Tumor Infection by Oncolytic Reovirus Primes Adaptive Antitumor Immunity

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Abstract **Purpose:** Early clinical trials are under way exploring the direct oncolytic potential of reovirus. This study addresses whether tumor infection by reovirus is also able to generate bystander, adaptive antitumor immunity.

Experimental Design: Reovirus was delivered intravenously to C57BL/6 mice bearing lymph node metastases from the murine melanoma, B16-tk, with assessment of nodal metastatic clearance, priming of antitumor immunity against the tumor-associated antigen tyrosinase-related protein-2, and cytokine responses. In an *in vitro* human system, the effect of reovirus infection on the ability of Mel888 melanoma cells to activate and load dendritic cells for cytotoxic lymphocyte (CTL) priming was investigated.

Results: In the murine model, a single intravenous dose of reovirus reduced metastatic lymph node burden and induced antitumor immunity (splenocyte response to tyrosinase-related protein-2 and interleukin-12 production in disaggregated lymph nodes). *In vitro* human assays revealed that uninfected Mel888 cells failed to induce dendritic cell maturation or support priming of an anti-Mel888 CTL response. In contrast, reovirus-infected Mel888 cells (reo-Mel) matured dendritic cells in a reovirus dose-dependent manner. When cultured with autologous peripheral blood lymphocytes, dendritic cells loaded with reo-Mel induced lymphocyte expansion, IFN-γ production, specific anti-Mel888 cell cytotoxicity, and cross-primed $CD8^+$ T cells specific against the human tumor-associated antigen MART-1.

Conclusion: Reovirus infection of tumor cells reduces metastatic disease burden and primes antitumor immunity. Future clinical trials should be designed to explore both direct cytotoxic and immunotherapeutic effects of reovirus.

Oncolytic viruses are self-replicating, tumor-selective viruses, which directly lyse cancer cells (1). Although most interest in both naturally occurring and genetically modified oncolytic viruses has focused on their direct oncolytic properties, there is accumulating evidence suggesting that tumor infection can also induce antitumor immunity (2–8).

The ability of the immune system to modify the immunogenicity and behavior of clinically evident tumors, and the host of mechanisms by which tumors can induce a state of immune tolerance, is becoming increasingly recognized (9). Although a range of tumor-associated antigens (TAA) have been identified (10), the presence of tumor-associated "danger" signals is critical to the generation of an antitumor immune response (11). Tumors commonly lack such signals, and it has been proposed that successful tumor immunotherapy will be dependent on their provision (12). Oncolytic virotherapy is expected to promote an inflammatory "dangerous" environment within the tumor, involving the release of proinflammatory cytokines, Toll-like receptor ligands, and an infiltration of innate immune cells (12–14). In addition, virally induced tumor cell lysis can release a wide range of TAA into the tumor microenvironment for uptake by professional antigen-presenting cells, such as dendritic cells, for adaptive T-cell priming. The immune consequences of oncolytic viral therapy are, however, finely balanced, with many viruses possessing immune evasion strategies involving the inhibition of dendritic cell maturation and/or function (15, 16). The ability of dendritic cells to take up and cross-present TAA in an appropriate costimulatory context to T cells is central to the generation of an effective adaptive antitumor cellular immune response (17).

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Translational Relevance

Oncolytic viruses are able to selectively lyse cancer cells and represent a promising novel approach to anticancer therapy. Despite widespread interest in their direct anticancer activity, only limited attention has been applied to the critical interaction between viral therapy and the immune system. Antiviral immune responses can limit the efficacy of oncolytic virotherapy by viral clearance; in contrast, viral oncolysis may release TAA in combination with "danger" signals leading to the generation of antitumor immunity. Reovirus is a naturally occurring oncolytic virus, currently in phase I and II clinical trials. This study shows that reovirus infection of tumor cells is immunogenic, activating dendritic cells and providing a source of TAA in an "dangerous" context to prime adaptive antitumor immune responses. On this basis, future clinical trials of oncolytic virotherapy should be designed to explore immunotherapeutic as well as direct cytotoxic efficacy.

Reovirus is a naturally occurring oncolytic virus and is a ubiquitous member of the Reoviridae family of nonenveloped double-stranded RNA viruses. Reovirus can be isolated from the respiratory and enteric tracts of humans, but infection is usually asymptomatic (18, 19). Nontransformed cells are relatively resistant to infection, whereas transformed cells with activated ras signaling pathways are permissive to reovirus via enhancement of viral uncoating, increased particle infectivity, and apoptotic release of viral progeny (20). Ras signaling pathways are aberrant in most human tumors, involving either activating Ras mutations or altered upstream or downstream pathways (21), such that reovirus has a broad range of activity against human tumors including breast, colon, ovary, brain, and hematologic malignancies in preclinical models (22, 23). Oncolytic reovirus has already entered early-phase clinical trials, administered intratumorally (24) and intravenously (25). The ability of reovirus infection of tumors to generate antitumor immunity has, however, not been fully addressed. In older murine studies combining reovirus with the chemotherapeutic agent BCNU, cured mice were protected from tumor rechallenge, implying an immune-mediated effect, although no mechanisms were defined (26).

This study first shows the ability of intravenous reovirus monotherapy to induce antitumor immunity, in addition to a reduction in metastatic disease burden, in an immunocompetent murine melanoma model of lymph node metastases. To determine the applicability of these findings to human cancer, we have also explored the immune consequences of reovirus infection of the human melanoma cell line Mel888. Reovirus-infected (but not uninfected) Mel888 cells activate dendritic cells phenotypically and functionally in a contact-dependent manner. Only dendritic cells loaded with reovirus-infected Mel888 cells prime an *in vitro* naive CTL response against Mel888, including cross-priming of CTL specific for the melanoma TAA, MART-1. These murine and human data support the role of reovirus as an immunogenic as well as directly cytotoxic therapy for human neoplasia, activating dendritic cells and priming effective antitumor immunity.

Materials and Methods

Reovirus. Reovirus type 3 Dearing strain was provided by Oncolytics Biotech and stored in the dark at neat concentrations in PBS at 4°C (maximum 3 months) or at -80°C (long-term storage). Virus titer was determined by a standard plaque assay using L929 cells.

Murine *in vivo* assays

Murine cells. Mouse B16-tk melanoma cells (H2-K^b) were derived from B16 cells by transducing them with a cDNA encoding the herpes simplex virus thymidine kinase gene (27). Cells were grown in DMEM (Life Technologies) supplemented with 10% (v/v) FCS (Life Technologies), L-glutamine (Life Technologies), and 1.25 μg/mL puromycin selection. Cell lines were routinely tested for *Mycoplasma* and found to be free of infection.

In vivo *studies.* All procedures were approved by the Mayo Foundation Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from Jackson Laboratories at ages 6 to 8 weeks. To establish subcutaneous tumors, 5×10^5 B16-tk cells were injected in 100 μL PBS into the flanks of mice (subgroups of three mice in each experiment). Ten days later, 5×10^8 plaque-forming units (pfu) reovirus or PBS was administered intravenously. Tumor draining lymph nodes and spleen were explanted after a further 10 days.

PCR screening for B16-tk tumor cells. Genomic DNA from lymph nodes was prepared with the DNeasy kit (Qiagen). DNA (10 ng) was amplified by PCR with primers specific for HSV-tk, which is stably integrated into the genome of B16-tk tumor cells. As a control, PCR was done with primers specific for a genomic fragment of the murine tyrosinase promoter. In all experiments, a mock PCR (without added DNA) was done to exclude contamination.

Puromycin-resistant colony outgrowth assay to detect metastatic B16-tk tumor cells. B16-tk tumor cells stably express the puromycin resistance gene, allowing for growth in puromycin. To select for viable B16-tk cells present at resection, 1×10^6 cells from dissociated lymph nodes were plated in six-well plates at 1.25 μg/mL puromycin. Every 2 to 3 days, cultures were washed and fresh puromycin-containing medium was added. Within 5 to 10 days, individual puromycin-resistant colonies were counted in wells.

ELISA for IFN-γ secretion. Day 10 splenocytes (1×10^6) were plated into 24-well plates in triplicate and incubated at 37°C with 5 μg/mL of appropriate peptide. Cell-free supernatants were collected after 48 h and tested by specific ELISA for IFN-γ according to the manufacturer's instructions (OptEIA IFN-γ kit; BD Biosciences). The synthetic, H-2Kb-restricted peptides tyrosinase-related protein-2 $(\text{TRP-2})_{180\text{-}188}$ SVYDFFVWL and control ovalbumin SIINFEKL were synthesized at the Mayo Foundation Core Facility.

Statistics. The two-sample unequal variance Student's t test was used for *in vitro* assays. Statistical significance was determined at the level of $P < 0.05$.

Human *in vitro* assays

Cell culture. Human melanoma cell lines Mel888, Mel624, Mewo, SK-Mel28, HT144, and MM96 and nonmelanoma tumor cell lines SW480, HCT116 (colorectal), MCF7 (breast), SKOV-3 (ovarian), EJ (bladder), and, SiHa (cervix) were grown in DMEM (Life Technologies) supplemented with 10% (v/v) FCS (Harlan Sera-Labs) and 1% (v/v) L-glutamine (Life Technologies). Cells were routinely tested for *Mycoplasma* and found to be free of infection.

Human dendritic cell generation. Peripheral blood mononuclear cells (PBMC) were obtained from buffy coats of healthy blood donors by Ficoll-Hypaque density centrifugation, and monocytes were isolated by plastic adherence as described previously (28). Immature dendritic cells were generated by culture in dendritic cell medium [RPMI 1640 (Life Technologies) supplemented with 10% (v/v) FCS, 1% L-glutamine, 800 units/mL granulocyte-macrophage colony-stimulating factor, and 500 units/mL interleukin (IL)-4 (R&D Systems)] for 5 days.

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Reovirus infection of Mel888 cells and dendritic cell coculture. Mel888 cells were seeded on day 1 and infected on day 2 at 0.1, 1, and 10 pfu reovirus per cell. After 18 h infection, Mel888 cells were harvested and cultured with dendritic cells at a 3:1 ratio in dendritic cell medium. Lipopolysaccharide (250 ng/mL; Sigma) was added where appropriate as a positive control for dendritic cell activation. Cocultures were harvested at 24 h. Cell-free supernatants were stored at -80°C. To test contact dependence, dendritic cells and tumor cells were separated by filters (0.4 μm) in Transwell plates (Corning).

Flow cytometry. Flow cytometry was done using a FACSCalibur (Becton Dickinson). Anti-human HLA-DR-FITC, CD80-PE, CD83-PE, CD86-PE, and CD40-PE (BD Pharmingen) were used for dendritic cell phenotype. Dendritic cells were identified in the mixed dendritic cell/tumor cell population by gating on HLA-DR-FITC-positive cells (Mel888 cells are class II negative).

Cytokine detection. Levels of IFN-γ, IL-4, IL-6, IL-10, IL-12p70, and tumor necrosis factor-α (TNF-α) in tissue culture supernatants were measured by ELISA using matched paired antibodies (all from BD Biosciences, except TNF-α from Biosource) according to manufacturers' instructions.

Dendritic cell viability. Dendritic cells were labeled with 1 μmol/L CellTracker Green (Invitrogen) before coculture with Mel888 cells, as above. Dendritic cells and tumor cells were harvested and stained with propidium iodide (Sigma) before flow cytometry with analysis gated on labeled dendritic cells.

Phagocytosis assay. Living dendritic cells and Mel888 cells were labeled with 1 μmol/L CellTracker Green and 5 μmol/L CellTracker Red (Invitrogen), respectively, before coculture at a 1:3 ratio. Double-positive cells were enumerated by flow cytometry. Subsequent incubation for 1 h with 75 nmol/L Lysotracker Blue (Invitrogen) was used to colabel late phagosomal and lysosomal structures. Living cells were visualized using a Zeiss Axiovert 200 inverted fluorescence microscope as described previously (29).

Generation of tumor-specific CTL. Immature dendritic cells were loaded with uninfected Mel888 cells or Mel888 cells infected for 18 h with 0.1 pfu/cell reovirus at a 1:3 ratio. Tumor-loaded dendritic cells were irradiated (30 Gy) and mixed with autologous PBMCs at a 1:10 to 1:30 ratio. CTL medium [RPMI 1640 supplemented with 7.5% (v/v) human AB serum (Sigma), 1% (v/v) L-glutamine, 1% (v/v) sodium pyruvate (Life Technologies), 1% (v/v) nonessential amino acids (Life Technologies), 1% (v/v) HEPES (Life Technologies), 20 μmol/L β-mercaptoethanol (Sigma)] was used in CTL cultures supplemented with 5 ng/mL IL-7 (R&D Systems) from day 1 and 30 units/mL IL-2 (R&D Systems) on day 4 only. Cultures were restimulated using the same protocol at weekly intervals. Cells were harvested at day 14 or 21.

^{51}Cr cytotoxicity assay. Cytotoxicity was measured using a standard 4 h ^{51}Cr release assay (30). Unlabeled K562 cells were added to tumor targets to reduce nonspecific killing. Supernatants (4 h) were counted in scintillation plates (Packard Biosciences). Percent lysis was calculated using the formula: % lysis = 100 × [(counts/min experiment) - (counts/min spontaneous release)] / [(counts/min maximum release) - (counts/min spontaneous release)].

CD107 lymphocyte degranulation assay. Lymphocyte degranulation was measured as described previously (31). CTL and tumor targets were incubated at a 1:1 ratio with anti-CD107a-FITC and anti-CD107b-FITC antibodies (BD Biosciences) with brefeldin A (Sigma) added at 10 μg/mL after 1 h. After a further 4 h, CTL were stained with anti-human CD8-PerCP and acquired by flow cytometry. To determine MHC class I restriction, a pan-MHC class I blocking antibody (Dako) or an isotype antibody (Dako) was added at 50 μg/mL throughout CTL/tumor target incubation.

Assessment of MART-1-specific lymphocytes. CTL were treated with Dead Cell Discrimination Kit (Miltenyi Biotec), labeled with MART-1-PE pentamer (ELAGIGITLV) or human negative control PE pentamer (Proimmune), counterstained with CD8-FITC, and fixed in 1% paraformaldehyde as per manufacturers' protocols. Analysis was done by flow cytometry, gating on live lymphocytes by excluding cells labeled with Dead Cell Discriminator.

Results

***Intravenous reovirus reduces lymph nodes metastases* in vivo.** A murine model of lymph node metastasis from an established tumor was used as described recently (5). In this model, the readouts are the clearance of metastases from lymph nodes draining the primary tumor, cytokine production, and associated generation of an immune response against a melanoma TAA (TRP-2). In the current work, a melanoma cell line encoding HSV-tk was used (27), with tumor detection

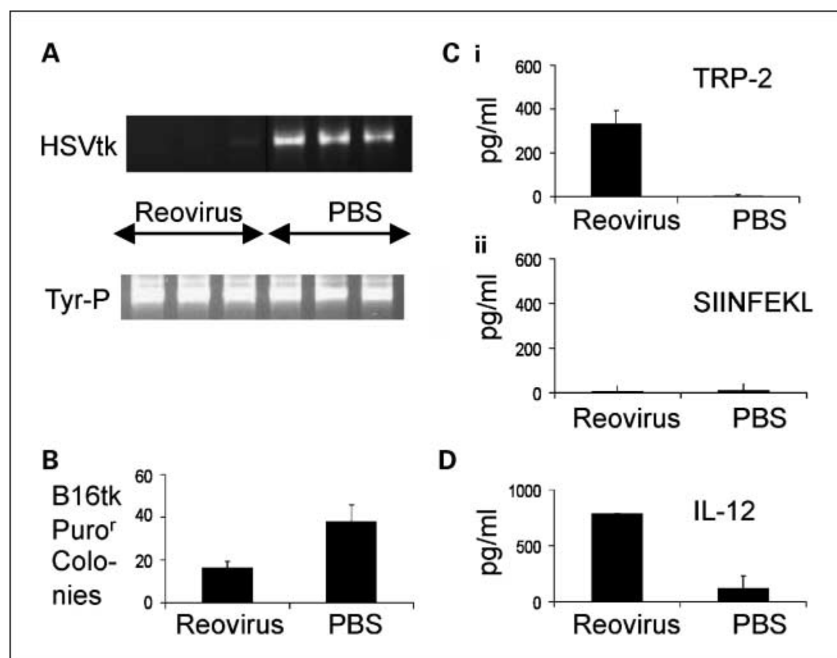

Fig. 1. Intravenously administered reovirus can reduce lymph node metastatic melanoma burden, prime antitumor immunity, and induce proinflammatory cytokines. C57BL/6 mice were seeded with subcutaneous B16-tk tumors (5×10^5 cells). Ten days later, mice were treated intravenously with 5×10^8 pfu reovirus or with PBS. Ten days after that, tumor draining lymph node and spleen were explanted, and lymph nodes were dissociated and plated overnight in culture. *A,* total genomic DNA from 10^6 lymph node cells was screened with primers specific for the HSV-tk gene. Equal loading of DNA was confirmed using primers specific for a genomic fragment in the tyrosinase gene promoter. *B,* 10^6 cells from the dissociated lymph node cultures were seeded in medium containing 1.25 μg/mL puromycin to select for viable B16-tk cells, which were present in the lymph node at the time of resection. Within 5 to 10 days, individual puro^r colonies appear, which were counted. *C,* splenocytes recovered at day 10 were pulsed, in triplicates of 750,000, with the synthetic TRP-$2_{180-188}$ SVYDFFVWL peptide (*i*) or with the irrelevant H-2Kb-restricted OVA SIINFEKL peptide (*ii*). Forty-eight hours later, supernatants were assayed by ELISA for IFN-γ. *D,* explanted tumor draining lymph node were dissociated and plated overnight in culture. Supernatants were assayed for IL-12. Representative of one of two independent experiments.

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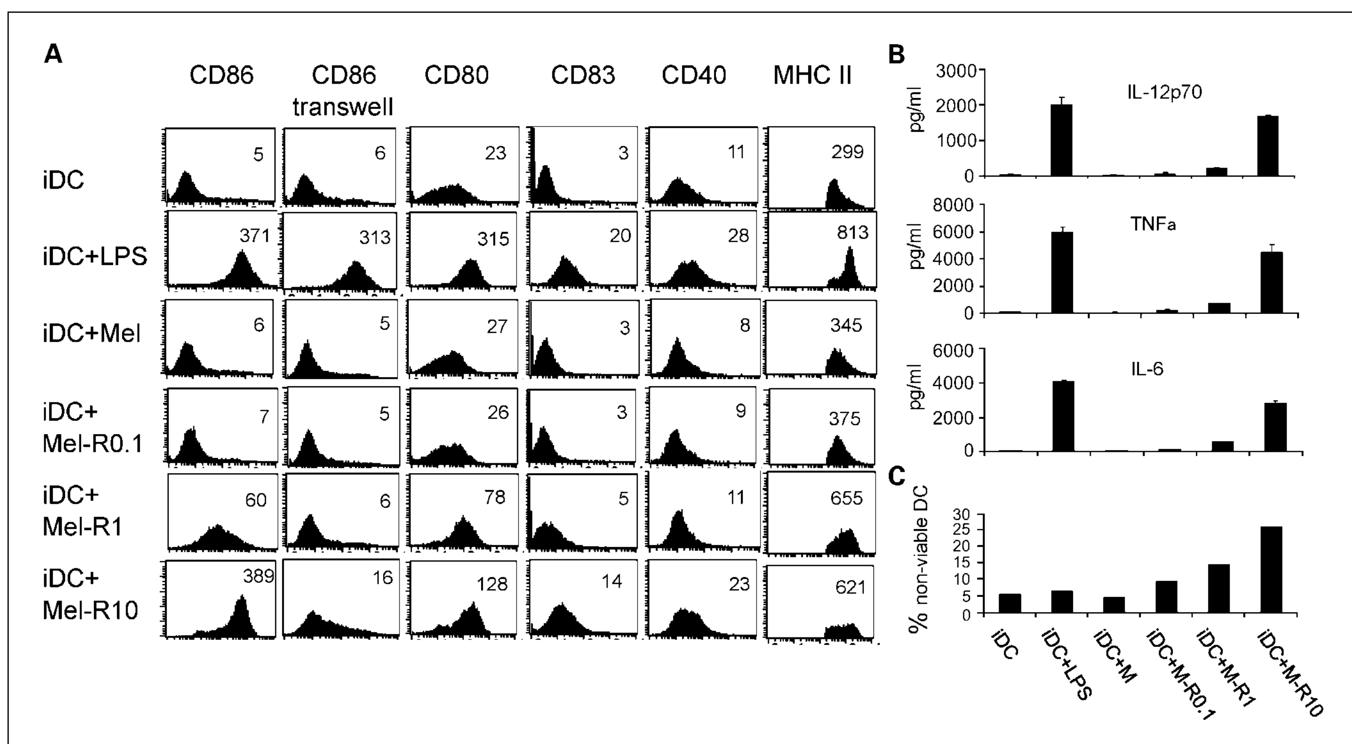

Fig. 2. Analysis of human dendritic cell phenotype, cytokine secretion, and viability after coculture with reovirus-infected Mel888 cells. Dendritic cells were incubated with lipopolysaccharide (250 ng/mL), uninfected Mel888 cells, or Mel888 cells infected with 0.1, 1, and 10 pfu reovirus/Mel888 cell at a 1:3 ratio for 24 h. *A,* surface expression of dendritic cell phenotypic markers, CD86, CD80, CD83, CD40, and MHC class II, was examined by flow cytometry. Median fluorescence intensity is shown in each plot. Contact dependence of phenotypic changes was examined using a 0.4 μm Transwell. Representative of at least four independent experiments. *B,* levels of IL-12p70, IL-6, and TNF-α in supernatant were determined by ELISA. Representative of six independent experiments. *C,* proportion of nonviable dendritic cells was examined by flow cytometry, gating on dendritic cells labeled with CellTracker Green, and stained with propidium iodide. Representative of two independent experiments.

measured by reverse transcription-PCR for the HSV-tk transgene and puromycin-resistant tumor colony outgrowth. B16-OVA, as studied previously (5), was not suitable, as the OVA-transfected line (unlike parental B16 and B16-tk) is relatively resistant to oncolysis by reovirus (data not shown). The mechanism of the resistance of B16-OVA to reovirus is unclear, although the mechanisms underlying sensitivity to reovirus are known to be complex (20). Ten days after seeding with subcutaneous B16-tk tumors, mice were treated intravenously with 5×10^8 pfu reovirus or PBS; 10 days later, tumor draining lymph nodes and spleen were isolated for analysis.

Semiquantitative PCR for the HSV-tk transgene indicated that intravenous delivery of reovirus alone induced a reduction (though not clearance) of the numbers of B16-tk cells that could be detected in the draining lymph nodes (Fig. 1A). These results were supported by a significant reduction in the number of puromycin-resistant B16-tk colonies grown from dissociated tumor draining lymph node cultures exposed to puromycin following treatment with intravenous reovirus (for pooled results of two independent experiments, $P = 0.015$; Fig. 1B). These data suggest that direct intravenous reovirus is able to significantly reduce the tumor burden in lymph nodes draining a primary B16-tk tumor in immunocompetent mice.

Reovirus oncolysis in lymphoid organs primes antitumor immunity and induces IL-12 in lymph nodes following* in vivo *delivery. The splenocytes recovered at day 10 were pulsed with synthetic TRP-$2_{180-188}$ peptide and an irrelevant SIINFEKL epitope of the ovalbumin antigen, and supernatants were assayed for IFN-γ after 48 h (Fig. 1C). A single intravenous dose of reovirus was able to prime significant anti-TRP-2 immune responses (Fig. 1C); no specific T-cell responses were seen toward the irrelevant SIINFEKL epitope (Fig. 1C). These data suggest that intravenous delivery of reovirus is effective at priming antitumor immunity through the breaking of tolerance to self tumor antigens. To investigate this further, the production of Th1 cytokines from explanted tumor draining lymph node was examined. Reovirus induced significant levels of IL-12 (Fig. 1D), although neither IL-6 nor TNF-α were detected (data not shown).

Taken together, these data indicate that *in vivo* a single dose of intravenous reovirus can reduce tumor metastatic burden and induce priming of an antitumor immune response in a fully immunocompetent murine system. To progress these studies toward clinical application, we next tested whether the observations made in the murine model also applied to human *in vitro* systems.

Effect of reovirus-infected Mel888 cells on human dendritic cell phenotype, function, and viability. Dendritic cells are the key antigen-presenting cells regulating adaptive immunity, and the interaction between tumor cells and dendritic cells is critical in determining the ability of dendritic cells to generate an effective immune response (32). To test the immunologic consequences of reovirus infection of tumor cells in a human *in vitro* system and to translate the murine data toward human application, the effect of reovirus infection of the human melanoma cell line Mel888 on dendritic cell phenotype, cytokine secretion, and

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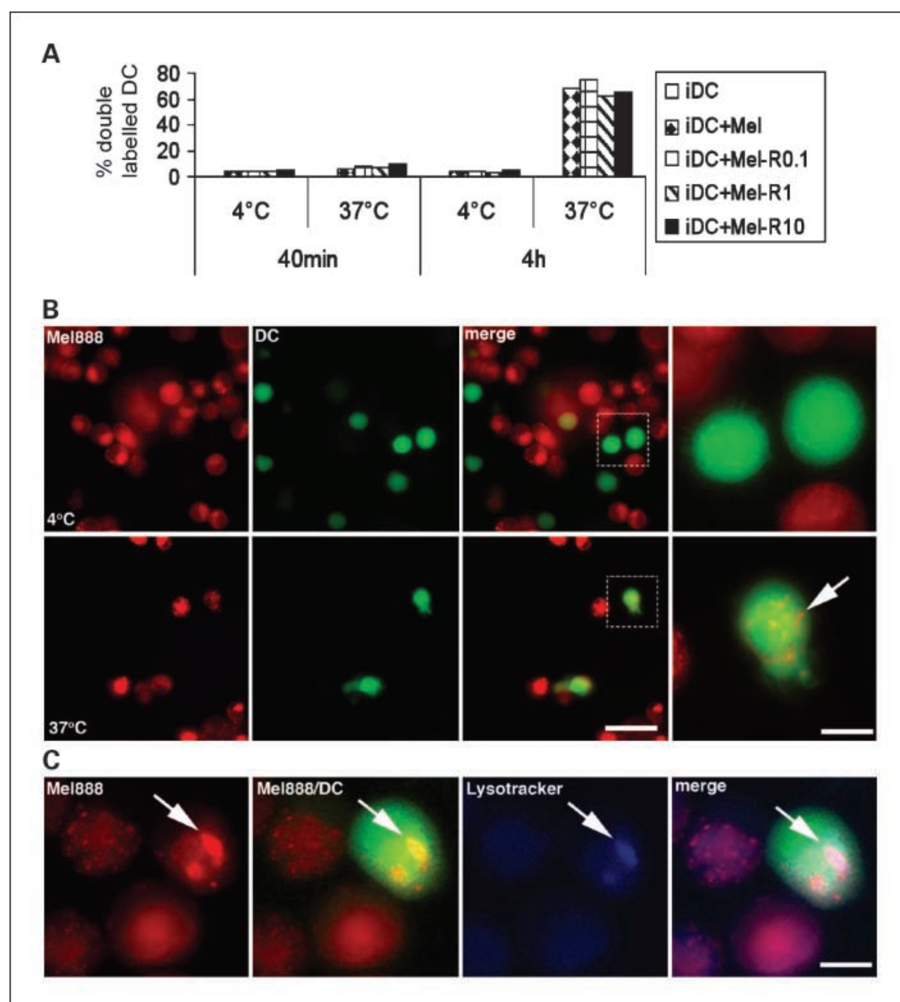

Fig. 3. Dendritic cells phagocytose uninfected and reovirus-infected Mel888 cells equally efficiently. Dendritic cells labeled with CellTracker Green were cocultured at 4°C and 37°C at a 1:3 ratio with Mel888 cells or Mel888 cells infected with 0.1, 1, or 10 pfu reovirus and labeled with CellTracker red. *A,* uptake of Mel888 by dendritic cells was examined by flow cytometry after coculture for 40 min or 4 h. The percentage of dendritic cells double-labeling red and green was assessed. Representative of six independent experiments. *B,* uptake of Mel888 tumor material by dendritic cells after 4 h at 37°C was confirmed by fluorescence microscopy of living cultures. At 4°C, no Mel888-derived material (*red*) was observed within dendritic cells (*green*), whereas at 37°C Mel888 material was observed to be internalized within dendritic cells (*arrow*). Bar, 40 μm. For zoomed image: bar, 10 μm. *C,* Mel888-derived material internalized within dendritic cells localized to late endosomal/lysosomal structures as shown by colocalization of red-dyed material with Lysotracker blue-labeled intracellular compartments within green-labeled dendritic cells (*arrow*). Bar, 10 μm. Representative of data from two independent donors. Similar images were obtained following 4 h coculture of dendritic cells with uninfected or reovirus-infected Mel888 cells.

viability was first examined. Recent data have confirmed that reovirus is cytotoxic to human melanoma cells, that infected cells secrete inflammatory cytokines (33), and that free reovirus directly matures dendritic cells (34). However, the effect of reovirus infection on the interaction between tumor cells and dendritic cells has not been addressed previously. First, immature dendritic cells were cocultured for 24 h with control Mel888 cells or Mel888 cells, which had been infected with 0.1, 1, and 10 pfu/cell reovirus 18 h previously; lipopolysaccharide was used as a positive control for dendritic cell activation. Dendritic cell maturation was examined by surface expression of CD86, CD80, CD83, CD40, and MHC class II (Fig. 2A) and secretion of the inflammatory cytokines IL12p70, TNF-α, and IL-6 (Fig. 2B).

Uninfected Mel888 cells had little effect on the immature dendritic cell phenotype. In contrast, reovirus-infected Mel888 cells induced dendritic cell maturation in a virus dose-dependent fashion (Fig. 2A). Although phenotypic changes were minimal with Mel888 cells infected with reovirus 0.1 pfu/cell, the dendritic cell phenotype following coculture with Mel888 cells infected at 10 pfu/cell was similar to that induced by lipopolysaccharide. To explore the mechanisms behind the maturation of dendritic cell phenotype, Transwell experiments were done to test whether this effect required cell-cell contact or was mediated by a soluble factor. As seen in Fig. 2A, the up-regulation of a representative activation marker, CD86, induced by reovirus-infected Mel888 cells was almost completely abrogated, indicating that dendritic cell maturation was dependent on contact between melanoma cells and dendritic cells.

As shown in Fig. 2B, immature dendritic cells, as expected, produced very low levels of IL-12p70, TNF-α, and IL-6. Coculture of dendritic cells with uninfected Mel888 cells did not affect production of these cytokines, whereas reovirus infection of Mel888 cells elicited a dose-dependent increase in all three.

Several oncolytic viruses including vaccinia virus (35) and herpes simplex virus-1 (36) have been reported to adversely effect dendritic cell viability. Therefore, the effect of reovirus-infected Mel888 cell coculture on dendritic cell viability was examined by propidium iodide staining of dendritic cells. As shown in Fig. 2C, there was some loss of dendritic cell viability, although toxicity was minimal at a reovirus dose of 0.1 pfu per Mel888 cell.

Reovirus does not alter phagocytic uptake of Mel888 cells into dendritic cell late endosomes/lysosomes. The infection of tumor cells by several viruses has been reported to enhance the phagocytosis of these cells by dendritic cells (7, 8). Therefore, phagocytic assays were done using differential cell labeling of dendritic cells and tumor cells with fluorescent dyes to allow

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analysis of uptake of Mel888 cells ± reovirus infection by dendritic cells. As shown in Fig. 3A, any association of tumor cells with dendritic cells was low after 40 min coculture, but after 4 h the majority of dendritic cells were associated with material from tumor cells; reovirus infection of Mel888 cells did not alter association of tumor with dendritic cells. Because flow cytometry is unable to determine whether dendritic cells were phagocytosing material from Mel888 cells, as opposed to closely associating with such material or adhering to intact tumor cells, fluorescence microscopy was also done as shown in Fig. 3B. Images showed uptake of Mel888 material (red), into dendritic cells (green), confirming phagocytosis. These images were similar regardless of whether Mel888 cells were infected with reovirus. To address the nature of the subcellular compartments to which tumor material localized after uptake by dendritic cells, microscopy was carried out after the incubation of cocultures with Lysotracker Blue, a dye that labels acidic late endosomal and lysosomal structures. Within dendritic cells, internalized tumor cell material clearly colocalized with Lysotracker Blue-labeled compartments, consistent with phagocytic uptake into appropriate compartments for priming (37).

Priming of tumor-specific CTL by dendritic cells loaded with reovirus-infected Mel888 cells. Next, the ability of reovirus-infected versus uninfected Mel888 cells to prime a human naive T-cell response was determined. Dendritic cells were loaded for 24 h with uninfected Mel888 cells (Mel-DC) or Mel888 cells infected with 0.1 pfu/cell reovirus (reo-Mel-DC). Autologous PBMC were then cocultured with tumor-loaded dendritic cells and restimulated with further dendritic cells loaded in the same way weekly. Despite minor effects on dendritic cell phenotype and function (Fig. 2A and B), this low reovirus concentration of 0.1 pfu/Mel888 cell was selected for these longer-term priming cultures due to the lack of toxicity to dendritic cells (Fig. 2C) and to avoid overwhelming dendritic cells with mounting viral antigen during replication. In addition, recent insights have suggested that phenotypic maturation per se is not the distinguishing feature of immunogenic versus tolerogenic dendritic cells (38).

To monitor PBMC proliferation during naive priming, trypan blue exclusion was used to determine the number of viable cells each week. The results of two donors representative of results in >10 experiments are shown in Fig. 4A. Consistent with previous data (30), PBMC stimulated with Mel-DC did not undergo any expansion. In contrast, stimulation with reo-Mel-DC consistently yielded more effector cells after 2 weeks of culture.

The activity of CTL generated by Mel-DC and reo-Mel-DC after 2 weeks of culture toward Mel888 cells and other melanoma and nonmelanoma targets was determined first using a standard ^{51}Cr release assay. Figure 4B shows the pattern of target killing in two donors, representing results typical of six independent experiments. CTL generated by stimulation by Mel-DC showed little cytotoxic activity. In contrast, CTL generated using reo-Mel-DC consistently exhibited high levels of specific cytotoxicity toward Mel888 cells, with up to 80% lysis observed. As shown, no significant cytotoxicity was observed toward a limited range of other cell lines in this ^{51}Cr release assay. To further assess the degree of specificity of these CTL, their degranulation was assessed using a CD107

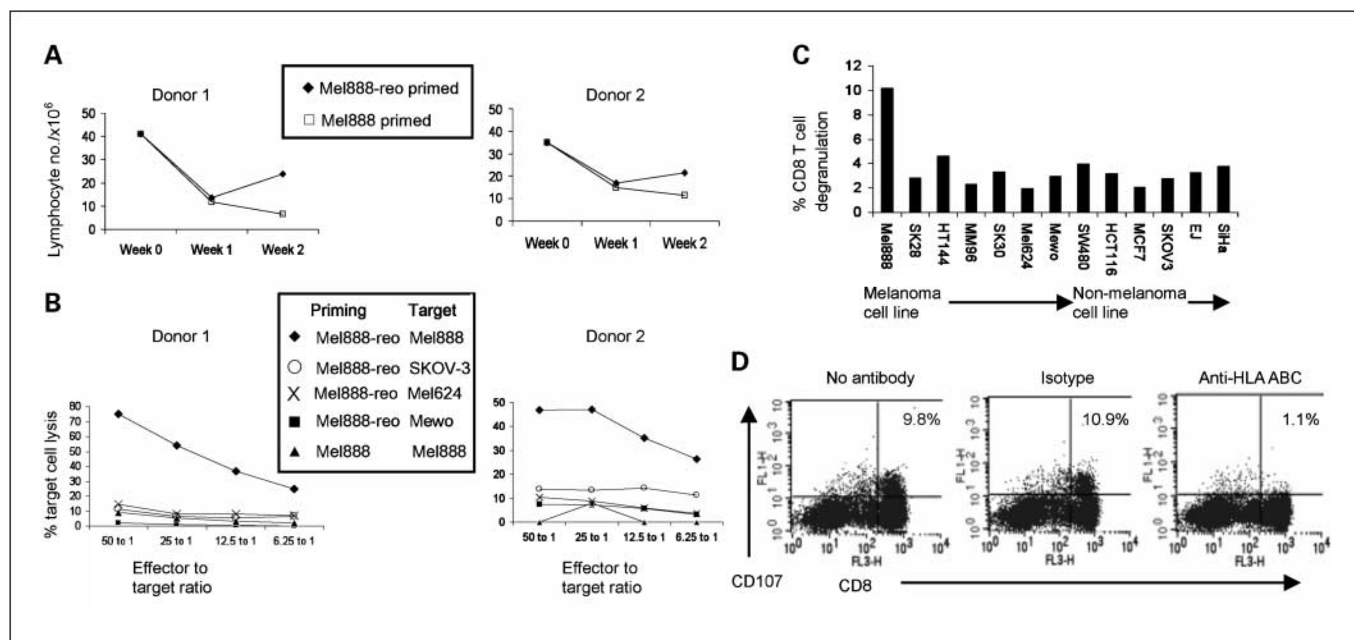

Fig. 4. Naive priming of human tumor-specific cytotoxic lymphocytes by dendritic cells loaded with reovirus-infected Mel888 cells. PBMC were incubated with autologous dendritic cells loaded overnight with Mel888 cells or Mel888 cells infected with 0.1 pfu reovirus per Mel888 cell (at 1:3 ratio), restimulated 7 d later, and assayed at 14 d. *A,* lymphocyte proliferation was determined by trypan blue exclusion. *B,* cytotoxicity of lymphocytes primed in the presence or absence of reovirus was determined by ^{51}Cr release assay using Mel888 cells and a range of melanoma and nonmelanoma cell lines as targets. Two experiments representative of at least six independent donors are shown in *A* and *B. C,* specificity of the activity of lymphocytes primed by dendritic cells loaded with reovirus-infected Mel888 cells toward Mel888 cells was further tested using the CD107 lymphocyte degranulation assay toward a larger panel of melanoma and nonmelanoma cell lines. *D,* a pan-MHC class I blocking antibody and isotype control was used to test the MHC class I restriction of degranulation, measured by the CD107 assay, toward Mel888 cells. The percentage of CD8 T cells degranulating is shown. Representative of at least four independent experiments.

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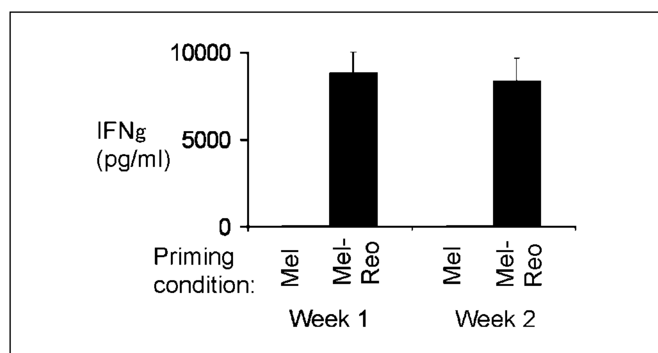

Fig. 5. Cytokine production in CTL cultures. *A,* levels of IFN-γ in CTL cultures were determined by ELISA each week. Data from one donor representative of nine.

expression assay (31) in response to a wider panel of melanoma and nonmelanoma cell lines. As shown in Fig. 4C, reo-Mel-DC-generated CTL exhibited no significant cross-reactivity to 12 other cell lines. Furthermore, CTL activity was MHC class I restricted as shown by significant reduction in the levels of degranulation against Mel888 cells in the presence of a pan-MHC class I blocking antibody (Fig. 4D).

Cytokine production in CTL priming cultures. The profile of cytokines produced within the CTL priming cultures was investigated to determine whether reo-Mel-DC polarized the immune response toward a Th1 or Th2 direction. Figure 5 shows that priming with reo-Mel-DC was associated with the production of high levels of the Th1 cytokine IFN-γ (up to 10,000 pg/mL). In contrast, IFN-γ was barely detectable when Mel-DC were used in priming cultures. Low levels of TNF-α and IL-6 were inconsistently produced, and no significant production of the Th2 cytokines IL-4 and IL-10 was found in either priming condition (data not shown). The high levels of IFN-γ produced are indicative of a Th1 skew induced by reo-Mel-DC.

Cross-priming of CTL with specificity toward a defined melanoma TAA by reo-Mel-DC. Although reo-Mel-DC generate CTL with specific MHC class I-restricted cytotoxicity toward Mel888 cells, it was not clear whether the anti-Mel888 activity was directed toward TAA in this allogeneic system. Dendritic cells loaded with whole tumor cells in this system have access to a host of TAA (many of which are likely to be as undefined) in addition to nontumor antigens (including viral) and allo-antigens. We therefore wished to address whether the reo-Mel-DC-induced CTL polyclonal population included cells specific for a TAA. MART-1 is one defined HLA-A2-restricted melanoma TAA expressed by Mel888 cells. Mel888 cells are HLA-A2 negative and therefore cannot present MART-1 directly to T cells (30). Hence, any expansion of T cells with specificity toward MART-1 on coculture with Mel888-loaded HLA-A2$^+$ dendritic cells (from the same donor as the T cells) must represent cross-priming against MART-1 mediated by dendritic cells. In view of the host of different antigens present in these priming cultures, the frequency of CTL generated with activity toward a particular TAA is likely to be very low. For this reason, priming cultures were done as previously but with a third identical stimulation step, and the frequency of MART-1-specific CD8$^+$ T cells generated after 2 and 3 weeks was determined using a MART-1-specific pentamer. As shown in Fig. 6, a small but significant expansion of MART-1-specific T cells was seen in reo-Mel-DC cultures, whereas no expansion of pentamer$^+$ CD8$^+$ T cells was seen in Mel-DC primed cultures. This suggests that reovirus infection of human tumor cells is able to support the priming of an effective antitumor CTL response, which includes CD8$^+$ T cells directed against relevant TAA.

Discussion

The interplay between host antiviral and antitumor immune responses is complex during oncolytic virotherapy. Antiviral responses limit the intratumoral replication and spread of viruses but also play an important role in reducing normal tissue toxicity by providing a barrier to normal tissue infection (39). In contrast, the development of antitumor immunity may enhance the efficacy of virotherapy (2–8). Virus-induced oncolysis is likely to release a wide range of tumor antigens from whole tumor cells, which may be taken up and cross-presented by infiltrating dendritic cells, and virally infected cells can be more effective at delivering nonviral antigen for *in vivo* cross-priming of APC than noninfected cells (40). Dendritic cells are known to be important mediators of early viral

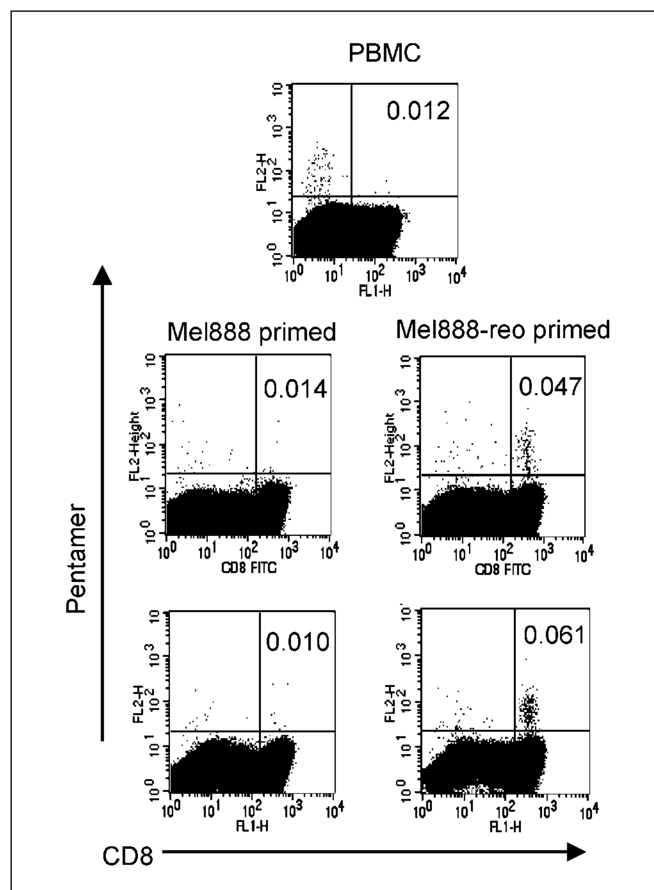

Fig. 6. Cross-priming of MART-1-specific CTLs by dendritic cells loaded with reovirus-infected Mel888 cells. HLA-A2$^+$ PBMCs were incubated with autologous dendritic cells loaded overnight with uninfected Mel888 cells (a HLA-A2$^-$ cell line) or reovirus-infected Mel888 cells as in Fig. 5. A total of three stimulations were done at 7-d intervals, with analysis on day 21. Initial PBMC and the lymphocyte populations generated after 2 and 3 wk in culture were examined by flow cytometry after labeling with a MART-1-specific pentamer and a negative control (which labeled no detectable cells; data not shown). Percentage of CD8$^+$ T cells labeled with the MART-1 pentamer. Representative of five independent experiments.

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recognition via pattern recognition receptors such as Toll-like receptors, which respond to viral RNA and DNA (41).

Reovirus is a promising naturally occurring oncolytic virus, which has already entered phase I and II clinical trials. In the data reported here, we have for the first time explored the ability of reovirus infection of tumor cells to support generation of adaptive antitumor immunity. Melanoma was chosen as the disease target for this study due to the susceptibility of melanoma to reovirus (33) and the potential immunogenicity of melanomas (42, 43). Initially, intravenous reovirus was administered using an established murine model of B16 melanoma lymph node metastasis (5). In this system, a single intravenous dose of reovirus reduced lymph node metastatic burden (Fig. 1A and B) and generated a response against the self-TAA TRP-2 (Fig. 1C; ref. 44). In addition, intravenous reovirus was associated with production of IL-12 (a cytokine with a key role in immune priming; ref. 45) in tumor draining lymph nodes (Fig. 2C).

Although murine models allow assessment of *in vivo* interactions between viruses and components of the immune system, they are limited in their application to human systems (46). For this reason, it was important to determine whether these findings also held true in a human *in vitro* model. Mel888 cells were chosen for testing, as they are inherently immunosuppressive (30), although potentially able to provide TAA for cross-priming dendritic cells in an appropriate immunostimulatory context (47).

Experiments coculturing tumor cells with dendritic cells confirmed that Mel888 cells alone are unable to induce dendritic cell phenotypic maturation or induce cytokine secretion. In contrast, reovirus-infected Mel888 cells activated dendritic cells phenotypically and functionally (Fig. 2A and B). The IL-12p70 and TNF-α secreted may, in particular, promote priming *in vivo*, with IL-12p70 linking the innate and adaptive arms of the immune system, activating NK cells, and directing the differentiation of Th1 helper T cells (45). These data show for the first time that reovirus-infected tumor cells activate dendritic cells. It is significant in this context that many viruses, including oncolytic viruses, conversely interfere with dendritic cell function (15, 16, 48, 49).

Transwell experiments showed that dendritic cell maturation induced by reo-Mel888 is dependent on direct dendritic cell contact with reovirus-infected tumor cells as opposed to a soluble factor (Fig. 2A). Although previous reports with oncolytic viruses have shown an enhancement of phagocytosis of tumor by dendritic cells following tumor infection (7, 8), we found no increased dendritic cell uptake of reovirus-infected tumor cells compared with noninfected cells (Fig. 3A and B). Previous studies have shown that cross-priming by cells infected with double-stranded RNA viruses requires phagocytosis of infected material and signaling via the double-stranded RNA receptor, Toll-like receptor-3 (40). Colabeling experiments (Fig. 3C) showed that tumor material taken up by dendritic cells colocalized with acidic late endosomal/lysosomal compartments. Significantly, Toll-like receptor-3 engagement has been shown to occur in an acidic environment in such late endosomal/lysosomal compartments (50). Overall, this would be consistent with a role for Toll-like receptor-3 receptor interactions in dendritic cell maturation in response to reovirus-infected Mel888 cells, and we are now further addressing these mechanisms in our laboratory.

By priming and restimulating autologous PBMC with dendritic cells loaded with reovirus-infected Mel888 cells, in the absence of other maturation factors, we have shown lymphocyte proliferation (Fig. 4A) and effective naive CTL priming (Fig. 4B) toward Mel888 cell targets, with minimal activity toward other melanoma and nonmelanoma targets (Fig. 4B and C). Importantly, in this allogeneic system, the response included cross-priming of CTL specific for a melanoma TAA, MART-1 (Fig. 6). Although the levels of MART-1-specific CTL generated were low, this is unsurprising in a system primed with whole tumor cells containing a host of antigens. Moreover, lymphocyte degranulation toward Mel888 cell targets was highly specific and MHC class I dependent (Fig. 5C and D). Notably, in the absence of reovirus infection, Mel888 cells consistently failed to expand lymphocytes or prime an antitumor response (Fig. 4A and B). High levels of the Th1 cytokine, IFN-γ, accumulated in reovirus-infected Mel888 cell primed CTL cultures (Fig. 5), consistent with generation of a Th1 response. These data are consistent with the detection of the Th1 cytokine, IL-12, from lymphocytes disaggregated from tumor draining lymph nodes in the *in vivo* murine model following intravenous reovirus (Fig. 1D). It is interesting to note that effective antitumor priming in the human *in vitro* system took place with a reovirus dose (0.1 pfu/Mel888 cell), which caused minimal dendritic cell maturation after 24 h coculture (Fig. 2A and B). Furthermore, these dendritic cells did not show any further increase in maturation over 48 h (data not shown). This reovirus dose was chosen in view of the detrimental effect of higher reovirus doses on dendritic cell viability (Fig. 2C) and also to maintain relevance to human therapy, in which several obstacles are likely to limit the dose, which can be delivered to tumors (1).

In summary, we have shown that reovirus infection of tumor cells is immunogenic, activating dendritic cells, and providing an antigen source in an appropriate dangerous context to prime a naive CTL response toward TAA in both *in vivo* murine and *in vitro* human model systems. These findings provide a powerful rationale for the design of future clinical studies with reovirus, and other oncolytic viruses, to explore both their cytotoxic and immunotherapeutic activity. Combination therapy manipulating virus delivery, antiviral and antitumor immune responses, provide an encouraging avenue for future preclinical and clinical development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tumor Infection by Oncolytic Reovirus Primes Adaptive Antitumor Immunity

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