicated in DC maturation and activation as well as being themselves effective as gene therapy strategies against murine tumor models in both autologous and allogeneic settings (6, 14–17). Surprisingly, in the same vaccination/challenge protocol described above for parental K1735, K1735-GM-CSF, and K1735-IL-12 were not only ineffective themselves at generating protection against a B16 challenge, but they also abrogated the benefit of DCs admixed with the parental K1735 vaccine (data not shown). This may be because IL-12 or GM-CSF produced locally within the vaccine alters the maturity, antigen acquisition, or migratory capacity of DC delivered in vivo, to prevent effective T-cell priming.

In summary, we have shown that adoptive transfer of DCs in both syngeneic and allogeneic murine tumor models can generate effective systemic antitumor immunity. Immature DCs cultured with GM-CSF alone were effective at priming immune responses when admixed with a primary tumor inoculum at the time of initial seeding or when injected into established tumors. In these syngeneic models, the effectiveness of adoptively transferred DCs correlated with the intrinsic immunogenicity of the model being tested. DCs could also render an allogeneic melanoma vaccine effective against subsequent challenge with a poorly immunogenic syngeneic melanoma. The importance of DC maturation stage to the effectiveness of such strategies is currently under investigation. The injection of DCs into established tumors or DCs combined with allogeneic tumor cell vaccines may be of significant clinical benefit because they require neither definition of specific tumor antigens nor manipulation of individual tumors in vitro. Adoptively transferred DCs may have a role as part of adjuvant treatment in the radical setting or as a therapy temporarily to control disease with minimum toxicity in advanced malignancy.

References

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