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# Adjuvant effects of formalin-inactivated HSV through activation of dendritic cells and inactivation of myeloid-derived suppressor cells in cancer immunotherapy

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**Use of adequate adjuvant is necessary for induction of effective antitumor immune responses. To develop an effective adjuvant for cancer immunotherapy, we selected formalin-inactivated (f)-HSV as an adjuvant component, and analyzed the mechanisms underlying its adjuvant effects. First, we found that f-HSV can induce the tumor antigen-specific CTLs by enhancing antigen cross-presentation by dendritic cells (DCs), mainly through TLR2, but not TLR9. Next, f-HSV was also found to prevent the accumulation of myeloid-derived suppressor cells (MDSCs). We demonstrated that the expansion of MDSCs in the blood and spleen during tumor progression required B cells producing the inflammatory angiogenesis factors, vascular endothelial growth factor (VEGF)-A and neuropilin-1 (NRP-1), a co-receptor for VEGF receptor-2 (VEGFR-2). Interestingly, the transmembrane-type NRP-1 on B cells changed to soluble-type NRP-1 (sNRP-1) by f-HSV treatment. We further showed that the sNRP-1 and VEGF-A secreted from B cells by f-HSV treatment could abrogate the immunosuppressive ability of MDSCs. These results suggest that f-HSV can enhance antitumor immune responses as an adjuvant, not only through activation of DCs, but also inactivation of MDSCs *via* B cells.**

For induction of specific immune responses against tumor antigens in cancer immunotherapy, use of effective adjuvant is important for enhanced activation of dendritic cells (DCs) and innate immunity. Recent studies on the functions of TLRs and their adaptors have facilitated elucidation of the molecular basis of adjuvant activity.[1] TLR-signaling induces the production of IFNs, chemokines and pro-inflammatory cytokines in DCs. These mediators then play a crucial role in the maturation of the DCs, in turn, resulting in activation of CTLs and NK cells.[2,3]

**Key words:** myeloid-derived suppressor cells (MDSCs), HSV, B cells
**Abbreviations:** t-MDSCs: tumor-associated myeloid-derived suppressor cells; t-B cells: tumor-associated B cells; f-HSV: formalin-inactivated herpes simplex virus; gD: glycoprotein D; DCs: dendritic cells; VEGF: vascular endothelial growth factor; TLR: Toll-like receptor; sNRP-1: soluble-type neuropilin-1; mAb: monoclonal antibody; OVA: ovalbumin; HA: hemagglutinin
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Herpes simplex virus type 1 (HSV-1) can activate the innate immune system through TLR2 in macrophages and myeloid DCs,[4] and through TLR9 in plasmacytoid DCs (pDCs).[5] In addition, HSV glycoproteins can activate DCs though binding to the mannose receptor or other lectins.[6] HSV glycoprotein D (gD) that binds to 2 molecules, nectin-1, a member of the Ig superfamily of proteins,[7] and herpes virus entry mediator (HVEM), a member of the TNF receptor family,[8] functions as a trigger of the IFNα production in DCs.[9] HSV gB that functions as a paired immunoglobulin-like type 2 receptor α (PILRα) ligand[10] activates DCs and NK cells.[11] Interestingly, UV-inactivated HSV-1 can also activate DCs, followed by the production of IL-12 and IFNα/β *in vivo*.[12,13] Furthermore, UV-inactivated HSV-2 also induces IFNα production *via* TLR9 in pDCs *in vitro*.[14] These reports suggest that DCs can not only be activated by live (active) HSV, but also by inactivated HSV.

Gr-1$^+$ CD11b$^+$ cells, called myeloid-derived suppressor cells (MDSCs), expand during tumor progression, and accumulate in blood, secondary lymphoid organs and tumor tissue. Accumulation of tumor-associated (t)-MDSCs which is considered as one of the main mechanisms of immune escape[15] is enhanced by the proinflammatory mediators such as IL-1β, IL-6, $PGE_2$, IL-13 and S100A8/A9.[16–19] The t-MDSCs were shown to inhibit T cell proliferation induced by CD3 ligation or CD3/CD28 co-stimulation *via* an MHC-independent mechanism requiring cell-cell contact.[20] It has also been reported that IFNγ production and the CTL-activity of

$CD8^+$ T cells are inhibited by t-MDSCs *via* an MHC-dependent mechanism.[21–24] Moreover, the high expression of arginase-1 in t-MDSCs was shown to be associated with TCR CD3ζ chain down-regulation and cell cycle arrest in T cells.[24,25]

In this study, to develop an effective adjuvant for cancer immunotherapy, we first analyzed the efficacy of formalin-inactivated HSV-1 (f-HSV) with incomplete Freund's adjuvant (IFA) (hereafter referred to as HSV-adjuvant) as an adjuvant for the enhancement of antigen-specific immune responses. Then, the mechanism(s) in the adjuvant effects of f-HSV was analyzed; in particular, the effects on the DCs and t-MDSCs. f-HSV was shown to enhance antigen cross-presentation by DCs mainly through TLR2, but not TLR9. Furthermore, HSV-adjuvant inhibited the expansion of t-MDSCs mediated by tumor-associated (t)-B cells in tumor-bearing mice *in vivo*. Interestingly, the neuropilin-1 (NRP-1) on t-B cells was changed to soluble-type NRP-1 (sNRP-1) by f-HSV *in vitro*. The natural NRP-1 is a 120 to 140 kD transmembrane glycoprotein receptor that binds with VEGF-A, and plays role as a co-receptor for VEGF receptor-2 (VEGFR-2). The NRP-1 that is critical for the dimerization of VEGFR-2 enhances the VEGFR-2-signal response when VEGF-A is sandwiched between NRP-1 and VEGFR-2.[26,27] Moreover, VEGF-A and NRP-1 are related to the accumulation of t-MDSCs *via* VEGFR-2 signaling.[28,29] In contrast, sNRP-1, a 90-kD secreted-type protein, acts as an antagonist of VEGFR-2 signaling because of a deletion in the "c" domain for dimer formation. It is reported that the increase of sNRP-1 results in antitumor effects *in vivo*.[30,31] In this study, we further analyzed the VEGFR-2 signaling between t-B cells and t-MDSCs. Our results suggest that sNRP-1 and VEGF-A secreted from t-B cells by f-HSV treatment may reduce the immunosuppressive actions of t-MDSCs.

## Material and Methods

### Cell lines

CT26 line derived from mouse colon adenocarcinoma (H-$2^d$), Meth A line from mouse fibrosarcoma (H-$2^d$), P815 line from mouse mastocytoma (H-$2^d$), mouse thymus lymphoma EL4 line (H-$2^b$) and E.G7 line (EL4 transfected with cDNA encoding OVA) were maintained in RPMI 1640 containing 5% heat-inactivated FCS, 2 mM L-glutamine, and penicillin-streptomycin. Vero cells, African green monkey kidney cells, (ATCC, Rockville, MD) were maintained in DMEM containing 10% heat-inactivated FCS and penicillin-streptomycin.

### Mice

BALB/c and C57BL/6 mice (females 6–7 wk old) were obtained from the SHIZUOKA LAB. ANIMAL CENTER (SLC) (Shizuoka, Japan), and the TLR9−/− mice (C57BL/6 background)[32] were obtained from the Nippon CLEA (Kanagawa, Japan). OT-I transgenic mice (OT-I Tg), kindly provided by Dr. Kosaka and Dr. Heath (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia),[33] were bred in a C57BL/6 background and showed expression of the $OVA_{257\text{-}264}/K^b$-specific TCR (Vα2/Vβ5) transgene in greater than 95% of the peripheral $CD8^+$ T cells. The expression of the transgene was checked by FACS analysis (BD Biosciences, San Diego, CA) after Vα2-FITC/CD8-PE-staining of the peripheral blood cells. All animal procedures were conducted with the approval of the Animal Care and Use Committee of Keio University School of Medicine.

### Ags and peptides

Inactivated Influenza A Virus (H1N1; New Caledonia/20/99 strain, Capricorn, Portland, ME) and OVA (PIERCE, Rockford, IL) were used as model antigen for the analyses of the adjuvant activity. The I-$E^d$-restricted HA-peptide comprising amino acids 124–136 (SVSSFERFEIFPK), the H-$2K^d$-restricted HA-peptide comprising amino acids 533–541 (IYSTASSL), the H-$2L^d$-restricted AH1-peptide derived from murine leukemia virus gp70 comprising amino acids 138–147 (SPSYVYHQF), the H-$2K^b$-restricted OVA-peptide comprising amino acids 257–264 (SIINFEKL) and the H-$2L^d$-restricted P815AB (P1A)-peptide comprising amino acids 35–43 (LPYLGWLVF) were synthesized by American Peptides.

### Purification of formalin-inactivated HSV-1

*Virus replication*. Vero cells were infected or not infected with HSV-1 (KOS strain) at a multiplicity of infection (MOI) of 0.01. After culture for 2 days, HSV-1-containing supernatants or Mock supernatants were recovered from the culture.

*Virus purification*. The HSV-1-containing supernatants were concentrated using the Midjet System (UFP-500-C-4A; GE Healthcare, Little Chalfont, Buckinghamshire, UK), and centrifuged in a 30–60% (w/w) sucrose gradient at 25,000 cpm with a Beckman SW28 rotor at 4°C for 18 hr. Then, HSV-1 was recovered from the 40 to 50% (w/w) sucrose gradient layer.

*Titration*. The titer of purified HSV-1 was analyzed by using the Vero cells (PFU/ml).

*Formalin inactivation*. After treatment with 0.1% formalin/PBS for 1 wk, the purified HSV-1 or Mock was replaced with 1% glycerin/PBS as 10-fold dilution with PBS of Glyceol (Chugai Pharmaceutical, Tokyo, Japan). Formalin-inactivated HSV-1 (f-HSV) was finally adjusted to the equivalent to $1 \times 10^9$ PFU/ml infectious HSV-1, and stored at −150°C.

### Immunization

HSV-adjuvant was prepared by f-HSV (equivalent to final $5 \times 10^8$ PFU/ml) in an emulsified form with an equal volume (1:1) of IFA (Difco, Detroit, MI). The control Mock-adjuvant was prepared in an emulsified form using mock with an equal volume (1:1) of IFA.

*H1N1 vaccine*. BALB/c mice were immunized on their footpads with H1N1 (10 μg/body) or PBS and HSV-adjuvant (equivalent to $10^7$ PFU/body or $10^8$ PFU/body) or Mock-

adjuvant. On day 7, a booster consisting of I-$E^d$-restricted $HA_{124-136}$ (50 μg/body) and $K^d$-restricted $HA_{533-541}$ (50 μg/body) with IFA was given, except to the PBS/Mock-adjuvant and PBS/HSV-adjuvant groups, which were administered PBS with IFA.

*AH1 peptide vaccine in the i.d. CT26 tumor model.* BALB/c mice were challenged by *i.d.* inoculation of CT26 ($1 \times 10^5$) and immunized on their footpads with AH1 (50 μg/body) and different adjuvants, namely, IFA, CFA or HSV-adjuvant (equivalent to $10^8$ PFU/body) on day 5, except in the control mice, which received PBS/IFA. A booster with AH1/IFA was administered, except in the control mice, which received PBS/IFA.

*AH1 peptide vaccine in the i.p. CT26 tumor model.* BALB/c mice were challenged by *i.p.* inoculation of CT26 ($5 \times 10^4$) and immunized on their footpads with AH1 (50 μg/body) and Mock- or HSV-adjuvant (equivalent to $10^8$ PFU/body) on day 3.

*OVA peptide vaccine in the i.p. E.G7 tumor model.* C57BL/6 mice were challenged by *i.p.*inoculation of E.G7 ($5 \times 10^6$) and immunized on their footpads with $OVA_{257-264}$ (50 μg/body) and Mock- or HSV-adjuvant (equivalent to $10^8$ PFU/body) on day 3.

### Cells purification

$CD8\alpha^+$ lymphoid DCs were enriched from the splenocytes in C57BL/6 mice using a MACS $CD8\alpha^+$ DC isolation kit (Miltenvi Biotec, Gladbach, Germany) (>98%). $CD11b^+$ myeloid DCs were enriched from the splenocytes of C57BL/6 mice using magnetic microbeads-conjugated anti-mouse CD11c mAb (Miltenyi Biotec) after removal of $B220^+$ cells and $CD8\alpha^+$ cells using BD IMag anti-mouse B220 mAb DM (BD Biosciences) and BD IMag anti-mouse CD8α mAb DM (BD Biosciences) (>96%). Gr-$1^+$ $B220^+$ plasmacytoid DCs were enriched from splenocytes in wild type or TLR9 −/− (C57BL/6 strain) mice using a MACS plasmacytoid DC isolation kit (Miltenyi Biotec) (>93%). γδT cells were purified from nu/nu (BALB/c strain) using streptavidin-conjugated BD IMag DM (BD Biosciences) after staining with biotinylated anti-mouse γδTCR mAb (eBioscience, San Diego, CA) (>96%). B cells were enriched from splenocytes in BALB/c or C57BL/6 mice using a MACS B cell lsolation kit (Miltenyi Biotec) (>98%). t-B cells, t-DCs and t-MDSCs were purified from splenocytes in E.G7 ($5 \times 10^6$) *i.p.*-inoculated C57BL/6 mice on day 15. To purify t-MDSCs, cells in the intermediate layer of a lymphoprep buffer (Fresenius Kabi Norge) were stained with a mixture of FITC-conjugated anti-mouse CD11b mAb (eBioscience), PE-conjugated anti-mouse Gr-1 mAb (eBioscience), and biotinylated anti-mouse Gr-1 mAb (eBioscience), followed by treatment with streptavidin-conjugated BD IMag DM (BD Biosciences). Gr-$1^+$ cells purified by BD IMag DM were showed as the phenotype of Gr-$1^+$ $CD11b^+$ (>98%) by FACS.

### $^{51}$Cr-release assay

The standard 4 h $^{51}$Cr-release assay was carried out to measure the tumor antigen-specific cytotoxic activity of the $CD8^+$ T cells. Targets were labeled with 1.85 MBq of sodium chromate-$^{51}$Cr in FCS (1 ml) (Amersham Biosciences, Buckinghamshire, UK) for 1 hr at 37°C. After extensive washing, the labeled targets ($5 \times 10^3$/well) were incubated for 4 hr with various numbers of $CD8^+$ T cells in 96 well U-bottom plates (Nunc, Roskilde, Denmark). After harvesting of the culture supernatant from each well using HARVESTING FRAMES (Nihon Molecular Devices, Tokyo, Japan), the $^{51}$Cr-release activity was measured by a scintillation counter auto well gamma system (Wallac 1480 Wizard 3, PerkinElmer Life Sciences). All assays were performed in triplicate. $^{51}$Cr-release was calculated as follows: [sample counts – spontaneous counts]/[maximum counts − spontaneous counts] × 100%.

### [$^3$H]-thymidine uptake assay

For the analysis of the cell proliferative activity, after culture for 3 days in 96-well U-bottomed plates (Nunc), the cells in each well were labeled with 18.5 kBq of [$^3$H]-thymidine (PerkinElmer Life Sciences, Boston, MA) for 6 hr and harvested onto filter mats, UniFilter plate 96 (Perkin-Elmer). The incorporated radioactivity was measured by scintillation counting on a Packed Top Counter (Perkin-Elmer).

### Hemagglutination inhibition (HI) assay

A 1:10 dilution of serum (100 μl) was treated with Receptor Destroying Enzyme (RDE) (300 μl) at 37°C overnight, and heat-inactivated at 56°C for 30 min to remove non-specific hemagglutination activity. HI-reactions were performed in accordance with the manufacturer's instructions for the HI test (RDE(II) SEIKEN, DENKA SEIKEN, Tokyo, Japan), and the HA-specific Ab titer was analyzed.

### Antigen cross-presentation assay

DCs ($5 \times 10^4$/well) isolated from wild type or TLR9−/− (C57BL/6 strain) were treated with 10 μg/ml OVA protein (PIERCE, Rockford, IL) and Mock or f-HSV ($10^5 \sim 10^7$ PFU/ml), with or without an anti-mouse TLR2 mAb (2 and 10 μg/ml) (FG purified 6C2; eBioscience), an anti-mouse TLR4/MD2 mAb (10 μg/ml) (FG purified MTS510; eBioscience) or an NA/LE isotype control Rat IgG2b (10 μg/ml) (BD Biosciences) for 90 min in 96-well flat-bottomed plates (Nunc). The DCs were then washed and irradiated at 50 Gy, followed by co-culture with $CD8^+$ T cells ($1 \times 10^5$/well) isolated from the OT-I Tg for 4 days. The cell proliferative activity of the OT-I $CD8^+$ T cells was analyzed by [$^3$H]-thymidine uptake assay, and the cross-presentation of OVA was estimated by the cell proliferative activity of OT-I $CD8^+$ T cells.

### Western-blot analysis

MDSCs ($1 \times 10^6$) or B cells ($1 \times 10^6$) were re-suspended in 50 μl of lysis buffer (1% Triton X-100, 10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). The lysates of the B cells were pre-treated for 5 hr at 37°C with PNGaseF (2500 U/ml) (New England Biolabs, Beverly, MA) to deglycosylation of the transmembrane protein. Then, these lysates were size-fractionated by SDS-12.5% PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane by electrotransfer. The membrane was incubated in blocking buffer (Tris-buffered saline containing 5% skim milk and 0.1% Tween 20) and then probed with anti-arginase-1 Ab, anti-VEGF Ab, anti-neuropilin mAb (Santa Cruz Biotechnology, Santa Cruz, CA), anti-NRP-1 mAb (Abnova, Taipei, Taiwan) or anti-β-actin mAb (Sigma-Aldrich, St. Louis, MO). The membrane was then washed and incubated with HRP-conjugated anti-rabbit IgG antibody or HRP-conjugated anti-mouse IgG1 antibody (MBL, Medical & Biological Laboratories, Nagoya, Japan). Proteins were visualized using an ECL kit in accordance with the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ).

### MDSCs-suppression assay

To induce CT26-specific CTLs, $CD8^+$ T cells were purified from AH1/HSV-immunized BALB/c on day 15 after CT26 *i.p.*-inoculation ($5 \times 10^4$), and co-cultured with AH1 (10 μg/ml)-pulsed myeloid DCs (irradiated by 50 Gy) for 7 d. OT-I CTLs specifically directed against target E.G7 (EL4 transfected with OVA cDNA) were established from $CD8^+$ T cells in OT-I Tg by co-culture with $OVA_{257\text{-}264}$-pulsed splenocytes (irradiated by 50 Gy) for 48–72 hr. The suppressive effect of the t-MDSCs against CTLs was evaluated by $^{51}Cr$-release assay using CT26 CTLs or OT-I CTLs as described in a previous report.[34] Briefly, t-MDSCs ($0.25–1 \times 10^5$/well) purified from BALB/c on day 15 after CT26 *i.p.*-inoculation ($5 \times 10^4$) were pulsed with AH1 (10 μg/ml) and added into the $^{51}Cr$-release assay using the effector CT26-specific CTLs ($1 \times 10^5$/well) and target CT26 ($1 \times 10^4$/well). t-MDSCs ($0.25–1 \times 10^5$/well) purified from tumor-bearing C57BL/6 on day 15 after E.G7 *i.p.*-inoculation ($5 \times 10^6$) were pulsed with $OVA_{257–264}$ (10 μg/ml) and added into the $^{51}Cr$-release assay using the effector OT-I CTLs ($1 \times 10^5$/well) and target E.G7 ($1 \times 10^4$/well). Recombinant mouse VEGF-A (10 ng/ml, PeproTech Inc, Rocky Hill, NJ), recombinant human NRP-1/Fc Chimera (10 ng/ml, R&D systems, Minneapolis, MN), t-B cells ($0.25–1 \times 10^5$/well) treated with Mock or f-HSV (equivalent to $10^7$ PFU/ml) for 2 hr, or the culture-supernatant (100 μl) of t-B cells ($2 \times 10^5$/well) treated with Mock or f-HSV (equivalent to $10^7$ PFU/ml) for 2 hr was also added in the $^{51}Cr$-release assay.

### ELISA

An anti-human NRP-1 (CUB Domain) Ab (x 2000, ECM Biosciences, Versailles, KY) or recombinant mouse VEGF (2 μg/ml, 450-32, PeproTec) was coated onto a 96-well flat-bottomed plate (Nunc) by incubation at 37°C for 2 hr. After blocking with 4% BSA for 2 hr, the culture supernatant or 4× diluted serum was incubated overnight at 4°C. After washing, the plate was incubated with the anti-human NRP-1 mAb 1B3 (200 ng/ml, Abnova), followed by that with HRP-conjugated anti-mouse IgG2a (50 ng/ml, MBL). The reacted proteins were analyzed by plate reader (Bio-Rad Laboratories, Hercules, CA) and the OD was read at 450 nm. Recombinant human NRP-1/Fc Chimera (R&D systems) was used as the standard.

### Statistical analysis

Comparisons between two groups was performed by Student's *t*-test using Microsoft Excel. Results in multiple groups were compared by one-way or two-way factorial analysis of variance (ANOVA) using ANOVA4 from the Web (http://www.hju.ac.jp/~kiriki/anova4/).

## Results

### Adjuvant effects of f-HSV in vivo

To develop an effective and safe adjuvant for cancer immunotherapy, we inactivated the infectious capacity of a wild-type HSV-1 (KOS strain), while maintaining the antigenicity, with a low concentration (0.1%) of formalin/PBS. First, to investigate the efficacy of the f-HSV as an adjuvant, mice were immunized with a inactivated influenza virus (H1N1-type) as a model antigen and the HSV-adjuvant. In the $^{51}Cr$-release assay, a linear increase of the CTL activity associated with an increase of the E:T ratios was observed in the groups that received H1N1-antigen, and the CTL-activity specifically directed against the H1N1-antigen was enhanced by the HSV-adjuvant (Fig. 1*a*; $p < 0.05$, ANOVA). The proliferation of $CD4^+$ T cells specifically directed against the influenza antigen hemagglutinin $(HA)_{124\text{-}136}$ was enhanced by the effect of H1N1-antigen, although no statistically significant difference in the effect of HSV-adjuvant was detected (Fig. 1*b*; $p = 0.0642$, ANOVA). Furthermore, the hemagglutination inhibition (HI) assay showed that the HA-specific Ab titers in the serum were also enhanced by the effect of H1N1-antigen, although no statistically significant difference in the effect of HSV-adjuvant was detected (Fig. 1*c*; $p = 0.3253$, ANOVA). These results suggest that the HSV-adjuvant can significantly enhance antigen-specific $CD8^+$ T cell responses and slightly enhance antigen-specific $CD4^+$ responses and Ab production by the B cells.

### Enhancement of tumor suppression and CTL induction by HSV-adjuvant *in vivo*

In order to investigate the efficacy of HSV-adjuvant in cancer immunotherapy, we analyzed the antitumor effect of peptide AH1 (a tumor antigen in CT26) vaccination with different adjuvants (IFA, CFA and HSV-adjuvant) in CT26-bearing mice. On day 5 after *i.d.* inoculation of CT26 (when tumor

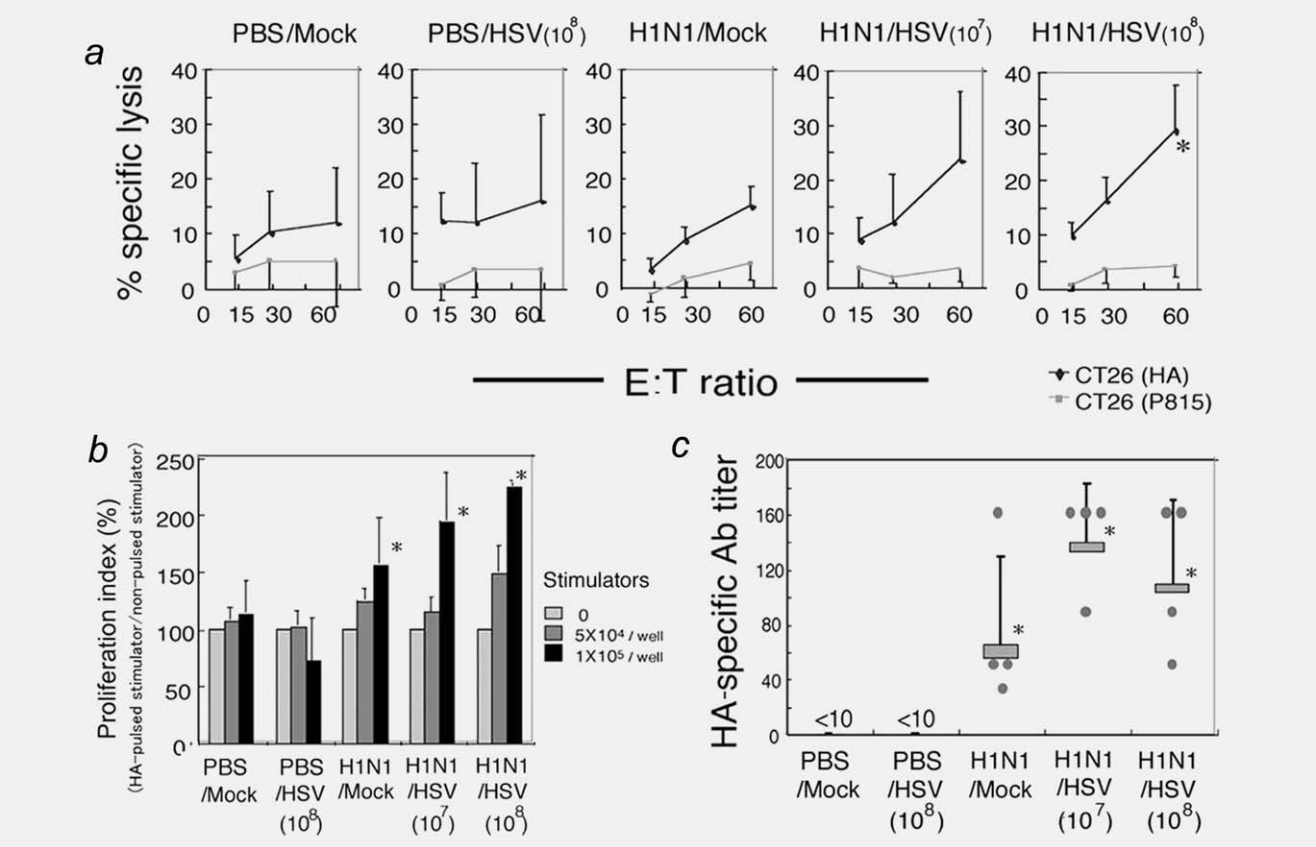



**Figure 1.** Enhancement of antigen-specific CTL activity by HSV-adjuvant. BALB/c mice were immunized on their footpads with PBS/Mock-adjuvant, PBS/HSV-adjuvant ($10^8$) (f-HSV (equivalent to $10^8$ PFU/body), formalin-inactivated Influenza A virus (H1N1) with Mock-adjuvant, H1N1 with HSV-adjuvant ($10^7$) or H1N1 with HSV-adjuvant ($10^8$). On day 7, a booster consisting of the $HA_{124-136}$ and $HA_{533-541}$-peptides with IFA was administered, except to the PBS/Mock and PBS/HSV groups. On day 14, the spleen and sera were harvested from the immunized mice. (*a*) The splenocytes from immunized mice were co-cultured *in vitro* with irradiated DCs pulsed with the peptide $HA_{533-541}$ (10 μg/ml) for 5 days. Purified $CD8^+$ T cells were used for the $^{51}$Cr-release assay. CT26 cells pulsed with 10 μg/ml of the peptide $HA_{533-541}$ [CT26(HA)] or peptide $P815_{35-43}$ [CT26(P815)], were used as the targets. Statistically differences in the results between H1N1/HSV($10^8$)-vaccinated and H1N1/Mock-vaccinated animals were observed at an E:T ratio of 60 (*, $p < 0.05$; *two-way ANOVA followed by Bonferroni t-test*, $n = 4$). Data indicate average ± SEM. (*b*) Purified $CD4^+$ T cells ($1 \times 10^5$/well) were cultured *in vitro* with irradiated splenocytes pulsed with $HA_{124-136}$ (10 μg/ml) as HA-pulsed stimulation (cell numbers; 0/well, $5 \times 10^4$/well or $1 \times 10^5$/well) or pulsed with PBS as non-pulsed stimulation (cell numbers; 0/well, $5 \times 10^4$/well or $1 \times 10^5$/well) for 72 hr. The proliferation index (%) of the $HA_{124-136}$-specific $CD4^+$ T cells was calculated by measurement of the [$^3$H]-thymidine uptake after the HA-pulsed stimulation and non-pulsed HA stimulation. Data indicate average + SEM. Statistical differences in the effect of H1N1 were detected. *, $p < 0.05$; two-way ANOVA ($n = 4$). (*c*) The titer of HA-specific Ab in the serum of each immunized mouse was analyzed by a HI-assay. Data indicates each titer (dot) and average + SEM. Statistical differences in the effect of H1N1 were detected. *, $p < 0.05$; two-way ANOVA ($n = 4$). The data represent two separate experiments with similar results.

size was approximately ϕ5 mm), the mice received AH1 vaccination with different adjuvants, followed 1 wk later by booster AH1/IFA immunization of all the mice except the control mice. In this *i.d.* tumor model, AH1/IFA did not produce any inhibition of CT26 tumor growth, since the *s.c.* implanted tumor grew very fast after the establishment of a ϕ5 mm tumor mass. In contrast, slightly greater suppression of the CT26 tumor growth was observed in the AH1/HSV-treated mice as compared with that in the mice treated with AH1/IFA or AH1/CFA, although there were no significant differences in the effect of HSV-adjuvant (Fig. 2*a*, $p = 0.0675$, ANOVA). The $^{51}$Cr-release assay revealed that a stronger CTL response against CT26 was induced by AH1/HSV than by AH1/IFA or AH1/CFA (Fig. 2*b*, at E:T ratio = 60; $p < 0.05$, ANOVA). These results showed that the effect of the AH1 antigen was inadequate (Figs. 2*a* and 2*b*). While, the effect of each adjuvant alone was analyzed in the tumor model established *s.c* inoculation, resulted in partial reduction of the tumor sizes in the HSV-adjuvant-treated mice as compared with that in the mice treated with IFA and CFA (data

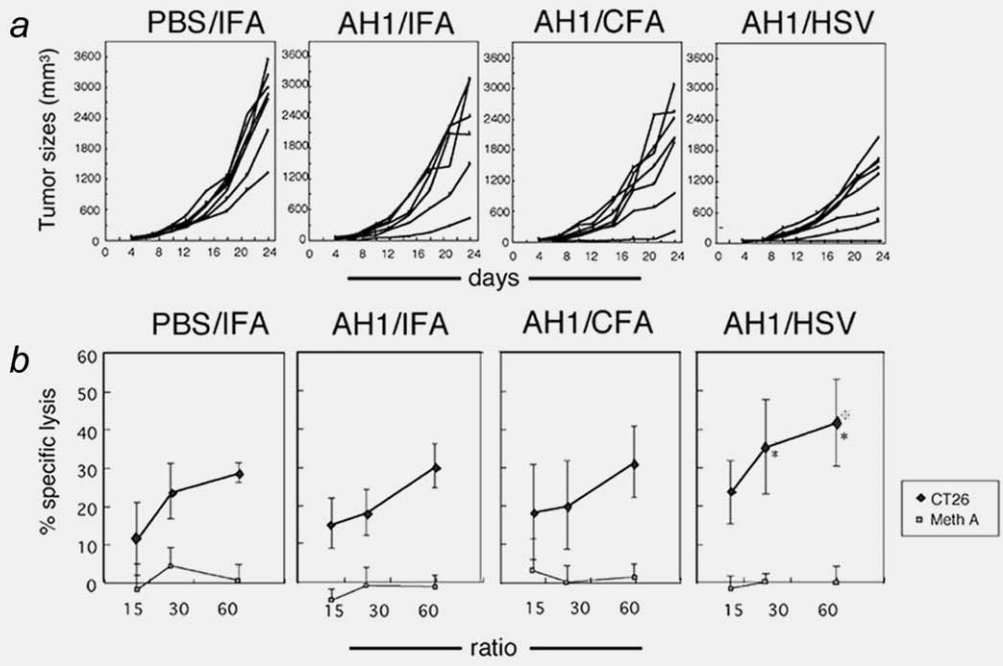



**Figure 2.** Enhancement of CTL against tumor antigen by HSV-adjuvant. BALB/c mice were challenged by *i.d.*-injection on the left flank of tumor CT26 ($1 \times 10^5$). The mice were immunized into the footpads with PBS/IFA, AH1/IFA, AH1/CFA or AH1/HSV (equivalent to $10^8$ PFU/body) on day 5. A booster in these groups was administered with AH1/IFA on day 12, while the control mouse received PBS/IFA again. (*a*) The tumor sizes were measured for 24 days after the tumor CT26-inoculation (*i.d.*) ($n = 7$). The tumor volume was calculated using the equation; volume = length $\times$ (width)$^2 \times 0.5$,[50] (*b*) The splenocytes from CT26-bearing mice on day 24 were cultured for 5 days with AH1-pulsed DCs. $^{51}$Cr-release assay was performed using purified CD8$^+$ T cells (targets, CT26 and Meth-A). Statistically significant differences in the results between the AH1/IFA and AH1/HSV groups at an E:T ratio = 30 and between the AH1/IFA or AH1/CFA and AH1/HSV groups at an E:T ratio = 60 were detected. *, E:T ratio = 30 and ※, E:T ratio = 60, $p < 0.05$; one-way ANOVA followed by Ryan's *q*-test ($n = 5$). Data indicate average ± SEM. The data represent two separate experiments with similar results.

not shown). These results suggest that the HSV-adjuvant can partially inhibit tumor development *via* enhancement of the AH1-specific CTL responses.

### Activation in f-HSV-treated myeloid DCs

To evaluate whether myeloid DCs (mDCs) are activated by f-HSV, we analyzed the expression of CD80 and CD86 as maturation makers in mDCs. Flow cytometry analysis reveled that the expression of CD80 and CD86 in mDCs was increased by treatment of f-HSV (CD80; 23.1% and CD86; 43.4%) compared with Mock (CD80; 12.9% and CD86; 24.1%) (Supporting Information Fig. 1). Then, to analyze the change in gene expression profile in mDCs by f-HSV, gene-chip analysis was performed using RNA extracted from mDCs after treatment of f-HSV or Mock. Out of approximately 39,000 genes analyzed, the expression of 162 genes in mDCs was upregulated more than 2-fold by f-HSV compared with Mock. The representative gene expression enhanced by f-HSV was detected such as TLRs-signal-mediated chemokines (*CCL3, CCL5, CXCL9, CXCL10, CXCL11* and *CCL22*), IFNs-related genes (*PNP, IGTP, IRG47, ISG54, IRF7, MAIL, IFRG15, IL-15, IL-12, ISG20, MX2, VIP, MyD88, STAT1* and *JAK2*), and cell cycle-related genes (*Npdc1, cyclin D1, cyclin D2* and *CYL-1*) (unpublished data). These results suggest that f-HSV can activate mDCs through TLRs signals.

### Enhancement by f-HSV of Ag cross-presentation by DCs *in vitro*

Since DCs have the capacity for antigen cross-presentation to deliver exogenous antigen on not only MHC class II, but also MHC class I, they can induce CTLs against tumor antigen. In this study, to analyze the effect of f-HSV on antigen cross-presentation by DCs *in vitro*, we used OVA as a model antigen and CD8$^+$ T cells from the OT-I transgenic mouse that carries a transgenic TCR specific for the H-2K$^b$-OVA$_{257-264}$ complex. The proliferative response of OT-I CD8$^+$ T cells induced by co-culture with OVA-pulsed DCs was significantly increased by f-HSV *in vitro*-treatment in a dose-dependent manner (Fig. 3*a*), suggesting that f-HSV may enhance the antigen cross-presentation ability of DCs. Furthermore, f-HSV enhanced the CTLs cross-priming of not only lymphoid DCs (CD11b$^{low}$ CD8$\alpha^+$ CD11c$^+$), but also myeloid DCs and pDCs (B220$^+$ Gr-1$^+$ CD11c$^+$) (Fig. 3*b*). The enhancement of CTLs cross-priming by f-HSV was inhibited by a blocking mAb specific for TLR2 (Fig. 3*b*), but not by a blocking mAb specific for TLR4 (data not shown).

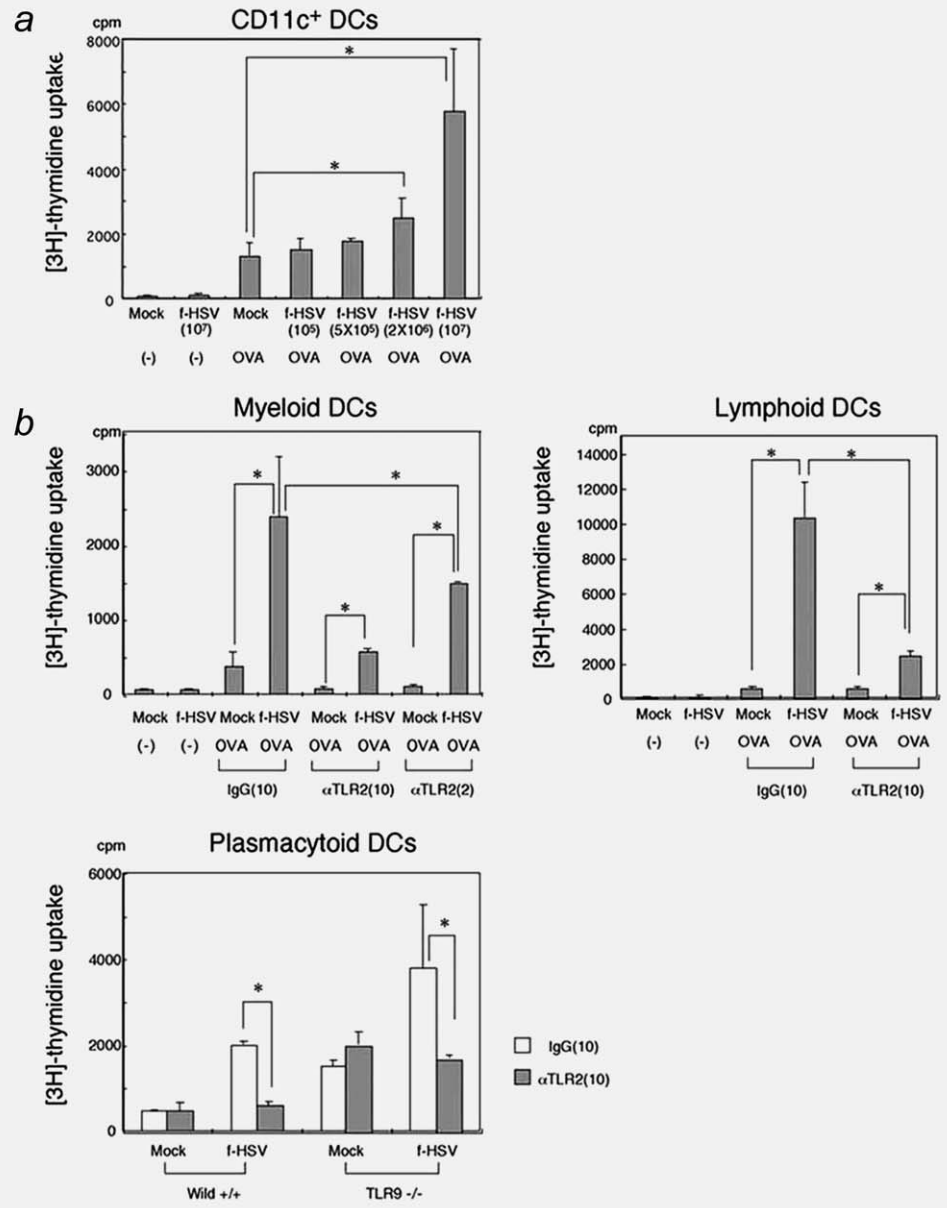



**Figure 3.** Enhancement of antigen cross-presentation in DCs by f-HSV *in vitro*. (*a*) The effect of f-HSV (equivalent to $10^5$–$10^7$ PFU/ml) in enhancing the antigen cross-presentation ability of DCs was analyzed by the antigen cross-presentation assay, as described in material and methods. (*b*) The effect of f-HSV (equivalent to $10^7$ PFU/ml) with anti-TLR2 mAb (2 or 10 μg/ml) or isotype control Rat IgG2b (10 μg/ml) on the antigen cross-presentation ability was analyzed by the same assay in myeloid CD11b$^+$ DCs and lymphoid CD8α$^+$ DCs derived from the C57BL/6 mice, and B220$^+$ pDCs derived from C57BL/6 mice or TLR9−/− mice ($n = 3$). *, $p < 0.05$; *t*-test. Data indicate average + SD. Results are representative of three independent experiments.

In addition, f-HSV could enhance the CTLs cross-priming of pDCs from TLR9 −/−, suggesting that the CTLs cross-priming in pDCs is not related to TLR9 signaling. These findings suggest that f-HSV can enhance the Ag cross-presentation by DCs through TLR2, but not TLR4 or TLR9.

### Inhibition of MDSCs accumulation by HSV-adjuvant *in vivo*

To analyze the effect of f-HSV on t-MDSCs, BALB/c mice were immunized with Mock-adjuvant (Mock), AH1 with Mock-adjuvant (AH1), HSV-adjuvant (HSV) or AH1 with HSV-adjuvant (AH1/HSV) on day 3 after *i.p.* inoculation of CT26 cells into mice. The weights of the *i.p.*-tumors and the number of Gr-1$^+$ CD11b$^+$ MDSCs in the spleen were analyzed on day 15 after tumor inoculation. In this *i.p.* tumor model, not only AH1/HSV, but also HSV-adjuvant produced inhibition of tumor growth as compared to that with Mock (Fig. 4*a*). Use of HSV as the adjuvant resulted in a statistically significant difference in the tumor weights ($p < 0.0005$,

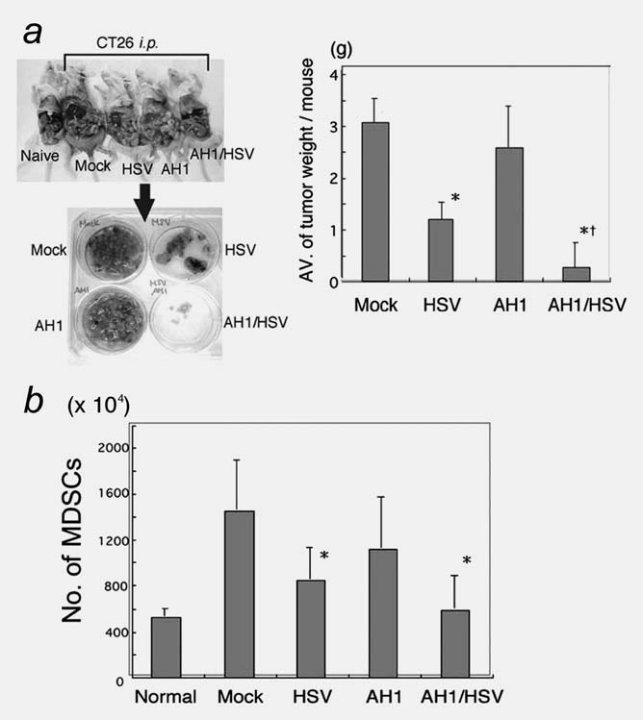



**Figure 4.** Inhibition of MDSCs expansion in tumor-bearing mice by HSV-adjuvant. (*a*) BALB/c mice were challenged by *i.p.*-inoculation of tumor CT26 cells ($5 \times 10^4$). On day 3 after tumor *i.p.*-inoculation, immunization was undertaken with PBS/IFA (Mock), PBS/HSV-adjuvant (HSV), AH1/IFA (AH1) or AH1/HSV-adjuvant (AH1/HSV). The weight of the *i.p.*-tumors was measured on day 15 ($n = 4$). HSV-adjuvant was used as equivalent to $10^8$ PFU/body. Statistical differences in the effect of HSV-adjuvant or AH1 were detected. *, $p < 0.005$, †, $p < 0.05$; two-way ANOVA. Data indicates average + SEM. (*b*) On day 15 after CT26-inoculation (*i.p.*), the number of Gr-$1^+$ CD$11b^+$ MDSCs (No. of MDSCs) in the spleen was analyzed by FACS ($n = 4$). Statistical differences in the effect of HSV-adjuvant were detected. *, $p < 0.005$; two-way ANOVA. Data indicates average + SEM. The data represent two separate experiments with similar results.

ANOVA). The number of t-MDSCs in the spleen was also reduced by HSV-adjuvant as compared with that in Mock (Fig. 4*b*). Use of HSV as the adjuvant resulted in a statistically significant difference in the number of t-MDSCs ($p < 0.05$, ANOVA), while there was no statistically significant difference in the number of MDSCs between the HSV and the AH1/HSV groups. Similar results were also observed in E.G7 (EL4 transfected with OVA cDNA)-inoculated (*i.p.*) C57BL/6 mice immunized $OVA_{257\text{-}264}$ with HSV-adjuvant (data not shown). In this tumor model, the spleen size and the number of total splenocytes were increased. Although no significant accumulation of MDSCs was found in the splenocytes of the tumor-bearing mice, an increase of the total number of MDSCs was observed. The decrease in the number of MDSCs in the spleen observed in the HSV- and AH1/HSV-treated groups in this study could be a consequence of the inhibition of "splenomegaly" associated with tumor mass development.

### Inhibition of the activity of t-MDSCs by f-HSV through t-B cells

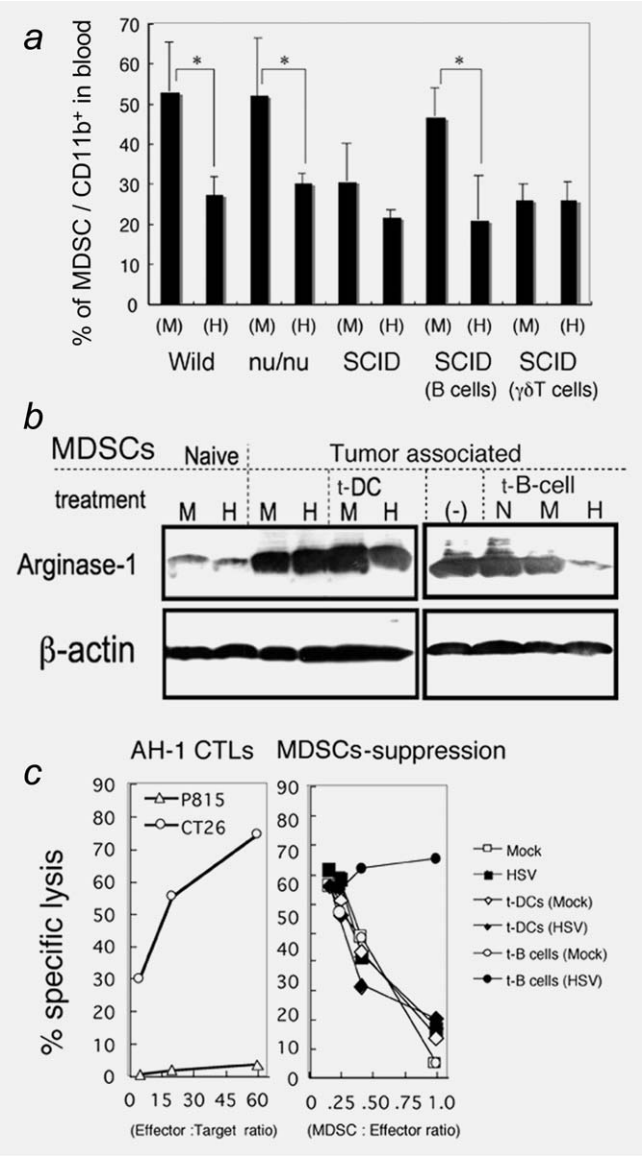
To analyze the mechanism(s) of decreases in t-MDSCs by f-HSV, Gr-$1^+$ CD$11b^+$ MDSCs in the blood were analyzed in the CT26-inoculated (*i.p.*) model using immunocomepetent mice (BALB/c), athymic nu/nu mice (lack of αβT cells and NKT cells), and SCID mice (lack of αβT cells, B cells, γδT cells, and NKT cells), after treatment with HSV-adjuvant. Expansion of t-MDSCs in the blood on day 15 after tumor CT26-inoculation (*i.p.*) was observed in the wild-type and nu/nu, but not in the SCID (Fig. 5*a*). Adoptive transfer of B cells, but not γδT cells, into the SCID led to expansion of the t-MDSCs during tumor progression. Moreover, treatment with HSV-adjuvant blocked the expansion of the t-MDSCs in this tumor model (Fig. 5*a*). These results indicate that the early expansion of t-MDSCs requires B cells *in vivo* and that the expansions are inhibited by HSV-adjuvant. The expansion of MDSCs in the blood might be detected in the relatively early stage of tumor development, while it might take a longer time to observe MDSC accumulation in spleen.

Next, to evaluate whether the inhibition of t-MDSCs by f-HSV involves DCs or B cells, the expression of arginase-1 in t-MDSCs were analyzed, because the arginase-1 expansion may be related to the suppressive activity of t-MDSCs against CTLs.[21–24] The t-B cells or t-DCs isolated from tumor CT26-inoculated (*i.p*) BALB/c were treated with Mock or f-HSV *in vitro*, and co-cultured with t-MDSCs. Then, the expression of arginase-1 in the t-MDSCs isolated from the mixed culture was evaluated by Western-blot analysis. A higher expression of arginase-1 was observed in the t-MDSCs as compared with that in naïve MDSCs, and the expression in the t-MDSCs was reduced by co-culture with f-HSV-treated t-B cells, but not f-HSV-treated t-DCs or f-HSV alone (Fig. 5*b*). No apparent difference in the arginase-1 expression in either the t-MDSCs or naïve-MDSCs was observed between the mock and f-HSV groups. In addition, the expression of arginase-1 in t-DCs or t-B cells was low, and did not change by treatment with f-HSV (data not shown). These results suggest that f-HSV may inhibit the activity of the t-MDSCs through t-B cells. We then analyzed whether t-B cells treated with f-HSV can inhibit the immunosuppressive activity of t-MDSCs against CTLs *in vitro*. In this analysis, $CD8^+$ T cells derived from the AH1/HSV-immunized CT26-bearing mice were used as CT26-specific CTLs. The CT26-specific CTLs specifically killed tumor CT26, but not tumor P815 (Fig. 5*c*). The MDSCs-suppression assay showed that AH1-pulsed t-MDSCs (isolated from CT26-inoculated mice) suppressed the tumor-killing activity of CT26-specific CTLs (Fig. 5*c*). Moreover, the immunosuppressive activity of t-MDSCs was blocked by co-culture with f-HSV-treated t-B cells, but not Mock-treated t-B cells, Mock- or f-HSV-treated t-DCs, Mock alone and f-HSV alone (Fig. 5*c*). These results indicate that f-HSV can

reduce the immunosuppressive ability of t-MDSCs *via* t-B cells.

### Secretion of sNRP-1 from B cells treated with f-HSV

To clarify the effect of f-HSV on the t-B cells in relation to the inactivation of t-MDSCs, we focused on the VEGF-mediated signaling since a recent report showed that $VEGF_{165}$ leads to the accumulation of MDSCs *via* VEGFR-2 in mouse blood and spleen.[28] Western-blot analysis revealed that both VEGF-A and NRP-1 (130 kD) were expressed at higher levels in t-B cells isolated from E.G7-inoculated mice than in naïve B cells (Fig. 6*a*). The densitometric analysis revealed that the production of VEGF-A in the t-B cells was 2.5-fold higher than that in naïve B cells. Interestingly, the secretion of sNRP-1 (90 kD) by t-B cells was increased by treatment with f-HSV *in vitro*. sNRP-1 was reported to function as an antagonist to VEGFR-2 signaling during tumor outgrowth.[30,31]




Our result in ELISA detected the increase of sNRP-1 in the culture supernatant of t-B cells ($2.74 \pm 0.21$ ng/$10^6$/h) and naïve B cells ($0.76 \pm 0.04$ ng/$10^6$/h) after f-HSV treatment (Fig. 6*b*). These results suggest that f-HSV treatment can change the transmembrane-type NRP-1 to sNRP-1 in B cells. Next, to investigate the effect of HSV *in vivo*, we analyzed the serum levels of sNRP-1 in the mice on day 1 after treatment with IFA or HSV-adjuvant on day 15 after CT26 inoculation, and demonstrated increase of the serum sNRP-1 by HSV, but not IFA (Fig. 6*c*). In particular, high levels of sNRP-1 were detected in the serum of tumor-rejected mice following HSV-adjuvant treatment.

### Inhibition of t-MDSCs by sNRP-1 and VEGF-A

To evaluate the effect of sNRP-1 on the immunosuppressive activity and survival of t-MDSCs isolated from E.G7-inoculated mice, the expression of arginase-1 and phospho (p)ERK-2 in the t-MDSCs were analyzed. ERK activation in VEGFR-2 signaling *via* VEGF-A and NRP-1 induces anti-apoptotic effect.[35] Western-blot analysis showed that the expressions of arginase-1 and pERK-2 in t-MDSCs were reduced by treatment with both recombinant sNRP-1 and

**Figure 5.** Inhibition of MDSCs activity by f-HSV through B cells. (*a*) The percentage of Gr-$1^+$ $CD11b^+$ MDSCs in the total population of blood $CD11b^+$ cells was analyzed by FACS on day 15 after CT26 ($5 \times 10^4$) *i.p.*-inoculation. The indicated each mice (BALB/c strain) on day 3 after CT26 *i.p.*-inoculation were immunized with HSV-adjuvant (equivalent to $10^8$ PFU/body) (H) or Mock (M) into the footpads ($n = 3$). Adaptive transfer of B cells ($7 \times 10^6$) or γδT cells ($7 \times 10^6$) into SCID mice (indicated as SCID (B cells) or SCID (γδT cells)) was performed one day before the *i.p.*inoculation of CT26. *, $p < 0.05$; *t*-test. Data indicates average ± SEM. (*b*) Western-blot analysis of arginase-1 expression in t-MDSCs. Naïve MDSCs (Naïve) were purified from naïve BALB/c. t-MDSCs, t-DCs and t-B cells were purified from BALB/c on day 15 after CT26 ($5 \times 10^4$)-*i.p.*-inoculation. The t-DCs and t-B cells were treated with Mock (M) or f-HSV (H) (equivalent to $10^7$ PFU/ml) for 2 h *in vitro*. Non-treatment (N) t-B cells were also prepared. Then, t-MDSCs were co-cultured for 2 hr with Mock (M), f-HSV (H), Mock-treated t-DCs (M), f-HSV-treated t-DCs (H), non-treated t-B cells (N), Mock-treated t-B cells (M) or f-HSV-treated B cells (H). The t-MDSCs were re-isolated from these mixed cell cultures, and analyzed by Western-blotting. (*c*) The immunosuppressive activity of t-MDSCs against CT26 CTLs was analyzed by the MDSCs-suppression assay as described in material and methods. Left panel shows the $^{51}$Cr-release assay of the CT26 CTLs against CT26 or P815. Right panel shows the the MDSCs-suppression assay using the same CT26 CTLs against CT26 (E:T ratio = 20). Mixture of t-MDSCs ($0.25-1 \times 10^5$/well) and Mock or f-HSV, mixture (ratio = 1) of t-MDSCs and t-DCs treated with Mock or f-HSV for 2 hr, or mixture (ratio = 1) of t-MDSCs and t-B cells treated with Mock or f-HSV for 2 hr were used in the MDSCs-suppression assay. The data represent two separate experiments with similar results.

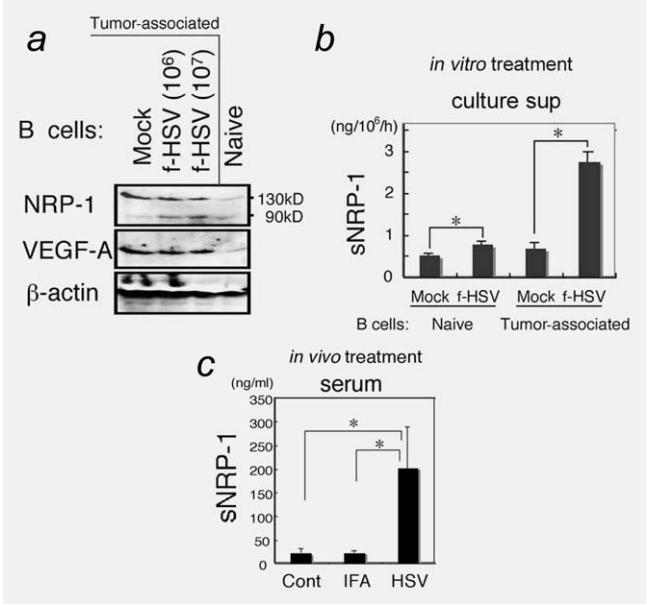



**Figure 6.** Increase of soluble-type neuropilin-1 (sNRP-1) in the B cells induced by f-HSV treatment. (*a*) Western-blot analysis for the expression of neuropilin-1 (NRP-1) and VEGF-A in naïve B cells and t-B cells after treatment with f-HSV (equivalent to $10^6$ PFU/ml or $10^7$ PFU/ml) or Mock *in vitro* for 2 hr. t-B cells were isolated from the E.G7 (*i.p.*)-bearing C57BL/6. Transmembrane-type NRP-1 (130 kD) and soluble-type (s)NRP-1 (90 kD) were detected after deglycosylation. (*b*) ELISA for sNRP-1 in the culture-supernatant of naïve B cells or t-B cells after treatment with Mock or f-HSV (equivalent to $10^7$ PFU/ml) for 2 hr *in vitro*. *, $p < 0.01$; *t*-test. Data indicates average + SD. The data represent two separate experiments with similar results. (*c*) ELISA for sNRP-1 in the serum of BALB/c mice on day 1 after administration of IFA or HSV-adjuvant (equivalent to $10^8$ PFU/body) on day 15 after CT26 inoculation and control serum of naïve BALB/c mice (Cont). *; $p < 0.05$; *t*-test. Data indicate average + SEM ($n = 3$). The data represent two separate experiments with similar results.

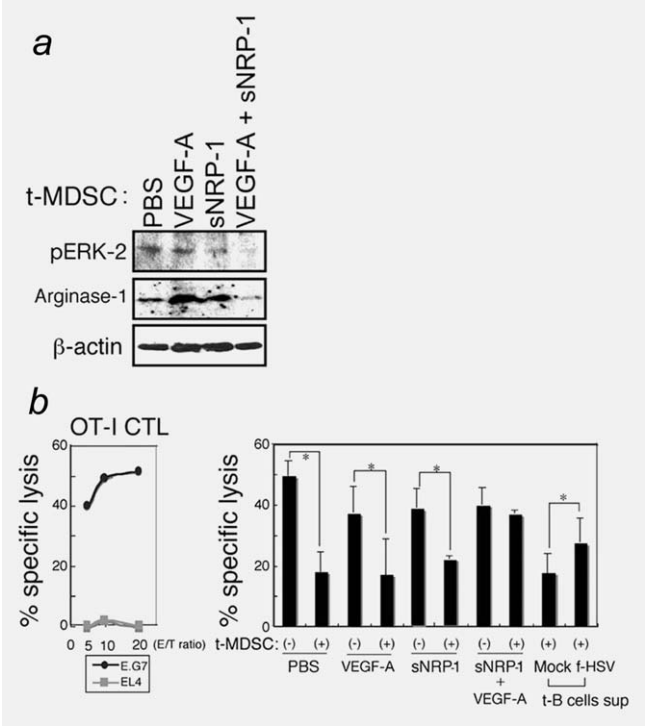



**Figure 7.** Inhibition of MDSCs activity by both sNRP-1 and VEGF-A. (*a*) Western-blot analysis of the expression of phospho (p)ERK-2 and arginase-1 in t-MDSCs after treatment with sNRP-1 (recombinant NRP-1/Fc; 10 ng/ml) and VEGF-A (recombinant VEGF-A; 10 ng/ml) for 2 hr *in vitro*. (*b*) The effect of VEGF-A, sNRP-1, and the culture-supernatant of t-B cells (t-B cells sup) treated with Mock or f-HSV on the t-MDSCs was analyzed by the MDSCs-suppression assay (Effector: Target ratio = 10 and MDSC: Effector ratio = 1), as described in material and methods. After t-B cells were isolated from E.G7 (*i.p.*)-bearing C57BL/6, the t-B cell sup was collected after f-HSV or Mock treatment *in vitro* for 2 h. Left panel shows the $^{51}$Cr-release assay of OT-I CTLs against E.G7 or EL4. Right panel shows the MDSCs-suppression assay using the same OT-I CTLs. *, $p < 0.01$; *t*-test. Data indicates average + SD. The data represent two separate experiments with similar results.

recombinant VEGF-A, but not by that with recombinant sNRP-1 or recombinant VEGF-A alone (Fig. 7*a*). The densitometric analysis normalized to the β-actin expression confirmed that the expression level of arginase-1 after sNRP-1 treatment did not differ significantly from that observed after PBS treatment. This result suggests that the inactivation of t-MDSCs by sNRP-1 require VEGF-A. sNRP-1 secreted from the t-B cells may induce apoptosis of MDSCs, consequently resulting in inhibition of MDSC expansion. Next, we then analyzed the effect of sNRP-1 and VEGF-A on the immunosuppressive activity of t-MDSCs against CTLs by the MDSCs-suppression assay. In this MDSCs-suppression assay, we used OVA as a model tumor Ag and $CD8^+$ T cells (OT-I CTLs) derived from the OT-I Tg as CTLs specific for $OVA_{257\text{-}264}$. The OT-I CTLs specifically killed tumor E.G7, but not EL4. $OVA_{257\text{-}264}$-pulsed t-MDSCs (isolated from E.G7-inoculated mice) suppressed the killing activity of OT-I CTLs *in vitro* (data not shown). The suppressive ability of t-MDSCs was reduced by both VEGF-A and sNRP-1, and also the culture-supernatant of t-B cells (t-B cells sup) treated with f-HSV (Fig. 7*b*). These results suggest that sNRP-1 and VEGF-A secreted from f-HSV-treated t-B cells may reduce the immunosuppressive ability of the t-MDSCs.

## Discussion

In this study, we showed that the HSV-adjuvant can enhance the antigen-specific CTL activity, and also slightly enhance $CD4^+$ T cell proliferation and Ab-production induced by vaccination with an antigen. Furthermore, f-HSV was also shown to enhance the antigen cross-presentation activity of myeloid DCs, lymphoid DCs, and pDCs *via* TLR2, but not TLR9 signaling. Although the antigen cross-presentation

activity was mainly enhanced in lymphoid $CD8^+$ DCs,[36] f-HSV was able to enhance antigen cross-presentation activity in all subtypes of DCs through TLR2, but not TLR9 signaling. A previous report showed that antigen cross-presentation of melanoma antigen in DCs was enhanced by HSV-1 VP22-fusion-MART-1 protein.[37] Although the relationship between TLR2 signaling and HSV-1 VP22 is still unclear, VP22 may be an important molecule of HSV-1 for enhancement of the antigen cross-presentation activity in DCs. On the other hand, living (active) HSV can inhibit the important signals for immune responses by intracellular HSV gene products in infected immune cells. HSV ICP47 and $U_S11$ inhibit the TAP-mediated loading of peptides on MHC class I molecules,[38] leading to down-regulation of MHC class I molecules on the surface of HSV-infected cells.[39] HSV $U_S3$ and $U_S5$ inhibit the production of granzyme B for CTL-killing activity in T cells.[40] f-HSV lacks infectious capacity because of formalin inactivation, therefore, its immunosuppressive functions might also be reduced.

Next, we showed that the early accumulation of t-MDSCs in spleen and blood during tumor progression was mediated by B cells. B cells play a crucial role in tumor progression through angiogenesis *via* VEGF-production[41] and also inhibit CTL-mediated antitumor immunity.[42] In this study, t-B cells showed higher expression levels of VEGF-A as compared with naïve B cells (Fig. 6*a*). Our results suggest that t-B cells may inhibit antitumor immunity *via* association with expansion of t-MDSCs, and also be involved in tumor angiogenesis through VEGF-A production at a high level.

The activation of t-MDSCs mediated by t-B cells was inhibited by HSV-adjuvant *in vivo*. The inactivation of t-MDSCs was shown to be involved in the sNRP-1 secretion from t-B cells treated with f-HSV. Moreover, we demonstrated that both sNRP-1 and VEGF-A are required to inhibit the immunosuppressive function of t-MDSCs. Yamada *et al.* reported a biological difference between the dimer of NRP-1 and monomer of sNRP-1.[43,44] Our data also suggest that the active signal in t-MDSCs may be induced by the VEGFR-2 dimer formation on the t-MDSCs binding to the NRP-1 dimer *via* VEGF-A. In contrast, the VEGF-A and monomer of sNRP-1 secreted from t-B cells following f-HSV treatment may bind to the VEGFR-2 monomer on the t-MDSCs, and then acts as an antagonist of VEGFR-2 signaling in the t-MDSCs. The affinity of VEGFR-2 may be stronger to the VEGF-A/sNRP-1 (monomer) complex than the VEGF-A/NRP-1(dimer) complex. Further studies of this affinity will be needed to clarify the precise mechanism(s) of suppression of the MDSCs by sNRP-1.

Additionally, we detected increase of the serum sNRP-1 following HSV-adjuvant treatment *in vivo* (Fig. 6*c*). The increase in the amount of sNRP-1 secreted following by HSV-adjuvant treatment is expected to cause not only MDSC inactivation, but also direct tumor mass inhibition.[30]

Oncolytic HSV-1 mutant that selectively replicate in actively dividing cells but not in terminally differentiated cells have already been applied for cancer therapy in the clinical setting.[45] We previously showed the effectiveness of a HSV-1 mutant for cancer immunotherapy.[46–49] Intratumoral injection of the HSV-1 mutant induces a strong antitumor immune response, resulting in inhibition of tumor growth. The antitumor immune response induced by the HSV-1 mutant was suggested to involve with DC activation. In this study, we demonstrated the efficacy of f-HSV as an adjuvant in cancer immunotherapy and also the mechanisms of immune modulation by f-HSV. To inhibit tumor growth, not only MDSC suppression, but also induction of CTL specific to the tumor antigen is required. Because of its efficacy and safety, it is expected that f-HSV will come to be employed in immunotherapy for the treatment of cancer patients.

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