Romero P, Cerottini JC, Waanders GA (1998) Novel methods to monitor antigen-specific cytotoxic T-cell responses in cancer immunotherapy. Mol Med Today 4:305–312

Rosenberg SA, Yang JC, Schwartzentruber DJ, Hwu P, Marincola FM, Topalian SL et al (1998) Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. Nat Med 4:321–327

Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P (1997) Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science 275:1790–1792

Sharkey MS, Lizee G, Gonzales MI, Patel S, Topalian SL (2004) CD4(+) T-cell recognition of mutated B-RAF in melanoma patients harboring the V599E mutation. Cancer Res 64:1595–1599

Somasundaram R, Swoboda R, Caputo L, Otvos L, Weber B, Volpe P et al (2006) Human leukocyte antigen-A2-restricted CTL responses to mutated BRAF peptides in melanoma patients. Cancer Res 66:3287–3293

Sumimoto H, Miyagishi M, Miyoshi H, Yamagata S, Shimizu A, Taira K et al (2004) Inhibition of growth and invasive ability of melanoma by inactivation of mutated BRAF with lentivirus-mediated RNA interference. Oncogene 23:6031–6039

Toda M, Iizuka Y, Kawase T, Uyemura K, Kawakami Y (2002) Immuno-viral therapy of brain tumors by combination of viral therapy with cancer vaccination using a replication-conditional HSV. Cancer Gene Ther 9:356–364

Udagawa M, Kudo-Saito C, Hasegawa G, Yano K, Yamamoto A, Yaguchi M et al (2006) Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and Bacillus Calmette-Guerin cell wall skeleton stimulation. Clin Cancer Res 12:7465–7475

Ueda R, Iizuka Y, Yoshida K, Kawase T, Kawakami Y, Toda M (2004) Identification of a human glioma antigen, SOX6, recognized by patients' sera. Oncogene 23:1420–1427

Ueda R, Ohkusu-Tsukada K, Fusaki N, Soeda A, Kawase T, Kawakami Y et al (2009) Identification of HLA-A2- and A24-restricted T-cell epitopes derived from SOX6 expressed in glioma stem cells for immunotherapy. Int J Cancer 126(4):919–929

van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B et al (1991) A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254:1643–1647

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

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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/STAT3$^{flox/flox}$ 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 adenoviral 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: 27–36.**

Dendritic cells (DCs) play a pivotal role in the induction of Ag-specific T cell immune responses (1). Immunotherapies using DCs have been attempted for various diseases. For cancer patients, a number of DC immunotherapies have been developed and evaluated in preclinical and clinical settings (2–4). However, the effectiveness of DC vaccines has been limited (5). Therefore, improvements in DC therapy are essential for successful cancer immunotherapy.

To induce efficient systemic antitumor immunity, even fully activated DCs are insufficient due to the following problems. First, DC function may be suppressed in cancer patients. Cancer cells produce a variety of immunosuppressive soluble factors, such as IL-6, IL-10, vascular endothelial growth factor (VEGF), and TGF-β1, which directly inhibit DC maturation (6). Immunosuppressive immune cells, such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages, and regulatory T cells (Tregs), often are generated in cancer patients, creating an immunosuppressive microenvironment that leads to the suppression of T cell responses directly or indirectly via the inhibition of DC functions (7).

STAT3 mediates cancer cell-initiated immune evasion signals in various immune cells (8, 9). Cancer-derived soluble factors, such as IL-10, IL-6, VEGF, or monocyte CSF, induce activation of STAT3, especially in myeloid cells, resulting in the systemic accumulation of MDSCs and the inhibition of DC differentiation (10) and activation (8). Inactivation of STAT3 signaling in hematopoietic cell-specific conditional knockout (CKO) mice (11) or by pharmacological inhibitors, such as JSI-124 (12) or CPA-7 (11), resulted in enhanced antitumor immune responses through the activation of various immune cells, such as DCs, and inactivation of immune suppressor cells, such as Tregs (11, 12). These observations provide a proof of principle for the strategy of STAT3 signaling inactivation in immune cells to enhance antitumor immune responses. In these studies, only systemic inactivation of STAT3 signaling was attempted, so the role of each

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Received for publication June 21, 2010. Accepted for publication March 29, 2011.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (19390355, 17016070, 21591445, and 23240128), Grants-in-Aid for Cancer Research from the Ministry of Health, Labor and Welfare, Japan (15-10, 19-7, and 21-6-4) for Second-Term Comprehensive 10-Year Strategy for Cancer Control, the Sagawa Foundation for Promotion of Cancer Research, the Uehara Memorial Foundation, the Yasuda Medical Foundation, and the Keio University Grant-in-Aid for Encouragement of Young Medical Scientists.

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Abbreviations used in this article: BMDC, bone marrow-derived dendritic cell; CKO, conditional knockout; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; MoDC, monocyte-derived dendritic cell; MOI, multiplicity of infection; poly-IC, polyinosinic-polycytidylic acid; shRNA, short hairpin RNA; SOCS-1, suppressor of cytokine signaling-1; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1002067

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/$STAT3^{flox/+}$, $STAT3^{flox/flox}$, and $STAT3^{flox/+}$ mice ($H\text{-}2^b$) were mated to generate LysMcre/$STAT3^{flox/flox}$ and control littermates (LysMcre/$STAT3^{+/+}$, $STAT3^{flox/flox}$, $STAT3^{flox/+}$, and $STAT3^{+/+}$). Six- to 7-wk-old C57BL/6 mice were purchased from Japan SLC (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 μg/ml streptomycin.

Generation of mouse bone marrow-derived DCs

Bone marrow cells were isolated from femurs and tibias, and after hemolysis, lineage-negative cells ($CD3^-CD11b^-B220^-Gr\text{-}1^-TER\text{-}119^-$) 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 2×10^5 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 IFN-γ 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

Splenic 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 2×10^3 irradiated (52 Gy) BMDCs derived from STAT3-CKO mice or control littermates on 96-well plates in triplicate wells. On day 5, 1 μCi [^{3}H]thymidine was added to each well, and after 18 h, the cells were harvested, and the incorporation of [^{3}H]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 2×10^3 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 3×10^5 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 littermates were incubated for 6 h with or without 1 μg/ml gp70 peptide (KSPWFTTL), 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 nontreated or pulsed with the cellular lysates of MC38 cells for 9 h, followed by LPS stimulation (1 μg/ml) overnight, then the irradiated (52 Gy) BMDCs (stimulators) were cocultured with splenic T cells (responders) from the vaccinated mice (PBS, control DCs, or 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)/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-CD4 (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 (HA5.2B7), and HLA-DR (IMMU357) mAbs or isotype-matched control mAbs (Beckman Coulter, Fullerton, CA). The surface expression of these molecules was analyzed using a FACSCalibur 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 Na_3VO_4], 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 Biomedicals, 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 against Hyperfilm ECL (GE Healthcare, Tokyo, Japan). The signal intensity of the blot was measured using a GS-800 Calibrated Densitometer (Bio-Rad).

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% CO_2 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 used for subsequent assays.

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 BfuAI 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′-GCCTCAAGATTGACCTAGA-3′; STAT3 #4, 5′-ATAGGAAGGTTTAAGGAGA-3′; GL3B (control anti-firefly luciferase), 5′-GTGCGCTGCTGGTGCCAAC-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 maturated with LPS (Sigma) at a concentration of 100 ng/ml. On day 7, the DCs (1×10^6 cells/ml in AIM-V) were pulsed with 1 μg/ml of either MART-$1_{27–35}$ (AAGIGILTV) or influenza matrix protein FluM$1_{58–66}$ (GILGFVFTL) peptides and incubated for 2 h at 37°C,

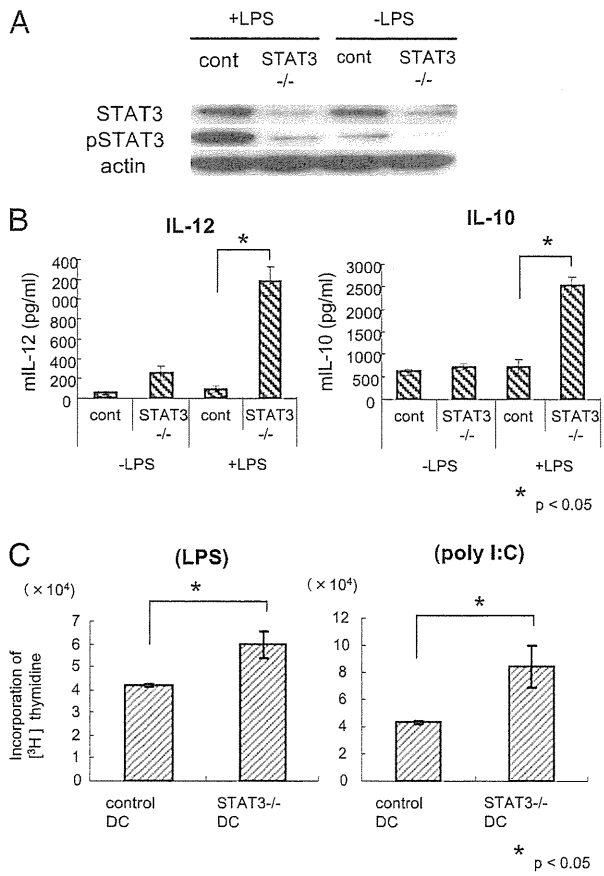

FIGURE 1. Characteristics of BMDCs derived from STAT3-CKO mice. *A*, Western blot analysis of BMDC STAT3 expression. Proteins were extracted from BMDCs before and after TLR stimulation (100 ng/ml LPS). *B*, ELISA of IL-12 (p70) and IL-10. Cytokines in the culture supernatants of either control or $STAT3^{-/-}$ BMDCs with or without LPS stimulation (20 h at a concentration of 100 ng/ml) were quantified by ELISA. Error bars indicate SE of five experiments. *C*, Allogeneic MLR. Irradiated BMDCs from control or $STAT3^{-/-}$ mice were mixed with splenic T cells from BALB/c mice at a T cell-to-DC ratio of 100:1 and incubated for 6 d. A total of 1 μCi [^{3}H]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. *$p < 0.05$.

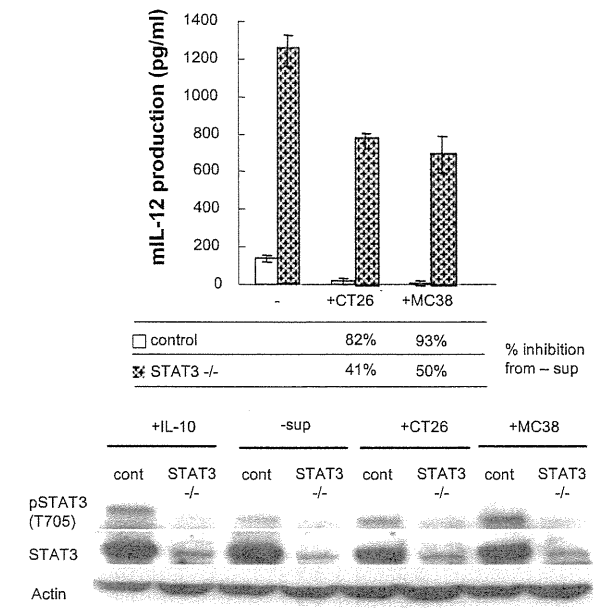

FIGURE 2. STAT3-CKO DCs were resistant to the STAT3-activating cancer-derived factors. *Upper panel*, Inhibition of IL-12 production in the presence of conditioned media of cancer cell lines. The BMDCs (control or $STAT3^{-/-}$) were stimulated with LPS in the presence or absence of 20% of culture supernatants 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)} × 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 ($HLA\text{-}A2.1^+$) and melanoma cell lines, 501mel, 526mel ($MART\text{-}1^+/HLA\text{-}A2.1^+$), and 888mel ($MART\text{-}1^+/HLA\text{-}A2.1^-$), were used as stimulator cells. T2 cells were preincubated with 1 μg/ml of either $MART\text{-}1_{27\text{–}35}$ or $FluM1_{58\text{–}66}$ peptide for 2 h at 37°C, then washed three times in PBS, before being used

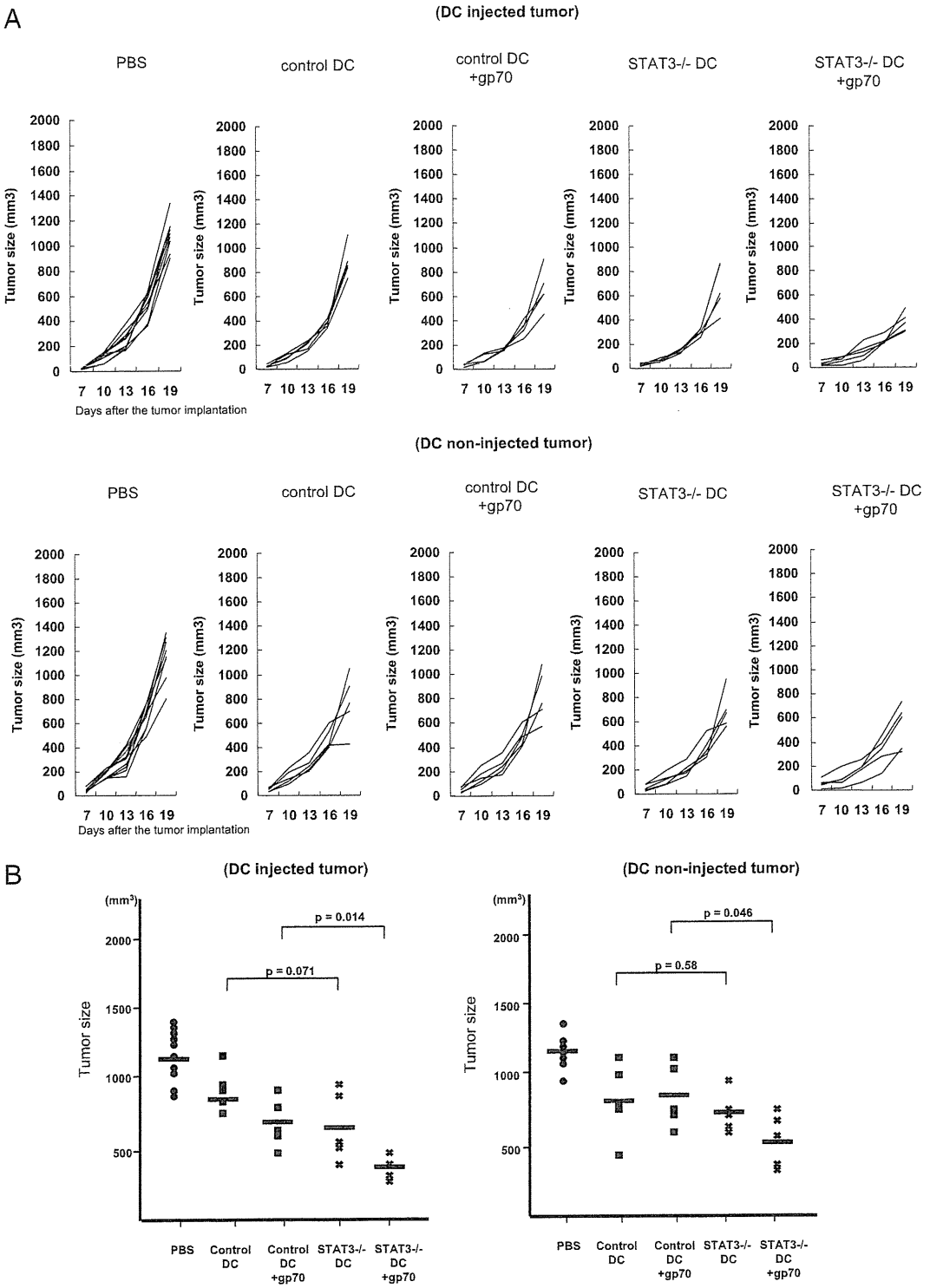

FIGURE 3. Therapeutic DC vaccination with $STAT3^{-/-}$ BMDCs. *A*, Comparison of tumor size. A total of 3×10^5 MC38 cells was implanted s.c. on the bilateral flanks of 6- to 7-wk-old C57BL/6 mice on day 0. On days 7 and 14, PBS (control), control immature BMDCs, or $STAT3^{-/-}$ immature BMDCs with 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-1_{27-35} peptide, T2 cells pulsed with Flu$M1_{58-66}$ 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% CO_2, 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. 1*A*). 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. 1*B*). IL-10 expression in STAT3-CKO BMDCs also was 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. 1*B*). 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-2^d) were mixed with BMDCs stimulated with LPS or poly-IC from either STAT3-CKO mice or control littermates (H-2^b) to perform allogeneic MLRs. MLR was increased significantly in STAT3-CKO BMDCs compared with control BMDCs (Fig. 1*C*), 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

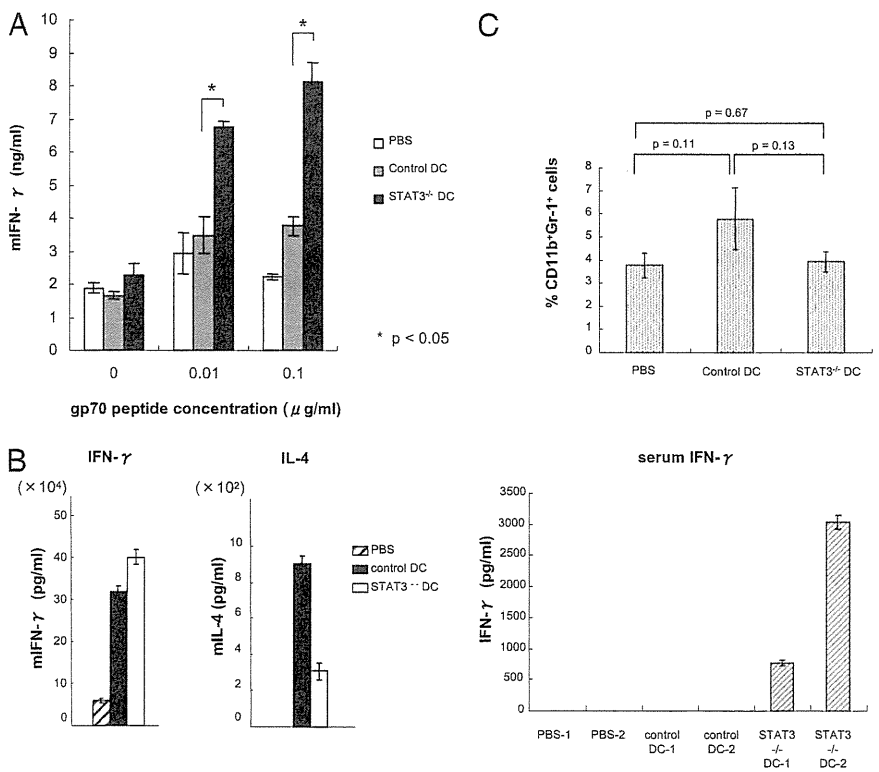

FIGURE 4. STAT3$^{-/-}$ BMDCs potently induced tumor Ag-specific T cells and systemic Th1 immune responses. *A*, Splenocytes derived from mice vaccinated with PBS, gp70 peptide-pulsed control, or STAT3$^{-/-}$ BMDCs (the splenocytes from two mice for each group were pooled) were restimulated in vitro with gp70 peptide at a concentration of 1 μg/ml for 5 d. Then, 2×10^5 T cells were incubated with 1×10^6 irradiated syngeneic splenocytes in the presence of gp70 peptides at concentrations of 0, 0.01, or 0.1 μg/ml for 24 h. IFN-γ released from the T cells was measured by ELISA. Error bars indicate SE of three experiments. *B*, *Left panel*, BMDCs derived from syngeneic mice were pulsed with tumor cell lysates of MC38 cells for 9 h on day 6 of culture, then stimulated with LPS (1 μg/ml). On day 7, the BMDCs were irradiated (52 Gy) and mixed with T cells derived from the vaccinated mice (PBS, control DCs, or STAT3-CKO DCs) at a responder-to-stimulator ratio of 1:2.5. IFN-γ and IL-4 in the culture supernatant were measured after 2 d of coculture. *Right panel*, The serum IFN-γ levels in the three vaccinated groups were measured by ELISA 19 d after tumor implantation. Two mice were evaluated for each group. *C*, The percentage of CD11b$^+$Gr-1$^+$ MDSCs infiltrating the tumors of mice from the three vaccinated groups. The bars indicate the mean percentage value of CD11b$^+$Gr-1$^+$ cells of three mice for each vaccinated group, and vertical bars indicate the SD. $^*p < 0.05$.

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 antitumor 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 (noninjected 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. 4*A*). 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. 4*B*). Large amounts of serum IFN-γ were detected only in mice administered STAT3-CKO BMDCs (Fig. 4*B*). 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. 1*B*). The percentage of $CD11b^+$ $Gr\text{-}1^+$ MDSCs in the tumors was not significantly different across the three groups (Fig. 4*C*), 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.

STAT3-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

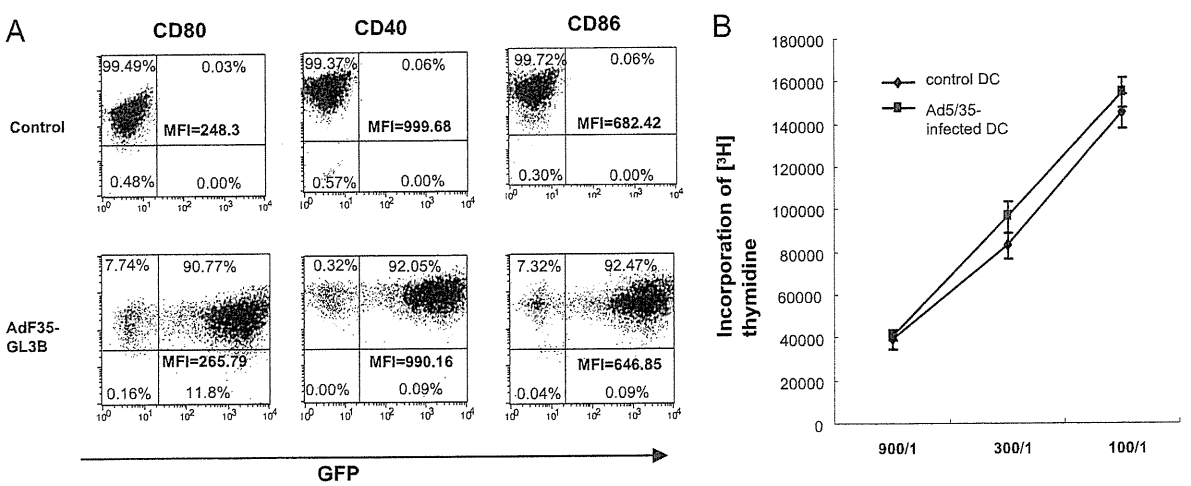

FIGURE 5. Adenovirus vector did not impair the maturation and allogeneic MLR of the human MoDCs. *A*, A fiber knob mutant of adenovirus vector (AdF35) expressing control shRNA (GL3B) and a GFP gene (AdF35-GL3B) efficiently transduced the GFP gene to human MoDCs without inhibition of maturation or T cell 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 identical 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. 6*A*). The expression levels of CD80,

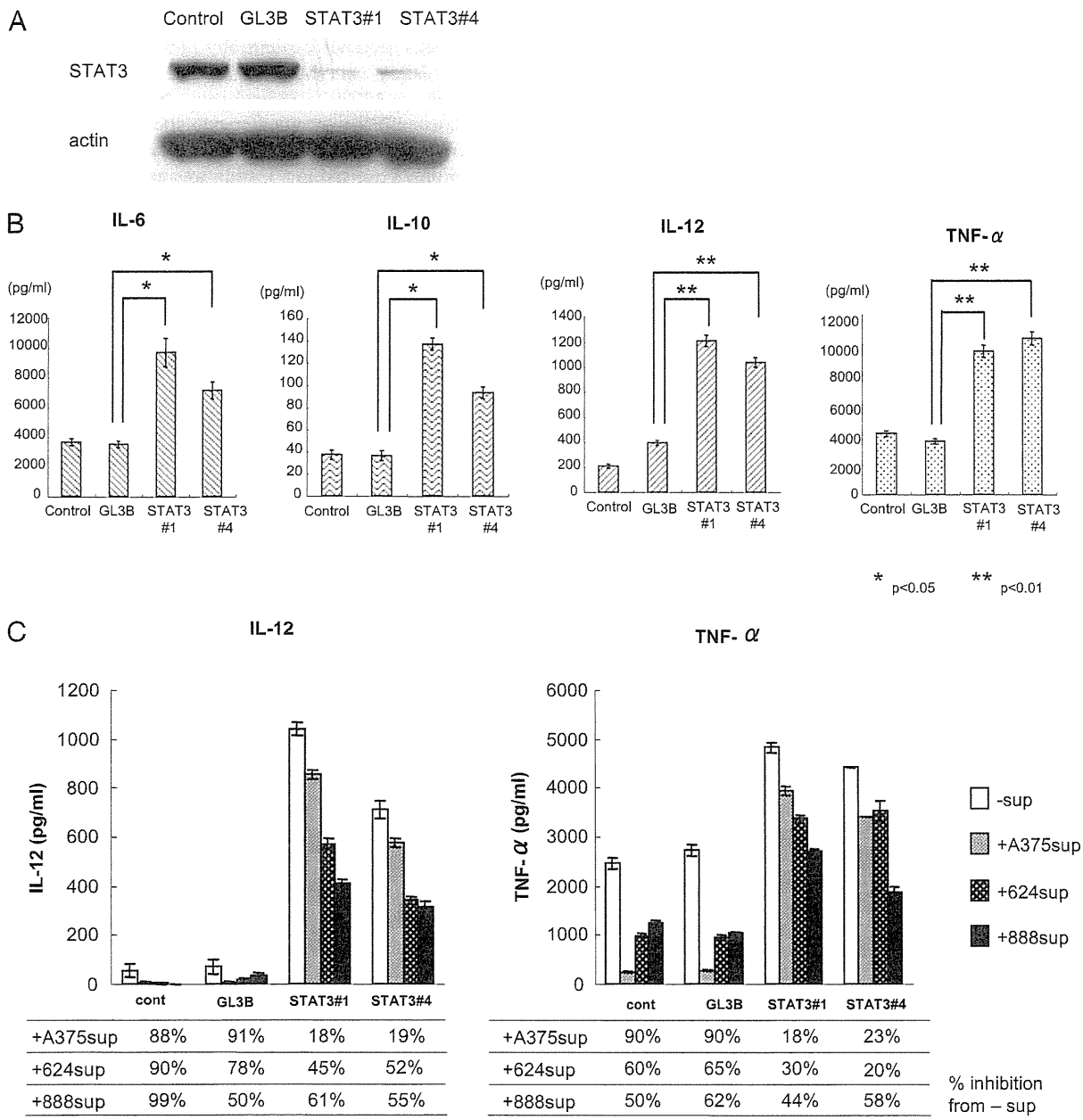

FIGURE 6. STAT3-depleted human MoDCs produced more inflammatory cytokines and were resistant to the cancer-derived factors. *A*, Western blot of STAT3. The protein was extracted from human MoDCs infected with AdF35-shRNA vectors (GL3B, STAT3 #1, or STAT3 #4) or control MoDCs. STAT3 protein was depleted 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)}×100%. 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. 6*B*). 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. 6*C*).

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

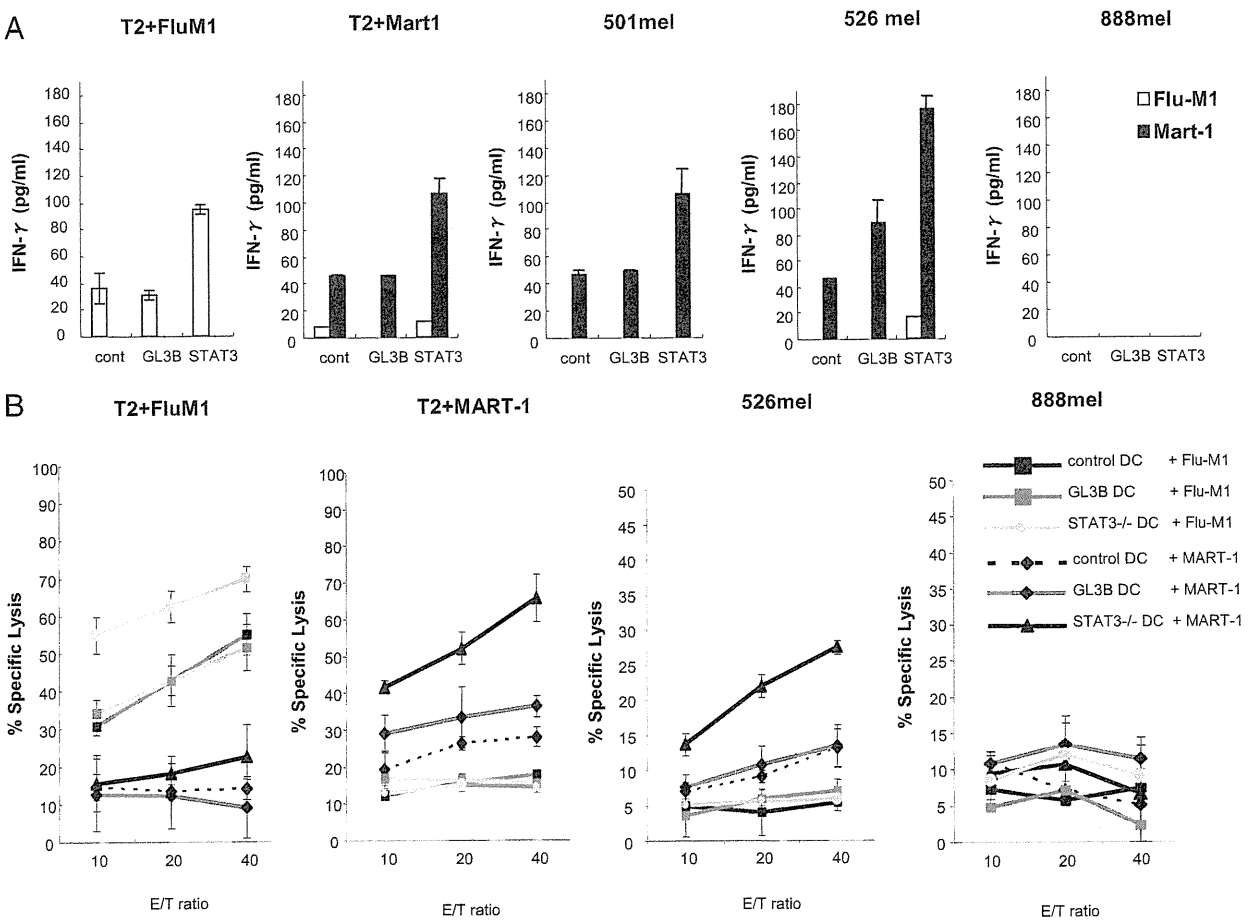

FIGURE 7. STAT3-depleted human MoDCs could efficiently induce Ag-specific T cells. *A*, IFN-γ ELISA. $CD8^+$ T cells (HLA-A*0201^+) were stimulated with peptide [MART-1_{27-35} (AAGIGILTV) or FluM1_{58-66} (GILGFVFTL)]-pulsed autologous DCs (uninfected (control) or infected with the adenovirus vectors (GL3B or STAT3 #1) three times weekly. Seven days after the third stimulation, the $CD8^+$ T cells were mixed with T2 cells pulsed with either MART-1_{27-35} or FluM1_{58-66} peptide or melanoma cell lines, 501mel, 526mel (MART-1^+/HLA-A2.1^+), or 888mel (MART-1^+/HLA-A2.1^-), at a responder-to-stimulator ratio of 1:1. The peptide-specific T cell responses were measured by IFN-γ ELISA. The open and shaded bars indicate the T cell responses primed with FluM1 and MART-1 peptides, respectively. The bars indicate the means of triplicate tests for each experiment, and the error bars indicate the SD. These data are representative of two independent experiments with similar results. *B*, [^{51}Cr] release assay. $CD8^+$ T cells were stimulated as described in *A*. Percentage specific lysis against different target cells is shown. Error bars indicate the SD values of the triplicate tests.

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 those of 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, adenoviral 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.

Acknowledgments

We thank Dr. Irmgard Förster for permission to use LysMcre transgenic mice and Dr. Chie Kudo-Saito and Dr. Tomonori Yaguchi for technical advice and helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

References

1. Steinman, R. M., and J. Banchereau. 2007. Taking dendritic cells into medicine. *Nature* 449: 419–426.
2. Figdor, C. G., I. J. de Vries, W. J. Lesterhuis, and C. J. Melief. 2004. Dendritic cell immunotherapy: mapping the way. *Nat. Med.* 10: 475–480.
3. Palucka, A. K., H. Ueno, J. W. Fay, and J. Banchereau. 2007. Taming cancer by inducing immunity via dendritic cells. *Immunol. Rev.* 220: 129–150.
4. Melief, C. J. 2008. Cancer immunotherapy by dendritic cells. *Immunity* 29: 372–383.
5. Kawakami, Y., T. Fujita, C. Kudo, T. Sakurai, M. Udagawa, T. Yaguchi, G. Hasegawa, E. Hayashi, Y. Ueda, T. Iwata, et al. 2008. Dendritic cell based personalized immunotherapy based on cancer antigen research. *Front. Biosci.* 13: 1952–1958.
6. Rabinovich, G. A., D. Gabrilovich, and E. M. Sotomayor. 2007. Immunosuppressive strategies that are mediated by tumor cells. *Annu. Rev. Immunol.* 25: 267–296.
7. Zou, W. 2005. Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat. Rev. Cancer* 5: 263–274.
8. Wang, T., G. Niu, M. Kortylewski, L. Burdelya, K. Shain, S. Zhang, R. Bhattacharya, D. Gabrilovich, R. Heller, D. Coppola, et al. 2004. Regulation of the innate and adaptive immune responses by Stat-3 signaling in tumor cells. *Nat. Med.* 10: 48–54.
9. Yu, H., M. Kortylewski, and D. Pardoll. 2007. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. *Nat. Rev. Immunol.* 7: 41–51.
10. Nefedova, Y., M. Huang, S. Kusmartsev, R. Bhattacharya, P. Cheng, R. Salup, R. Jove, and D. Gabrilovich. 2004. Hyperactivation of STAT3 is involved in abnormal differentiation of dendritic cells in cancer. *J. Immunol.* 172: 464–474.
11. Kortylewski, M., M. Kujawski, T. Wang, S. Wei, S. Zhang, S. Pilon-Thomas, G. Niu, H. Kay, J. Mulé, W. G. Kerr, et al. 2005. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. *Nat. Med.* 11: 1314–1321.
12. Nefedova, Y., P. Cheng, D. Gilkes, M. Blaskovich, A. A. Beg, S. M. Sebti, and D. I. Gabrilovich. 2005. Activation of dendritic cells via inhibition of Jak2/STAT3 signaling. *J. Immunol.* 175: 4338–4346.
13. Yoshimura, A., T. Naka, and M. Kubo. 2007. SOCS proteins, cytokine signalling and immune regulation. *Nat. Rev. Immunol.* 7: 454–465.
14. Takeda, K., B. E. Clausen, T. Kaisho, T. Tsujimura, N. Terada, I. Förster, and S. Akira. 1999. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 10: 39–49.
15. Fukao, T., M. Tanabe, Y. Terauchi, T. Ota, S. Matsuda, T. Asano, T. Kadowaki, T. Takeuchi, and S. Koyasu. 2002. PI3K-mediated negative feedback regulation of IL-12 production in DCs. *Nat. Immunol.* 3: 875–881.

16. Marine, J. C., D. J. Topham, C. McKay, D. Wang, E. Parganas, D. Stravopodis, A. Yoshimura, and J. N. Ihle. 1999. SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* 98: 609–616.
17. Alexander, W. S., R. Starr, J. E. Fenner, C. L. Scott, E. Handman, N. S. Sprigg, J. E. Corbin, A. L. Cornish, R. Darwiche, C. M. Owczarek, et al. 1999. SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* 98: 597–608.
18. Hanada, T., K. Tanaka, Y. Matsumura, M. Yamauchi, H. Nishinakamura, H. Aburatani, R. Mashima, M. Kubo, T. Kobayashi, and A. Yoshimura. 2005. Induction of hyper Th1 cell-type immune responses by dendritic cells lacking the suppressor of cytokine signaling-1 gene. *J. Immunol.* 174: 4325–4332.
19. Shen, L., K. Evel-Kabler, R. Strube, and S. Y. Chen. 2004. Silencing of SOCS1 enhances antigen presentation by dendritic cells and antigen-specific anti-tumor immunity. *Nat. Biotechnol.* 22: 1546–1553.
20. Cheng, F., H. W. Wang, A. Cuenca, M. Huang, T. Ghansah, J. Brayer, W. G. Kerr, K. Takeda, S. Akira, S. P. Schoenberger, et al. 2003. A critical role for Stat3 signaling in immune tolerance. *Immunity* 19: 425–436.
21. Staveley-O'Carroll, K., E. Sotomayor, J. Montgomery, I. Borrello, L. Hwang, S. Fein, D. Pardoll, and H. Levitsky. 1998. Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc. Natl. Acad. Sci. USA* 95: 1178–1183.
22. Bogen, B. 1996. Peripheral T cell tolerance as a tumor escape mechanism: deletion of CD4+ T cells specific for a monoclonal immunoglobulin idiotype secreted by a plasmacytoma. *Eur. J. Immunol.* 26: 2671–2679.
23. Sinha, P., V. K. Clements, S. K. Bunt, S. M. Albelda, and S. Ostrand-Rosenberg. 2007. Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. *J. Immunol.* 179: 977–983.
24. Sumimoto, H., S. Yamagata, A. Shimizu, H. Miyoshi, H. Mizuguchi, T. Hayakawa, M. Miyagishi, K. Taira, and Y. Kawakami. 2005. Gene therapy for human small-cell lung carcinoma by inactivation of Skp-2 with virally mediated RNA interference. *Gene Ther.* 12: 95–100.
25. Mizuguchi, H., and M. A. Kay. 1998. Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum. Gene Ther.* 9: 2577–2583.
26. Hoentjen, F., R. B. Sartor, M. Ozaki, and C. Jobin. 2005. STAT3 regulates NF-kappaB recruitment to the IL-12p40 promoter in dendritic cells. *Blood* 105: 689–696.
27. Mizuguchi, H., and T. Hayakawa. 2002. Adenovirus vectors containing chimeric type 5 and type 35 fiber proteins exhibit altered and expanded tropism and increase the size limit of foreign genes. *Gene* 285: 69–77.
28. Sumimoto, H., F. Imabayashi, T. Iwata, and Y. Kawakami. 2006. The BRAF-MAPK signaling pathway is essential for cancer-immune evasion in human melanoma cells. *J. Exp. Med.* 203: 1651–1656.
29. Clausen, B. E., C. Burkhardt, W. Reith, R. Renkawitz, and I. Förster. 1999. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 8: 265–277.
30. Udagawa, M., C. Kudo-Saito, G. Hasegawa, K. Yano, A. Yamamoto, M. Yaguchi, M. Toda, I. Azuma, T. Iwai, and Y. Kawakami. 2006. Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and Bacillus Calmette-Guerin cell wall skeleton stimulation. *Clin. Cancer Res.* 12: 7465–7475.
31. Tacken, P. J., I. J. de Vries, K. Gijzen, B. Joosten, D. Wu, R. P. Rother, S. J. Faas, C. J. A. Punt, R. Torensma, G. J. Adema, and C. G. Figdor. 2005. Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti-DC-SIGN antibody. *Blood* 106: 1278–1285.

特集　進化するがん免疫療法(ワクチン療法，細胞療法，抗体療法)

ヒトがん細胞に対する免疫応答機構と免疫制御の可能性*

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Key Words：tumor antigen, immune escape, immunotherapy, vaccine, adoptive immunotherapy

はじめに

臨床で認められるがんは，長年にわたるがんの形成過程で免疫学的に選択されて，すでに免疫抵抗性や免疫抑制性を獲得して，免疫から逃避してきたがん細胞の集団である．このようながんに対して，免疫療法が可能であるのか．本稿では，最近のヒト腫瘍免疫学の進歩により理解されているヒト腫瘍免疫応答機構と免疫制御によるがん治療の可能性について議論してみたい．

臨床でみられるがん細胞はすでに免疫防御機構から逃れて増殖している

がんの形成過程では，発生初期から，がん細胞と免疫細胞とその他の間質細胞との相互作用が起こる．骨髄から動員されがん組織に浸潤するマクロファージや間葉系幹細胞などは，がん細胞を排除するどころか，TNF-αなどのサイトカインやケモカインの分泌などを介して，むしろがん細胞の増殖浸潤能の促進や血管新生の誘導により，がんの進展を促進する[1]．しかし，NK細胞やT細胞は，免疫監視機構として働き，がん細胞を排除する(図1)．マウスの化学発がん実験での経時的な免疫細胞とがん細胞の動態解析は，がんの進展・排除だけでなく，がん細胞と免疫細胞が共存する状況を経て，基本的に遺伝子不安定性を持つがん細胞は変化して免疫抵抗性や免疫抑制性を獲得し，免疫防御機構から逃れて(免疫逃避)増殖していくことを明らかにした[2]．現在，遺伝子ノックアウトマウスなど，特定の免疫分子や細胞を低下させたマウスを用いて，その細胞分子機構の詳細が解明されつつある．

免疫不全マウスではがんの発生は増加するが，発生したがん細胞は，正常な免疫機構を持つマウスに発生するがん細胞と比べて，免疫細胞に簡単に拒絶される．ヒトでも，免疫不全状態でしばしば発生するEBウイルス関連リンパ腫は，T細胞治療などで治療しやすいのに対して，通常の免疫状態の患者に発生するEBウイルス関連リンパ腫は免疫抵抗性である．これは，がん形成過程で免疫監視機構が働き，がん細胞の免疫感受性が規定されることを示しており，免疫編集(immune-editing)と呼ばれる[2]．実際，臨床でみられるがん細胞は免疫抵抗性や免疫抑制性を獲得している．がん細胞自体の免疫学的性質の変化に加えて，がん細胞は，抗腫瘍T細胞の消失や不応答性(アナジー)や抗腫瘍免疫抑制性細

* Immune regulation to human cancer cells and its control for cancer treatment.

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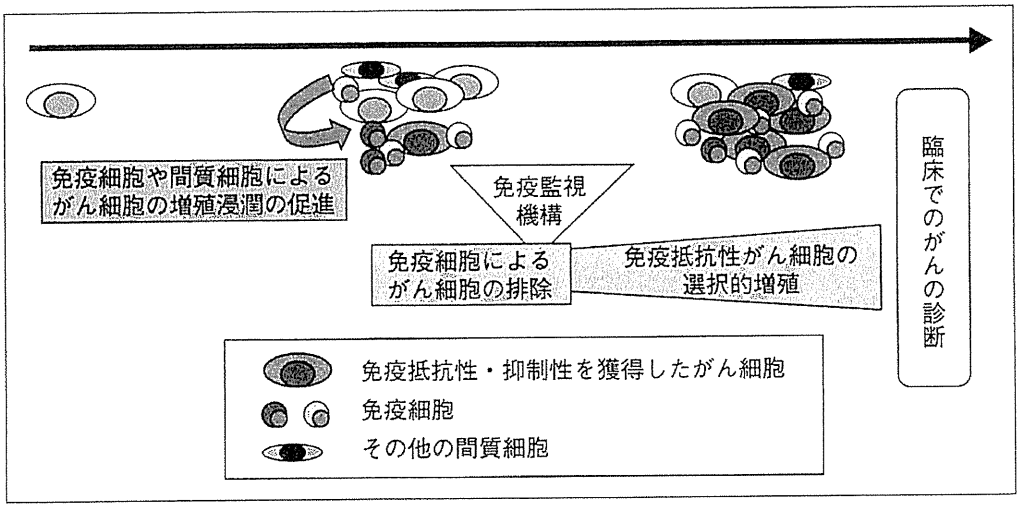

図1　免疫編集によるがん細胞の免疫抵抗性・抑制性の獲得
遺伝子不安定性を持つがん細胞は，がん形成過程で，NK細胞やT細胞などの免疫細胞による免疫監視機構により，がん細胞の排除や免疫逃避が起こり，臨床でみられるがん細胞はすでに免疫抵抗性や抑制性を獲得している．また，マクロファージなどの免疫細胞や間質細胞はがん細胞の増殖浸潤を促進する場合がある(矢印)．

胞群の増加などの患者の免疫系レパトアの変化を起こす．

担がん生体では，がん細胞に対する正と負の免疫応答が起こる

担がん生体では，さまざまな免疫細胞がネットワークを形成して，がん細胞に対して正と負の免疫応答を起こす(図2)[3]．自然免疫系細胞では，マクロファージはがん細胞の増殖進展や免疫抑制作用により，がん進展の促進作用もあるが，T細胞などが分泌するIFN-γなどで活性化されると抗腫瘍エフェクターとしても働く．NK細胞，NK-T細胞などは免疫監視機構に関与するだけでなく，IFN-γ産生などを介して，T細胞などの獲得免疫系の活性化促進作用を持ち，抗腫瘍T細胞誘導におけるアジュバント的役割も果たすので，α-galactosyl ceramideを用いたNKT細胞活性化を介した抗腫瘍T細胞の誘導も試みられている．しかし，NK・NK-T細胞の，大きな進行がんに対する抗腫瘍効果は弱いと考えられている．

樹状細胞(DC)は，腫瘍抗原を提示して生体内でナイーブT細胞を誘導する専門的抗原提示細胞として，クローナル増殖による強力な作用を発揮できる獲得免疫系への免疫応答の橋渡しとして重要である[4]．DCは，T細胞の活性化/不活性化，Th1/Th2/Th17/TregなどのT細胞分化の方向性を規定する点でも，抗腫瘍免疫ネットワークの最終出力の方向性にかかわる重要な細胞の一つである．DCは，大きくランゲルハンス細胞や真皮DCなどの骨髄系DC(myeloid DC, conventional DC；cDC)と，扁桃などに多く存在してウイルス感染に対して多量のタイプI-IFNを産生する形質細胞様DC(plasmacytoid DC；pDC)に分類される．cDCはがん組織で腫瘍抗原を取り込んで成熟化してリンパ節に遊走し，抗腫瘍T細胞の誘導を行う．pDCも分泌するIFNαなどにより抗腫瘍T細胞の誘導を促進する．末梢血単球からGM-SCF, IL4などで体外誘導したcDCはがんワクチンとして利用されている．

T細胞やB細胞などの獲得免疫系は，抗原特異的に増殖して，メモリー機構を持ち，強力な免疫応答を起こすことが特徴である．マウスにがん細胞などを免疫して作製した抗ヒト腫瘍モノクローナル抗体は，すでに臨床試験で抗腫瘍効果が認められ標準治療として確立されているが，がん患者の体内でB細胞が産生する抗体の抗腫瘍効果については明らかでなく，むしろ抗腫瘍免疫応答を妨げるとの報告もある．

T細胞は，動物モデルやヒト悪性黒色腫などでは強い抗腫瘍効果を持つことがわかっている．T細胞受容体は，がん細胞表面上の抗原ペプチ

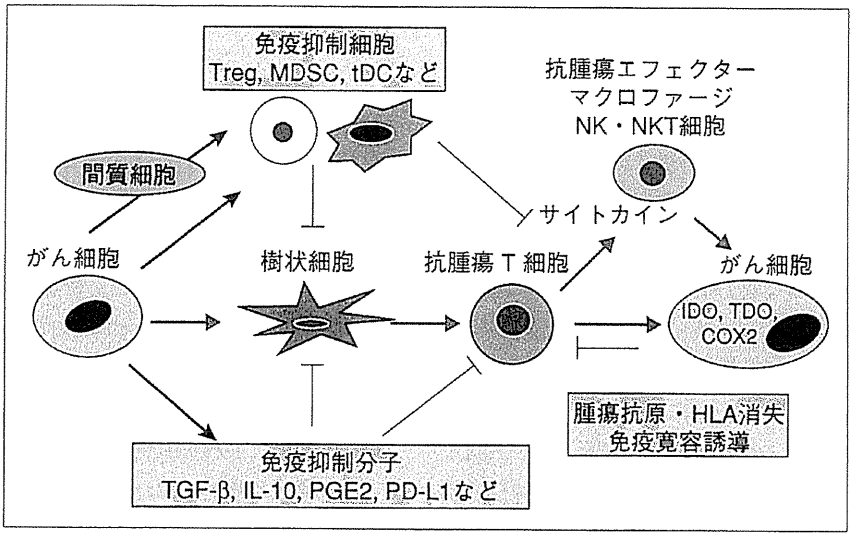

図２　がん細胞に対する正負の免疫応答
担がん生体では，抗腫瘍免疫応答に対して，正（ヘルパーＴ細胞やエフェクター細胞）と負（免疫抑制分子や免疫抑制細胞）に作用する免疫細胞・分子群が存在する．また，HLAや腫瘍抗原の消失などにより，がん細胞の免疫抵抗性が獲得される．

ド・MHC複合体を認識するが，抗原ペプチドは，細胞内蛋白からも由来するので，Ｔ細胞は抗体と異なり，がん細胞内の変化をも検出することができる．がん反応性Ｔ細胞が認識する腫瘍抗原には，自己がん細胞だけに発現する固有抗原と他のがん細胞にも発現する共通抗原がある．固有抗原として，がん細胞の遺伝子変異に由来する変異ペプチド，共通抗原として，MART-1悪性黒色腫抗原やPSA前立腺がん抗原，proteinase-3白血病抗原などの組織特異的蛋白，MAGEやNY-ESO-1などのがん精巣抗原，WT-1やhTERTなどのがん高発現抗原，HPV-E6/E7子宮頸がん抗原などのがんウイルス抗原などがある[5]．同種造血幹細胞移植下では，ドナーＴ細胞やNK細胞が，レシピエントがん細胞の同種抗原を腫瘍抗原として認識する．

抗腫瘍Ｔ細胞にはMHCクラスⅠ・ペプチド複合体を認識するCD8$^+$Ｔ細胞とMHCクラスⅡ・ペプチド複合体を認識するCD4$^+$Ｔ細胞がある．造血器腫瘍や一部の固形がんはMHCクラスⅡを発現するので，CD4$^+$Ｔ細胞に直接認識される場合があるが，多くの固形がんはMHCクラスⅡを発現しないので，CD8$^+$Ｔ細胞が主にがん細胞の認識や傷害にかかわる．CD4$^+$Ｔ細胞は機能的に，ヘルパーＴ細胞と免疫抑制性の制御性Ｔ細胞（Treg）に分けられる．ヘルパーＴ細胞は，活性化Ｔ細胞が発現するCD40LによるCD40陽性樹状細胞の抗原提示・Ｔ細胞活性化能の促進，CD8$^+$Ｔ細胞の誘導維持やがん組織へ浸潤の促進，IL2やIFNγなどのサイトカイン分泌による，マクロファージ・NK細胞・NKT細胞などのエフェクター細胞の活性化などにより，抗腫瘍免疫応答にかかわる．サイトカイン産生能の違いにより，抗腫瘍免疫応答に重要なIFNγやIL2を産生するTh1，慢性炎症促進作用によりがん発生進展促進作用を持つTh17，Th1抑制により抗腫瘍免疫応答の抑制作用を持つIL4などを産生するTh2に分けられ，それぞれ抗腫瘍免疫応答に異なる役割を果たす．担がん生体の抗腫瘍免疫抑制に関与するTregは，FOXP3陽性内在性Treg，FOXP3陽性誘導性Treg，IL10産生性Tr1，TGFβ産生性Th3などに分類される．

担がん生体では免疫抑制環境が構築されている

臨床で見つかるがんは，すでに免疫抵抗性や免疫抑制性を獲得したがん細胞の集団である．ヒトのがんは，１つの細胞から由来したにもかかわらず，長年かけて遺伝子異常も性質も不均一な集団となっている．がん細胞の遺伝子異常が異なれば，免疫学的特性も異なる場合があり，免疫療法を考える場合，症例ごとの免疫学的性

質の違いも考慮する必要がある．また，抗腫瘍免疫応答を考える場合，がん組織，そこから最初にリンパ流が到達し，本来，抗腫瘍T細胞が誘導される場であるセンチネルリンパ節，また，免疫抑制性細胞や間葉系幹細胞の供給源であり，抗腫瘍メモリーT細胞が維持される骨髄などの，がん関連微小環境という解剖学的な観点が重要である．がん組織では，がん細胞・免疫細胞・その他間質細胞の相互作用により，がん細胞増殖浸潤促進・免疫抑制的な免疫病態が形成されている．また，センチネルリンパ節には，がん組織から免疫抑制性サイトカインや制御性T細胞などの免疫抑制細胞が流入して免疫抑制環境が構築されて，抗腫瘍免疫応答が誘導されにくくなっている．

がん細胞は，多様な免疫抵抗性・抑制性機構を獲得しているが，それらは本来，自己免疫反応を抑制するための機構や，外来異物に対する免疫反応を，異物排除後に元の状態に戻すためのネガティブフィードバック機構であり，がん細胞がそれらを免疫逃避機構として悪用している．抗腫瘍免疫応答の抑制は，樹状細胞やT細胞など，さまざまな段階に対して起こっており，最終的に，抗腫瘍T細胞の増殖活性化の抑制，抗原特異的不応答(アナジー)，細胞死(アポトーシス)などが起こる．

がん細胞には，腫瘍抗原やHLAなどの抗原の処理提示にかかわる分子の異常が認められ，T細胞の認識から逃避する(図2)[6]．腫瘍抗原は多数あるので，その消失はあまり問題にならないが，HLA消失によるT細胞認識からの逃避は大きな問題である．HLA低発現の機序として，β2ミクログロブリン遺伝子のホットスポットの遺伝子変異によるHLAクラスI全消失や各種転写機構の問題によるHLA発現低下が認められる[8]．後者の場合，IFN-γやHDAC阻害剤などによりHLAの発現回復が認められる場合があり，がん種によっては，HLA発現誘導法を併用した免疫療法が期待されている．

がん細胞は多様な免疫抑制機構を持つ(図2)[6]．がん細胞は，TGF-β，IL10, VEGF, IL6, MICA/Bなどの免疫抑制分子を分泌したり，PD-L1やFasLやCD200やILT7Lなどの免疫抑制性の膜分子を発現したり，トリプトファン欠乏や代謝産物キヌレニンによりT細胞機能を抑制するトリプトファン代謝酵素IDOやTDOや，免疫抑制活性を持つPGE2の産生にかかわるCOX2などの細胞内酵素を発現して，担がん生体で，局所性・全身性の免疫抑制環境を構築する．また，がん細胞は，制御性T細胞(Treg)，骨髄由来免疫抑制細胞(myeloid derived suppressor cell；MDSC)，M2マクロファージ，寛容性DC(tolerogenic DC；tDC)，pDC, タイプII NKT細胞，免疫抑制性γδT細胞など，多様な免疫抑制細胞群を誘導する．がん組織で産生されたCCL21やCCL22などのケモカインは，それぞれCCR7やCCR4などを発現するTregなどの免疫抑制細胞をがん組織やセンチネルリンパ節などに引き寄せる．本来，抗腫瘍活性を持ちうる免疫細胞も，がん微小環境の中では，サイトカインなどの影響を受けて，免疫抑制性細胞に分化してしまう場合があることが問題である．

担がん生体では，がん細胞の遺伝子・シグナル異常(MAPK, STAT3, PI3-AKT, Wnt-β-catenin, NF-κB, Snailなど)を起点として，複数の免疫抑制カスケードが作動して，多様な機序による免疫抑制病態が形成される[7]．たとえば，がん細胞のSTAT3など遺伝子異常・シグナル亢進によりがん細胞はIL10やTGF-βを産生して，直接的にTregを誘導するだけでなく，tDCの誘導を介してもTregを誘導する．TGF-βやIL10は，がん組織に浸潤するTregやMDSCなどの免疫抑制性細胞からも産生される．同様に，がん細胞のNF-κBなどの異常により産生されるIL6は，DC機能を抑制するだけでなく，MDSCの骨髄からの動員やMDSCのarginase発現を促す．また，同時に産生されるIL8は，好中球からのarginase分泌を促進して，アルギニン欠乏やT細胞受容体機能障害などを介してT細胞の増殖活性化を阻害する．実際，がん患者では，血液中のTGF-β・IL10・IL6・IL8・arginaseの増加と予後不良とに相関が認められている．

この免疫抑制病態改善のためには，免疫抑制カスケードを遮断する必要がある(図2)．一つの方法として，最終的に抑制作用を持つ分子や細胞を阻害・除去することである．たとえば，

TGF-βの吸着による除去，抗体を用いたTregの除去，IDOやTDO，COX2の阻害剤の投与などである[9]．別の方法として，免疫抑制カスケードの起点であるがん細胞の遺伝子・シグナル異常を分子標的薬やsiRNAなどで阻害する方法がある．たとえば，マウスモデルでは，STAT3阻害剤やJAK阻害剤による抗腫瘍免疫応答の増強が報告されている[10]．TK阻害剤sunitinibの腎がん患者への投与では，腎がん増殖抑制効果に加えて，MDSCやTregの減少が報告されている[11]．TK阻害剤dasatinibの慢性骨髄白血病患者への投与では，やはり白血病減少作用に加えて，Treg低下やT細胞やNK細胞のオリゴクローナル増殖が認められ，予後を向上させるとの報告がある[12]．悪性黒色腫では，共通変異BRAFによるMAPKシグナル亢進は，IL10，VEGF，IL6などの複数の免疫抑制性サイトカインの産生に関与するので，最近開発されたが変異BRAF阻害剤による免疫病態の改善が期待される[7]．

IL10，VEGF，IL6などの免疫抑制サイトカインは，DCやMDSCに対して，そのSTAT3の活性化を介して免疫抑制活性を発現させるので，STAT3阻害剤は，がん細胞だけでなく，免疫細胞に対しても直接作用して，その機能増強や免疫抑制性細胞への分化の阻害により，抗腫瘍免疫応答を増強する作用も持つ[10]．他のシグナル阻害剤も，同様にがん細胞と免疫細胞の両方に作用して抗腫瘍免疫を増強する場合があることが示されている．がん細胞の分子異常は，症例ごとに異なるので，主要な免疫抑制機序は症例ごとに異なる可能性もあり，個別化治療の必要性も考えられる．また，化学療法剤は一般的に免疫抑制作用を持つが，その中にも，抗腫瘍免疫応答の誘導に有用なものが報告されている．たとえば，gemcitabineのMDSC阻害作用やアンスラサイクリン系薬剤やoxaliplatinなどが持つ，がん細胞表面へのcalreticulin発現誘導による，その受容体を持つ樹状細胞への腫瘍抗原取り込み促進作用や，がん細胞からのHMGB1分泌によるTLR4などを介した樹状細胞の成熟活性化の促進作用などである[13][14]．これらの化学療法剤や分子標的薬は，がん細胞の増殖抑制・傷害作用を持ち，がん細胞の破壊は内在性腫瘍抗原のDCへの提供につながるが，さらに上記の特有な免疫増強作用により，内在性腫瘍抗原に対する抗原スプレッディングを効率的に誘導できる可能性があり，現在，適切な分子標的薬や化学療法剤の免疫療法への併用が検討されている．

がんは，不均一な細胞集団からなるが，化学療法耐性でがん再発の原因になるがん幹細胞や，転移の原因となる上皮間葉転換(epithelial mesenchymal transition；EMT)は免疫学的にも特有な性質を持つので，免疫療法においても十分に考慮する必要がある．がん幹細胞は，免疫療法による排除が期待され，われわれもヒト脳腫瘍幹細胞が発現し，T細胞に認識されるSOX6抗原を同定している[15]．しかし，悪性黒色腫症例で，強力な養子免疫療法により完全寛解になったあとに，数年ごとに再発を繰り返し，がん幹細胞様の休止期がん細胞が強力な免疫療法後も残存した可能性が考えられる症例を経験しており，がん幹細胞は免疫療法にも抵抗性を持つ場合があると考えている．EMTは，E-cadherin低下による正常上皮からの離脱，細胞運動能上昇や蛋白分解酵素分泌により，がんの浸潤転移に関与する．われわれは，TGF-β-Snail誘導性EMTでは，がん細胞浸潤能の亢進に加えて，TGF-β，IL10，TSP-1などの免疫抑制性サイトカインの産生が亢進して，DC機能低下やTreg誘導などの免疫抑制活性が上昇して，さらに転移が促進される可能性を見出した[16]．今後，免疫療法におけるこれらのがん細胞亜集団の意義をさらに検討していく必要がある．

抗腫瘍免疫ネットワークの総合的な制御による効果的な免疫療法開発の可能性

すでにさまざまな免疫療法が実施されているが，標準的治療として十分に確立されているのは，抗体療法と同種造血幹細胞移植と一部の能動免疫法だけである(図3)．今後，新しい免疫療法を改善して標準治療として確立していく必要がある．われわれが先駆的に行ったヒト腫瘍抗原の同定は，HLAテトラマー技術の開発とともに，患者体内での抗腫瘍免疫応答の定量的・定性的な解析を可能にし，免疫療法施行後にがん細胞排除に至る各段階での具体的な問題点が

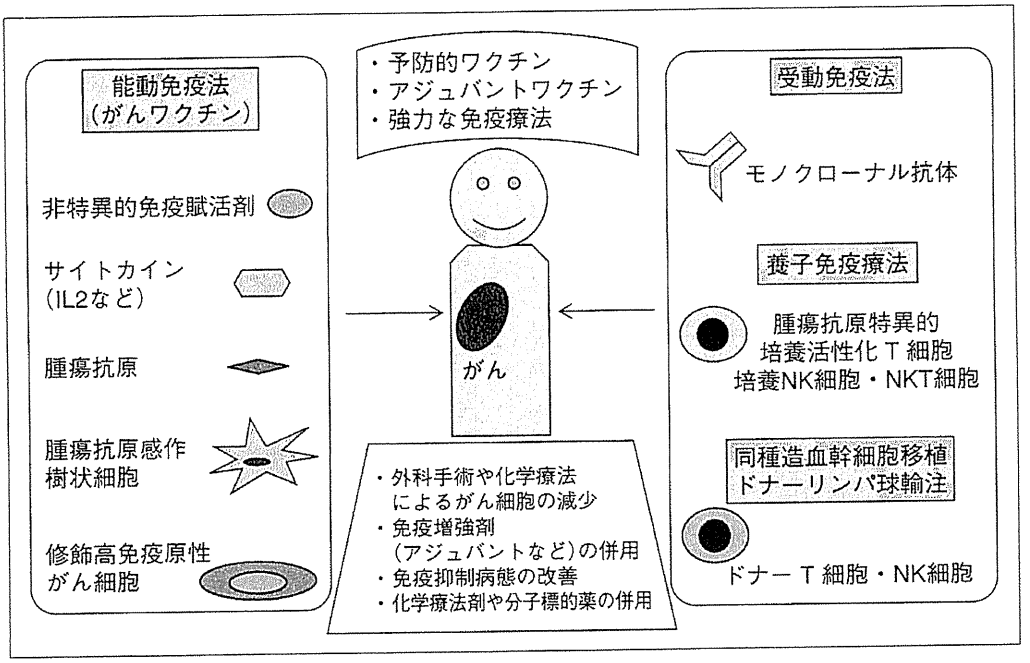

図３　がん免疫療法

がん免疫療法には，患者体内で抗腫瘍免疫応答を誘導する能動免疫法(がんワクチン)と，抗体などの最終エフェクターを投与する受動免疫法，抗腫瘍培養Ｔ細胞などを投与する養子免疫療法，同種造血幹細胞移植などの同種抗原に対する免疫療法などがある．

明らかになりつつある．われわれは臨床試験の解析結果から，効果的な免疫療法の開発のためには，抗腫瘍免疫ネットワークの以下のポイントの制御と総合的な免疫制御が重要と考えている(図４)[17][18]．

1．内在性腫瘍抗原に対する免疫誘導を起こす生体内腫瘍破壊法の開発

がんワクチン臨床試験の解析で得られた重要な知見の一つは，免疫抗原ではなく，内在性の腫瘍抗原に対するＴ細胞が新たに誘導されて(抗原スプレッディング)，生体内のがん排除に重要なことである[19]．上記のように，特定の抗がん剤は，がん破壊による内在性腫瘍抗原の放出に加えて，その後の免疫誘導を促進する可能性が示唆されている．ほかに，放射線照射や凍結融解や熱凝固法や光線療法などの物理的手段による破壊，抗腫瘍抗体やがん細胞破壊性ウイルスなどが検討されている[20]．

2．適切な腫瘍抗原の同定

免疫療法に理想的な腫瘍抗原の条件として，多くの患者に，がん幹細胞を含むすべてのがん細胞に高発現するが，正常細胞では限局的な発現しかせず，がん細胞の増殖生存にかかわるために抗原消失が起こりにくく，免疫誘導能が高いことが考えられるが，すでに免疫編集を経たがん細胞は，これらの条件をすべて満たすわけではなく，少しでもこれに近い腫瘍抗原を選択して使用することになる．

3．樹状細胞機能増強法の開発

生体内で腫瘍抗原を樹状細胞上のFc受容体やHsp受容体などにターゲッティングして抗原取り込みを促進させる方法や，樹状細胞を成熟活性化させる，サイトカインやTLRなどの異物センサーを刺激するアジュバントの開発が進められている．

4．Ｔ細胞の生体内増殖活性化法の開発

IL2, IL7, IL15, IL21などのＴ細胞増殖性サイトカインや，4-1BBなどのＴ細胞の副刺激分子に対するアゴニスト抗体などを用いて，適切なヘルパーＴ細胞サブセットや，cytotoxic T lymphocytes(CTL)などの抗腫瘍エフェクター細胞の生体内増殖活性化を促進する方法が開発されている．

5．担がん生体の免疫抑制病態の改善法の開発

IFNやHDAC阻害剤などを用いたがん細胞のHLA発現回復や，がん細胞が産生誘導するTGF-β, IL10, PD-L1, IDO, TDO, COX2などの免疫抑

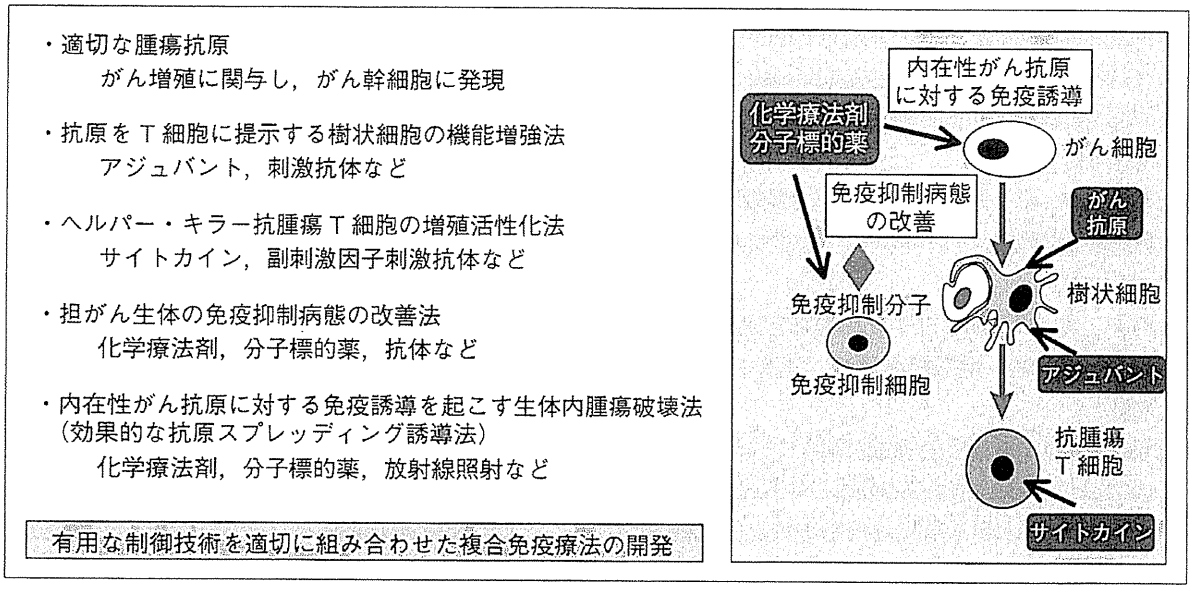

図４　複合免疫療法開発のために必要な免疫制御技術
効果的ながん免疫療法の開発のためには，①適切な生体内腫瘍破壊，②適切な腫瘍抗原の使用，③適切な樹状細胞の制御，④適切なヘルパーＴ細胞サブセットの増殖活性化，⑤適切な抗腫瘍エフェクター免疫細胞の増殖活性化，⑥適切な免疫抑制環境の是正などを組み合わせた総合的な免疫制御が必要である．

制分子やTregなどの免疫抑制細胞の阻害，免疫抑制カスケードの上流であるがん細胞の分子・シグナル異常の阻害剤などの開発が進められている．

6．複合的な免疫療法の開発

現在までに実施されている総合的な免疫療法の成功例として，cyclophosphamideやfludarabineなどのリンパ球抑制薬剤や全身性放射線照射などの前処置後に，抗腫瘍培養Ｔ細胞をIL2とともに投与して，がんワクチンで免疫する方法がある．この前処置により，免疫抑制性細胞も抗腫瘍免疫細胞も減少するが，他の免疫細胞による利用の減少も関与して上昇したIL7やIL15が関与するhomeostatic expansion機構が働き，投与したＴ細胞は，生体内で長期に増殖し続けて，体内で強力な抗腫瘍効果を発揮する．この方法により，多発転移巣を持つ進行悪性黒色腫患者93症例中20例が完全寛解になり，3～8年の経過観察でも１例しか再発していないという驚異的な治療効果が報告された[21]．さらに，抗腫瘍Ｔ細胞から単離したＴ細胞受容体遺伝子，あるいは抗体の可変領域をＴ細胞受容体の定常領域に結合させたキメラ抗原受容体（chimeric antigen receptor；CAR）をウイルスベクターで末梢血リンパ球に導入して人工的に作製した抗腫瘍リンパ球を用いた養子免疫療法が実施され，悪性黒色腫以外に，滑膜肉腫，大腸がん，悪性リンパ腫，白血病などに対しても治療効果が認められている[22][23]．

おわりに

近年，ヒト腫瘍抗原同定などのヒト腫瘍免疫学の進歩により，やっと患者体内での抗腫瘍免疫応答の解析が可能になり，腫瘍免疫病態の解明，そして免疫療法の科学的開発が可能になってきた．免疫療法の効果が期待できるがんがどれほどであるかはまだ不明であるが，一部のがんでは，すでに進行がんの根治も可能であることが明らかになっている．さらなるヒト腫瘍免疫学の発展と制御法の開発による抗腫瘍免疫ネットワークの総合的な制御により，より効果的ながん免疫療法の開発ができることを期待したい．

文　献

1）河上　裕．なぜ，今自然免疫か？―自然免疫系細胞によるがん進展の促進とその制御によるがん治療の可能性―．Cancer Frontier 2009；11：91.
2）Smyth MJ, Dunn GP, Schreiber RD. Cancer

immunosurveillance and immunoediting : the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. Adv in Immunol 2006 ; 90 : 1.

3) 河上　裕．癌に対する免疫応答と免疫療法．日本医師会雑誌特別号「わかりやすい免疫疾患」2005；134：84.

4) 河上　裕．樹状細胞による免疫制御と臨床応用．稲葉カヨ・編．実験医学増刊．東京：羊土社；2008.

5) Kawakami Y, Fujita T, Matsuzaki Y, et al. Identification of human tumor antigens and its implications for diagnosis and treatment of cancer. Cancer Sci 2004 ; 95 : 784.

6) 河上　裕，住本秀敏，工藤千恵，ほか．がん細胞による免疫抑制・抵抗性の分子機構とその制御．実験医学 2009；27：2213.

7) Sumimoto H, Imabayashi F, Iwata T, Kawakami Y. The BRAF-MAPK signaling pathway is essential for cancer immune evasion in human melanoma cells. J Exp Med 2006 ; 203 : 1651.

8) Restifo NP, Esquivel F, Kawakami Y, et al. Identification of human cancers defective in antigen processing. J Exp Med 1993 ; 177 : 265.

9) Phan, GQ, Yang JC, Sherry RM, et al. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci USA 2003 ; 100 : 8372.

10) Kortylewski M, Kujawski M, Wang T, et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat Med 2005 ; 11 : 1314.

11) Ko JS, Zea AH, Rini BI, et al. Sunitinib mediates reversal of myeloid-derived suppressor cell accumulation in renal cell carcinoma patients. Clin Cancer Res 2009 ; 15 : 2148.

12) Kreutzman A, Juvonen V, Kairisto V, et al. Mono/oligoclonal T and NK cells are common in chronic myeloid leukemia patients at diagnosis and expand during dasatinib therapy. Blood 2010 ; 116 : 772.

13) Suzuki E, Kapoor V, Jassar AS, et al. Gemcitabine selectively eliminates splenic Gr-1＋/CD11b＋ myeloid suppressor cells in tumor-bearing animals and enhances antitumor immune activity. Clin Cancer Res 2005 ; 11 : 6713.

14) Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. Nature Med 2007 ; 13 : 54.

15) Ueda R, Ohkusu-Tsukada K, Fusaki N, et al. Identification of HLA-A2- and A24-restricted T-cell epitopes derived from SOX6 expressed in glioma stem cells for immunotherapy. Int J Cancer 2010 ; 126 : 919.

16) Kudo-Saito C, Shirako H, Takeuchi T, Kawakami Y. Cancer metastasisi is accelerated through immunosuppression during EMT of cancer cell. Cancer Cell 2009 ; 15 : 195.

17) 河上　裕．がん細胞と免疫系の相互作用の分子機構とその制御．実験医学 2009；27：2170.

18) 河上　裕．がん免疫ネットワークの総合的制御によるがん治療の可能性．固形癌の最新治療―癌治療への新たな取組み―．日本臨床 2010；68：1094.

19) Lurquin C, Lethé B, De Plaen E, et al. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 2005 ; 201 : 249.

20) Udagawa M, Kudo-Saito C, Hasegawa G, et al. Enhancement of immunologic tumor regression by intratumoral administration of dendritic cells in combination with cryoablative tumor pretreatment and Bacillus Calmette-Guerin cell wall skeleton stimulation. Clin Cancer Res 2006 ; 12 : 7465.

21) Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res 2011 ; 17 : 4550.

22) Robbins PF, Morgan RA, Feldman SA, et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. J Clin Oncol 2011 ; 29 : 917.

23) Kalos M, Levine BL, Porter DL, et al. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Transl Med 2011 ; 3 : 95.