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Enhanced Cancer Immunotherapy Using STAT3-Depleted Dendritic Cells with High Th1-Inducing Ability and Resistance to Cancer Cell-Derived Inhibitory Factors

Tomoko Iwata-Kajihara,* Hidetoshi Sumimoto,* Naoshi Kawamura,* Ryo Ueda,* Tomomi Takahashi,* Hiroyuki Mizuguchi,† Makoto Miyagishi,‡ Kiyoshi Takeda,§ and Yutaka Kawakami*

STAT3 signaling constitutes an important negative feedback mechanism for the maintenance of immune homeostasis, a suppressive signal for the Th1 immune response in murine macrophages, and a cancer immune evasion signal in various immune cells. The strategy for STAT3 signal inhibition should be considered, because these features could impede effective cancer immunotherapy. We have evaluated the effects of STAT3 inactivation in dendritic cells (DCs) on immune responses in mice and humans. DCs derived from LysMcre/STAT3flox/flox mice displayed higher cytokine production in response to TLR stimulation, activated T cells more efficiently, and were more resistant to the suppression of cytokine production by cancer-derived immunosuppressive factors compared with DCs from control littermates. Antitumor activities of STAT3-depleted and control DCs were compared by intratumoral administration of gp70 Ag peptide-pulsed DCs in the therapeutic MC38 tumor model. Intratumoral administration of STAT3-depleted DCs significantly inhibited MC38 tumor growth of both injected and nontreated remote tumors. The inhibition was accompanied by an increase in gp70-specific T cell response as well as in systemic Th1 immune response. STAT3-depleted human DCs with adenosiviral STAT3 short hairpin RNA were also capable of producing more cytokines with TLR stimulation and more resistant to cancer-derived factors, and they induced tumor Ag-specific T cells more efficiently than control DCs. The identified role of DC STAT3 signaling in both in vivo therapeutic tumor models in mice and in vitro-specific T cell induction in humans indicates that STAT3-inactivated DCs may be a promising approach for cancer immunotherapy. The Journal of Immunology, 2011, 187: 000–000.
immune cell was unclear in the enhanced antitumor immune response. It was not shown whether STAT3-inactivated DCs could be sufficient to enhance antitumor immune responses.

Second, DC activation is usually self-limiting through intracellular negative feedback mechanisms that involve suppressor of cytokine signaling-1 (SOCS-1) (13), STAT3 (14), and PI3K (15). These negative feedback mechanisms are important for the maintenance of immune homeostasis and the prevention of excessive, deleterious immune responses [e.g., fatal T cell activation with systemic organ inflammation in SOCS-1 knockout mice (16, 17) or lethal endotoxin shock due to cytokine storms in STAT3-CKO mice (14)]. However, this autoregulatory mechanism also may limit the maximal ability of DCs to activate T cell responses to relatively weakly immunogenic, human tumor Ags. The blockade of the SOCS-1 negative feedback mechanism resulted in enhanced DC activation with higher antitumor responses (18, 19).

Third, STAT3 ablation in macrophages was shown to prevent immune tolerance (20) and enhance Th1 activity (9), both of which are favorable features for evoking antitumor immune responses, because tumor-bearing hosts often suffer from anergy to tumor Ags (21, 22) and are deviated to Th2-dominant immune responses (23). These observations suggest the possibility that STAT3-inactivated DCs may augment antitumor immunity through resistance to cancer-derived suppressive factors, induction of higher tumor-specific T cell responses, and enhanced Th1 immune responses.

We demonstrate that STAT3-depleted DC vaccination induces effective systemic antitumor effects through high Ag-specific T cell responses accompanied by systemic Th1 immune responses in a murine tumor model. Furthermore, we demonstrate that STAT3-depleted DCs induce Ag-specific T cell responses in vitro efficiently in humans, thus providing a rationale for the development of immunotherapy using STAT3-inactivated DCs for future clinical trials.

Materials and Methods

**Mouse lines**

STAT3-CKO mice have been described previously (9, 20). LysMcre/STAT3flox/+ mice and STAT3flox/flox mice (H-2b) were mated to generate LysMcre/STAT3flox/flox mice. Six- to 7-wk-old C57BL/6 mice were purchased from Tokyo, Japan. All of the mice were maintained in specific pathogen-free conditions and used upon approval by the Animal Care and Use Committee of the Keio University School of Medicine.

**Cell lines**

CT-26 and MC38 (both murine colon carcinoma cell lines), A375 (a human melanoma cell line, purchased from American Type Culture Collection, Manassas, VA), 624mel and 888mel (human melanoma cell lines, kindly provided by Dr. S.A. Rosenberg, National Cancer Institute) were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin.

**Generation of mouse bone marrow-derived DCs**

Bone marrow cells were isolated from femurs and tibias, and after homogenization, lineage-negative cells (CD3, CD11b, B220, Gr-1, TER-119) were isolated by using Mouse Hematopoietic Progenitor (Stem) Cell Enrichment Set-DM (BD Biosciences, Japan) according to the manufacturer’s instructions. The hematopoietic progenitor cells were cultured in RPMI 1640 supplemented with 10% FBS and 10 ng/ml mouse GM-CSF (PeproTech EC, London, U.K.) at a concentration of 2 × 10^7 cells/ml in six-well plates. On days 4 and 6, half of the medium was replaced with fresh complete medium. On day 7, the cells were harvested and used in subsequent experiments.

**ELISA**

Murine IL-12 (p70), IL-10, and TGF-β1 in the culture supernatant of bone marrow-derived DCs (BMDCs) were quantified by ELISA (BD OptEIA, BD Biosciences, Japan) according to the manufacturer’s instructions. Murine IFN-γ and IL-4 produced by T cells or found in the serum were measured by ELISA (Mouse IFN-γ CytoSet; Life Technologies, Carlsbad, CA, and BD OptEIA; BD Biosciences, respectively). The detection ranges of mouse IL-12 (p70), mouse IL-10, mouse TGF-β1, mouse IFN-γ, and mouse IL-4 were 62.5–4000, 31.3–500, 62.5–4000, 31.3–500, and 7.8–500 pg/ml, respectively. Human IL-6 and VEGF (DuoSet, R&D Systems, Minneapolis, MN), IL-12 (p70), and TNF-α (BD OptEIA) in the culture supernatant of human DCs were measured by ELISA. Human IL-6 and VEGF produced by T cells was measured by ELISA (Endogen, Rockford, IL). The detection ranges of IL-6, VEGF, TNF-α, and IFN-γ were 9.38–600, 31.3–2000, 7.8–500, and 5–3800 pg/ml, respectively.

**Allogeneic MLR**

Splenetic T cells were isolated from BALB/c mouse splenocytes by using CD90.2 MicroBeads and autoMACS (Miltenyi Biotec KK, Japan), and 2 × 10^5 T cells were mixed with 2 × 10^5 irradiated (50 Gy) BMDCs derived from STAT3-CKO mice or control littersmates on 96-well plates in triplicate wells. On day 5, 1 μCi [3H]thymidine was added to each well, and after 18 h, the cells were harvested, and the incorporation of [3H]thymidine was quantified using TopCount NXT (Perkin Elmer Japan, Japan). In humans, CD3+ T cells were isolated from PBMCs of healthy volunteers by using CD3 MicroBeads and autoMACS (Miltenyi Biotec). A total of 2 × 10^5 T cells was mixed with 2 × 10^5 irradiated (50 Gy) human monocyte-derived DCs (MoDCs), and subsequent assays were the same as those used for mouse allogeneic MLR.

**DC vaccination experiment**

A total of 3 × 10^6 MC38 cells was injected s.c. on the bilateral flanks of 6- to 8-wk-old C57BL/6 mice on day 0. On days 7 and 14, BMDCs from STAT3-CKO mice or control littersmates were incubated for 6 h with or without 1 μg/ml gp70 peptide (KSPWFITTL), a MC38 self tumor Ag-derived immunodominant T cell epitope. Then, a total of 2 × 10^6 BMDCs resuspended in 100 μl RPMI 1640 was injected into tumors on the right flank. PBS was used as a control vaccine. The size of the bilateral tumors was measured every 3 d in three perpendicular diameters (longest diameter, width, and height), and the tumor volume was calculated as the product of the three diameters.

**Detection of tumor-specific T cell response by IFN-γ release**

Spleens from two mice vaccinated with STAT3-CKO BMDCs, control BMDCs, or PBS were harvested on day 19, and the splenocytes were cultured in RPMI 1640 supplemented with 10% FBS and restimulated with 1 μg/ml gp70 peptide for 5 d. The splenic T cells then were collected and incubated with irradiated (60 Gy) syngeneic splenocytes in the presence of gp70 peptide at concentrations of 0, 0.01, and 0.1 μg/ml for 24 h. The amount of IFN-γ secreted into the culture supernatant was quantified by ELISA.

**Th1 versus Th2 immune responses**

The syngeneic BMDCs were non-treated or pulsed with the cellular lysates of MC38 cells for 9 h, followed by LPS stimulation (1 μg/ml) overnight, then the irradiated (50 Gy) BMDCs (stimulators) were cocultured with splenic T cells (responders) from the vaccinated mice (PBS, control DCs, STAT3-CKO DCs) at a responder-to-stimulator ratio of 1:2 for 48 h. IFN-γ and IL-4 in the culture supernatant were measured by ELISA.

**Flow cytometric analysis**

For MDSCs, tumors were digested with 1% (w/v) collagenase type IV (Sigma, Tokyo, Japan)/300 U/ml DNase (Sigma) in 0.1% (w/v) hyaluronidase V (Sigma) at 37°C for 1 h, then the dead cells were removed using a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient, and viable cells were stained with PE-conjugated Gr-1 (RB6-8C5) and FITC-conjugated CD11b (M1/70) mAb or isotype-matched control mAbs (BD Biosciences). Tregs were stained with FITC-conjugated anti-CD3 (RM4-5) Ab (BD Biosciences), allophycocyanin-conjugated anti-CD25 (7D4) Ab (BD Biosciences), and PE-conjugated anti-Foxp3 (FJK-16S) Ab (eBiosciences) or isotype-matched control Abs by using a Foxp3 Staining Buffer Set (eBiosciences) according to the manufacturer’s instructions. Human MoDCs were stained with PE-conjugated CD1a (BL6), CD83 (HB15A), CD86 (HAS.2B7), and HLA-DR (IMMU357) mAbs or isotype-matched control mAbs (Beckman Coulter, Fullerton, CA). The surface expression of these molecules was analyzed using a FACS caliber and CellQuest Pro (BD Biosciences).

**Western blot analysis**

Cell lysates were prepared by incubating cells on ice for 30 min in lysis buffer (20 mM Tris-HCl (pH 7.5), 12.5 mM β-glycerophosphate, 2 mM...
EGTA, 10 mM NaF, 1 mM benzamide, 1% NP-40, and a protease inhibitor mixture (complete, EDTA-free [Roche, Germany]) and 1 mM NaN3O4, then centrifuged at 15,000 rpm at 4°C for 10 min. The supernatant (cell lysate) was collected, and the protein concentration was measured using a DC protein assay kit (Bio-Rad, Hercules, CA). The protein was subjected to 10% SDS-PAGE electrophoresis, then transferred onto a nitrocellulose Immobilon-P membrane (Millipore, Tokyo, Japan) using a Trans-Blot SD cell (Bio-Rad). Anti-STAT3 (BD Biosciences) or anti-actin (Sigma) was used as the primary Ab, and goat anti-mouse IgG-HRP (Cappel MP Bio-medicals, LLC, Solon, OH) or goat anti-rabbit IgG-HRP (Cappel) was used as the secondary Ab. The blot was immersed in SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), then exposed using as the secondary Ab. The blot was immersed in SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), then exposed used as the secondary Ab. The blot was immersed in SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), then exposed used as the secondary Ab. The blot was immersed in SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), then exposed used as the secondary Ab. The blot was immersed in SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), then exposed used as the secondary Ab. The blot was immersed in SuperSignal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL), then exposed.

**Human MoDCs**

Heparinized blood collected from healthy volunteers was subjected to a Lymphoprep gradient to obtain PBMCs. The CD14+ cells were isolated from the PBMCs using CD14 MicroBeads and autoMACS (Miltenyi Biotec). The CD14+ cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 ng/ml GM-CSF (PeproTech), and 50 ng/ml IL-4 (PeproTech). On day 2, half of the culture medium was replaced with fresh complete medium. On day 3, the cells were collected and infected with adenovirus vectors at 100 or 150 multiplicity of infection (MOI) at 37°C, 5% CO2 for 2 h, then washed three times in PBS to remove the residual virus. On day 5, LPS was added at a concentration of 1 µg/ml, and on day 6, the cells and culture supernatant were quantified by ELISA. Error bars indicate SE of five experiments.

**Adenovirus vectors**

Adenovirus vectors expressing short hairpin RNA (shRNA) have been described previously (24). These vectors contain Ad5/35 chimeric fiber protein for efficient gene transduction to DCs. Double-stranded oligonucleotides encoding shRNA templates were subcloned into the two FbaA1 sites downstream of the human U6 promoter in the shuttle plasmid, pHMCMV-GFP-U6i, which has both an shRNA expression unit and an enhanced GFP expression unit. shRNA target sequences for the indicated genes were as follows: STAT3 #1, 5'-GGGTCAGAATTGACGCTAAG-3'; STAT3 #4, 5'-ATAGGAAGTTTTAAGGAAGT-3'; GL3B (control anti-firefly luciferase), 5'-GGCGGCGCTGCTGTCGCTC-3'. The adenovirus vectors expressing double-stranded oligonucleotides and the GFP gene were constructed by an improved in vitro ligation method as described (25). The (adenovirus) vectors then were propagated in 293 cells, and the postinfection viral titers were evaluated by GFP expression (transducing unit/µl). The virus particle to transducing unit ratio was in the range of 0.28–0.34.

**MART-1- or Flu-specific T cell responses by IFN-γ release**

Human MoDCs were used as stimulators for peptide-specific T cell induction. HLA-A*0201+ healthy volunteers were the donors of DCs and T cells. The preparation and infection of DCs with the adenovirus vectors (AdF35-GL3B or AdF35-STAT3 #1) were described above. On day 6 of the culture, the DCs were matured with LPS (Sigma) at a concentration of 100 ng/ml. On day 7, the DCs (1 x 10^6 cells/ml in AIM-V) were pulsed with 1 µg/ml of either MART-127–35 (AAGIGILTV) or influenza matrix protein FluM158–66 (GILGFVFTL) peptides and incubated for 2 h at 37°C, with 1 µCi [3H]thymidine was added to each well for the last 18 h, and the incorporation was quantified. Error bars indicate SD of triplicate wells. These data are representative of two independent experiments with similar results. *p < 0.05.

**FIGURE 1.** Characteristics of BMDCs derived from STAT3-CKO mice. A, Western blot analysis of BMDC STAT3 expression. Proteins were extracted from BMDCs before and after TLR stimulation (100 ng/ml LPS). ELISA of IL-12 (p70) and IL-10. Cytokines in the culture supernatants of MC38 or CT26 cell lines. IL-12 levels in the culture supernatant were assessed by ELISA. Error bars indicate SD of three experiments. Percentage inhibition of IL-12 production was shown in the bottom of the graph by calculating as follows: % inhibition = ([IL-12 level without conditioned media} - (IL-12 level with conditioned media})/ (IL-12 level without conditioned media}) x 100%. Lower panel, Western blot analysis of p-STAT3 of BMDCs in the presence or absence of conditioned media of cancer cell lines. BMDCs were stimulated with supernatants of CT26 and MC38 murine cancer cell lines or IL-10 (as a positive control for p-STAT3) for 15 min.

**FIGURE 2.** STAT3-CKO DCs were resistant to the STAT3-activating cancer-derived factors. Upper panel, Inhibition of IL-12 production in the presence of conditioned media of cancer cell lines. The BMDCs (control or STAT3-/-) were stimulated with LPS in the presence or absence of 20% of culture supernatants of MC38 or CT26 cell lines. IL-12 levels in the culture supernatant were assessed by ELISA. Error bars indicate SD of three experiments. Percentage inhibition of IL-12 production was shown in the bottom of the graph by calculating as follows: % inhibition = ([IL-12 level without conditioned media} - (IL-12 level with conditioned media})/ (IL-12 level without conditioned media}) x 100%. Lower panel, Western blot analysis of p-STAT3 of BMDCs in the presence or absence of conditioned media of cancer cell lines. BMDCs were stimulated with supernatants of CT26 and MC38 murine cancer cell lines or IL-10 (as a positive control for p-STAT3) for 15 min.
then irradiated (56 Gy) and washed three times in PBS. The peptide-pulsed DCs (6 × 10^5 cells/well) were mixed with the autologous CD8\(^+\) T cells (2 × 10^5 cells/well), which were isolated from PBMCs using CD8 MicroBeads and autoMACS (Miltenyi Biotec) in 24-well plates (2 ml/well). IL-2 then was added at a concentration of 12 IU/ml to the cultures. T cell stimulation was repeated every 7 d, totaling three times. Seven days after the third stimulation, the responder T cells were collected and incubated with stimulators at a responder-to-stimulator ratio of 1:1 (1 × 10^5 cells for ELISA and 5 × 10^5 cells for ELISPOT assay). For each experiment, triplicate wells were prepared. Peptide-pulsed T2 (HLA-A2.1\(^+\)) and melanoma cell lines, 501mel, 526mel (MART-1\(^+\)/HLA-A2.1\(^+\)), and 888mel (MART-1\(^+\)/HLA-A2.1\(^-\)), were used as stimulator cells. T2 cells were preincubated with 1 \(\mu\)g/ml of either MART-1\(_{27-35}\) or FluM1\(_{58-66}\) peptide for 2 h at 37˚C, then washed three times in PBS, before being used.

**FIGURE 3.** Therapeutic DC vaccination with STAT3\(^{-/-}\) BMDCs. **A**, Comparison of tumor size. A total of 3 × 10^5 MC38 cells was implanted s.c. on the bilateral flanks of 6- to 7-wk-old C57BL/6 mice on day 0. On days 7 and 14, PBS (control), control immature BMDCs, or STAT3\(^{-/-}\) immature BMDCs with or without gp70 peptide pulsation were injected into the tumor on the right flank (injected tumor), and the tumor size of both flanks was measured every 3 d (n = 5 or 6; in PBS, n = 10). These data are representative of four independent experiments with similar results. **B**, Tumor size on day 19. The sizes of bilateral tumors were compared among the five therapeutic groups.
as stimulators. Twenty-four hours after the T cell stimulation, the quantity of IFN-γ released into the culture supernatant was measured by ELISA.

$^{51}$Cr release assay

The MART-1- or Flu-specific T cells were induced as described above. The effector T cells were mixed with $^{51}$Cr (NEN Life Science Products, Boston, MA)-labeled target cells, including T2 cells pulsed with MART-127–35 peptide, T2 cells pulsed with FluM158–66 peptide, 526mel cells, or 888mel cells at an effector-to-target ratio of 10, 20, or 40, in triplicate wells. After 4 h of incubation at 37°C, 5% CO2, radioactivity levels in the culture supernatant were quantified with an automatic γ-counter (1480 Wizard γ-counting system; Wallac, Turku, Finland). Percentage specific lysis was calculated according to conventional evaluation methods.

Statistical analysis

All of the statistical analyses were performed using unpaired Student t tests, and $p$ values < 0.05 were regarded as significant.

Results

STAT3-deficient DCs are more potent to activate T cells and are resistant to inhibitory factors from cancer cells

To characterize STAT3-inactivated DCs, the phenotypes, cytokine production, and susceptibility to cancer-derived soluble factors of BMDCs from STAT3-CKO mice were evaluated. BMDCs from STAT3-CKO mice showed almost complete loss of STAT3 protein compared with BMDCs of control littermates (Fig. 1A). LPS stimulation increased IL-12 secretion by 7.9-fold in STAT3-CKO BMDCs compared with control BMDCs (1003.8 ± 196.5 versus 126.8 ± 25.5 pg/ml) (Fig. 1B). IL-10 expression in STAT3-CKO BMDCs was also increased by 3.5-fold compared with that in control BMDCs (2506.9 ± 268.4 versus 717.1 ± 333.3 pg/ml), whereas no significant difference in TGF-β1 production was observed (data not shown) (Fig. 1B). The increase in IL-12 and IL-10 production by the LPS-stimulated STAT3-CKO BMDCs can be explained by decreases in STAT3-mediated negative feedback against NF-κB signaling, as reported previously (26). However, surface expression of DC maturation markers, including CD80, CD86, MHC class II, and CD40, was similar between STAT3-CKO BMDCs and control BMDCs following LPS stimulation or polyinosinic-polycytidylic acid (poly-IC) stimulation (data not shown). To compare the T cell activation ability of these DCs, splenic T cells derived from BALB/c mice (H-2d) were mixed with BMDCs stimulated with LPS or poly-IC from either STAT3-CKO mice or control littermates (H-2b) to perform allogeneic MLRs. MLR was increased significantly in STAT3-CKO BMDCs compared with control BMDCs (Fig. 1C), although immunosuppressive IL-10 levels increased along with IL-12 levels in STAT3-CKO BMDCs, indicating that the net influence of STAT3 depletion is favorable to T cell proliferation.

Interestingly, IL-12 production by STAT3-CKO BMDCs was less inhibited by culture supernatants of multiple murine cancer cell lines (MC38 and CT-26), which produce STAT3-activating cytokines such as VEGF (data not shown), compared with that of wild-type BMDCs (Fig. 2). Almost no STAT3 phosphorylation was...
observed after the addition of cancer supernatants to STAT3-CKO DCs. IL-12 production was less inhibited, although an increase in STAT3 phosphorylation was observed after the addition of cancer supernatants to control DCs, which showed strong inhibition of IL-12 production. These results indicate that the inhibition of IL-12 production by DCs is dependent partly on STAT3 activation by cancer supernatants (Fig. 2). Thus, STAT3-deficient DCs are more resistant to the inhibitory effects of cancer cells. These results suggest that STAT3-depleted DCs could be useful for the efficient induction of tumor-specific T cells even in the immunosuppressive tumor microenvironment.

Intratumoral vaccination with STAT3-depleted DCs induced potent antitumor effects

On the basis of the observed characteristics of STAT3-CKO BMDCs (higher T cell stimulatory activity and more resistance to cancer-derived immunosuppressive factors), we then evaluated their ability as therapeutic anticancer vaccines. Subcutaneous MC38 colon carcinomas were established on the bilateral flanks of C57BL/6 mice. Immature BMDCs pulsed with or without gp70 peptide (a MHC class I-restricted immunodominant epitope of MC38) were injected intratumorally on the right flank (injected tumor) twice on days 7 and 14 after tumor inoculation, whereas no treatment was performed for the tumor on the left flank (non-injected tumor). The longer diameter of the tumor ranged from 4 to 5 mm on day 7 in all of the groups. The tumor growth was inhibited significantly by the administration of gp70 peptide-pulsed STAT3-CKO BMDCs compared with gp70 peptide-pulsed control BMDCs in both DC-injected tumors (p = 0.014) and noninjected tumors (p = 0.046) on day 19 (Fig. 3). When DCs without gp70 peptide pulse were used, antitumor effects were not significantly different between control DCs and STAT3-CKO DCs on both DC-injected (p = 0.071) and noninjected (p = 0.580) tumors. These results indicate that the antitumor activity of gp70 peptide-pulsed STAT3-CKO DCs may not be explained simply by increased cytokine production; rather, it may be caused by enhanced induction of tumor Ag-specific T cells that are activated by the administration of gp70 peptide-pulsed DCs.

The significant inhibition of tumor growth on the noninjected tumor with STAT3-CKO DC vaccination (p = 0.046) also indicates that a STAT3-depleted DC vaccine could be sufficiently potent for the induction of a systemic antitumor immune response. Fewer antitumor effects on the noninjected tumor than those on the injected tumor may be explained by additional local immune responses caused by increased cytokine production by the injected DCs at the injected tumors. The systemic T cell response was supported by a greater induction of gp70-specific T cell responses in mice administered STAT3-CKO BMDCs compared with that in either control PBS- or control BMDC-administered mice (Fig. 4A). Interestingly, splenic T cells from mice administered STAT3-CKO BMDCs produced more IFN-γ but less IL-4 when stimulated by BMDCs pulsed with MC38 tumor lysates (Fig. 4B). Large amounts of serum IFN-γ were detected only in mice administered STAT3-CKO BMDCs (Fig. 4B). These results indicate that intratumoral injection of STAT3-depleted DCs effectively induced Th1 antitumor immune responses in vivo, possibly through an increase in IL-12 production (Fig. 1B). The percentage of CD11b+ Gr-1+ MDSCs in the tumors was not significantly different across the three groups (Fig. 4C), and Foxp3+ CD4+ Tregs in the tumor were very much under the detection limit (<0.01%) in all three groups (data not shown), indicating that STAT3-depleted DCs enhance antitumor T cells not by decreasing these immunosuppressive cells. These results indicate that STAT3-depleted DCs could be useful for cancer immunotherapy possibly due to the cells’ resistance to cancer-derived immunosuppressive effects even in the tumor microenvironment, their induction of systemic Th1 immune responses, and their greater T cell stimulatory ability.

STATT3-depleted human MoDCs were capable of producing higher amounts of cytokines and inducing higher specific T cell responses and were resistant to cancer cell-derived inhibitory factors

On the basis of the enhanced antitumor effects of the STAT3-depleted murine DCs, we evaluated whether STAT3 signaling has similar functions in human DCs. STAT3 depletion was achieved in human DCs using recombinant adenovirus vectors

FIGURE 5. Adenovirus vector did not impair the maturation and allogeneic MLR of the human MoDCs. A, A fiber knob mutant of adenovirus vector (AdF35) expressing control shRNA (GL3B) and a GFP gene (AdF35-GL3B) efficiently transduced the GFP gene to human MoDCs without inhibition of maturation or T cell stimulatory capacity. Upper panel, Control MoDCs. Lower panel, MoDCs infected with AdF35-GL3B at 100 MOI on day 3 of culture. Both DCs were maturated with LPS (100 ng/ml) on day 5, and surface expression of CD80, CD40, and CD86 as well as GFP expression were evaluated on day 6. Mean fluorescence intensity of the positive population (control, left upper quadrant; AdF35-GL3B, right upper quadrant) of each surface marker is indicated. These data are representative of three independent experiments with similar results. B, Allogeneic MLR was compared between control DCs and AdF35-GL3B–infected DCs. These data are representative of three independent experiments with similar results.
genetically engineered to express shRNA against STAT3 under the control of the human U6 promoter, in addition to a GFP gene under the control of a CMV promoter to monitor gene transduction. Adenovirus vectors with a serotype 5 backbone were used. In these vectors, the fiber knob was replaced by that of the serotype 35 adenovirus (chimeric adenovirus; AdF35) for efficient gene transduction to hematological cells expressing CD46, including DCs (27). The AdF35 vector could efficiently transduce the GFP gene into human MoDCs without inhibiting DC maturation or allogeneic MLR (Fig. 5). We selected two shRNA target sequences (STAT3 #1 and STAT3 #4) specific for the STAT3 gene for efficient depletion (Fig. 6A). The expression levels of CD80,

![Image](image1)

**FIGURE 6.** STAT3-depleted human MoDCs produced more inflammatory cytokines and were resistant to the cancer-derived factors. A, Western blot of STAT3. The protein was extracted from human MoDCs infected with AdF35-shRNA vectors (GL3B, STAT3 #1, or STAT3 #4) or control MoDCs. STAT3 protein was depleted by infection of the two shRNA vectors, STAT3 #1 and STAT3 #4. These data are representative of three independent experiments with similar results. B, Inflammatory cytokine production was increased in human MoDCs with STAT3 depletion. IL-6, IL-12 (p70), TNF-α, and IL-10 produced from LPS-stimulated human MoDCs were measured by ELISA. Production of all cytokines was increased significantly with STAT3-inactivated human MoDCs. These data are representative of six independent experiments with similar results. C, STAT3-deficient human MoDCs were resistant to tumor-derived factors. Human MoDCs were nontreated or pretreated with 20% conditioned medium of A375, 624mel, or 888mel human melanoma cell lines, then stimulated with LPS at a concentration of 100 ng/ml. IL-12 or TNF-α production was suppressed significantly in human MoDCs preconditioned with melanoma culture supernatants. The suppression of cytokine production was compensated in the STAT3-depleted DCs compared with that in control (uninfected) DCs or DCs infected with control adenovirus (GL3B). Percentage inhibition of IL-12 or TNF-α production was shown in the bottom of the graph by calculating as follows: % inhibition = [(cytokine level without conditioned media) − (cytokine level with conditioned media)]/(cytokine level without conditioned media)\times100%. Vertical bars indicate SD of triplicate experiments. These data are representative of three independent experiments with similar results. *p < 0.05, **p < 0.01.
CD86, and HLA-DR after LPS stimulation were similar among the DCs infected with the three adenovirus vectors expressing shRNA [GL3B (control shRNA, anti-firefly luciferase shRNA), STAT3 #1, or STAT3 #4] (data not shown). However, production of cytokines, including IL-6, IL-12, TNF-α, and IL-10, after TLR4-activating LPS stimulation was significantly higher in the STAT3-depleted MoDCs than that in the uninfected control MoDCs or GL3B shRNA-transduced MoDCs (Fig. 6B). Similar results were obtained by TLR3-activating poly-IC stimulation (data not shown). These results indicate that STAT3 signaling negatively regulates cytokine production not only in murine DCs but also in human DCs.

The human melanoma cell lines A375, 624mel, and 888mel produce STAT3-activating immunosuppressive cytokines, including VEGF, IL-6, and IL-10. Supernatants derived from these melanoma supernatants inhibit the ability of human MoDCs to produce inflammatory cytokines, such as IL-12 and TNF-α (28, 29). As with murine STAT3-depleted DCs, human STAT3-depleted MoDCs treated with adenoviruses STAT3 #1 or STAT3 #4 also were relatively resistant to the inhibitory effects of the melanoma culture supernatants on LPS-stimulated TNF-α or IL-12 production by MoDCs (Fig. 6C).

We then evaluated the ability of STAT3-depleted human MoDCs to induce Ag-specific CD8+ T cells in vitro. Peripheral blood CD8+ T cells were stimulated with autologous MoDCs pulsed with either HLA-A*0201-restricted influenza FluM1 or melanoma Ag MART-1 epitope peptide, with or without AdF35-shRNA vector infection (GL3B or STAT3 #1). Seven days after the third stimulation, IFN-γ secretion and cytolytic activity of the recovered T cells were evaluated against the peptide-pulsed T2 cells and melanoma cell lines. As shown in Fig. 7, the MART-1-stimulated CD8+ T cells responded specifically to 501mel and 526mel cell lines expressing both MART-1 and HLA-A*0201 but not to MART-1+/HLA-A*0201+ 888mel cells, as demonstrated by data gathered using IFN-γ secretion or cytolytic assays. Similar results also were obtained by ELISPOT assay (data not shown). Induction of these tumor Ag-specific T cells was augmented significantly by stimulation with STAT3-depleted DCs infected with AdF35-STAT3-shRNA vectors compared with that with the control GL3B-infected DCs (Fig. 7). Thus, STAT3 depletion also enhanced the T cell stimulatory activity of human DCs as APCs, as observed in mouse DCs. Therefore, human DCs with inactivated STAT3 may be just as useful as murine DCs when used for DC vaccination to enhance antitumor responses, due to their increased
T cell stimulatory ability and resistance to cancer-induced immunosuppression.

Discussion

DCs are professional APCs that activate naive T cells in vivo; thus, the efficient use of DCs in cancer immunotherapy has been exploited in preclinical studies and clinical trials (1–5). One important target molecule that maximizes DC ability is STAT3, because it is involved in DC suppression by cancer cell-derived cytokines and the cytokine negative feedback loop in DCs.

Immune evasion is one of the major problems in the development of cancer immunotherapy. The STAT3 signal in immune cells appears to be one of the therapeutic targets to overcome cancer immune evasion. The significance of systemic STAT3 inactivation in various immune cells to enhance antitumor immunity has been proposed by using hematopoietic cell-specific STAT3-CKO mice (11) or pharmacological inhibitors (11, 12). However, the role of each immune cell type (e.g., DCs) in the augmented antitumor immune response has not been well investigated. In addition, systemic inactivation of STAT3 involves the potential risk of disturbing immune homeostasis, leading to lethal systemic inflammation as shown in the myeloid cell-specific STAT3-CKO mice (14). Therefore, in this study, we have clarified the role of STAT3-depleted DCs in antitumor immune responses. We have shown the negative roles of STAT3 in DCs in the induction of T cell immune responses, particularly in tumor-bearing hosts, and we have demonstrated effective immunotherapy using STAT3-inactivated DCs that did not display harmful adverse inflammatory responses.

We have reported previously that intratumoral DC injection following tumor cryoablation, which destroys tumor cells and the immunosuppressive tumor microenvironment, is an effective method to induce immune responses to multiple endogenous tumor Ags, leading to efficient in vivo tumor elimination (30). However, without tumor cryoablation, the antitumor effects of intratumoral DC injection were reduced. In this study, we tested antitumor activity of STAT3-depleted DCs by intentionally injecting the cells into the immunosuppressive tumor microenvironment without cryoablation because STAT3-depleted DCs are relatively resistant to tumor-derived immunosuppressive cytokines. The specific antitumor T cell responses were increased significantly with STAT3-CKO BMDCs compared with those of control BMDCs, indicating that STAT3 depletion in DCs is effective in augmenting antitumor T cell responses even in the immunosuppressive tumor microenvironment.

The administration of STAT3-depleted DCs induced systemic Th1-type immune responses, as shown by the increases in serum IFN-γ and IFN-γ production by T cells, possibly because of increased IL-12 production by the STAT3-depleted DCs. The Th1 shift is a favorable characteristic for DC-based cancer immunotherapies. Although we found that IL-10 production also was increased in STAT3-depleted DCs, the enhanced IFN-γ production by T cells indicated that the net balance of various cytokines resulted in efficient Th1 antitumor immune responses.

In the mouse model with hematopoietic cell-specific STAT3 depletion, the tumor-infiltrating Tregs were reduced compared with the control mice (11). Yet, we did not observe significant infiltration of Tregs in the MC38 tumors, and in our immunotherapy model, there was no difference in the number of tumor-infiltrating MDSCs followed by DC vaccination with or without STAT3 depletion. The enhanced antitumor immune responses do not appear to be due to the reduction of immunosuppressive immune cells, such as MDSCs and Tregs, in this tumor model. Although the significance of immune cell STAT3 signaling in the suppression of antitumor immune responses was demonstrated in mice, the role of STAT3 in human DCs for T cell activation has not been evaluated. Thus, in this study, we applied STAT3 RNA interference to evaluate the potential roles of STAT3 in human DCs and found the same in vitro characteristics in human DCs as in mouse DCs (e.g., enhanced cytokine production, resistance to cancer-derived immunosuppressive factors, and augmented T cell-inducing activity by STAT3 inactivation). These findings provide a proof of principle for the use of STAT3-inactivated DCs in cancer immunotherapy in the clinic. In this study, adenosviral shRNA was used to deplete STAT3; however, better methods, such as ligand or Ab conjugation (31) to deliver small interfering RNA to DCs, may be preferable in future clinical trials due to the potential attenuation of DC function in high MOI adenovirus infections and viral protein interference with tumor Ag presentation.

In summary, we demonstrated the negative role of STAT3 for DC activation in both mice and humans and that STAT3 is an attractive target for the development of effective cancer immunotherapies that do not induce harmful inflammatory responses. STAT3-inactivated DCs, which display favorable characteristics for the induction of antitumor immunity, including enhanced T cell stimulatory activity with Th1 deviation and resistance to cancer-derived immunosuppressive factors, may be useful in the improvement of current DC-based cancer immunotherapies.

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Disclosures

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References


