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Tumor immunotherapy using bone marrow-derived dendritic cells overexpressing Toll-like receptor adaptors

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Running title: **Adoptive transfer of TLR adaptor-expressing DC**

Abbreviations:

DC, dendritic cell; IRES, internal ribosomal re-entry sequence; mDC, myeloid dendritic cell; MLR, mixed lymphocyte reaction; pDC, plasmacytoid dendritic cell; TLR, Toll-like receptor

Introduction

Myeloid dendritic cells (mDCs) have a potency to produce cytokines/interferons (IFNs), present antigens and up-regulate co-stimulatory molecules, which can lead to antibody production and activation of T cells and NK cells (1). Antigen-presenting mDCs play an important role in triggering immune responses to cancer and infectious diseases (1). mDCs express Toll-like receptors (TLRs) that recognize bacterial or viral ligands and deliver signals for mDC maturation (2). MyD88 (3,4) and TRIF/TICAM-1 (5,6) are major TLR adaptor molecules, and transduce downstream signals by their over-expression.

TLRs have been identified as a family of receptors that consist of >10 protein members both in humans and mice (2,7). Each TLR recognizes different microbial pattern molecules. When the TLR recognizes its ligand, it signals through the adaptor molecule that associates with the cytoplasmic domain of the TLR (2,7). These adaptors are involved in activation of transcription factors. In mDCs, NF- κ B and IRF-3 are activated (8,9) and in plasmacytoid dendritic cells (pDCs) NF- κ B and IRF-7 are activated (10). Production of cytokines (IL-6, IL-12, TNF α etc.) and IFN- α /IFN- β are regulated by NF- κ B and IRF-3/IRF-7, respectively (11). Thus, the adaptors participate in selective activation between the different functions of mDCs. Ultimately, CTL induction and NK activation are dependent upon the adaptor pathways in mDCs (12,13).

The presence of a variety of effector modes induced by TLRs suggests that in maturation of mDCs, the TLR signals act differentially on their functional output depending upon the adaptors selected. In fact, cytokine/interferon profiles, CD83/CD86 up-regulation and NK/CTL induction represent markers for differential maturation of mDCs. Thus, TLR ligands such as polyI:C (13), CpG (14,15), BCG-CWS (16), and imiquimod (17,18) can induce distinct immune responses and may act as adjuvants with multiple functions. Some TLR ligands have in fact been applied with tumor antigen for immunotherapy against cancer. On the other hand, recent reports have suggested that TLRs are expressed by various cells (19) such as NK (20), B (21) and regulatory T cells (22), in addition to the DCs. Thus, TLR ligands directly affect not only the initial response of DCs but also later activation and regulation of various immune cells.

mDCs are the most useful tool in cancer immunotherapy, because they can induce lymphocyte proliferation that would damage the host tumor in an antigen-specific manner.

Several models of DC therapy for cancer have been proposed. The models include mDCs expressing cytokines by gene-transfection, mDCs loading tumor antigens and mDCs stimulated by adjuvants such as TLR ligands. The effectiveness of mDC therapies has been tested in animals, and some of them have been evaluated for clinical applications. These studies encouraged us to establish methods for inducing tumor-specific CTL responses and reciprocal activation of NK cells.

In this communication, we generated mouse mDCs that constitutively express MyD88 or TICAM-1 and applied them to antitumor immunotherapy in a mouse syngeneic tumor implant model. Our results suggest that each adaptor confers different responses on the mDCs *in vitro* and in tumor-bearing mice *in vivo*. Adoptive transfer of mDCs that overexpress MyD88 or TICAM-1 into tumor-bearing mice induced retardation of tumor growth in the mouse implant tumor.

Materials and Methods

Reagents

FCS was purchased from Bio Whittaker (Walkersville, MD). Mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferon (IFN)- γ were from PeproTech EC, Ltd (London, UK). Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4) was supplied by SIGMA/Aldrich Chemical company (St. Louis, MO). Lympholyte-M was obtained from Cedarlane (Ontario, Canada). Enzyme-linked immunosorbent assay (ELISA) kits for IL-6, IL-12p40 and IFN- α were from Biosource (Camarillo, CA). [3 H]-thymidine was from Amersham Biosciences (Piscataway, NJ). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD86 and isotype control antibodies (American Hamster IgG (FITC)) were purchased from eBioscience (San Diego, CA). Mouse CD90 microbeads were from Miltenyi Biotec (Auburn, CA).

Cells

B16D8 was established in our laboratory as a sub-line of the B16 melanoma cell line (23). This sub-line was characterized by its low or virtually no metastatic properties when injected subcutaneously into syngeneic C57BL/6 mice (24). This B16 subline barely expressed MHC class I but in response to IFN- γ expressed it to a certain extent (13). Cells were incubated with 100 U/ml of IFN- γ for 24 hrs at 37 °C for surface-expression of class I, and used as target cells.

Construction of lentiviral vector and preparation of virus particle

We used a gene-expression kit, Lenti-viral system (Invitrogen, Carlsbad, CA). At first, to check transfection efficiency, a sequence of hrGFP with the multi-cloning site and IRES was cloned from pIRES-hrGFP-1a vector (Stratagene, LA Jolla, CA) by PCR and placed into the cloning site of pLenti6/V5-D-TOPO vector (Invitrogen) by TOPO-cloning system. This vector was named as pLenti-IRES-hrGFP.

Mouse *Ticam-1* and *Myd88* cDNAs were isolated from cDNA library of RAW cell as described previously (5). In most experiments, a cDNA encoding the N-terminal region of TICAM-1 (TICAM-1 N-terminal) (1-550 amino acids) (25) was employed instead of the full-length TICAM-1 cDNA, since the full-length TICAM-1 expression led to cell apoptosis within 8 h after transfection (26). The cDNAs were subcloned and sequenced for confirmation. The cDNAs encoding MyD88, full length of TICAM-1 and N-terminal

region of TICAM-1, were ligated into pLenti-IRES-hrGFP vector at the site of *EcoRI* and *XhoI* and named as pLenti-MyD88-IRES-GFP, pLenti-TICAM-1-IRES-GFP, pLenti-TICAM-1N-IRES-GFP. The primer sequence used for PCR are as follows: MyD88, sense 5'- ggaattcaccatgtctgcgggagacccccg-3', antisense 5'-ccgctcgaggggcagggacaaagccttggc-3'; TICAM-1, sense 5'- ggaattcaccatggataaccagggccttc-3', antisense 5'- ccgctcgagctctggagtctcaagaagggg-3'; TICAM-1N-terminal (1-550), antisense 5'- ccgctcgaggggttctggcctctgcgcttt-3'; multi-cloning site and IRES-hrGFP, sense 5'-caccatgggaattcgcgcgctcgactcaggagact-3', antisense 5'-ttacaccactcgtgcaggctgccagggg-3'. PCR was performed 30 cycles as follows: denature for 30 sec at 95 °C, anneal for 30 sec at 55 °C and extend for 1 min at 72 °C by using KOD plus enzyme (TOYOBO, Japan). Samples were extended for an additional 5 min at 72 °C.

Four plasmids (one of these pLenti vectors, pLP1, pLP2, pLP/VSVG) were transfected into 293FT packaging cells, and the viral particles for transfection were prepared according to the manufacturer's protocol. The virus particles were concentrated by centrifugation of 20,000 rpm at 4 °C for 2 hrs. After the precipitation was resuspended in RPMI1640 as the virus concentrated solution, the solution was subdivided to store at -80 °C.

Transfection efficiency

The transfection efficiency was assessed by fluorescence of GFP ligated in the vector of the lentivirus, and the rate of the positive cell was determined by FACS in comparison with that of non-infected cells. The indicated volume of virus concentrated solution was added to 293FT cells (5×10^5 cell/500 μ l of culture medium with 5 μ g/ml polybrene (Specially Media, Phillipsburg, NJ) in 24 well plate) and incubated for 12 hrs. These cells were cultured for 36 hrs additionally in fresh medium after wash. Then, the fluorescence intensities of the cells were assessed by FACS. Transduction efficiency of mDCs were analyzed under the same conditions as that of 293FT cells.

MLR assay.

Mouse mDCs were derived from bone marrow of C57BL/6 and appropriately manipulated as described in the text. mDCs (5×10^4 cells) were cultured with 10^5 CD90-positive T cells isolated from spleen of BALB/c mice by MACS beads in 96-well

cell culture plates in 200 μ l of culture medium for 72 hrs. Half of the medium was replaced with fresh medium containing [3 H]-thymidine (1 μ Ci/well). Then the cells and medium were harvested separately using a cell harvester, and the radioactivity was measured by MicroBeta (Perkin Elmer, Foster City, CA) with solid scintillator.

Preparation of mouse bone marrow-derived mDCs

Wild-type female C57BL/6 and BALB/c mice were purchased from Japan Clea (Tokyo, Japan). Bone marrow-derived mDCs were prepared as reported (27) with minor modifications. mDCs were cultured in RPMI-1640 containing 10 ng/ml mouse GM-CSF, 50 μ M 2-ME, 10 mM HEPES and 10% FCS.

FACS analysis and ELISA

For FACS analysis of CD86 expression levels, cells were suspended in PBS containing 0.1% sodium azide and 1% FCS and then incubated for 30 min at 4 °C with fluorescence-labeled mAbs. Cells were washed and their fluorescence intensities were measured by FACS. For FACS analysis to check transduction efficiency, cells were fixed with 0.5% paraformaldehyde after wash, and resuspended in PBS. For ELISA, samples were stored at -80 °C. Their levels were measured according to the manufacture's protocol.

Dendritic cell (DC) therapy

The protocol of animal studies is shown in Figure 4a. The doses of virus preparations were adjusted by titration assay to make 50% of 293FT cells GFP positive (ED50). The ED50 doses of virus were cultured with mouse mDCs for 12 hrs as in the conditions employed for 293FT cells (5×10^5 cells/500 μ l in a 24-well plate). mDCs were then washed and suspended in fresh culture medium for additional culture for 12 hrs (total 24 hrs). Tumor debris was prepared from B16 cells by 3-cycle freeze-thaw and the B16 debris (corresponding to 2.5×10^5 B16 cells/well) was co-incubated with lentivirus-bearing mDCs for last 6 hrs. After mDCs were loaded with tumor debris, cells were washed, and then suspended in PBS (10^6 cells/200 μ l). For tumor implantation, C57BL/6 mice were shaved at the flank and injected subcutaneously with 200 μ l of 6×10^5 syngeneic B16D8 melanoma cells in PBS (indicated as Day 0). Thereafter, mDC treatment was performed 3 times on day 12, 16 and 19; mDCs (10^6 cells) bearing TLR adaptor and tumor debris were injected subcutaneously around the transplanted tumor in mice. Tumor volumes were

measured at regular intervals using a caliper every 2days. Tumor volume was calculated using the formula: Tumor volume (cm^3) = (long diameter) x (short diameter) x (short diameter) x 0.4.

Cytotoxic assay

Debris of B16D8 cells (10^6 cells) was prepared by freeze-thaw for immunization. C57BL/6 mice were subcutaneously immunized with the debris twice on day -14 and -7. On day 0, the mice were sacrificed and CD8⁺ splenocytes (containing CTL and part of NK) were isolated with MACS beads by positive selection. To test the effect of antigen pulse on mDC-mediated tumor cytotoxicity of mouse splenocytes, mDCs were transduced with TICAM-1 N or MyD88 as above and in Fig. 4a. Adaptor-expressing mDCs thus prepared were incubated for 24 hrs with the splenocytes in the presence or absence of the antigen (B16 debris). Then, B16D8 cells pretreated with IFN- γ and labeled with ^{51}Cr (12) were added to the mDC-splenocyte mixture at the E/T ratio=100. 18 hrs later, B16D8 killing was determined by ^{51}Cr release.

Results

Since mDCs proliferate poorly in culture and retrovectors do not efficiently transduce mDCs, a Lenti-viral gene expression system (Invitrogen, Carlsbad, CA) was used to introduce TLR adaptors into mouse mDCs. pLenti-IRES-GFP vector from pLenti6/V5-D-TOPO vector (Invitrogen) and pIRES-hrGFP-1a vector (Stratagene, LaJolla, CA) were prepared for this purpose (Figure 1a). The empty vector was used as a control. cDNAs of mouse TLR adaptors, *Myd88* and *Ticam-1*, were inserted into the multicloning site of this vector (Figure 1a).

It has been reported that the C-terminal region of TICAM-1 recruits receptor-interacting protein 1 (RIP1) and caspases leading to apoptosis within 8 hrs after gene transduction (26). In fact, most of the 293FT packaging cells with full-length TICAM-1 died within 24 hrs after transduction (data not shown). To circumvent cell apoptosis, the cDNA encoding an N-terminal region (1-550 a.a.) of TICAM-1 including the start methionine and the Toll-IL-1 homology (TIR) domain was ligated into the vector (Figure 1b). This region is sufficient for recruiting NAK-associated protein 1 (NAP1) followed by activation of the promoter of IFN- β (25) without inducing apoptosis. When virus particles were prepared by transfection of the pLenti-TICAM-1N-IRES-GFP into

293FT cells, the cells did not die within 24 hrs.

Virus titer was evaluated by ED50 of 293FT cells, where the amount of virus that induced GFP expression in 50% of 5×10^5 293FT cells suspended in 500 μ l of culture media was determined (Figure 2a). Then, using the same ED50 dose, mDCs were infected with lentivirus. The transfection efficiencies of control virus, virus with *Myd88*, and virus with *Ticam-1* were approximately 60%, 50%, and 40%, respectively (Figure 2b). After treatment of mDCs with virus particles, up-regulation of the CD86 co-stimulatory molecule was observed, irrespective of the inserted cDNA. When mDCs infected with virus (i.e. GFP-positive cells) were stimulated with lipopolysaccharide (LPS), the levels of CD86 were further up-regulated (Figure 2b). GFP-negative and lentivirus-negative mDCs were barely mature according to the CD86 expression levels. Slight CD86 up-regulation was observed in GFP-positive mDCs expressing *Ticam-1*, but their CD86 levels were similar to that observed in mDCs transduced with *Myd88*-bearing or the control virus. The up-regulation of CD86 was not a specific response to the overexpression of MyD88 in mDCs.

We also examined the production of cytokines or IFNs by mDCs transduced with MyD88 or TICAM-1 since cytokine/IFN are possible maturation markers for mDCs. High levels of IL-6 and IL12p40 were predominantly detected in the culture supernatants of MyD88-expressing mDCs (Figures 3a, b), whereas the production of IFN- α was augmented in the culture supernatant of TICAM-1-expressing mDCs (Figure 3c). In the MLR experiment, no large difference between these two mDCs was observed. In mDCs expressing either MyD88 or TICAM-1, MLR was slightly augmented compared to the mock-infected mDCs (Figure 3d).

mDCs transduced with the TLR adaptor gene were employed as a DC therapy for cancer in a mouse model, and the therapeutic effect of the mDCs on tumor regression was examined. The schedule of mDC manipulation/therapy is shown in Fig. 4a. The manipulated mDCs retarded the growth of implanted tumors (Figure 4b). This antitumor effect was negligible in the control group consisting of mDCs infected with control virus. However, an antitumor effect was observed in the group consisting of mDCs that expressed the TICAM-1 N terminal region. Furthermore, the group consisting of mDCs transfected with MyD88 triggered a more significant retardation of tumor growth than the group consisting of mDCs transfected with TICAM-1.

We tested whether the adaptor-transduced mDCs augment antigen-dependent or

nonspecific cytotoxicity. Splenocytes containing CD8⁺ lymphocytes were prepared from C57BL/6 mice immunized with B16 debris. The splenocytes were incubated with mDCs expressing MyD88 or TICAM-1 and their cytotoxic activities were measured with B16D8 cells that had been treated with IFN- γ for MHC class I expression. MyD88 in mDCs tended to augment antigen-specific lymphocyte-mediated B16 cytotoxicity whereas TICAM-1 in mDCs up-regulated B16 cytotoxicity in an antigen-independent manner (Figure 4c). Thus, the MLR potency *in vitro* does not simply reflect the *in vivo* tumor regression activity by mDCs. MyD88 and TICAM-1 allow mDCs to mature differentially inducing cytotoxic lymphocytes mainly in an antigen-dependent and an independent fashion, respectively.

Discussion

Following infection with bacteria and viruses, TLRs recognize microbial components and activate mDCs (2,7). IFNs and cytokines that are produced by activated mDCs induced the inflammatory response leading to activation of the immune system. Maturation of mDCs is characterized by CD83/CD86 levels and induction of effector cells. It is becoming clear that antitumor responses arise after mDCs prime with antigen as well as sense inflammatory signals. However, how TLRs participate in sensitizing mDCs to induce an antitumor effect remains unclear (28). This study firstly focused on the difference between the MyD88 and TICAM-1 (TRIF) pathway in the induction of antitumor potential in mDCs. The second point of this study is that not only mDCs but also cancer cells express TLRs that promote tumor cell growth in response to inflammatory stimuli (29). Methods for more specific activation of TLRs exclusively on mDCs should be explored.

Here, we propose a method for specifically activating mDCs by introducing TLR adaptor molecules into mouse mDCs using a lentivirus vector. The differential activation phenotype was obtained depending on the adaptor employed. CD86 was up-regulated in mDCs expressing TICAM-1 but not in mDCs expressing MyD88. MyD88 allowed mDCs to induce IL-6 and IL12p40 and augmented MLR, whereas TICAM-1 (N-terminal) facilitated mDCs by potentially inducing type I IFN and augmenting MLR. Perhaps, the MLR activity does not always reflect *in vivo* antitumor activity: MyD88 preferentially matures mDCs with ability to induce antigen-dependent lymphocyte-mediated cytotoxicity whereas TICAM-1 matures mDCs to induce NK-like

cytotoxicity. Either of these mDCs exhibited *in vivo* tumor-regression activity irrespective of their phenotypic differences. Hence, the level of CD86 does not always reflect the antitumor potency of mDCs.

Although the retardation of tumor growth was significant in this mDC adoptive-transfer therapy, we observed low protein-expression efficacy (~50%) and apoptosis-inducing properties of mDCs. Since these technical problems were particularly prominent in TICAM-1-expressing mDCs, we employed the TICAM-1 N-terminal construct. Regardless of these technical problems, TICAM-1 N-terminal confers unique properties on mDCs, which are consistent with previous reports using the full-length TICAM-1, suggesting that polyI:C-mediated TLR3 activation results in cross-priming and mDC-mediated NK activation (30,31). The antitumor potential due to activation of the TLR3-TICAM-1 pathway may be closely linked to mDC-mediated NK activation.

Our previous findings using TLR adaptor gene-knock out mice indicated that in mDCs, the TICAM-1 pathway is involved in NK activation (13), while the MyD88 pathway solely induces CTL resulting in tumor regression (12). We obtained these results using C57BL/6 mice with B16 melanoma, and have employed the same system in the present study. TICAM-1 directly binds to TLR3 (5) and activates virus-activated kinases via NAP1 (25). It has been reported that IRF-3 and the IFN- β promoter are constitutively activated by over-expression of TICAM-1 (5). Thus, TICAM-1 is crucial in this pathway. Indeed, in mDCs the protein level of TICAM-1 is usually minimal, and is therefore insufficient to induce type I IFNs (32). The antitumor potential of TICAM-1-expressing mDCs may be attributable to induction of type I IFN and antitumor effectors including NK. In fact, the cells harvested from the draining lymph nodes of tumor-implanted mice with mDCs expressing TICAM-1, exhibited cytotoxicity against YAC-1 (an NK target) and parental B16 melanoma (data not shown).

The functional interpretation of the data for mDCs over-expressing MyD88 is more complicated. Since MyD88 is an adaptor molecule of not only the TLR but also IL-1 and IL-18 receptors (3,4), mDCs expressing MyD88 are implicated in more complex inflammatory reactions. Consistent with this, adoptive transfer of MyD88-expressing mDCs induced a superior antitumor effect than TICAM-1-expressing mDCs. However, since no appropriate method for determining CTL activity in the lentiviral mDC manipulation system, we were unable to detect the antitumor CTL activity generated in mice receiving DC therapy (data not shown).

The lentivirus vector is an efficient tool for gene delivery into differentiated cells (33). TLR adaptors and tumor antigens can be inserted into this vector and overexpressed in mDCs. Efficiency of transfection can be easily monitored by insertion of the internal ribosomal re-entry sequence (IRES) and GFP downstream of the objective genes. If the tumor antigen is already known, the antigen sequence can be inserted downstream of the *TLR* gene and IRES insertion site. CTL activation following antigen uptake, occurs through cross-presentation by mDCs stimulated with TLR ligands or adaptor input. Although the mechanism by which cross-priming is induced in TLR-treated mDCs is uncertain, tumor antigens are efficiently presented on MHC class I by the normal antigen-processing pathway. Taken together, *in vivo* adoptive transfer studies show that these mDCs are able to regress syngeneic tumors that have been implanted in mice.

DC therapy has been developed with various modifications. For example, vaccine enhancement has been accomplished by the addition of a foreign helper protein KLH (34). Potent protective immunity has been induced by administration of tumor RNA-pulsed mDCs (35). An effective therapeutic potential has been obtained using mDCs with a modified *IL-12* gene (36). These last two reports suggest that the intratumoral route is more effective than the other extratumoral routes (35,36). Here, we propose a strategy of specifying the mode of maturation of mDCs by manipulating TLR signaling. It is possible that a more effective therapeutic gain is obtained by further revision of our administering protocol, schedule, route, and the number of mDCs used.

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Figure legends

Figure 1: Preparation of lentivirus particles containing TLR adaptor gene.

a) Construction of pLenti-IRES-GFP vector. Sequence of multi-cloning site and hrGFP isolated from pIRES-hrGFP (stratagene) by PCR were constructed into pLenti6/V5 D-TOPO vector (Invitrogen) by TOPO-cloning system. b) Isolation of mouse TICAM-1 N-terminal region. Full-length of mouse TICAM-1 was composed of 732 amino acids. The TIR domain, which participates in molecular association with TLR3, spans the region of 399-548 amino acid. The DNA sequence of TICAM-1 (1-550 amino acid) was isolated as TICAM-1 N-terminal.

Figure 2: Transfection efficiency of the lentivirus preparation.

a) Titration assay of lentivirus prepared with 293FT cells. Titration assay was performed under the conditions of manufacturer's protocol (5×10^5 cells/ 500 μ l/ 24well plate). Indicated volumes of the virus preparations were added to cells and incubated for 12 hrs. Then, cells were washed and cultured in fresh medium for 36 hrs. The cells were collected and analyzed by FACS. Percentages of GFP-positive cells are shown in the inset. b) Lentiviral transfection into bone marrow-derived mDCs. The ED50 doses of the virus preparations were determined with 293FT cells and the ED50 doses were added to mDCs as in 293FT cells. The percentages of GFP positive cell (left), and mean fluorescence intensities of CD86 expression in GFP-negative (middle) and GFP-positive cells (right) are shown. One of three similar experiments is shown.

Figure 3: The characterization of TLR adaptor over-expressed mDCs.

The production of IL-12p40 (a), IL-6 (b), interferon- α (c) by TLR adaptor-overexpressing mDCs (a&b:48 hrs, c:24 hrs). mDCs overexpressing MyD88 produced IL-12p40 and IL-6, but the mDCs overexpressing TICAM-1 produced interferon- α . (d) Allogeneic mixed lymphocyte reaction assay. CD90-positive T cells derived from BALB/c mice were co-cultured with mDCs derived from the bone marrow of C57BL/6 mice for 72 hrs. Overexpression of TLR adaptors in mDCs were confirmed by Ab staining (not shown). $p < 0.05$ in MyD88-expressing mDCs, while $p = 0.14$ in TICAM-1-expressing mDCs by student t-test.

Figure 4: Application of mDCs overexpressing TLR adaptors to DC therapy for

cancer. a) The protocol of DC therapy. mDCs overexpressing MyD88 or TICAM-1 were prepared as in Figure 2. Tumor debris was prepared from B16 cells by 3-cycle freeze-thaw and co-incubated with lentivirus-bearing mDCs for last 6hrs. b) Effect of mDCs overexpressing TLR adaptor on regression of implanted tumor. Manipulated mDCs (10^6 cells) were injected intraperitoneally into C57BL/6 mice three times per week (open arrows). Tumor growth was monitored in mouse groups treated with mDCs having indicated lentiviruses. c) B16 cytotoxicity by mouse splenocytes was enhanced by adaptor-transduced mDCs. CD8⁺ effector cells were isolated from the spleen of C57BL/6 mice immunized with debris of B16 cells that had been treated with IFN- γ . Adaptor-transduced mDCs were manipulated as in Fig. 4a. Effector cells and manipulated mDCs were co-cultured in the presence (solid bars) or absence (open bars) of B16 debris for 24 hrs. Then, the ^{51}Cr -labeled B16 cells were added as target and incubated for additional 18 hrs at the E/T ratio=100. Cytotoxicity was determined by the released ^{51}Cr .

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