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Irradiation enhances human T cell function by up-regulating CD70 expression on antigen-presenting cells *in vitro*

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Abstract

In addition to the direct killing of tumor cells, radiation therapy can alter the balance of immune cells *in vivo* due to the differential radio-sensitivity of different cell types. The addition of adjuvant radiation therapy prior to adoptive cell transfer therapy has been shown to enhance antitumor responses in both mouse models and clinical trials. The current study examines the effects of *in vitro* irradiation on the phenotype and function of human antigen-presenting cells. The results indicated that irradiation up-regulated CD70 expression on both B cells and mature dendritic cells (DCs). Expression of CD70 on mature DCs was enhanced in a dose-dependent manner, while under the same conditions, no significant up-regulation of CD80, CD86, or CD40 was observed. The levels of expression of CD70 induced on mature DC by irradiation correlated highly with the ability of those cells to stimulate T cell proliferation and IFN- γ production. Furthermore, significant reductions in T-cell proliferation and IFN- γ production were seen when CD70 expression on DCs was partially reduced using shRNA, as well as when DCs were incubated with a blocking anti-CD70 antibody. Radiation therapy may therefore enhance T cell activation *in vivo* through the CD27 pathway by virtue of its ability to up-regulate the expression of CD70 on antigen-presenting cells.

Keywords

radiation; TBI; CD70; CD27; mature human DCs; co-stimulation; human T cells; adoptive cell transfer; cancer immunotherapy

Introduction

In the treatment of cancer, radiation therapy is employed to eliminate tumor cells by direct killing. The selective cytotoxicity of radiation therapy can also directly or indirectly affect the survival and function of immune cells, and emerging evidence suggests that radiation can alter the immune response by modulating tumor-induced immune tolerance, thus enhancing the anti-tumor effects of radiation therapy (1).

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Disclosures

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In the tumor microenvironment, radiation could alter the balance of immune cell populations. For instance, compared to B cells and naïve T cells, memory T cells and natural killer cells, as well as DCs, are relatively resistant to radiation (2, 3). Evidence suggests that radiation can enhance cross-priming and presentation of tumor antigens to the immune system, and can elevate major histocompatibility complex (MHC) expression on DCs (3). In addition, radiation may cause apoptotic tumor cells to send “danger” signals to induce DC activation, thus promoting cytokine production and up-regulation of costimulatory molecules (4).

DC activation can up-regulate costimulatory molecules ligands (5). The CD70 molecule, which represents the only known ligand of CD27, promotes T-cell survival, plays an important role in determining the size of effector and memory T-cell populations, and has been shown to break CD8⁺ T cell tolerance (6-8). Minimal expression of CD70 is observed on resting cells, whereas activated B cells and DCs, as well as activated T cells, can express relatively high levels of CD70 (9, 10). Evidence suggests that the ability of CD40 activated DCs to promote effective CD8⁺ T-cell responses is mediated, at least in part, by signals delivered through the CD70 pathway (11). In addition, other costimulatory molecules do not appear to completely compensate for a lack of CD70 (12). In recent studies, CD27 expression on CD8⁺ T cells in adoptively transferred TILs was associated with clinical responses, suggesting that the CD27/CD70 costimulatory pathway is important for effective T-cell antitumor response (13 and Huang, manuscript submitted). Stimulation of murine DCs with toll-like receptors (TLR) ligands, which induce DC activation and maturation, has also been shown to up-regulate CD70 expression (14). In a recent study, CD40L was shown to minimally up-regulate CD70 expression upon the stimulation of DC, whereas stimulation with a combination of CD40L and TLR ligands such as poly I:C and CpG oligonucleotides significantly up-regulated CD70 expression (15). Transgenic mice generated with a CD70 construct under the control of the CD19 promoter constitutively express high levels of CD70 on B cells, which lead to the enhanced generation of effector T cells but a progressive decrease in the number of B cells in bone marrow, spleen and lymph nodes (16). These observations point to the need for tight control of CD70 expression, and may help to explain why expression of CD70 is limited to activated cells.

Studies in mouse model systems as well as clinical trial data provide evidence that radiation can promote tumor immune responses. Radiation therapy administered prior to cell transfer therapy can improve the efficacy of antitumor responses in a mouse tumor model (17). This effect is not primarily due to direct effects of irradiation on tumor cells, but rather appears to result from enhanced persistence of activated effector T cells. In recent clinical trials carried out in the Surgery Branch of the National Cancer Institute, a response rate of 51% was observed in patients who received non-myeloablative chemotherapy (NMA) treatment alone prior to the adoptive transfer of autologous tumor infiltrating lymphocytes (TIL), while a response rate of 72% was seen in patients who received NMA plus 1200 cGy of TBI (18). The enhanced response rate in the patients receiving TBI, although not statistically significant, suggests that further depletion of patients' endogenous T cells, in particular T regulatory cells, and alteration of APC function may have an added benefit to patients. Although irradiated APCs have been routinely used to carry out *in vitro* stimulation of both mouse and human T cells, the factors responsible for the enhanced stimulatory capacity of irradiated cells have not been fully explored in previous studies.

The present study investigated the effects of *in vitro* irradiation on the phenotype and function of DC. The results provide evidence that up-regulation of CD70 expression on irradiated DCs leads to enhanced activation of T cells in response to alloantigen and in response to specific peptide stimulation. These results provide further support for the continued use of irradiation in combination with immunotherapy in cancer therapies in the

hopes that this may lead to more effective in vivo T cell responses and increased clinical response rates.

Materials and Methods

Cells

PBMCs from healthy donors were isolated by LSM® Ficoll density gradient centrifugation (MP Biomedicals, Cleveland, OH). Immature DCs were derived from monocytes, which were isolated from PBMC samples by removing non-adherent cells, the adherent cells were then cultured in medium containing 1000 U/ml of GM-CSF and 1000 U/ml of IL-4 for 5 days. Mature DCs were derived from immature DCs by adding 1000U/ml of CD40L for 24 hours. CD3⁺ cells were obtained from healthy donor PBMCs using Miltenyi cell separation beads (Miltenyi Biotec, Auburn, CA).

Preparation and maturation of DCs

Monocytes were cultured in X-VIVO15™ containing 10% human serum and 1000 U/ml GM-CSF (PeproTech, Rocky Hill, NJ) and 1000 U/ml IL-4 (PeproTech). After 6 days, a portion of the immature DCs were used for experiments; the rest were continuously cultured in medium with 2 µg/ml CD40L (Immunex, Seattle, WA) or CD40L plus 1 µg/ml LPS (Sigma, St. Louis, MO). After 24 hours, these cells were harvested and used for experiments.

Irradiation

Using a GC 1000 irradiator (MDS Nordion, Ottawa, ONT), whole PBMCs or DCs were irradiated with between 0 and 7000 cGy at a delivery rate of 625 rad/min.

Antibodies and FACS analysis

Human anti-CD70-PE, anti-CD40-APC, anti-CD83-APC, anti-CD80-PE, anti-CD86-PE, anti-CD3, and anti-CD8 were purchased from BD Biosciences (San Diego, CA). Anti-CD11c-PE-Cy7 and anti-CD27-APC were obtained from eBioscience (San Diego, CA). Blocking antibody for human CD70 (clone BU69) was purchased from Ancell (Bayport, MN). The lineage cocktail 1 (lin 1) contains FITC-conjugated antibodies include CD3, CD14, CD16, CD19, CD20, and CD56. Peripheral blood dendritic cells can be distinguished from other leucocytes by their lack of staining with lin 1. FACS samples were run on FACSCalibur or Canto II (BD Biosciences) and data analysis was performed using FlowJo software (TreeStar, Ashland, OR).

T-cell proliferation assay

T-cell proliferation was determined by CFSE dilution analysis. CD3⁺ cells were labeled with CFSE (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The labeled CD3⁺ T cells were then co-cultured with irradiated immature or mature DCs (T:DC = 5:1). The level of CFSE in CD3⁺ cells was analyzed by flow cytometry 5 days after the co-culture.

Cytokine assay

Mature DCs were irradiated at varying doses, then placed into 48-well plates at 10⁶/ml. Supernatants were sent to SearchLight (Thermo Fisher Scientific, Waltham, MA) for multiple cytokine analyses. Peptide stimulation was performed by pulsing HLA-A2⁺ DCs with 10 µM of MART-1₂₆₋₃₅ or a negative control peptide for 2 hours, and co-culturing in cytokine-free medium with 2 samples of freshly thawed post-TIL transfer therapy PBMCs

for 18 hours. These PBMCs mainly contained MART-1⁺CD8⁺ cells. Cytokine release was measured by ELISA.

Lentiviral vectors and DC transductions

A set of 4 CD70 (NM_001252) shRNA lentiviral transduction particles (catalog number SHVRS; clones TRCN0000007840, TRCN0000007841, TRCN0000007843, and TRCN0000011202) and a MISSIONTM non-target shRNA control vector (catalog number SHC002) were purchased from Sigma-Aldrich (St. Louis, MO). CD14⁺ monocytes were cultured in X-VIVO15TM (Cambrex) containing 10% FBS with 1000 U/ml GM-CSF (PeproTech) and 1000 U/ml IL-4 (PeproTech) for 2 days. The cells were then replaced with supernatants containing each of the 5 virus vectors (MOI:1), along with 10 µg/ml protamine sulphate. The plates were centrifuged at 1000 × g at 32°C for 2 hours. The next day, cells were placed in fresh medium containing IL-4 and GM-CSF. Seventy-two hours after transduction, DC maturation reagents were added; 24 hours later, DCs were cocultured with T cells for proliferation analysis and cytotoxic assay.

Statistical analysis

Statistical analysis was performed using an unpaired T test. A p value of < 0.05 was considered statistically significant.

Results

In vitro irradiation of PBMCs induces CD70 expression on subpopulations of cells

To analyze the influence of *in vitro* irradiation on immune cells, PBMCs were irradiated *in vitro* with doses that ranged between 1000 and 7000 cGy. The results showed that while only a relatively small percentage of the CD20⁺ B cell population expressed CD70 prior to irradiation, treatment with the minimum dose of irradiation that was tested, 1000 cGy, resulted in the up-regulation of CD70 expression on nearly all of the treated B cells (Fig. 1A). Higher doses of irradiation appeared to lead to lower levels of CD70 expression on B cells as compared with 1000cGy, an effect that may have resulted from the increased apoptosis that was observed in the cultures of B cells treated with higher doses of irradiation (data not shown). Further analysis demonstrated irradiation induced up-regulation of CD70 expression on mature DCs present within the population of PBMCs, and that this increase was linearly correlated with the dose of radiation administered to the cells (Figs. 1B and 1C). Irradiation also increased the percentage of DCs that co-expressed CD70 and CD40 (Fig. 1D), although it did not significantly alter the total percentage of CD40 positive DCs (Fig. 1E). Irradiation did not up-regulate cell surface expression of the co-stimulatory ligands CD80 and CD86 or the maturation marker CD83 on DCs (Fig. 1F).

DC maturation alone does not up-regulate surface expression of CD70

The observation that up-regulation of CD70 expression appeared to occur only on mature DCs that expressed the CD83 marker lead to further studies to evaluate the effects of DC maturation on cell surface expression of CD70. The analysis of DCs present in fresh melanoma samples indicated that while a proportion of the cells expressed the maturation marker CD83, essentially none of the cells expressed CD70 (Fig. 2A). Treatment of DCs with CD40 ligand (CD40L), as well as a variety of TLR ligands, induced DC maturation, as demonstrated by the enhanced expression of CD83 on the treated cells (Fig. 2B). Nevertheless, none of the maturation reagents that were tested significantly up-regulated the expression of CD70 on DCs (Figs. 2B).

Irradiation of DCs enhances their ability to promote T cell proliferation and IFN- γ production

To determine whether the up-regulation of CD70 expression observed on irradiated DCs can enhance T-cell function, further experiments were carried out with purified DCs that were matured using CD40L and LPS. As previously shown, increasing doses of radiation lead to progressive increases in the expression of CD70 on DCs (Fig. 3A), the DCs were co-cultured with CFSE-labeled MHC-mismatched CD3⁺ T cells for 5 days. The results demonstrated that increasing doses of radiation resulted in progressively enhanced T cell proliferation in response to alloantigen stimulation, and this increase correlated with increases in CD70 expression that were observed on DCs treated with higher doses of radiation ($R^2 = 0.82$, $P < 0.02$) (Figs. 3B and 3C). Peptide-pulsed DCs were then co-cultured with PBMC from 2 patients who received autologous TIL that were reactive with the HLA-A2 restricted MART-1 immunodominant T cell epitope. Peripheral blood obtained following adoptive transfer contained high levels of persistent MART-1 reactive T cells (Pt#9 and Pt#10 in (19)). The results demonstrated that un-irradiated DCs stimulated the release of relatively low levels of IFN- γ from un-cultured PBMC obtained from the 2 patients, whereas irradiated DCs stimulated that release of significantly higher levels of IFN- γ from the 2 PBMC samples (Fig. 3D). These findings provide further evidence that the up-regulation of CD70 expression on DCs mediated by irradiation enhances their antigen presenting cell activity.

Irradiation-induced IL-12 and IL-23 productions from DCs in vitro

It has previously been shown that DC maturation induces production of IL-12 (20). Analysis of DC supernatants one day following irradiation revealed that increased doses of irradiation led to the secretion of increasing levels of IL-12 and IL-23 (Fig. 4), demonstrating that irradiation of DCs can lead to increased production of cytokines, which in turn may act to enhance T cell function.

Irradiation-induced CD70 expression on DCs is at least partially responsible for enhanced T-cell function

After demonstrating that induction of CD70 on irradiated DCs correlated with enhanced T cell function, studies were carried out to determine whether CD70 up-regulation was directly responsible for the elevated T cell function. Blocking the interaction of CD70 with CD27 using an anti-CD70 antibody was found to significantly decrease the proliferation of T cells in response to alloantigenic stimulation (Fig. 5A). Additional studies were then carried out by inhibiting CD70 expression using specific shRNAs, which decreased the levels of CD70 induced by irradiation on CD83⁺ DCs by up to 67% (Fig. 5B). The diminished levels of CD70 expression led to a decrease in T cell proliferation in response to alloantigen stimulus by approximately 50% and decreased the release of IFN- γ from persistent MART-1 reactive T cells present in the peripheral blood of a patient who received MART-1 reactive TIL by 40% or more (Fig. 5C and 5D). These data confirm that irradiation-induced CD70 expression on DCs is at least partially responsible for enhanced T-cell function.

Taken together, these results indicate that irradiation can alter the expression of CD70 by mature DCs in a way that enhances the function of T cells that interact with these DCs.

Discussion

Pre-clinical and clinical studies have demonstrated that combining immunotherapy with other cancer treatment modalities can produce a synergistic antitumor response. For instance, adding radiation treatment prior to adoptive cell transfer therapy has been shown to improve the antitumor efficacy of the cell therapy in B16 mice (17). In our most recent clinical trial employing total body irradiation (TBI) as an adjuvant to cell therapy, 72% of

patients treated with chemotherapy and TBI showed an objective response, compared to 48.8% for patients receiving cyclophosphamide and fludarabine alone without radiation (21). Although there were too few patients in this trial ($n = 25$) to draw any definitive conclusions, these findings suggest that radiation may enhance the therapeutic effect of adoptively transferred T cells.

The selective cytotoxicity of radiation therapy alters the balance of immune cell populations, but is dependent on the sensitivity of particular cell types to radiation. DCs, which are relatively radio-insensitive, are more likely to survive radiation therapy than lymphocytes. In addition, previous studies have suggested that radiation can alter the function of DCs by increasing antigen presentation and processing, and by enhancing MHC expression on DCs. Tumor cells killed by radiation may also lead to the activation of DCs through the release of “danger” signals, thus increasing cytokine production and expression of surface costimulatory molecules such as CD80, CD86, 4-1BB, and CD70. In this study, we sought to determine whether radiation therapy given prior to adoptive TIL transfer could improve T-cell function, and if so, to identify the immunological bases of this enhanced functionality. We focused on the expression of CD70 on APCs post-radiation, since we had previously demonstrated that the frequency and number of CD27-expressing CD8⁺ T cells in infusion TILs for adoptive transfer therapy was highly correlated with clinical response (13). The CD27/CD70 signaling pathway has previously been shown to play a role in T cell proliferation, survival, and effector function. In addition, preclinical studies have shown that CD70 expression on DCs plays a key role in CD40-dependent CD8 T-cell response, and lack of CD70 cannot be completely compensated for by other costimulatory molecules.

Given the importance of understanding the mechanisms underlying the beneficial effects of radiation on the adoptive cell transfer therapy, in this study we investigated the potential influence of CD27/CD70 signaling on the immune cell populations after *in vitro* radiation. When CD70 expression was examined in different populations of human PBMCs following *ex vivo* irradiation, the majority of B cells were found to up-regulate CD70 expression after receiving 1000 cGy. Although activated B cells can function as APCs and may play an important role in providing help to the adoptively transferred T cells, the combined effects of NMA chemotherapy with TBI administered to patients lead to a nearly complete absence of lymphocyte populations in peripheral blood as well as in tumors at the time of T-cell administration. In a recent study, cyclophosphamide was found to increase the numbers of immature dendritic cells (DCs) in blood, which was associated with enhanced antigen-specific responses of the adoptively transferred CD8⁺ T cells (22). In addition, significant levels of DC cell death were not observed two days following *in vitro* treatment with 3000 cGy (data not shown), further demonstrating the relative insensitivity of DCs to irradiation.

Analysis of myeloid lin1⁻, CD11c⁺, and DR⁺ DCs revealed that both mature (CD83⁺) and immature (CD83⁻) DCs expressed low or undetectable levels of CD70 prior to irradiation. At increasing doses of radiation, mature (CD83⁺) DCs linearly up-regulated surface expression of CD70, while immature (CD83⁻) DCs did not, indicating that CD70 can be induced by irradiation solely on mature DCs. Irradiation did not, however, appear to alter the maturation status of DCs. The ability of different activation signals to mediate up-regulation of CD70 expression on DCs was then examined. Stimulation of murine bone marrow derived DCs with CpGs or CD40L has been shown to up-regulate CD70 expression (14); however, we observed that, while the combined stimulation of TLRs and anti-CD40 can induce maturation of human DCs, it did not up-regulate CD70 expression. Similarly, *in vitro* culturing of DCs with TLR ligands that have previously been shown to lead to the maturation and activation of human DCs (such as TLR2, TLR3, TLR4, and TLR9) had no affect on CD70 expression. Results presented in a recent report indicate that CD70 can be induced on myeloid and plasmacytoid DCs that were directly purified from human PBMC.

In this study, thymic stromal lymphopoietin (TSLP) was used in combination with TLR or/ and CD40L (15). Under the experimental conditions used in our study however, incubation with TLR ligands did not appear to induce CD70 expression, whereas CD70 expression was up-regulated following irradiation of mature DCs. These findings indicate that the expression of CD70 on human DCs is tightly controlled. Furthermore, *in vitro* irradiation produced no changes in CD80, CD86, and CD40, which implies that up-regulation of CD70 may play a significant role in the enhanced responses observed against irradiated DCs. Further studies indicated that the level of surface expression of CD70 on mature irradiated DCs was correlated with the ability of the DCs to stimulate T cell proliferation in response to allo-antigenic stimulation. In addition, irradiated enhanced the ability of DCs that were pulsed with the MART-1 peptide to stimulate the release of IFN- γ from persistent peptide reactive T cells that were present in the peripheral blood of patients who received the adoptive transfer of autologous MART-1 reactive TIL. This correlation led us to determine if CD70 expression on DCs played a direct role in enhancing T cell function. An antibody that blocks the interaction of CD27 and CD70 inhibited the ability of irradiated DCs to induce the proliferation of T cells in response to allo-antigenic stimulation by almost 40%. Further analysis using shRNA to knock down CD70 expression induced by irradiation showed significant inhibition of alloantigen-induced T cell proliferation as well as the IFN- γ release of T cells in response to specific peptide stimulation. These data provide further evidence that expression of CD70 on DCs induced by irradiation plays a role in enhancing the function of T cells.

One of the central goals of cancer immunotherapy is to efficiently activate tumor-specific T cells by breaking immune tolerance to tumors. Many of the antigens targeted by tumor reactive T cells appear to be self-antigens that may induce a state of partial T cell tolerance, and the majority of tumors do not appear to express high levels of costimulatory signals. Activation of DCs by radiation can up-regulate CD70 expression, as shown in this report, which may then lead to enhanced T cell activation. Our results confirm previous studies showing DC maturation lead to IL-12 production but also demonstrate that irradiation further enhances IL-12 production, a cytokine that promotes T_H1 cell differentiation, expansion and IFN- γ production, as well as NK cell activation (23, 24). Moreover, irradiation of DCs can increase secretion of IL-23, a cytokine is closely related to IL-12 (25). *In vitro* irradiation also up-regulated the expression of MAPK4, a molecule that plays a critical role in the cell signaling pathway upon activation (data not shown). These data indicate that CD70 expression on DCs enhances the release of IFN- γ from adoptively transferred T cells.

As a result of the limitations on pursuing *in vivo* studies on human subjects, this investigation focused mainly on *in vitro* irradiation of human DCs and its influence on T cell function. The delivery rate of radiation used in these *in vitro* studies was significantly higher than that administered to patients in the Surgery Branch receiving TBI as a part of clinical adoptive cancer trials (18), and thus the *in vivo* effects of irradiation on DCs are difficult to determine. Treatment with TBI could induce a variety of factors that would not present in *in vitro*, and the *in vivo* environment could provide an opportunity for these factors to synergize. In a recent report in a mouse model system, TBI was found to enhance the serum levels of LPS as well as multiple inflammatory cytokines (26), and in humans, elevated levels of IL-7 and IL-15 were found in post-treatment sera from cancer patients receiving TBI (18). Mature DCs, which are susceptible to CD70 up-regulation, are enriched by LPS stimulation, and activation of CD4⁺ T cells by IL-15 enhances CD40L expression, which can also promote DC maturation (27). These factors may effectively lower the dose of irradiation needed to increase the number of CD70 expressing DC *in vivo*. When purified DC were irradiated with 200cGy twice a day for three days (total 1200cGy), which partially

mimics the *in vivo* clinical protocol, the levels of CD70 were similar to those observed on DCs receiving a single dose of 1200cGy (data not shown).

Following irradiation, DCs that have up-regulated CD70 might interact with CD27 expressing T cells in tumor, draining lymph node or other lymphoid organs; however, there is a limited availability patient biopsies following radiation treatment. These interactions could occur inside tumors, as mature DCs were observed in samples of un-cultured melanomas obtained prior to treatment (Fig.2A).

Taken together, the *in vitro* data presented in this report provide support for *in vivo* studies aimed at determining the effects of irradiation on CD70 expression on populations of antigen presenting cells to determine if expression of this co-stimulatory marker is correlated with improved anti-tumor immunity and clinical response to therapy. This correlation, if observed, would provide support for the hypothesis that the CD27/CD70 signaling pathway is involved with the enhancement of T cell functions that are observed following irradiation.

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Figure 1. Expression of CD70 on subpopulations of cells in PBMCs following irradiation (A) Up-regulation of CD70 on B cells post-irradiation. PBMCs were irradiated *in vitro* at indicated doses. Cells were plated in culture medium without cytokines, and analyzed by FACS 18 hours later. (B) Expression of CD70 on DCs was analyzed by gating the lin1 negative, CD11c⁺, and PI⁻ cells present in PBMC. The data are representative of three individual experiments. (C) Correlation between irradiation dose and CD70 expression on DCs. Data represent 2 PBMC samples. (D and E) Irradiation increased CD70 expression on CD40⁺ DCs, but did not influence CD40 expression. PBMCs were treated with different doses of irradiation *in vitro* as described above, and DCs were analyzed by FACS after 2

days. **(F)** Results from 2 representative PBMC samples show that CD80 and CD86 expression on DCs were not altered by irradiation of PBMCs.

Figure 2. DC maturation did not up-regulate CD70 expression

(A) Four fresh melanoma tumors obtained prior to adoptive transfer therapy were analyzed by FACS. Cells were gated for $\text{lin}1^-$, $\text{CD}11\text{c}^+$, and PI^- populations. Expression of CD70 was not observed on mature DCs present within the tumor samples. (B) No up-regulation of CD70 was observed on DCs matured by CD40L and TLRs. Monocytes were isolated from 2 PBMC samples by removing non-adherent cells. Adherent cells were then cultured in medium containing 1000 U/ml of GM-CSF and 1000 U/ml of IL-4 for 5 days. On day 6, the cells were placed into 7 wells and treated with 7 separate TLR ligands. FACS analysis was performed 48 hours later by examining CD83 and CD70 expression on $\text{lin}1^-$, $\text{CD}11\text{c}^+$, and PI^- populations. Irradiation at a dose of 3000 cGy was administered 24 hours after CD40L was added.

Figure 3. Up-regulating CD70 on mature DCs enhanced adoptively transferred T-cell effector function and promoted T-cell proliferation

(A) Up-regulation of CD70 on mature DCs. Monocytes were isolated from 2 PBMC samples by washing out non-adherent cells. Adherent cells were then cultured in medium containing 1000 U/ml of GM-CSF and 1000 U/ml of IL-4 for 5 days. On day 6, 1000 U/ml of CD40L was added. Cells were irradiated with the indicated doses after 24 hours (top row) or 5 days (bottom row). FACS analysis was carried out by analyzing CD70 expression on $\text{lin}1^-$, $\text{CD}11c^+$, and PI^- populations and the % of $\text{CD}70^+\text{CD}83^+$ cells were indicated (B) Irradiation of mature DCs enhanced alloantigen-stimulated T cell proliferation. DCs were prepared as in (A) and co-cultured with CFSE-labeled $\text{CD}3^+$ T cells derived from 3 MHC-

mismatched PBMC samples. After 5 days, FACS analysis was performed by gating of CD3⁺ and PI⁻ T cells. **(C)** CD70 expression on DCs correlated with dose of *in vitro* irradiation (MFI: mean fluorescence intensity). **(D)** Irradiation of DCs promoted IFN- γ release in adoptively transferred CD8⁺ T cells. Irradiated DCs were prepared from HLA-A2⁺ PBMCs as described in **(A)** and pulsed with MART-1 peptide for 2 hours. DCs were then co-cultured with PBMC from 2 patients obtained either 19 or 59 days following the adoptive transfer of MART-1 reactive TIL, samples that were previously shown to contain high levels of MART-1 reactive T cells (Pt#9 and Pt#10, respectively, in(19)). Analysis of IFN- γ release was carried out 18 hours later. The results from 1 of 3 representative experiments are shown.

Figure 4. Irradiation increased IL-12 and IL-23 release from mature DCs
(A) IL-12 release from irradiated DCs. DCs were derived from PBMC samples as described in Figure 2. Eighteen hours after adding CD40L, the cells were irradiated and plated 2×10^6 /ml in medium without cytokine. After 24 hours, supernatants were harvested for cytokine analysis. **(B)** IL-23 release from 1 of 3 representative experiments.

Figure 5. Inhibiting CD70 decreases T-cell proliferation

(A) T-cell proliferation was decreased by blocking irradiation-induced CD70 expression on DCs. Three DCs derived from monocytes were differentiated by IL-4 (1000 U/ml) and GM-CSF (1000 U/ml). Anti-CD70 and isotype control (20 μ g/ml) were added to the culture before DCs were matured by CD40L (2 μ g/ml), LPS (1 μ g/ml), and irradiation (1000 cGy). Purified MHC-mismatched CD3⁺ T cells were labeled with CFSE and the labeled cells were co-cultured with the DCs. FACS analysis was performed 1 day later. (B) CD70 expression induced by irradiation of mature DCs was inhibited by shRNA. Monocytes were isolated from 2 HLA-A2⁺ PBMC samples, differentiated to immature DCs using GM-CSF and IL-4 for 2 days, and transduced with lentiviral shRNA, an inhibitor of CD70 (non-target shRNA and 4 clones: TRCN 0000007840, TRCN 0000007841, TRCN 0000007843, and TRCN 0000011202). After 3 days, cells underwent maturation and irradiation, and FACS analysis was performed by examining CD70 expression on DCs. (C) shRNA inhibition of CD70 led to reduced IFN- γ production. The 2 DC cultures described in (B) were pulsed with MART-1₂₆₋₃₅ peptide for 2 hours and co-cultured with a PBMC sample derived from a melanoma patient 59 days after transfer of MART-1-specific infusion TILs (Pt 10 in (19)). (D) Inhibiting CD70 decreased T-cell proliferation. The 2 treated DCs described in (B) were co-cultured with CFSE-labeled CD3⁺ T cells, and FACS analysis was done 5 days later. The results from 1 of 2 representative experiments are shown.