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Protective Antitumor Immunity Induced by a Costimulatory Thalidomide Analog in Conjunction with Whole Tumor Cell Vaccination Is Mediated by Increased Th1-Type Immunity

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Thalidomide and its novel T cell costimulatory analogs (immunomodulatory drugs) are currently being assessed in the treatment of patients with advanced cancer. However, neither tumor-specific T cell costimulation nor effective antitumor activity has been demonstrated in vivo. In this study, we assessed the ability of an immunomodulatory drug (CC-4047/ACTIMID) to prime a tumor-specific immune response following tumor cell vaccination. We found that the presence of CC-4047 during the priming phase strongly enhanced antitumor immunity in the vaccinated group, and this correlated with protection from subsequent live tumor challenge. Protection was associated with tumor-specific production of IFN-γ and was still observed following a second challenge with live tumor cells 60 days later. Furthermore, CD8+ and CD4+ splenocyte fractions from treated groups secreted increased IFN-γ and IL-2 in response to tumor cells in vitro. Coculture of naive splenocytes with anti-CD3 mAb in the presence of CC-4047 directly costimulated T cells and increased Th1-type cytokines. Our results are the first to demonstrate that a costimulatory thalidomide analog can prime protective, long-lasting, tumor-specific, Th1-type responses in vivo and further support their ongoing clinical development as novel anti-cancer agents. The Journal of Immunology, 2002, 168: 4914–4919.

Recent reports have highlighted the resurgence of thalidomide (Thd) as a clinically effective drug (1–3). A number of studies have highlighted its efficacy in diseases ranging from leprosy (4) and HIV-associated cachexia (5, 6) to some cancers (7–11). Numerous mechanisms of action have been reported, including TNF-α inhibition, T cell costimulation, and antiangiogenic effects (12–14). Indeed, Thd’s activity against multiple myeloma may be due to a combination of all of these activities (7, 15) as well as direct antitumor activity (8). However, it is Thd’s ability to act as a T cell costimulator that is of most interest in an immunotherapeutic clinical setting (12). The administration of a clinically effective dose of Thd can be associated with significant side effects. Novel Thd analogs have been designed and synthesized to improve efficacy and limit side effects. These compounds have been shown to segregate into at least two distinct classes; selective cytokine inhibitory drugs, consisting of phosphodiesterase type 4 inhibitors, and immunomodulatory drugs (IMiDs), thought to act similarly to Thd via an as yet unknown mechanism(s) (16–22). Both groups of analogs contain compounds that are potent TNF-α inhibitors, although T costimulatory activity is limited to the latter group (22). The clinical development of IMiDs was initiated in 1999, and one has already completed phase I trials and entered phase II trials for multiple myeloma (23). The ability to costimulate T cells has been associated with an increased Th1-type cytokine response, in particular IFN-γ and IL-2 (12).

Recent studies have demonstrated the importance of IFN-γ in animal tumor immunotherapy models (24–27). IFN-γ acts in various ways on host and tumor cells, causing tumor regression by regulating different aspects of the immune response, such as up-regulation of MHC class I and II molecules (28), orchestrating leukocyte-endothelial interactions (28, 29), effects on cell proliferation (30), and anti-angiogenesis (31, 32). Other data showed that tumor suppressor functions of the immune system were critically dependent on IFN-γ (24). Although IFN-γ has been found to promote tumor evasion of the immune system by down-regulating the tumor Ag gp70 (25), subsequent findings showed that IFN-γ production from CD4+ T cells inhibits tumor angiogenesis and thus promotes tumor regression in a similar model (26). Effective antitumor responses have been found to be dependent on IFN-γ receptor expression either by tumor cells (26) or by nonhemopoietic host cells (27) depending on the model.

In this study, we examined the influence of a potent costimulatory IMiD (CC-4047) on the CT26 autologous murine model of ectopic colorectal cancer and the K1735 allogeneic murine vaccine model for B16F10 melanoma. Vaccination with CT26 or K1735 cells alone provided partial immunity from a subsequent live challenge with CT26 and B16F10 cells, respectively. However, protection from live tumor challenge was strongly enhanced by incorporating CC-4047 into the vaccination protocol. Subsequent investigation using the autologous model found that protection was
associated with the generation of a tumor-specific Th1-type immune response and led to long-lasting immunity.

**Materials and Methods**

**Animals**

Normal BALB/c and BALB/c nude mice (nu/nu) and C57BL/6 mice were purchased from Harlan (Loughborough, U.K.). Mice were age and sex matched for individual experiments. All procedures were conducted in accordance with the Home Office and institutional guidelines.

**Cell culture**

CT26 is an N-nitroso-N-methylurethane-induced colon epithelial tumor cell line that is syngeneic for H-2K^d^ BALB/c mice (33). B16-F10 (H-2^b^) and K1735 (H-2^d^) marine melanoma lines have been used previously in this laboratory (34). The A20 cell line, a mouse lymphoma syngeneic for BALB/c, was obtained from American Type Culture Collection (Manassas, VA). CT26 and B16F10 lines were grown in DMEM essential medium, while K1735 and A20 lines were grown in RPMI 1640 supplemented with 10% (v/v) FCS, 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) all from Sigma-Aldrich, Poole, U.K.). The cells were passaged two or three times per week by detaching cell lines with 0.05% trypsin/0.02% EDTA and for in vivo inoculation were washed three times in PBS. The cell lines were monitored routinely and found to be free of Mycoplasma infection.

**In vivo injection of tumor cells**

For the tumor cell vaccination, cells were prepared in sterile PBS and irradiated to 150 Gy using an IBL437C irradiator (CIS Biointernational, GIF/Yvette, France). CT26 cells (1 × 10^6/mouse) or K1735 cells (5 × 10^6/mouse) were injected s.c. in a volume of 200 μl into the left flank. Control mice received 200 μl of PBS. Fourteen days later, s.c. tumors were established by injection of 200 μl of live CT26 cells (5 × 10^6) or B16F10 cells (5 × 10^5) into the right flank of BALB/c or C57BL/6, respectively. Animals were examined daily until the tumor became palpable; thereafter, the diameter was measured in two dimensions, twice weekly, using calipers. Animals were killed when tumor size was ~1.5 × 1.5 cm in two perpendicular directions.

**Drug preparation and administration**

The Thd analog CC-4047 (Celgene, Warren, NJ) was dissolved in a final concentration of 0.5% (v/v) Tween 80 at 37°C. CC-4047 was administered to mice via an i.p. injection at 4 days and again at 7 days following the tumor cell vaccination in a 10-ml/kg volume at a dose of either 5 or 50 mg/kg. For in vitro studies, CC-4047 was used at a final concentration of 10 μg/ml in 0.05% DMSO (v/v).

**CTL assay**

Fresh spleen cell suspensions were obtained from unvaccinated mice or mice that had received the CT26 vaccine with or without CC-4047. The CTL assay was conducted as previously described (34). Briefly, splenocytes were prepared by removing spleens from the relative groups and teasing them apart, and RBC were lysed with RBC lysis buffer (Gentra Systems, Minneapolis, MN), and the manufacturer's instructions were followed accordingly. Briefly, the precoated mAb plate was washed with 200 μl of sterile culture medium twice for 20 min each time. One hundred microliters of effector cells (2 × 10^6 cells/ml) from the various groups was added to the appropriate wells and mixed with 50 μl of irradiated CT26 cells or A20 cells (1 × 10^6 cells/ml) for 20 h at 37°C with 5% CO2. For titration experiments, splenocytes were mixed with tumor cells at ratios of 4:1, 2:1, and 1:1. To prevent rolling of T cells, the plate was secured in the incubator. Following the incubation period, the plate was washed four times with wash buffer, and 100 μl of detection buffer A was added to each well and incubated at 4°C overnight. The plate was then washed three times with buffer, and 15 μl of detection mixture B (containing streptavidin-alkaline phosphatase) was added to each well and incubated for 2 h at room temperature. The reaction was stopped using wash buffer, and the plate was washed three times before addition of 100 μl of reagent C (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chromogen) for 1 h at room temperature. The plate was rinsed with double-distilled H2O and allowed to dry completely before analysis using the Zeiss KS ELISPOT imaging system (Imaging Associates, Thame, U.K.).

**Flow cytometric analysis of tumor cells**

CT26 tumor cells were treated for 48 h with or without CC-4047 (10 μg/ml). Cells were then trypsinized, washed, and surface stained (20 min, 4°C) with anti-MHC class I PE (H-2K^d^, clone SF1-1.1.), CD80 PE (B7.1, 16-10A1), or CD86 PE (B7.2, GL1; all from BD Pharmingen, Oxford, U.K.) and appropriate isotype-matched controls. Upon flow cytometric analysis, tumor cells were gated according to their forward vs side scatter properties and displayed as single-color histograms. For each sample, 5000 gated cells were acquired and analyzed on a FACScan (BD Biosciences, Oxford, U.K.).

**Cytokine detection**

Splenocytes were stimulated in two experimental systems.

**Cross-linking of the TCR by immobilized monoclonal anti-CD3 in vitro.**

The anti-CD3 Ab (BioSource International, Nivelles, Belgium) was diluted in PBS (10 μg/ml) and coated onto flat-bottom polystyrene tissue culture plates by overnight incubation at 4°C. A single-cell suspension of naive splenocytes was obtained as previously described, and 500 μl of the splenocyte suspension (2 × 10^6 cells/ml) was coincubated with CC-4047 (10 μg/ml) or DMSO at 37°C. The supernatants were assessed 24 or 48 h later for the detection of IFN-γ, IL-2, GM-CSF, IL-10, and IL-4 by ELISA (BD Pharmingen).

**Tumor-specific stimulation of Th1-type cytokines.**

To assess the ability of T cells from vaccinated mice to produce IL-2 or IFN-γ, cultures of splenocytes from the different groups were set up in a total volume of 2 ml at a concentration of 2 × 10^6/ml and stimulated with 1 × 10^6 irradiated CT26 cells for 48 h at 37°C. The protein transport inhibitor GolgiPlug (BD Pharmingen) was added to cells for the final 5 h of culture to allow for intracellular accumulation of cytokines. At the end of culture, cells were counted, and Fc Block (BD Pharmingen) was added for 5 min at 4°C before surface staining (20 min, 4°C) with anti-CD3ε PerCP (145-2C11) and anti-CD8α FITC (53-6-7; BD Pharmingen). Cells were then fixed and permeabilized using the BD Pharmingen Cytofix/Cytoperm kit and stained (30 min, 4°C) with anti-IL-2-PE (JES 6-5H4) or anti-IFN-γ PE (XMG1.2) with appropriate isotype-matched and compensation controls. Upon flow cytometric analysis, T cell subsets were gated on CD3^+^ cells (5000 events), and cytokine production from either CD8^+^ or the negative fraction containing CD4^+^ cells was displayed as a density plot.

**Statistical analyses**

Data from the animal studies were analyzed using the log rank test. Other data were analyzed using one-way ANOVA, followed by Dunnett’s post hoc test.

**Results**

CC-4047 strongly enhances the partially protective antitumor effect of whole tumor cell vaccination and generates long-lasting immunity against subsequent live tumor challenge

To assess whether a potent costimulatory IMiD could protect against tumor development, we used CC-4047 in an autologous tumor vaccine animal model. Fig. 1a shows that the CT26 vaccine alone led to only 20% survival/tumor-free mice 60 days after the live challenge (p < 0.05). In contrast, the additional treatment with CC-4047 (50 mg/kg) conferred 80% survival, with 60% of mice still tumor free (p < 0.01). The lower dose of CC-4047 (5 mg/kg) provided 80% survival, of which 40% were tumor free
We found that CC-4047 had no effect in unvaccinated mice. Thd in combination with vaccination provided 40% tumor-free mice (data not shown). In repeated experiments using CC-4047 at 50 mg/kg, we found up to 90% tumor-free mice at the end of 60 days. CC-4047 was also found to significantly enhance protection \((p < 0.01)\) in the B16F10 model, providing 30% protection over vaccine alone (Fig. 1b).

Mice that remained tumor-free for 60 days after CC-4047 and autologous tumor cell vaccination were subsequently rechallenged with live CT26 tumor cells alone (Fig. 1c). These animals were fully protected from tumor development \((p < 0.01)\). In contrast, all naive (nonvaccinated) mice developed tumors by day 18. This strongly suggested that protected animals had developed long-lasting immunity to the tumor cells and that priming was strongly enhanced by costimulatory IMiD.

Protective antitumor activity following CC-4047 costimulation of autologous vaccination requires the presence of T cells

To determine the requirement for T cells in the protective antitumor response in normal BALB/c mice, we repeated the exact experimental conditions in BALB/c nude mice. All animals in this study developed tumors by day 12 after the live challenge and were sacrificed (Fig. 1d). The results clearly show that CC-4047 did not protect these mice from tumor development. Therefore, T cells were essential in the protective antitumor effect observed in normal mice. Furthermore, this also suggests that neither anti-angiogenic activity nor increased NK activity can account for all of the antitumor activity of CC-4047 in this setting.

**Costimulation by CC-4047 enhances the tumor-specific effector response via increased production of IFN-\(\gamma\), and this correlates with in vivo protection**

The tumor-specific CTL response was assessed from mice vaccinated with/without CC-4047 and unvaccinated mice. The data showed that vaccinated mice with/without CC-4047 had a CTL response to the target CT26 cells (Fig. 2a). However, vaccinated mice treated with CC-4047 had a slightly improved CTL response compared with the vaccinated group. ELISPOT data clearly demonstrated that numbers of IFN-\(\gamma\)-secreting splenocytes were significantly increased \((p < 0.05)\) in the vaccinated group, and to a greater extent in the group receiving vaccine and CC-4047, in response to CT26 cells (Fig. 2b). Furthermore, the specificity of this result was confirmed by varying the ratio of splenocytes to CT26 cells and the lack of IFN-\(\gamma\) secretion in response to the A20 cell line.

CC-4047 has no direct antitumor effect on growth inhibition or on the expression of MHC class I or costimulatory molecules (B7.1 and B7.2)

CC-4047 did not influence normal growth rates of CT26 cells in vitro, ruling out any possible protective effect due to direct cytotoxic effects (data not shown). Furthermore, surface expression of
MHC class I (already evident on CT26 cells) was not increased on treatment with CC-4047 in vitro. Similar assessment of the co-stimulatory molecules B7.1 (CD80) and B7.2 (CD86) was also undertaken (Fig. 3). There was some baseline expression of B7.1 on CT26 cells, which may contribute to the tumor's partial immunogenicity. However, CC-4047 did not alter the expression of B7.1, nor did it induce the expression of B7.2. Therefore, CC-4047 is likely to enable priming of T cells independently of direct co-stimulatory effects on the tumor cells.

CC-4047 induces Th1-type immunity in splenocytes from protected mice in vivo and induces a Th1 type cytokine response when cocultured with naive splenocytes in vitro

To determine whether there were any differences in the Th1 cytokine profiles of these mice, their spleens were removed and assessed for intracellular IFN-γ and IL-2 production. Vaccinated mice given CC-4047 showed significant increases in IFN-γ and IL-2 production compared with mice that received vaccine alone (Fig. 4). This indicates that drug treatment can induce a Th1-type response that is still evident following two live tumor challenges. This would also explain a potential mechanism of action of increasing cell numbers via the production of IFN-γ and IL-2. Furthermore, the increases in both cytokines were evident in the population containing CD4+ cells and to a lesser extent in CD8+ T cell populations.

Stimulation of naive splenocytes with immobilized anti-CD3 mAb induced IFN-γ (6982 pg/ml), IL-2 (125 pg/ml), GM-CSF (323 pg/ml), IL-4 (362 pg/ml), and IL-10 (6997 pg/ml) production. CC-4047 induced a consistent increase in IFN-γ, IL-2, and GM-CSF at 48 h (expressed as a percentage of that in anti-CD3-stimulated cultures). Furthermore, the drug significantly inhibited IL-10 secretion while causing a slight decrease in IL-4 (Fig. 5). Cytokine concentrations were low at 24 h; only IL-2 secretion was at similar levels at both 24 and 48 h (100 and 125 pg/ml, respectively). These data show that IMiDs can provide a...
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The protection in the relatively nonimmunogenic B16F10 model is long-lasting and is most likely due to the successful priming of tumor-specific memory T cells, evident upon second challenge. Although CTL activity was observed, there were no significant differences between the vaccinated groups with or without CC-4047, suggesting that protection is not reliant on an improved CTL response. However, it should also be noted that the ELISPOT assay is more sensitive than CTL assays for the assessment of effector cells (38). Also, it is possible that IFN-γ production may synergize with CTL to provide an optimum response. Inhibition of tumor angiogenesis by CD4+ T cells has been shown to be dependent on IFN-γ (26). Our data revealing increased production of IFN-γ in the CD4+ fraction of splenocytes in comparison to CD8+ T cells supports this hypothesis.

The use of adjuvants has been used in cancer vaccine models to improve Ag presentation and thus potentiate immune recognition of tumor-associated Ags (39). These adjuvants (such as bacillus Calmette-Guérin and Mycobacterium vaccae) act by enhancing immune activation in a nonspecific and highly inflammatory manner and notably boost Th1-type, rather than Th2-type, immunity. This may have some relevance to colorectal cancer, as a marked inhibition of Th1-type responses has been noted in patients with early colorectal cancer (40).

Many autologous tumor vaccine animal models use gene therapy approaches to improve antitumor responses. Cells transfected with appropriate cytokines such as IL-2 and GM-CSF have demonstrated the potential of cancer vaccines in a number of animal models (41). However, the present study provides the first evidence that treatment with a pharmacological agent can lead to T cell costimulation in vivo, resulting in Th1-mediated antitumor immunity. CC-4047 did not affect the B7.1 or B7.2 costimulatory molecules on CT26 cells. Moreover, the decrease in IL-10 observed following coinubcation in CC-4047 suggests that another costimulatory signal to naive T cells, resulting in the secretion of cytokines associated with Th1-mediated immune responses.

Discussion

This is the first report to demonstrate the ability of an investigational drug to induce T cell costimulation and subsequent protection against a live tumor challenge in a cancer vaccine model. We have shown that a costimulatory Thd analog (CC-4047) significantly enhances the partial antitumor immunity elicited by tumor cell vaccination. However, in unvaccinated mice the compound had no effect, indicating synergy between IMiDs and the vaccination protocol. A smaller, but significant, effect was also observed with Thd, in agreement with comparative in vitro experiments (data not shown).

Previous studies have shown that the CT26 tumor is moderately immunogenic, since, compared with naive mice, whole cell vaccination provides improved protection from a subsequent live tumor challenge (35). The protection we observed with CC-4047 is long-lasting and is most likely due to the successful priming of tumor-specific memory T cells, evident upon second challenge. The protection in the relatively nonimmunogenic B16F10 model by CC-4047 indicates that this finding is not merely peculiar to the CT26 cell line.

The fact that CC-4047 is only present during the priming phase and not during live tumor challenge rules out a direct antitumor effect. Furthermore, nude mice are not protected in this model, thereby demonstrating the importance of T cell memory. Recent evidence has suggested that the administration of IMiDs to patients with myeloma may augment NK activity (15). However, the lack of protection in nude mice suggests that NK cell activity is not solely responsible for the protective effects observed in this model. Interestingly, CC-4047 demonstrates significant anti-angiogenic effects using in vitro angiogenesis assays (K. Dredge, unpublished observations). However, although this may be relevant in a treatment setting, this is highly unlikely to influence tumor growth during subsequent live challenge in this study.

As a single agent, Thd has demonstrated notable activity in relapsed or refractory multiple myeloma, glioblastoma multiforme and chronic graft-versus-host disease, with preliminary evidence of activity in various solid tumors, such as colorectal cancer (36). It has been suggested that Thd can act against multiple myeloma by enhancing cell-mediated immunity via direct stimulation of cytotoxic T cells (7), probably with concomitant induction of the Th1 cell response resulting in the secretion of IFN-γ and IL-2. Indeed, Thd appears to act as a costimulator to T cells that have received signal 1 via the TCR. In previous studies stimulation of purified T cells with anti-CD3 Abs in the absence of signal 2 induced only minimal T cell proliferation. However, the addition of Thd to this cell culture system resulted in a concentration-dependent increase in proliferative responses. This response was accompanied by enhanced IL-2 and IFN-γ production. Moreover, in the absence of anti-CD3 there was no T cell proliferative response to Thd, indicating that the drug alone is not mitogenic (37). IMiDs have also been found to markedly stimulate T cell proliferation as well as IL-2 and IFN-γ production (22). We have previously found that this Thd analog enhances costimulation to human T cells more so than the parent compound (J. B. Marriott, unpublished observations).

In our study, CC-4047 provided the costimulatory signal to naive splenocytes stimulated with anti-CD3 mAb, increasing Th1-type cytokines while producing inhibitory effects on Th2-type cytokines. We hypothesize that in vivo costimulation of T cells occurs in the priming phase of the immune response, resulting in a larger population of IFN-γ-secreting effector and memory T cells. However, CC-4047 does not enhance IL-12 production in naive splenocytes stimulated with LPS in vitro or in the lymph nodes of vaccinated/CC-4047-treated mice in vivo (data not shown). The costimulatory activity of IMiDs on T cells is known to be independent of APC, since isolated CD8+ T cells can be activated (12). Therefore, production of IL-12 by APC may not be an absolute requirement for this activity.

The IFN-γ production following CC-4047 treatment in vaccinated mice correlated with protection following live tumor cell challenge. Although CTL activity was observed, there were no significant differences between the vaccinated groups with or without CC-4047, suggesting that protection is not reliant on an improved CTL response. However, it should also be noted that the ELISPOT assay is more sensitive than CTL assays for the assessment of effector cells (38). Also, it is possible that IFN-γ production may synergize with CTL to provide an optimum response. Inhibition of tumor angiogenesis by CD4+ T cells has been shown to be dependent on IFN-γ (26). Our data revealing increased production of IFN-γ in the CD4+ fraction of splenocytes in comparison to CD8+ T cells supports this hypothesis.

FIGURE 5. CC-4047 can provide a costimulatory signal to T cells from naive mouse splenocytes stimulated with immobilized anti-CD3 mAb. Increased secretion of IFN-γ, IL-2, and GM-CSF was detected. However, IL-10 and, to a lesser extent, IL-4 were decreased following coinubcation with CC-4047, indicating the induction of a cytokine response associated with Th1-type immunity. Data are expressed as the mean percentages of anti-CD3-stimulated controls (with SEs) following anti-CD3 stimulation with CC-4047. Unstimulated samples secreted low or undetectable levels of all cytokines (data not shown), demonstrating that CC-4047 alone had no effect on cytokine secretion. Data are representative of at least two independent experiments.

The use of adjuvants has been used in cancer vaccine models to improve Ag presentation and thus potentiate immune recognition of tumor-associated Ags (39). These adjuvants (such as bacillus Calmette-Guérin and Mycobacterium vaccae) act by enhancing immune activation in a nonspecific and highly inflammatory manner and notably boost Th1-type, rather than Th2-type, immunity. This may have some relevance to colorectal cancer, as a marked inhibition of Th1-type responses has been noted in patients with early colorectal cancer (40).
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