Immune responses to human melanoma have been extensively studied, and various immunotherapies have been developed (1–4). One of the major problems contributing to immunological rejection of cancer is immune evasion through various mechanisms (5). Immune evasion is one of the malignant phenotypes that allows cancer cells to survive in the host. It is important to understand the molecular mechanisms of immune evasion to allow the development of cancer immunotherapy. Most cancer cells produce several immune suppressive factors that allow them to evade the host immune system (6, 7). The mechanisms for the production of these effector molecules in cancer cells have not been investigated well, although oncogenic STAT3 signaling has recently been reported to result in immune evasion through production of immune suppressive factors in various murine cancers (8, 9). In this study, we have evaluated the role of mitogen-activated protein kinase (MAPK) signaling in the production of various immune-suppressive cytokines. MAPK activation occurs frequently in human cancers due to various genetic changes, and MAPK signaling is also important in the regulation of cytokine signal pathways. Using a MAPK inhibitor and lentiviral BRAFV600E RNA interference (RNAi), we demonstrated that the MAPK pathway, along with the STAT3 pathway signaling, is essential for immune evasion by human melanomas that have constitutively active MAPK signaling and is a potential molecular target for overcoming melanoma cell evasion of the immune system.
control DMSO treatment did not affect the production of IL-10 and VEGF mRNAs, and only slightly increased the level of IL-6 mRNA. An 18-h exposure to U0126 also suppressed the production of IL-10, VEGF, and IL-6 at the protein level, indicating that activated ERKs may be responsible for suppressing local immune responses against melanoma by producing these immunosuppressive factors (Fig. 1 c). In addition, the suppression of ERK phosphorylation and the inhibition of IL-6, IL-10, and VEGF production occurred even after reducing the concentration of the U0126 treatment to 25 μM. The expression was detected by ELISA with the culture supernatants. The ratio of viability of U0126-treated cells DMSO-treated cells at the harvest was 77%. The cytokine production was normalized to the value of control DMSO-treated cells based on the cell count. Western blot showed strong inhibition of ERK phosphorylation, but not of STAT3 protein, or its phosphorylation at Ser727 and Tyr705. (d) Decreased production of IL-10 and VEGF from three melanoma cell lines with the BRAFV600E mutation, 624mel, 888mel, and 928mel after a 18-h treatment with U0126 at a concentration of 25 μM, which was detected by ELISA with the culture supernatants. The ratios of viability of U0126-treated cells DMSO-treated cells at the harvest were 95% for 624mel, 103% for 888mel, and 100% for 928mel. The cytokine production was normalized to the value of control DMSO-treated cells based on the cell count. Western blot showed strong inhibition of ERK phosphorylation, but not of STAT3 protein. Slight decrease of Ser727 phosphorylation of STAT3 was observed in 888mel and 928mel cells, but not in 624mel cells. These results are representative of three or four independent experiments with similar results.
RNAi was transfected into three melanoma cell lines, A375, 888mel, and 624mel, using the lentivirus vectors encoding short hairpin RNA for either firefly luciferase mRNA (GL3B, as control) or BRAFV600E mRNA (BRAF#1') at 50 or 100 multiplicity of infection. At 5 or 6 d after the infection, proteins were extracted and subjected to Western blot analysis. Profound decrease of phosphorylation of ERK1/2 with decrease of the BRAF protein was observed by the BRAFV600E-specific RNAi. No significant difference in STAT3 protein and phosphorylation of STAT3 at Ser727 or Tyr705 was observed. A slight decrease of phosphorylation at Ser727 was observed in 888mel and 624mel cells after BRAF RNAi; 5 or 6 d after the lentivirus infection, the equal number of the melanoma cells was dispersed at a density of 1–2 × 10^6 cells/ml, and the culture supernatants after 18 h were subjected to ELISA for IL-6, IL-10, or VEGF. Profound decrease of IL-6, IL-10, and VEGF was observed. One representative result of two or three independent experiments with similar results is shown.

Figure 2. Decreased production of immunosuppressive factors IL-6, IL-10, and VEGF from three melanoma cell lines with the BRAFV600E mutation by BRAFV600E-specific RNAi. The three melanoma cell lines with the BRAFV600E mutation, A375mel, 888mel, and 624mel, were infected with the lentivirus vectors encoding short hairpin RNA for either firefly luciferase mRNA (GL3B, as control) or BRAFV600E mRNA (BRAF#1') at 50 or 100 multiplicity of infection. At 5 or 6 d after the infection, proteins were extracted and subjected to Western blot analysis. Profound decrease of phosphorylation of ERK1/2 with decrease of the
Next, the potential effects caused by the soluble factors induced by the MAPK and STAT3 signaling in the supernatant of the A375 cultures upon the maturation of DCs were investigated. The supernatant from the cultured A375 melanoma cells contains soluble factors that inhibit human monocyte-derived DC (MoDC) maturation when stimulated with Toll-like receptor ligands such as LPS. The addition of the A375 culture supernatants to MoDC cultures at a final concentration of 10–20% significantly decreased the production of inflammatory cytokines, including IL-12 and TNF-α, in MoDCs as well as CD1a and CD86, CD40, or HLA-DR on MoDCs.
Supernatants from the other three melanoma cell lines produced the same results when added to the MoDC cultures (not depicted). The suppressive activity of the melanoma cell supernatants stems from the production of IL-6, IL-10, and VEGF because the addition of specific antibodies for these factors reduced the suppressive activity of the A375 culture supernatants (Fig. 4a). Disparities between our observations and the previously reported results concerning murine B16 melanoma cell supernatant with an activated STAT3 inhibiting the expression of MHC class II and CD40 may be explained by different tumor cells or species (8).

The suppressive activity of the A375 culture supernatants on the IL-12 and TNF-α production of the LPS-treated MoDCs was restored by pretreating the A375 cells with either RNAi targeting BRAFV600E alone, STAT3 alone, or both BRAFV600E and STAT3 (Fig. 4b). Although the STAT3 RNAi appeared to reduce the suppressive activity of the A375 culture supernatants more than the BRAFV600E RNAi, the difference may be simply caused by different RNAi activities. However, it is important to note that no additive and synergistic effects by the RNAi simultaneous-targeting BRAF and STAT3 were observed; again, indicating the essential roles of both signaling pathways in the production of suppressive factors on DC maturation. Although activation of DCs by the supernatant from murine CT26 colon cancer cell line transfected with a dominant-negative form of STAT3 possibly through the increased production of proinflammatory cytokines had been reported, activation of DCs was not observed in this study. It may be explained by an incomplete inhibition of BRAF and STAT3, no augmented proinflammatory cytokine production in the BRAF shRNA-transfected melanoma cells, or by differences in tumor cells or species.

In summary, we have demonstrated for the first time an essential role of the MAPK signaling along with the STAT3 pathway on the production of various immunosuppressive factors in melanoma cell lines with constitutively active MAPK due to the common BRAFV600E mutation. Thus, the MAPK pathway may be a potentially significant molecular target for overcoming immune evasion of various cancers because it is frequently activated in several types of cancers, including melanoma without BRAFV600E mutation (16, 17).

MATERIALS AND METHODS

Cell lines and reagents. A375, 624mel, 888mel, and 928mel were cultured in RPMI 1640 media supplemented with 10% FBS, penicillin, and streptomycin. Stock solution of MEK inhibitor U0126 (Sigma-Aldrich) was made by dissolving the U0126 in DMSO.

Quantitative RT-PCR. RT-PCR was performed by using TaqMan PCR assay probe/primer sets (Assay-on-Demand, Gene Expression Products; Applied Biosystems) for IL-6, IL-10, VEGF, and GAPDH by using ABI PRISM 7900HT (Applied Biosystems), and a relative quantitative method was applied for the target mRNA, which was normalized by the control GAPDH mRNA.

ELISA. Human VEGF, IL-6, IL-12, and TNF-α were measured by using the DuoSet ELISA Development System (R&D Systems), and human IL-10 was measured by using the BD OptEIA kit (BD Biosciences).
Lentivirus-mediated RNAi. HIV lentiviral vectors for shRNA expression were prepared as described previously (11). The shRNA target sequences were as follows: for mutated BRAF (V600E), (BRAF#1) GCTACAGAGAACATCTCGAT; for STAT3, (STAT3#4) ATAGAGGCTTAAAGGAGA; and for firefly luciferase (control shRNA, GL3B) GTTGCGCTGCTGTCGTTGGCAAC. The construction of an HIV-U6-BRAF/STAT3-GFP vector with the tandem arranged two shRNA units was similar with the previous report (18).

5–6 d after the transduction, the melanoma cells were analyzed for the cytokine production and signaling molecules by ELISA and Western blot analysis, and the equivalent transduction was confirmed by the GFP expression from the vector (11) by flow cytometry analysis.

EMSA. 5 μg of nuclear extracts was mixed with 5× binding buffer, poly d (I–C), and [γ-32P] ATP-labeled STAT3 double-stranded oligonucleotide probe with or without either unlabeled wild (5′-GATCCCTGGAATTCCTGATCATGATCATGATCATGC-3′) or mutant (5′-GATCCCTGGAATTCCTGATCATGATCATGATCATGCC-3′) STAT3 double-stranded oligonucleotide probe. The mixed samples were then subjected to electrophoresis in a 6.5% polyacrylamide gel. The radioactivity on the gel was developed by using a BAS imaging system (FUJI).

Western blot. Anti-BRAF (Santa Cruz Biotechnology, Inc.), anti-ERK2 (Santa Cruz Biotechnology, Inc.), anti-pERK1/2 (Cell Signaling), anti-STAT3 (Cell Signaling), anti–phospho-STAT3 (Ser727; Cell Signaling), and anti–phospho-STAT3 (Tyr705; Cell Signaling) antibodies were used for the first antibody. A horseradish peroxidase–conjugated anti-IgG antibody was used for the second antibody.

STAT3 reporter assay. 0.4 μg pSTAT3-3A-Luc (CLONTECH Laboratories, Inc.) with 0.4 μg pRL-SV40 were cotransfected to 2–4 × 10^5 melanoma cells with or without BRAF RNAi. 24 h after the transfection, the cells were analyzed for the firefly and renilla luciferase activity. Each firefly luciferase activity was normalized to renilla luciferase activity.

DC induction. Human CD14+ monocytes isolated by using CD14 MicroBeads (Miltenyi Biotech) were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 ng/ml GM-CSF, and 50 ng/ml IL-4 with or without the conditioned medium of the A375mel cells transduced with HIV lentiviral vectors. On day 5 of the culture, LPS was added at 100 ng/ml for 5–6 d after the transduction, the melanoma cells were analyzed for the firefly and renilla luciferase activity. Each firefly luciferase activity on the gel was developed by using a BAS imaging system (FUJI).

We thank Dr. M. Miyagishi and Dr. K. Taira, Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Tokyo, Japan for helping us generate shRNA lentiviruses. We thank Dr. N. Tsukamoto for technical advice. We thank Dr. H. Miyoshi (RIKEN Tsukuba Institute, Japan) for providing us with pCMV-VSV-G-RSV-Rev and pCAG-HIVgp for the HIV vector. We also thank Dr. H. Kurashige and Dr. K. Hayashi for the tandem arranged two shRNA units was similar with the previous report (18).

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