Protective Immunity Induced by Tumor Vaccines Requires Interaction between CD40 and Its Ligand, CD154

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Abstract

Interactions between CD40 and its ligand, CD154 (CD40L, gp39), have been shown to play a central role in the regulation of humoral immunity. Recent evidence suggests that this ligand-receptor pair also plays an important role in the induction of cell-mediated immune responses, including those directed against viral pathogens, intracellular parasites, and alloantigens. The contribution of this ligand-receptor pair to the development of protective immunity against syngeneic tumors was evaluated by blocking the in vivo function of CD154 or by studying tumor resistance in mice genetically deficient in CD40 expression (CD40−/−). In the former case, anti-CD154 monoclonal antibody treatment inhibited the generation of protective immune responses after the administration of three potent tumor vaccines: irradiated MCA 105, MCA 105 admixed with Corynebacterium parvum adjuvant, and irradiated B16 melanoma cells transduced with the gene for granulocyte macrophage colony-stimulating factor. Confirmation of the role of CD40/CD154 interactions in tumor immunity was provided by the overt tumor susceptibility in CD40−/− mice as compared to that in CD40+/+ mice. In this case, wild-type but not CD40-deficient mice could be readily protected against live TSA tumor challenge by preimmunization with TS/A admixed with C. parvum. These findings suggest a critical role for CD40/CD154 interactions in the induction of cellular immunity by tumor vaccines and may have important implications for future approaches to cell-based cancer therapies.

Introduction

Contemporary approaches to improve tumor vaccine efficacy have been to identify factors that can act as adjuvants that enhance tumor immunogenicity. Factors such as bacterial adjuvants, lymphokines, and costimulatory molecules combined physically or genetically with tumor have all been shown to enhance tumor vaccine potency (1–4). Although many of these tumor vaccines are very effective at preventing the growth of subsequently injected challenge tumors, most vaccines have had a minimal effect on established disease in murine models. Likewise, when used in an adjuvant setting after the primary tumor has been resected, randomized prospective studies in humans have failed to demonstrate a survival benefit. Therefore, a continued search for elements of the immune system that are involved in the promotion of tumor immune responses is warranted. Given the fact that CD40/CD154 interactions have been shown to be essential in a spectrum of cell-mediated immune responses (5–9), the role of these interactions in the generation of protective immunity to syngeneic tumors was studied.

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The induction of protective immunity to tumors involves the processing and presentation of tumor antigens by APCs, resulting in the clonal expansion and differentiation of tumor-specific CD4+ and CD8+ T cells. Strategies to improve the efficiency with which APCs process and present antigen have been shown to enhance tumor immunity. For example, the most potent vaccine in the B16 melanoma tumor model system consists of tumor cells engineered to secrete GM-CSF (1), a cytokine that recruits antigen-presenting DCs to the tumor site and induces both MHC class I and II expression as well as an increase in the B7-costimulatory molecules CD80 (B7.1) and CD86 (B7.2) (10–12). Enhanced expression of these two costimulatory molecules most likely facilitates the clonal expansion and differentiation of tumor-specific T cells by triggering CD28 on the T-cell surface (13). Although GM-CSF can promote DC maturation, it has also been shown that triggering via CD40 can greatly enhance the ability of DCs expanded in GM-CSF to up-regulate both CD80 and CD86 as well as trigger inflammatory T-cell responses in vitro (14). Therefore, the role of CD40 in the generation of protective tumor immunity to GM-CSF-transduced tumors was evaluated, and the results presented in this study confirm that CD40/CD154 interactions play a critical role in the immune response to a GM-CSF-transduced melanoma vaccine.

In addition to antigen presentation and the expression of costimulatory molecules, APCs must also elaborate the appropriate spectrum of cytokines to guide the T-cell response to one that will ultimately elicit protection of the host. In the context of protection to tumor cells, it is believed that inflammatory T-cell responses (Th1) and CD8+ T cells are most beneficial (15–16). It has been shown that IL-12 secretion by DCs after cognate interaction with Th cells is CD40-dependent (17) and plays a pivotal role in the differentiation of naive T cells toward the Th1 phenotype (18–19). Furthermore, systemic administration of IL-12 has been shown to mediate the regression of established tumor (20), and tumor vaccines engineered to secrete IL-12 greatly enhance the generation of protective tumor immunity (2). Therefore, it is reasonable to conclude that CD40-mediated IL-12 production may also play a role in the regulation of inflammatory cytokines that lead to optimal tumor-specific responses.

Thus, CD40-mediated events seem central in the regulation of many of the properties that APCs need to acquire to initiate inflammatory T-cell responses, namely the expression of costimulatory molecules and the production of inflammatory cytokines. The data presented here indicate that in vivo blockade of CD40/CD154 interactions via anti-CD154 mAb treatment or by genetic disruption of the gene for CD40 prevents the generation of protective immunity after the administration of a variety of potent tumor vaccines. The cellular

3 The abbreviations used are: APC, antigen-presenting cell; GM-CSF, granulocyte macrophage colony-stimulating factor; DC, dendritic cell; IL-12, interleukin 12; Th, helper T; SRBC, sheep RBC; Hlg, hamster immunoglobulin; CTL, cytotoxic T cell; mAb, monoclonal antibody; MBF, myelin basic protein.
Materials and Methods

**Mice.** Six to eight-week-old female C57BL/6, BALB/c, and BALB/c SCID mice were obtained from the National Cancer Institute (Bethesda, MD). CD40-deficient mice (21) backbred to the BALB/c background were a gift from Dr. Nancy Phillips (University of Massachusetts, Worcester, MA). Animals were maintained in a pathogen-free animal facility at Dartmouth Medical School.

**Cell Lines and Cell Culture.** MCA 105 is a methylcholanthrene-induced fibrosarcoma of B6 origin (22) and was kindly provided by Dr. Steven Rosenberg (National Cancer Institute). B16-F10 melanoma cells and GME-CSF-transduced B16 cells (1) are of B6 origin and were obtained from Dr. Glenn Dranoff (Dana-Farber Cancer Institute). The TS/A adenocarcinoma cell line of BALB/c origin (23) was kindly provided by Dr. Michael Lotze (University of Pittsburgh, PA). Cells were maintained at 37°C/5% CO2 in DMEM (Mediatech, Herndon, VA) or RPMI (Biowhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 2 mM glutamine (Sigma, St. Louis, MO), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma).

**Vaccine Protection Studies.** For experiments involving irradiated tumor cell vaccination, cells were suspended at 107 cells/ml in HBSS (Sigma) and irradiated at 3500 cGy. On day 0, cells suspensions were injected s.c. in 100 μl volume in the left flank. MR1, hamster anti-murine CD154 mAb (24), was purified by DEAE high-performance liquid chromatography from ascites fluid, and Hlg, used as a control antibody, was similarly purified from hamster serum (Accurate Chemical and Scientific Corp., Westbury, NY). Anti-CD154 or Hlg (200 μg) was administrated by i.p. injection on days −3, 0, 3, 6, and 9. On day 14, a live tumor challenge was administered s.c. in 100 μl of HBSS in the right flank, and tumor diameters were measured starting 1 week after live tumor injection using a caliper. Tumors were measured every 3 days, and mice whose tumors reached a diameter of over 2 cm were sacrificed as per IACUC guidelines.

For experiments involving *Corynebacterium parvum*-enhanced tumor vaccines, live cells were suspended at a final concentration of 2 × 106/ml in HBSS admixed with *C. parvum* (Coparvax/Wellcome Foundation Ltd., London, United Kingdom) at a final concentration of 1 mg/ml. Cells were injected in a total volume of 50 μl into the right hind footpad of female BALB/c mice, whereas control mice were injected with 50 μg of *C. parvum* alone in HBSS. On day 7, mice were anesthetized with pentobarbital, and the hind foot was amputated just below the hairline. On day 14, mice were challenged with live tumor in 200 μl of HBSS s.c. in the right flank. Fisher’s exact test was used to compare proportions of mice surviving.

**Results**

Initial experiments were performed using the fibrosarcoma line MCA 105 as a vaccine (25). Adoptive transfer studies using antibody depletion techniques demonstrated that T cells are the therapeutic effector cells generated by immunization with MCA 105 plus *C. parvum* and that both CD4+ and CD8+ lymphocytes are required for optimal effectiveness (22). Like many other murine tumors (1), s.c. injection of irradiated MCA 105 will result in the generation of protective immunity. C57BL/6 mice were given i.p. injections of either anti-CD154 or Hlg as described in “Materials and Methods.” On day 0, mice were vaccinated with media alone (mock vaccination) or with 9 × 105 irradiated MCA 105 cells. Mice were then challenged on the opposite flank with 1 × 106 live MCA 105 tumor cells on day 14. As indicated in Fig. 1, five of six mice receiving control Hlg after vaccination with MCA 105 (group 3) remained tumor-free for at least 100 days, whereas none of the mice (zero of six) receiving anti-CD154 were alive by day 30 (group 4; P = 0.02). The survival of the mice receiving vaccine plus anti-CD154 was the same as that of those mice that received the mock vaccination and either Hlg or anti-CD154 (groups 1 and 2). The challenge tumors in the groups receiving the mock vaccine and either anti-CD154 or Hlg showed similar tumor growth kinetics, indicating that treatment with anti-CD154 itself does not lead to enhanced tumor growth (data not shown).

To assess whether the effect of anti-CD154 blocked the development of tumor-specific T cells and/or interfered with their effector function, mice (groups 5 and 6) were given the live tumor challenge 3 weeks after the last injection of anti-CD154, a period of time that has been shown to allow for clearance of >95% of the antibody from the serum (26). As shown in Fig. 1, we observed that six of six mice receiving Hlg showed long-term survival (group 5), whereas zero of six mice treated with anti-CD154 were protected (group 6). To confirm the absence of anti-CD154 in mice at 5 weeks, separate mice were injected with SRBCs plus anti-CD154 or Hlg and assessed for their ability to generate an anti-SRBC humoral response as described previously (26). The anti-SRBC titer was the same in both treatment groups by 5 weeks, indicating that there was no remaining bioactivity.
of the anti-CD154 at this time (data not shown). Because no detectable anti-CD154 is present in the serum at the time of the 5-week tumor challenge, the lack of protective immunity in the mice treated with anti-CD154 must be due at least in part to an inhibition of the development of a protective antitumor response.

Murine tumor model systems often rely on the use of classical adjuvants such as C. parvum to enhance the generation of effector cells after vaccination (27). Moreover, C. parvum-enhanced tumor vaccines have been shown to be at least as effective as cytokine-transduced tumor cells in the generation of protective immunity (3). To evaluate whether CD40/CD154 interactions are also important when using this adjuvant to enhance tumor vaccination, mice were injected in the footpad with 9 \times 10^5 live MCA 105 tumor cells admixed with 50 \mu g of C. parvum and either control Hlg or anti-CD154 as described in “Materials and Methods.” One week after tumor vaccination, mice were anesthetized, and the hind foot was amputated just below the hairline. On day 14, mice were subsequently challenged with 1 \times 10^8 live MCA 105 tumor cells s.c. in the flank and followed for tumor growth to assess vaccine efficacy. These data are representative of two such experiments.

### Fig. 2. Protection induced by C. parvum-enhanced MCA 105 vaccine is blocked by anti-CD154 (MR1). Anti-CD154 or control Hlg was administered to C57BL/6 mice as described in “Materials and Methods.” On day 0, mice were vaccinated with 100 \mu l of media or with 9 \times 10^5 live MCA 105 tumor cells mixed with C. parvum at 1 mg/ml. On day 7, mice footpads were amputated. On day 14, mice were challenged with 1 \times 10^8 live MCA 105 tumor cells s.c. in the flank and followed for tumor growth to assess vaccine efficacy. These data are representative of two such experiments.

CD40/CD154 interactions and tumor immunity

In addition to the use of bacterial adjuvants, there are numerous studies that indicate that the transduction of tumor cells with various costimulatory molecules and cytokines can also enhance the generation of systemic immune protection when these modified cells are used as vaccines (4, 15). One of the most effective of these vaccines involves tumors engineered to secrete GM-CSF (1), a cytokine known to mature both DCs and macrophages for efficient antigen presentation to both CD4+ and CD8+ T cells (28, 29). To assess whether the effects of the CD40/CD154 blockade were generalizable to other tumor systems and to test whether anti-CD154 could inhibit this particularly potent tumor vaccine, C57BL/6 mice were given control Hlg or anti-CD154 as described in “Materials and Methods” and then vaccinated s.c. with 9 \times 10^5 irradiated B16 melanoma cells transduced with GM-CSF (1). Mice were challenged with live parental B16 cells s.c. in the opposite flank, and tumor growth was followed. As seen in Fig. 3, 67% of the mice receiving the B16-GM-CSF vaccine and Hlg were protected out to 90 days. In contrast, none of the mice given the B16-GM-CSF vaccine and treated with anti-CD154 survived beyond day 30, similar to those mice injected with a mock vaccine (HBSS) and treated with Hlg or anti-CD154 (data not shown).

To confirm the critical role of CD40/CD154 interactions in the induction of protective immunity by tumor vaccination, similar vaccine studies were performed in CD40-deficient mice (21). Wild-type BALB/c or CD40-deficient mice bred to the BALB/c background were vaccinated with 9 \times 10^5 syngeneic TS/A tumor cells admixed with 50 \mu g of C. parvum adjuvant in the footpad, and limbs were subsequently amputated 7 days later. One week after footpad amputation, mice were challenged with 1 \times 10^8 live TS/A tumor cells s.c. in the right flank. As seen in Fig. 4, 100% of the wild-type BALB/c mice remained tumor-free out to 100 days, whereas none of the CD40-deficient mice showed long-term survival after live tumor challenge. CD40-deficient mice receiving the TS/A vaccine grew...
tumor with kinetics similar to that of BALB/c mice injected with a mock vaccine (HBSS). These data indicate that the absence of CD4 severely impairs the ability of mice to generate protective immunity in response to tumor vaccination and confirm the observations made with anti-CD154 administration in Figs. 1–3.

Discussion

The data presented here demonstrate that CD40/CD154 interactions are critical for the induction of protective immunity after tumor vaccination. Treatment with anti-CD154 at the time of vaccine administration completely blocked systemic protection afforded by three very effective tumor vaccine regimens (both an irradiated and C. parvum-enhanced MCA 105 vaccine as well as a B16 melanoma vaccine engineered to secrete GM-CSF). In addition, CD40-deficient mice exhibited an impaired immune response after administration of a C. parvum-enhanced TS/A vaccine, confirming the role of CD40/CD154 interactions in tumor immunity.

One hypothesis that explains why CD40/CD154 interactions are essential for the development of protective immunity to tumors is that CD154 function is critical for optimal presentation of tumor-specific antigens as well as for the production of cytokines that will support an inflammatory response. The induction of protective tumor immunity in vivo to both the B16-GM-CSF and MCA 105 vaccines is dependent on both CD4+ and CD8+ T cells, as indicated by antibody depletion studies (1, 22). In addition, anti-CD8+ plus complement treatment of effector cells in vitro indicates that the predominant cell type responsible for actual lytic activity toward targets is CD8+ CTL (1, 30). Because the expression of CD154 is restricted almost exclusively to activated CD4+ Th cells (31), it follows that blockade of protective immunity by anti-CD154 administration most likely involves an initial dysregulation of Th cell function that ultimately leads to an impaired protective CD8+ CTL response. The exact nature of this Th cell function as it relates to the generation of tumor-specific CTLs most likely includes the production of cytokines such as IL-12 and IFN-γ but may also involve the ability of Th cells to activate those APCs responsible for effectively presenting tumor antigen to naive CD8+ CTLs. DCs and/or macrophages, the so-called professional APCs, are the cells that have been postulated to be critical for naive CTL priming (32), and the functional expression of CD40 in these two cell types is well documented (33, 34).

Evidence from other systems suggests that both CD4+ and CD8+ T-cell responses are impaired in CD40-deficient mice (5–8, 35), and one mechanism by which blockade of CD40/CD154 interactions leads to deficiencies in T-cell priming involves the inability of Th cells to up-regulate the expression of the B7-costimulatory molecules on the surface of APCs via CD40. For example, cytokine production and cytolytic activity induced by adenoviral infection in wild-type mice is CD80- and CD86-dependent, and deficits in these responses observed in CD154−/− mice can be restored by the administration of anti-CD40 mAb, accompanied by an increase in splenic CD86 expression (7). In addition, CD154-deficient, MBP T-cell receptor transgenic mice, which are normally susceptible to experimental allergic encephalomyelitis induction by MBP injection, can be given the disease if CD80-transgenic splenocytes are injected before immunization with MBP (6). The administration of anti-CD154 at the time of vaccination in our tumor model system may prevent tumor-specific Th cells from activating APCs at the tumor site or in the draining lymph node via CD40, resulting in insufficient B7/CD28-costimulatory signals to both Th cells and CTLs and a subsequent blockade of protective immunity.

In light of the important relationship between CD40 and the B7-costimulatory pathway, it is interesting that the administration of anti-CD154 blocks protective immunity induced by the C. parvum-enhanced MCA 105 and the B16-GM-CSF vaccines (see Figs. 2 and 3, respectively). GM-CSF has been shown to up-regulate both CD80 and CD86 on DCs (11), and histochemical analysis of the inoculation site of melanoma cells engineered to secrete GM-CSF reveals significant DC infiltration and CD86 expression at the periphery of the tumor that is not observed with the parental tumor (10). In addition, the adjuvant effect of C. parvum most likely includes the response of certain immune cells to bacterial endotoxin within the adjuvant, namely the up-regulation of the B7-costimulatory molecules on both macrophages and B cells (34, 36). Our data may suggest that the level of CD80 and CD86 induced by these two adjuvant-enhanced vaccines on APCs presenting tumor antigen is not optimal for T-cell priming, and further up-regulation of these molecules via CD40 signaling is required. Alternatively, there may be other molecules induced specifically by CD40 on APCs that are critical for proper T-cell priming to tumor antigen. Whether anti-CD154 administration has any effect on the levels of CD80 and CD86 expressed on APCs at the B16-GM-CSF tumor site is under active investigation in our lab.

Although insufficient T-cell costimulation may explain the inability of mice to generate protective tumor immunity in the absence of CD40/CD154 interactions, improper T-cell differentiation may also play an important role. Recent studies have shown that triggering CD40 on DCs leads to IL-12 secretion (37, 38), a cytokine known to be important in skewing naive, Th0 cells to the Th1 (IFN-γ-producing) rather than the Th2 (IL-4-producing) phenotype (18). There is evidence that suggests that the ability of anti-CD154 to block inflammatory responses may be largely due to its ability to block IL-12 production in vivo. For instance, blockade of Th1 cell-mediated diseases such as 2,4,6-trinitrobenzene sulfonic acid-induced colitis by anti-CD154 can be circumvented by the administration of recombinant IL-12 (39). Similarly, both CD154- and CD40-deficient mice fail to generate a protective Th1 cell-like response to Leishmania major infection (8, 19), although disease progression can be ablated by the administration of recombinant IL-12 (8). The effect of anti-CD154 on
the generation of protective tumor immunity may reflect its ability to skew primed T cells to the Th2 phenotype via the blockade of IL-12 production. Such a shift in Th cell cytokine profiles might then create an environment that is inappropriate for the generation of tumor-specific CTLs, resulting in failure to clear the live tumor challenge.

Blockade of tumor immunity by anti-CD154 may involve both the priming and effector stages of T-cell function. Previously, it has been shown that clearance of the biological activity of anti-CD154 from the serum of mice after in vivo administration occurs within 3 weeks (26). The observation that mice challenged with live tumor 3 weeks after the last anti-CD154 injection still show impaired tumor protection (Fig. 1, group 6) suggests that CD40/CD154 interactions may play an important role in priming T cells to tumor antigen. The data, however, do not rule out the possibility that CD40-mediated events are also important at the effector stage. Studies indicate that the production of nitric oxide intermediates by macrophages after interaction with Th cells is mediated by CD40 (40), and triggering CD40 on monocytes leads to an overall increase in tumoricidal activity (34). In addition, CD40 signaling has been shown to induce inflammatory cytokine production by both dendritic Langerhans cells and monocytes (14, 34, 41) as well as to induce the secretion of MIP-1α (14), a chemokine known to preferentially induce the migration of CD8+ T cells (42).

CD40-mediated inflammatory cytokine and chemokine production by macrophages and dendritic Langerhans cells may play a significant role in the clearance of live tumor in our model system. This may involve not only tumor destruction by macrophages but also the induction of CTL trafficking to the site of tumor challenge for effective clearance to occur. The inability of CD40-deficient or anti-CD154-treated mice to generate protective tumor responses might be restored by the administration of exogenous IL-12 at the time of vaccination or by the use of tumor cell vaccines engineered to secrete IL-12 and/or to express high levels of the B7-costimulatory molecules on their surface. Alternatively, the expression of membrane-bound or -soluble forms of CD154 on a nonimmunogenic tumor such as B16 may be an effective way of enhancing the nominal immune response induced by this tumor line when it is used as a vaccine. Such studies are ongoing and should better define the mechanisms by which the blockade of CD40/CD154 interactions leads to impaired cellular immunity to tumor antigen.

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