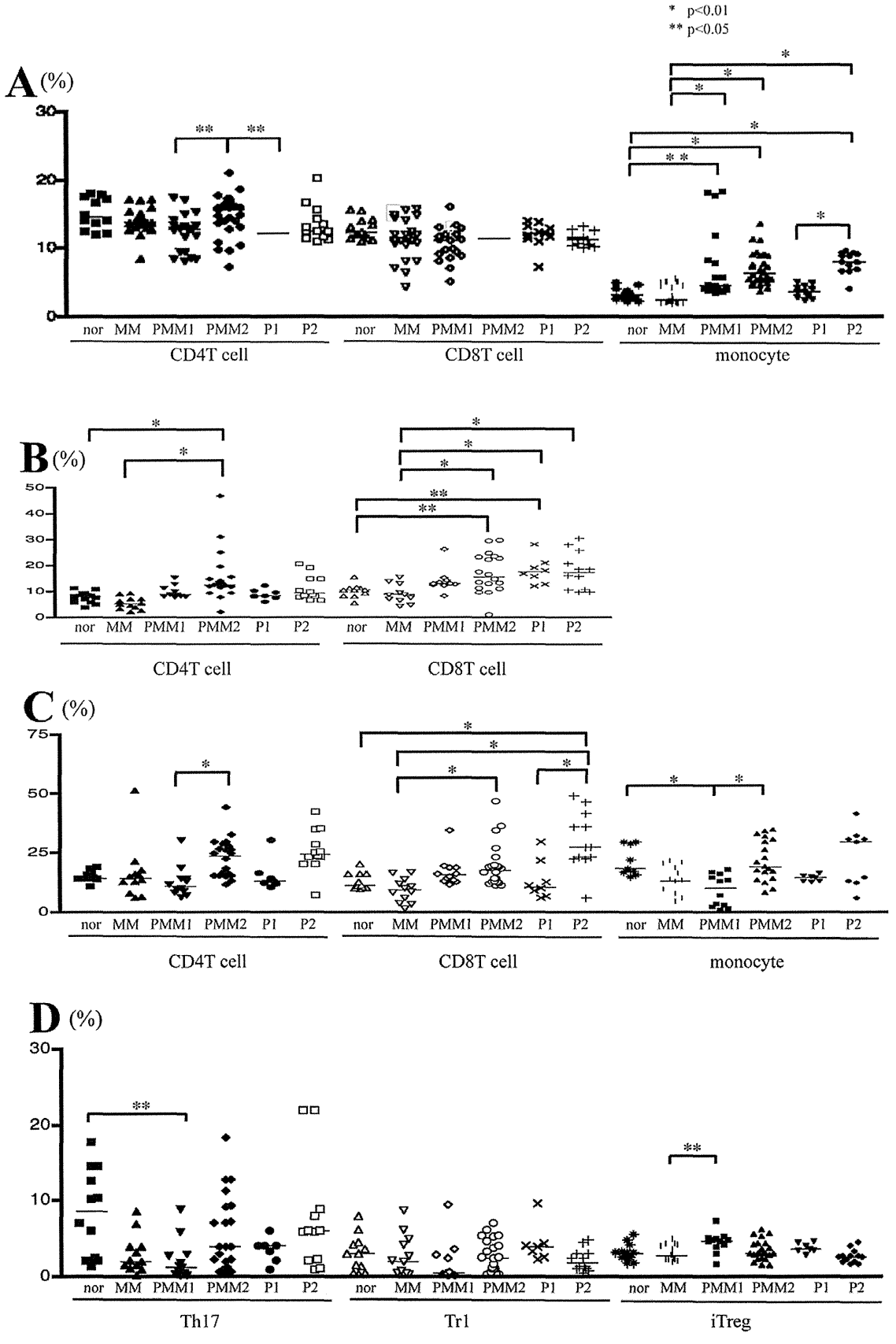
* p<0.01
** p<0.05
A (%)
B (%)
C (%)
D (%)
nor MM PMM1 PMM2 P1 P2
CD4T cell
CD8T cell
monocyte
Th17
Tr1
iTreg

Figure 2. Intracellular cytokine and Foxp3 expression in spleen cells. A, Phenotypical analysis: the ratios of the $CD4^+$ T cells to total lymphocytes, $CD8^+$ T cells to total lymphocytes and $CD14^+$ monocytes to total splenic mononuclear cells are shown. The percentage of $CD14^+$ monocytes was significantly increased in the PMM2, PMM1, and P2 groups compared to the MM and control groups, respectively. **B,** The percentage of IFN-γ^+CD4^+ and IFN-γ^+CD8^+ T cells among the total $CD4^+$ and $CD8^+$ T cells, respectively. In PMM2 mice, the percentage of both populations was significantly elevated compared to the MM and control mice. $CD8^+$IFN-γ^+ T cells were also increased in each of the P1 and P2 groups. **C,** The percentage of TNF-α^+CD4^+, TNF-α^+CD8^+ T cells and TNF-α^+ monocytes among the total $CD4^+$, $CD8^+$ T cells, and monocytes, respectively. In PMM2 mice, the percentage of TNF-α^+CD8^+ T cells was significantly elevated compared to MM. TNF-α^+CD8^+ T cells were also increased in the P2 group. **D,** The percentages of Th17, Tr1 and iTreg in $CD4^+$ T cells. The percentages of IL-17^+CD4^+ (Th17) cells and IL-10^+CD4^+ (Tr1) cells among the total $CD4^+$ cells, and the ratios of the Foxp$3^+CD4^+CD25^{high}$ T cells (iTreg) to $CD4^+$ T cells, were unchanged by *P. acnes* treatment.
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The percentages of IFN-γ^+CD4^+ and IFN-γ^+CD8^+ T cells among the total number of $CD4^+$ and $CD8^+$ T cells were significantly elevated in PMM2 mice compared with MM and control mice (Fig. 2B). $CD8^+$IFN-γ^+ T cells were also increased in the P1 and P2 groups. Similar results were observed in TNF-α expressing $CD8^+$ T cells (Fig. 2C). The ratio of IL-17^+CD4^+ (Th17) cells and IL-10^+CD4^+ (Tr1) cells among the total number of $CD4^+$ cells remained unchanged. The ratio of Foxp$3^+CD4^+CD25^{high}$ T (iTreg) cells was also not changed by *P. acnes* treatment (Fig. 2D).

Cytokine mRNA expression in skin lesions

Quantitative RT-PCR was performed to investigate the cytokine mRNA expression in the tumor lesions. The RNA expression of IFN-γ T-bet, IL-12p35, TNF-α and MIP2 was significantly increased in PMM2 and P2 mice compared with the MM group. No significant change was found in the mRNA expression of IL-12p40, IL-17 or IL-10 among the six groups (Fig. 3).

Characterization of tumor infiltrating lymphocytes

Single cell suspensions of TIL from PMM2 were analyzed. Abundant IFN-γ^+CD8^+ and IFN-γ^+CD4^+ cells were detected (Fig. 4). There was also an infiltration of TNF-α^+CD4^+ and TNF-α^+CD8^+ cells. On the other hand, Th17 and Tr1 cells were fewer in the TILs. The number of infiltrating cells was very limited in non-treated MM and could not be assessed.

Discussion

In the present study, the effects of intra-tumor *P.acnes* vaccination (ITPV) on the growth of melanoma skin lesions was evaluated, and found that the growth of seeded melanoma cells was suppressed. ITPV successfully controlled melanoma progression *in vivo* by an induction of Th1 type cytokines, including TNF-α and IFN-γ in both the skin and the systemic circulation. The clinical benefit of vaccination was associated with subcutaneous granuloma formation. Tumor cells were not detected in the granulomas. The measured tumor size was significantly decreased in the vaccine-treated group compared with the control groups. The tumor size may have been underestimated because of the granuloma volume. However, granuloma formation is an immunological event that is related to augmented phagocytic activity as well as cellular cytotoxic activity. Granuloma formation thus plays an important role in effective anti-tumor immunotherapy. In the present study, we found that ITPV promotes the activation of TNF-α and IFN-γ producing cells in the skin tumor lesions. IL-12, TNF-α and IFN-γ are known to be effective anti-tumor cytokines. However, individual cytokines are reported to exert only limited clinical effects, and thus they have been most commonly used in combination with chemotherapy. Unlike the *in vitro* study results, the effect of recombinant cytokine therapy *in vivo* is limited, in large part due to the very short biological half-life of recombinant cytokines. A branched-chain polyethylene glycol moiety attached interferon alfa-2a (peginterferon alfa-2a) has been used to prolong the biological half-life [12]. Previous studies have shown that *P. acnes* enhances the anti-tumor activity of monocytes/macrophages [13,14,15] and the tumoricidal function of NKT and NK against melanoma [15]. In ITPV, *P. acnes* is phagocytosed and processed by monocytes/macrophages, which are present inside and around the tumors, and the persistent secretion of cytokines and chemokines from these cells leads to granuloma formation.

IL-12 is an antitumor cytokine that activates NK and cytotoxic T cells, thereby promoting strong anti-tumor activity by inducing IFN-γ [16,17]. In the present study, we found increased local expression of IL-12p35 in ITPV. IL-12 is a heterodimeric cytokine containing IL-12p35 and IL-12p40 that binds to a specific receptor. On the other hand, free IL-12p40 forms sulfide-linked homodimers that block IL-12 function both *in vitro* and *in vivo* [18]. Enhanced expression of IL-12p35, but not IL-12p40, is suggested to have the potential to exert favorable therapeutic effects against tumors.

P. acnes binds to TLR2 on monocytes and dendritic cells, leading to activation of the IL-12 promoter [19]. IL-12 activates STAT-4 and T-bet transcription factors in T cells and NK cells. T-bet binds to the IFN-γ gene promoter and increases the production of IFN-γ [7]. In this study we found that IFN-γ induces cytotoxic effects by activating $CD8^+$ T cells, NK cells and B cells. It also induces chemokines, including CXCL9 (MIG) and CXCL10 (IP-10) that suppress vascular proliferation.

Recombinant IL-12 has been used as an anti-cancer therapy, but with unsuccessful results, eliciting systemic side effects and only limited clinical benefit. Local administration of IL-12 for therapeutic purposes has been suggested to improve the outcome in certain cancers. To limit the expression of IL-12 and prolong local IL-12 secretion, IL-12 plasmid vaccination has been administered for metastatic melanomas [20]. In this regard, ITPV has the advantage of persistently inducing IL-12 expression at the site of injection; this is followed by infiltration of TNF-α and IFN-γ producing T cells into the lesions, resulting in tumor suppression.

Granuloma formation is a characteristic feature of *P. acnes* vaccination, with the accumulation of monocytes being required for this activity. Potent IFN-γ expression occurs after *P. acnes* administration and leads to granuloma formation. Granuloma is a persistent source of Th1 type cytokines *in vivo*. An increase in MIP2 (CXCL2) was detected in *P. acnes*-treated skin lesions. This chemokine is secreted by monocytes and macrophages, and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells. MIP2 is one of the chemokines involved in granuloma formation. On the other hand, granuloma formation and ulceration have been considered as side effects in systemic anti-cancer vaccine trials. Since melanoma is a cutaneous malignancy, no special technique is required for accurate intralesional administration of the vaccine. Accumulated phagocytes in the granuloma may additionally contribute to the effective removal of tumor cells in combination with cytotoxic lymphocytes. Based on these observations, granuloma or ulceration is still considered to be relevant to successful cutaneous tumor immunotherapy.

In addition to its local effects, ITPV may also exert systemic anti-tumor activity. After ITPV, TNF-α and IFN-γ producing

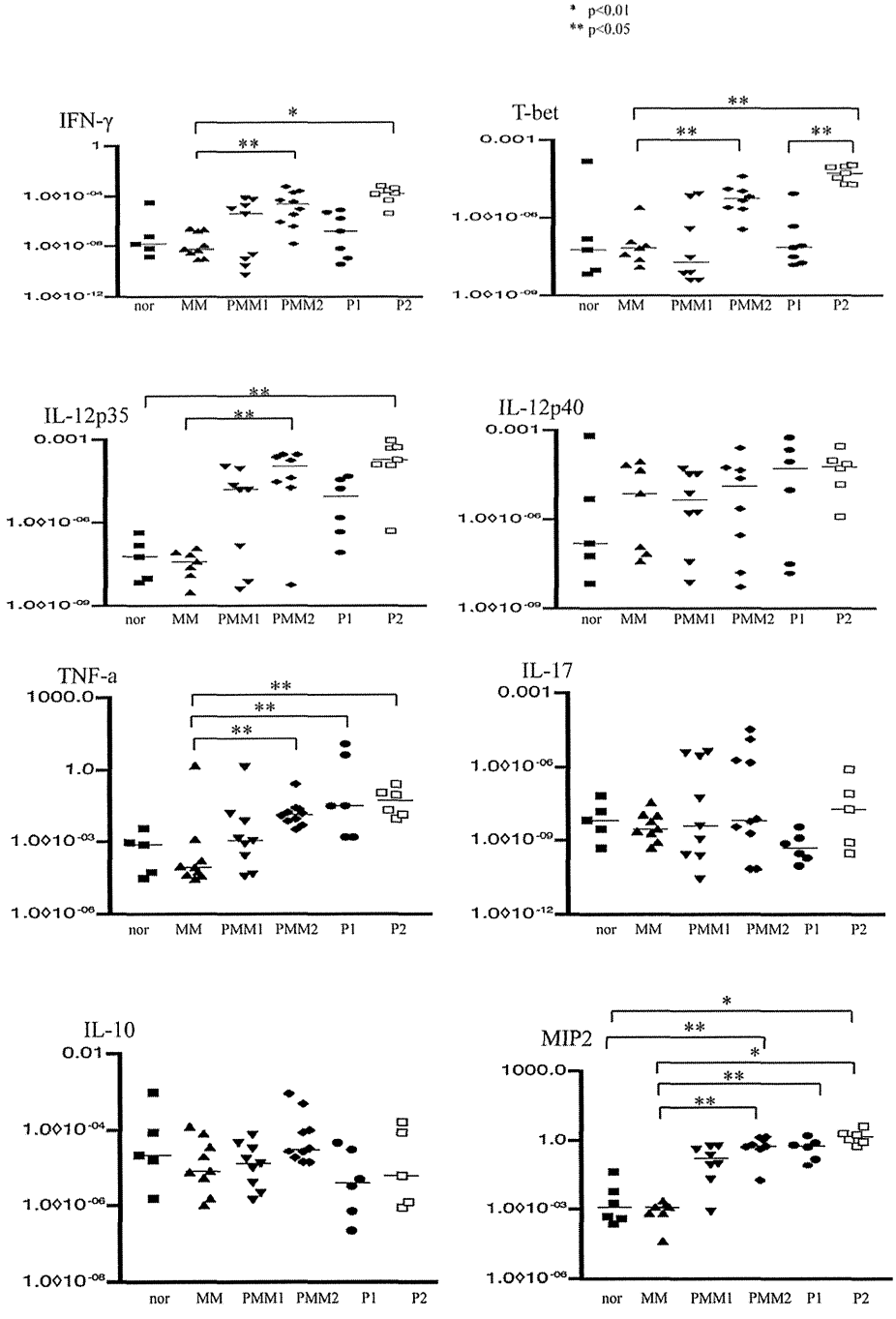

Figure 3. Cytokine mRNA expressions in the skin lesions. Quantitative RT-PCR was performed to determine the cytokine mRNA expression in the tumor lesions. The IFN-γ T-bet, IL-12p35, TNF-α and MIP2 mRNA levels in PMM2 and P2 mice were significantly increased compared to MM mice. No significant change was found in the IL-12p40, IL-17 or IL-10 mRNA levels among the six groups of mice.
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$CD8^+$ T cells were increased in the spleen and skin. However, melanoma-specific cytotoxicity of CD8 T cells was not increased in the spleen or draining lymph nodes in *P. acnes* injected melanoma-bearing mice (Fig. S1), suggesting that most of the cytotoxic CD8 T cells may be recruited into the injected skin lesions. Consistent with the previous reports [21,22], the number of splenic monocytes was also increased by ITPV. The role of the systemic immune response in the mechanism of distant metastasis remains unclear. Previous studies have suggested that augmentation of anti-tumor cytokine expression in spleen cells have preventive effects against distant metastasis.

In the present study, mice received either a single dose or two doses of vaccination. We injected *P. acnes* on day 0 and/or on day 14 into the cutaneous tumor lesions. Even the single therapy on

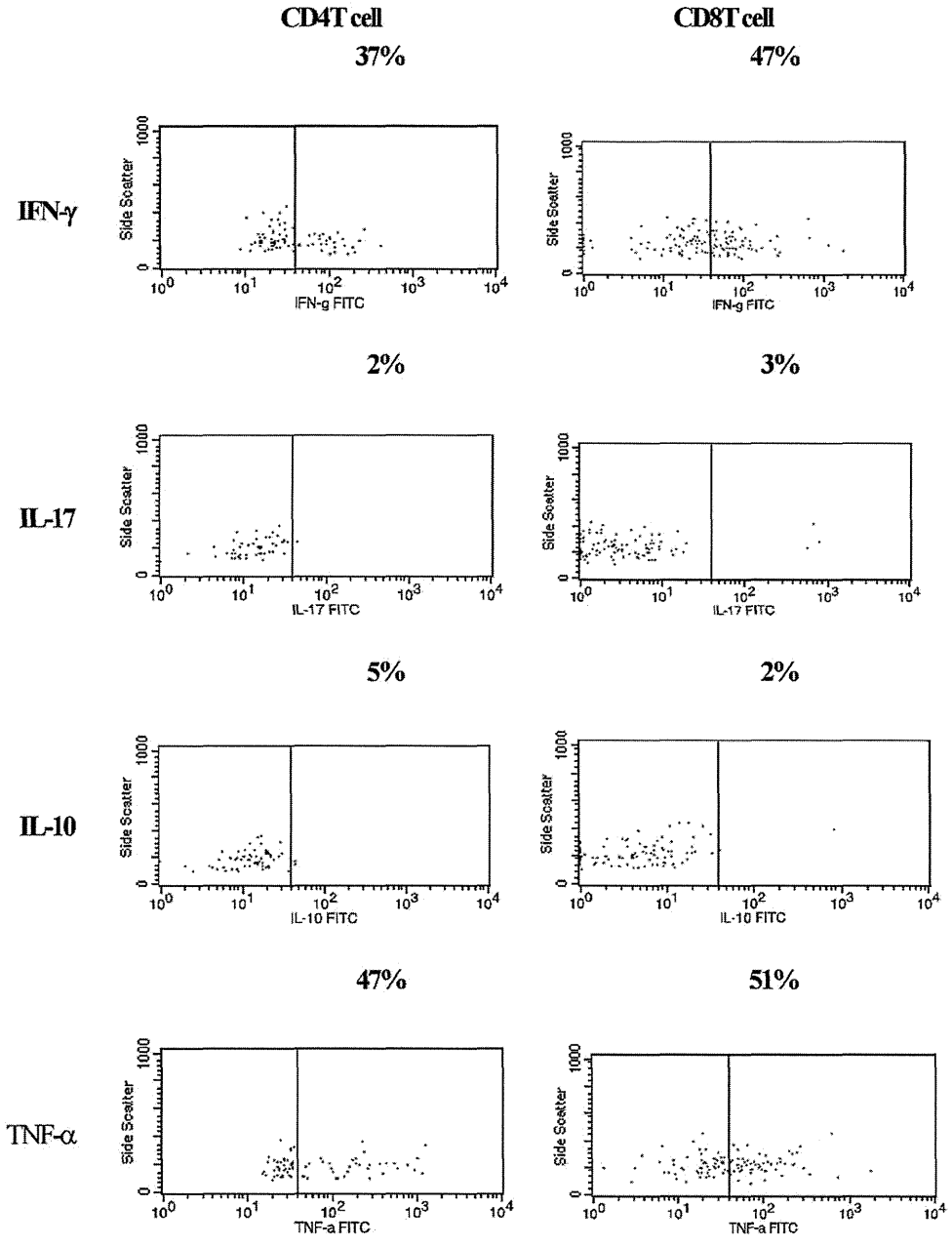

Figure 4. Characterization of the tumor infiltrating lymphocytes. One half of tumor infiltrating T cells from PMM2 tumor lesion were TNF-α producing cells. IFN-γ^+CD4$^+$ and IFN-γ^+CD8$^+$ T cells were also present in the tumor. By contrast, very few Th17 cells or Tr1 cells were detected in TILs. The number of infiltrating cells in the non-treated melanoma tumors was very limited, and thus was not analyzable. Representative results from five experiments are shown.
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day14 significantly suppressed the growth of melanoma cells. However, priming on day 0 followed by a second vaccination on day 14 resulted in a more potent growth inhibitory activity compared to the single vaccination. Early intervention before full tumor development may have the advantage of inducing enhanced Th1 type anti-tumor activity compared to vaccination after established melanoma growth. A second vaccination induced a booster effect on the activation of the cytokine network.

Th17, Tr1 and iTreg cells play critical roles in the regulation of the immune system. In the present study, *P. acnes* vaccination shifted the Th1/Th2 balance toward a dominant Th1 immune response. Th17 is involved in Th1-associated diseases such as psoriasis. However, we found no changes in the protein or RNA expression of IL-17 in the present immunotherapy, as IL-17 was undetectable in TIL. Therefore, it is unlikely that Th17 was involved in the beneficial effect of *P. acnes* vaccination therapy.

Tr1 and iTreg cells are known to regulate the inflammatory response. Tr1 regulatory cells produce IL-10 and play a critical role in the suppression of allergic diseases [11,23]. It was reported that *P. acnes* therapy increases iTreg cells by stimulating TLR2 in

Th2-mediated diseases [19,24]. By contrast, suppression of iTreg cells has been associated with successful cancer immunotherapy [25]. Interestingly, neither Tr1 cells nor iTreg cells were elevated in the present study, suggesting that they are not involved in the mechanistic effect of this therapy. Unlike allergic mice, which have a Th2 dominant response, cancer-bearing mice may have different immunological backgrounds in response to *P. acnes* vaccination. Further investigation is required to clarify the precise mechanism of the *P. acnes* mediated immune responses.

In conclusion, the results of this study showed that ITPV successfully suppresses MM, and that the beneficial effect of this therapy depends on the induction of granuloma formation along with the secretion of IL-12, IFN-γ and TNF-α. Further investigation is required before this treatment comes into use in clinical practice. *P. acnes* vaccine is a promising candidate as an adjuvant therapy of melanoma.

Supporting Information

Figure S1 The cytotoxicity of CD8$^+$ T cells prepared from spleen or draining lymph node was analyzed using three methods. The first method is chromium release assay, and the second is viability detection by flow cytometry using Live/Dead cell-mediated cytotoxicity kit (Molecular probes, Carlsbad, CA). Finally DHL cell cytotoxicity assay kit (AnaSpec Corporate Headquarter, San Jose, CA) was used to detect the release of Lactate Dehydrogenase (LDH) from targeted melanoma cells. Spleen and draining lymph node samples were taken from melanoma and *P. acnes*-injected mice: melanoma cell was free in the dorsal skin, *P. acnes* only injected mice, and normal control mice. Single cell suspensions were prepared by mechanical mincing, and after passing through a 70-μm-pore mesh, the cells are washed and resuspended in PBS. After Ficoll separation, the cells were washed and resuspended in RPMI1640 medium containing 10% FBS. CD8 T cells were purified using magnetic beads, and co-cultured with B16 melanoma cells at three different effector cell/target cell ratio (12.5:1, 25:1, 50:1) according to previous reports. Chromium release assay **A,** 6 hours incubation LN CD8 T cells. **B,** 6 hours incubation splenic CD8 T cells. **C,** 15 hours incubation LN CD8 T cells. **D,** 15 hours incubation splenic CD8 T cells. When there was injury of targeted melanoma cells, chromium was released. Analysis of apoptotic melanocytes using live/dead viability detection system by flow cytometry **E,** 8 hours incubation LN CD8 T cells. **F,** 8 hours incubation splenic CD8 T cells. Analysis of lactate dehydrogenase (LDH) released from targeted melanoma cells **G,** 8 hours incubation LN CD8 T cells **H,** 8 hours incubation splenic CD8 T cells. Melanoma and *P. acnes*-injected mice: ●, *P. acnes* only injected mice: ■, and normal control mice: ▲. CD8 T cells melanoma-specific cytotoxicity was not increased in *P. acnes* injected melanoma-bearing mice, suggesting that most cytotoxic CD8 T cells was recruited into the injected skin lesions.
(TIFF)

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Author Contributions

Conceived and designed the experiments: KY HM. Performed the experiments: KT WL YM TA TN HK. Analyzed the data: MK IK. Contributed reagents/materials/analysis tools: KT WL YM TA TN HK. Wrote the paper: KY ECG HS HM.

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UV irradiation of immunized mice induces type 1 regulatory T cells that suppress tumor antigen specific cytotoxic T lymphocyte responses

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We previously showed that exposure to UV radiation after immunization suppresses Th1 and Th2 immune responses, leading to impaired Ab and allo-immune responses, but the impact of UV radiation after immunization on anti-tumor immune responses mediated by tumor-specific $CD8^+$ T cell responses remains less clear. Furthermore, the exact phenotypic and functional characteristics of regulatory T cell population responsible for the UV-induced immunosuppression still remain elusive. Using the MBL-2 lymphoma cell line engineered to express OVA as a surrogate tumor Ag, here we demonstrate that UV irradiation after tumor Ag-immunization suppresses the anti-tumor immune response in a manner dependent on the immunizing Ag. This suppression was mediated by interleukin (IL)-10 released from $CD4^+CD25^+$ T cells, by which impaired the induction of cytotoxic T lymphocytes (CTL) able to kill Ag-expressing tumor cells. In addition, we generated a panel of T cell clones from UV-irradiated and non-irradiated mice, and all of the clones derived from UV-irradiated mice had a Tr1-type regulatory T cell phenotype with expression of IL-10 and c-Maf, but not Foxp3. These Tr1-type regulatory T cell clones suppressed tumor rejection *in vivo* as well as Th cell activation *in vitro* in an IL-10 dependent manner. Given that suppression of Ag-specific CTL responses can be induced in Ag-sensitized mice by UV irradiation, our results may imply that exposure to UV radiation during premalignant stage induces tumor-Ag specific Tr1 cells that mediate tumor-Ag specific immune suppression resulting in the promotion of tumor progression.

Tumor Immunology

UV radiation is regarded as one of the most significant environmental factors affecting human health. In addition to the direct effect of UVB on DNA that induces specific gene mutations,[1] several lines of evidence indicate that the immune suppressive effects of UV radiation are involved in skin cancer development by impairing tumor immune responses that can destroy developing skin tumors.[2] Skin tumors developed in UV-irradiated host are highly immunogenic and destroyed when injected in normal syngeneic hosts, but they grow progressively in immunosuppressed or UV-irradiated recipients.[3,4] It has been reported that UV irradiation impairs the ability of antigen-presenting cells (APCs) to induce protective antitumor immune responses.[5–7] It also has been reported that a potential correlation between UV-irradiation and risks of not only melanoma but also non-Hodgkin's lymphoma and colon cancer.[8,9] In addition, exposure to UV radiation significantly impairs resistance to various infectious agents such as bacteria, parasites, viruses and fungi.[10,11] Paradoxically, these undesirable effects of UV radiation on immune responses are used therapeutically in patients with cutaneous T cell lymphoma, autoimmune diseases and graft-*vs.*-host disease,[12,13] and may be useful in patients after organ transplantation and those with asthma.[14–16] Several groups, including our own, have demonstrated that UV-induced regulatory T cells include $CD3^+DX5^+$ NKT cells,[17] $CD4^+CD25^+$ T cells coexpressing CTLA-4, glucocorticoidinduced TNF-related protein (GITR) and neuropilin-1,[18] $CD4^+Foxp3^+$ T cells[19,20] and $CD4^+Foxp3^-$ T cells.[21] However, the difficulties associated with discrimination of these cells based on the molecules that are also expressed on effector T cells impeded previous attempts to fully characterize UV-induced regulatory T cells.

Key words: IL-10, regulatory T cell, Tr1, UV, CTL
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In most studies of UV-induced immunomodulation, naive animals are exposed to UV radiation before immunization, and only a few studies have examined immunosuppressive effects of UV irradiation after immunization.[22,23] We recently showed that mice exposed to UV irradiation 1 week after immunization exhibited reduced Th1- and Th2-driven Ab responses and prolonged allograft survival in an Ag-specific manner.[15,16,21] In addition, we showed that UV irradiation after immunization led to the generation of $CD4^+$ T cells producing interleukin (IL)-10 and interferon (IFN)-γ, *i.e.*, Tr1-type regulatory T cells, and Ag-dependent secretion of IL-10 was responsible for the observed immunosuppression. However, a fraction of the generated $CD4^+$ T cells was also $Foxp3^+$ T cells capable of suppressing a variety of immune responses.[24] Moreover, it is increasingly clear that precancerous cells and malignant cells are eliminated by immune system before they develop detectable tumors,[25] which also might be negatively regulated by regulatory T cells induced by UV irradiation. Therefore, we wished to determine whether UV radiation after immunization have impaired $CD8^+$ cytotoxic T lymphocytes (CTL) mediated anti-tumor immune responses, which might promote tumor progression, and, if so, whether $CD4^+Foxp3^-$ T cells are responsible for this suppression. Using an OVA-expressing tumor cell line, we show in our study that UV irradiation of OVA-immunized animals promotes tumor survival in an Ag-specific manner. In addition, we found that IL-10 produced by $CD4^+CD25^+$ T cells in these mice suppresses the generation of tumor-specific CTL. Furthermore, we established a panel of T cell clones from UV-irradiated mice and clearly demonstrate that UV irradiation induces the development of Tr1-type regulatory T cells expressing the transcription factor c-Maf, but not Foxp3, and these cells mediate UV-induced suppression of tumor immunity. Potential relevance of present findings to the promotion of tumor progression in premalignant stage will be also discussed in the context of tumor immunosurveillance.

Material and Methods

Mice

Female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan) and maintained under specific pathogen free conditions at the Institute for Laboratory Animals of Mie University. Mice were 8–10 weeks old at the beginning of each experiment. All experiments were approved by the Ethics Committee for Animal Experimentation at Mie University.

Tumor cell lines

MBL-2 is a Moloney MuLV-induced lymphoma cell line of C57BL/6 origin that does not express major histocompatibility complex (MHC) class II antigen. MBL-2 cells expressing OVA (MBL-2/OVA) were generated by transfection of pcDNA3.1 (Invitrogen) containing the cDNA encoding full-length OVA protein. Expression of OVA was confirmed by anti-OVA Ab staining. EL-4 is a thymoma cell line of C57BL/6 origin. All cell lines were cultured in RPMI1640 containing 10% FCS, 5×10^{-5} M 2-ME, 100 μg/ml streptomycin and 100 U/ml penicillin.

In vivo tumor growth

Mice were injected s.c. with $1–5 \times 10^6$ tumor cells. Tumor growth was monitored twice a week, and tumor size was determined as the mean length of two right-angled diameters measured using microcalipers.

Immunization, UV-irradiation and adoptive cell transfers

Mice were immunized i.p. with 100 μg OVA (Grade V) or hen egg-white lysozyme (HEL), both obtained from Sigma Aldrich, emulsified with 100 μl of IFA (Gibco BRL). The UV source was a bank of three unfiltered UV lamps (UVP, CA) with a 280–350 nm emission spectrum of which 67% was UV-B. After their dorsal fur was clipped (10 cm^2), mice were exposed to 23 kJ/m^2 of UV radiation 1 week after immunization as described.[16,21] Control mice were treated similarly but were not exposed to UV radiation. T cell subpopulations or T cell clones in were transferred via tail vein into recipient mice that had been immunized with OVA in IFA 7 days previously. In some experiments, mice were injected i.p. with 100 μg of anti-IL-10 (JES5-2A5).

Cell preparation

$CD4^+$ T cells and $CD8^+$ T cells were prepared from total splenocytes as described.[21,26] T cell-depleted spleen (TDS) cells were prepared from untreated syngeneic mouse splenocytes as described,[27] treated with 50 μg/ml mitomycin C (MMC; Kyowahakko, Co.), and used as APCs.

Establishment of T cell clones

$CD4^+$ T cells (5×10^6 cells/ml) from OVA-immunized and irradiated or sham-treated mice were cultured with OVA (100 μg/ml) and MMC-treated syngeneic spleen cells (1×10^6 cells/ml) in the presence of IL-2 (10 U/ml of recombinant human IL-2, Ajinomoto Co.). After 10 days, T cell clones were established from these cultures by limiting dilution. T cell clones that grew in the presence, but not absence, of OVA were further maintained by Ag stimulation and cultivation in the medium containing 10 U/ml IL-2. T cell clones were used for experiments at least 10 days after the last Ag stimulation.

ELISPOT assay

The number of IFN-γ secreting OVA-specific $CD8^+$ T cells was assessed by ELISPOT assays as previously described.[26] Briefly, purified $CD8^+$ T cells (1×10^5 cells) were cultured for 24 hr with 1×10^5 MMC-treated MBL-2/OVA or syngeneic TDS together with OVA in 96-well nitrocellulose-backed 96-well plates pre-coated with rat anti-mouse IFN-γ (R4-6A2, BD Pharmingen). Spots were developed using biotinylated anti-mouse IFN-γ (XMG1.2, BD Pharmingen), alkaline

phosphatase-conjugated streptavidin (Vector Labs) and alkaline phosphatase substrate (5-bromo-4-chloro-3-indolyl-phosphate, Sigma Aldrich).

Cytotoxic assay

Purified $CD8^+$ T cells (1×10^6 cells/ml) were cultured with MMC-treated syngeneic spleen cells (2×10^6 cells/ml) as feeder cells and MBL-2/OVA (1×10^5 cells/ml) in the presence of recombinant human IL-2 (20 U/ml, Ajinomoto). After 7 days of culture, cells were harvested and their cytotoxicity was determined by a standard 4-hr ^{51}Cr-release assay, as described.[28]

In vitro regulatory assay

$CD4^+$ T cells isolated from spleens of mice immunized 14 days previously (OVA-Th) at 2×10^5 cells/150 μl/culture were cultured alone or mixed with 1×10^5 cells of UP4-7, a T cell clone from UV-irradiated mice, in the presence of 3×10^5 TDS and 100 μg/ml OVA in a 96-well plate. Proliferation of the $CD4^+$ T cells was evaluated by pulsing with 0.5 μCi ^{3}[H]-TdR for the last 6 hr of the 72-hr culture. To assess whether UP4-7 cells function as regulatory cells through direct cell contact or through release of soluble factors, transwell experiments were performed. $CD4^+$ T cells from OVA-immunized mice were seeded at 2×10^6 cells/1 ml/culture in the lower chamber of a 24-well plate. UP4-7 cells (1×10^6 cells/well) were either cultured in the lower chambers directly in contact with the target cells or in the upper chambers separated from the target cells by a 0.4 μm-pore membrane (CORNING), which allows the diffusion of small molecules, such as cytokines, but not cells. Both chambers contained MMC-treated TDS (1×10^6 cells) and 100 μg/ml OVA. On day 4, 100 μl of cell suspension from the lower chambers was transferred to wells of a 96-well plate, and the proliferative response was evaluated by pulsing with 0.5 μCi ^{3}[H]-TdR for 12 hr.

Reverse transcription polymerase chain reaction

Total cellular RNA was reverse transcribed into cDNA and IL-4, IL-10, IFN-γ, TGF-β, Foxp3, c-Maf, and hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA accumulation were analyzed by amplification of the target sequence using number of cycles within the linear range of exponential amplification as described.[21] Primer sequences for the amplification of c-Maf mRNA were: forward, 5′-GTGC AGCAGAGA-CACGTCCT-3′; and reverse, 5′-CAACTAGCA AGCC-CACTC-3′, and those for other mRNAs were as described.[21]

Cytokine enzyme-linked immunosorbent assay

Levels of IL-4, IFN-γ and IL-10 in culture supernatants were assayed by enzyme-linked immunosorbent assay (ELISA) as described.[29]

Statistics

Data shown are either representative of multiple experiments or display the combined data of all experiments as indicated in the figure legends. Bar graphs and error bars show mean values and standard deviation (SD), respectively. All statistical analyses were performed using StatView (SAS Institute) software. Differences between groups of more than three were analyzed by the one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test. The frequencies of tumor rejection were compared by chi square test. Single measurement comparison between two groups was evaluated by the two-tailed Student's t-test. A p value of < 0.05 was considered significant. All experiments were performed at least twice.

Results

UV irradiation after immunization leads to Ag-specific suppression of anti-tumor immunity

We previously showed that UV irradiation given after immunization suppresses Ab and alloimmune responses against immunizing Ags,[15,16,21] but it remained unknown whether these observations were also true for anti-tumor immune responses. Using the MBL-2 lymphoma cell line engineered to express OVA as a surrogate tumor Ag (MBL-2/OVA), we assessed the effect of UV-irradiation given after OVA immunization on the growth of transplanted tumors. C57BL/6 mice were immunized with OVA on day 0, underwent UV irradiation on day 7, and were inoculated s.c. with MBL-2/OVA or MBL-2 on day 14. As shown in Figure 1*a* and Table 1, when MBL-2 or MBL-2/OVA cells were implanted into unimmunized, non-irradiated mice, some animals rejected implanted tumors while other did not. When animals were immunized with OVA before implantation, rejection of MBL-2/OVA cells was significantly enhanced while that of MBL-2 cells was unaffected. In contrast, UV irradiation significantly inhibited rejection of both tumor cell lines in unimmuized mice. Immunization with OVA before UV irradiation led to a further decrease in rejection of MBL-2/OVA cells, but not MBL-2 cells. Thus, these data argue that UV irradiation after immunization leads to an Ag-specific suppression of the anti-tumor immune response.

UV irradiation after immunization suppresses the Ag-specific $CD8^+$ cell responses

$CD8^+$ CTL play an essential role in the control of tumor growth including MBL-2 tumor,[30,31] therefore, we next examined whether UV irradiation of OVA-immunized mice suppressed the generation of effector CTL capable of killing MBL-2/OVA tumor. $CD8^+$ T cells from OVA-immunized and MBL-2/OVA challenged mice efficiently lysed MBL-2/OVA cells *in vitro* but not unrelated EL-4 cells. In contrast, $CD8^+$ T cells isolated from OVA-immunized, UV-irradiated and MBL-2/OVA-challenged mice exhibited significantly reduced cytotoxic activity against MBL-2/OVA cells (Fig. 1*b*). Consistent with this, the number of IFN-γ^+ cells in $CD8^+$ T cell fraction from UV-irradiated mice after *in vitro* stimulation with OVA in the presence of APC or MBL-2/OVA were

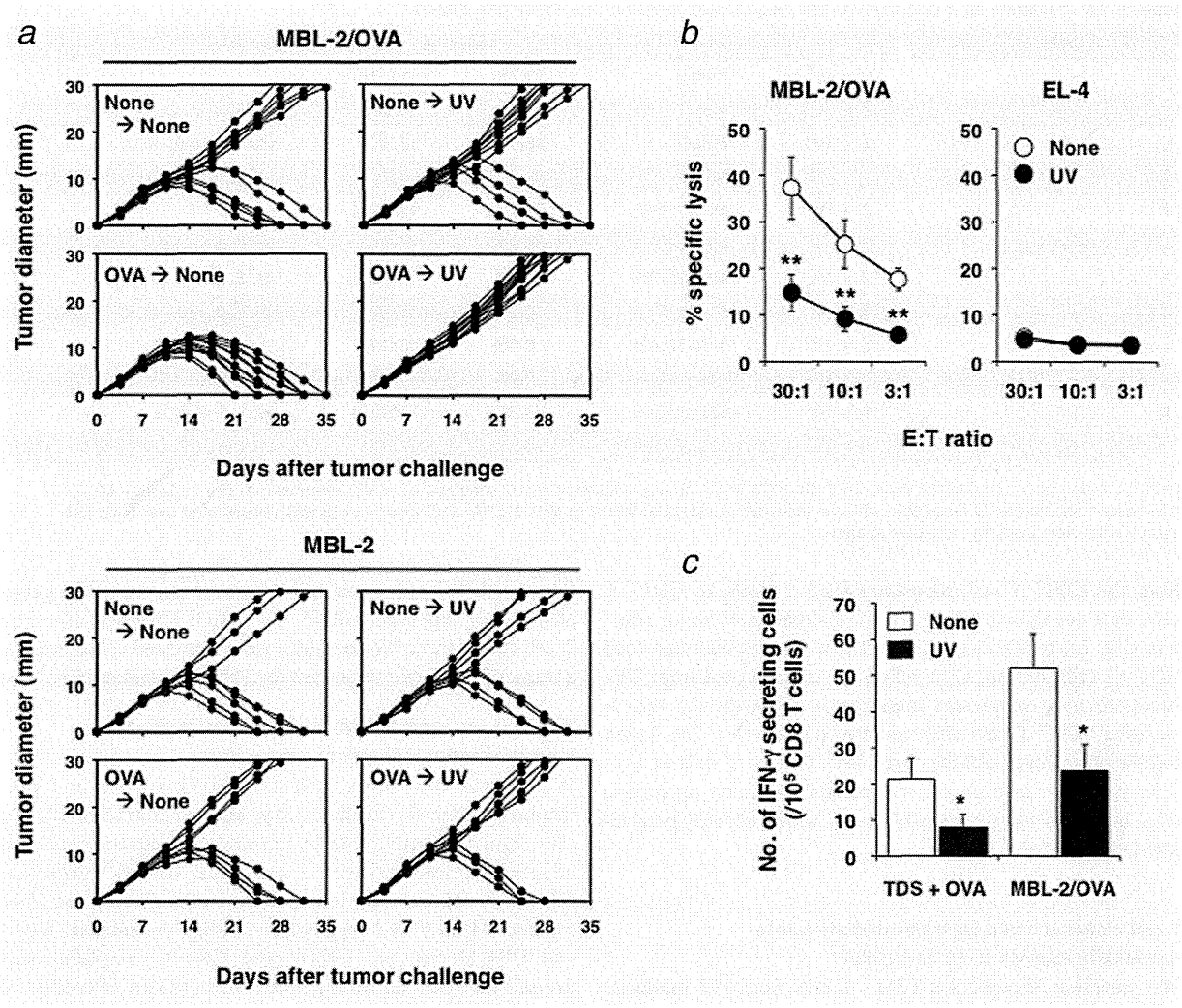


Figure 1. UV irradiation after immunization impairs the Ag-specific anti-tumor response. (*a*) Groups of mice ($n = 10$) were immunized with OVA (OVA) or PBS (None) in IFA on day 0, UV (UV) or mock (None) irradiated on day 7 and inoculated with 2×10^6 MBL-2/OVA (upper panels) or 1×10^6 MBL-2 cells (lower panels). Tumor size was monitored twice a week. (*b* and *c*) Groups of mice ($n = 3$–5) were immunized with OVA in IFA on day 0, UV- or mock-irradiated on day 7 and inoculated with 2×10^6 MMC-treated MBL-2/OVA on day 14. On day 21, $CD8^+$ T cells were isolated from these mice and individually assayed for cytolytic activity against MBL-2/OVA or EL-4 by ^{51}Cr release (*b*) and IFN-γ production after cultivation with T cell-depleted spleen (TDS) cells plus OVA or MBL-2/OVA by ELISPOT (*c*) in triplicate cultures. The results are expressed as mean ± standard deviation (SD) of between animals ($n = 3$ in A and $n = 5$ in B per group). Closed circles and bars, $CD8^+$ T cells from UV-irradiated mice; open circles and bars, $CD8^+$ T cells from mock-irradiated mice. Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test in *b* and Student's *t*-test in *c*. n.s., not significant; $*p < 0.05$; $**p < 0.01$. The data shown are representative of two to three experiments with similar results.

also significantly reduced as compared to those from non-irradiated mice (Fig. 1*c*).

Transfer of $CD4^+$ T cells from mice exposed to UV after immunization suppresses $CD8^+$ T cell-mediated anti-tumor immune response

To determine whether $CD4^+$ T cells were responsible for observed suppression of anti-tumor immunity, we adoptively transferred different T cell populations isolated from OVA-immunized and UV-irradiated mice into recipient mice that had been immunized with OVA. Although the mice immunized with OVA efficiently rejected MBL-2/OVA tumors, the adoptive transfer of whole T cells and $CD4^+$ T cells, but not $CD8^+$ T cells, from OVA-immunized and UV-irradiated mice completely suppressed MBL-2/OVA tumor rejection (Fig. 2*a*, left). In addition, co-administration of anti-IL-10 at the time of $CD4^+$ T cell transfer completely abrogated the loss of immune rejection (Fig. 2*a*, right). Furthermore, depletion of $CD25^+$ cells

Table 1. UV irradiation after OVA immunization suppresses rejection OVA-expressing tumors

	OVA	UV	Tumor cells		Rejection		Statistics
A	–	–	1×10^6	MBL-2	73.3%	(11/15)	
B	–	+	1×10^6	MBL-2	40.0%	(6/15)	*vs.* A, $p < 0.01$
C	+	–	1×10^6	MBL-2	58.3%	(7/12)	*vs.* A, $p = 0.19$
D	+	+	1×10^6	MBL-2	33.3%	(4/12)	*vs.* B, $p = 0.29$; *vs.* C, $p < 0.01$
E	–	–	2×10^6	MBL-2/OVA	54.2%	(13/24)	
F	–	+	2×10^6	MBL-2/OVA	29.2%	(7/24)	*vs.* E, $p < 0.01$
G	+	–	2×10^6	MBL-2/OVA	100.0%	(24/24)	*vs.* E, $p < 0.01$
H	+	+	2×10^6	MBL-2/OVA	0.0%	(0/24)	*vs.* F, $p < 0.01$; *vs.* G, $p < 0.01$
I	–	–	5×10^6	MBL-2/OVA	0.0%	(0/12)	
J	–	+	5×10^6	MBL-2/OVA	0.0%	(0/5)	
K	+	–	5×10^6	MBL-2/OVA	83.3%	(10/12)	*vs.* I, $p < 0.01$
L	+	+	5×10^6	MBL-2/OVA	16.7%	(2/12)	*vs.* J, $p < 0.05$; *vs.* K, $p < 0.01$

C57BL/6 were either untreated or immunized with OVA in IFA on day 0 followed by UV irradiation or mock treatment on day 7. On day 14, these mice were subcutaneously inoculated with the indicated numbers of MBL-2 or MBL-2/OVA, and tumor growth was documented over time. Chi-square tests were used for statistical analysis.

from the $CD4^+$ T cell population (12.3 ± 0.8% of $CD4^+$ T cells, data not shown) also led to the restoration of tumor rejection (Fig. 2*a*, right). Finally, ELISPOT assays revealed that total $CD4^+$ T cells, but not $CD4^+CD25^-$ T cells, isolated from UV-irradiated mice suppressed induction of OVA-specific IFN-γ-secreting $CD8^+$ T cells after adoptive transfer (Fig. 2*b*). Taken together these data indicate that $CD4^+$ $CD25^+$ T cells in UV-irradiated mice mediate the suppression of tumor Ag-specific CTL induction via secretion of IL-10, which allowed progressive tumor growth.

T cell clones derived from UV-irradiated mice universally express IL-10 and c-Maf

We previously showed that $CD4^+$ T cells from UV-irradiated mice produce more IL-10 and less IFN-γ compared to $CD4^+$ T cells isolated from non-irradiated mice. These $CD4^+$ T cell populations contained $Foxp3^+$ T cells, but multicolor FACS analysis revealed that $CD4^+Foxp3^-$ population was responsible for IL-10 production. To overcome the limitations of using bulk $CD4^+$ T cells containing $Foxp3^+$ and $Foxp3^-$ T cells as the source of regulatory T cells, we established multiple $CD4^+$ T cell clones from OVA immunized, UV-irradiated (UP4-1 ~ UP4-12) or non-irradiated (UN4-1 ~ UN4-12) mice and analyzed their cytokine production profiles. As shown in Figure 3*a*, all T cell clones from UV-irradiated mice produced large amounts of IL-10 and substantial amounts of IFN-γ, but did not produce detectable levels of IL-4, when cultivated with TDS cells (TDS) in the presence of OVA. In contrast, most T cell clones from non-irradiated mice produced IFN-γ alone, and some produced a combination of IL-4 and IL-10. Similar results were obtained when cytokine expression was assessed by reverse transcription polymerase chain reaction (RT-PCR) of mRNA in these cells (Fig. 3*b*). There were substantial differences between the cytokine production profiles of cells derived from irradiated and non-irradiated mice, but none of the generated clones expressed mRNA for Foxp3. However, all of the T cell clones from UV-irradiated mice expressed c-Maf mRNA, a transcription factor important for Tr1 cell differentiation.[32]

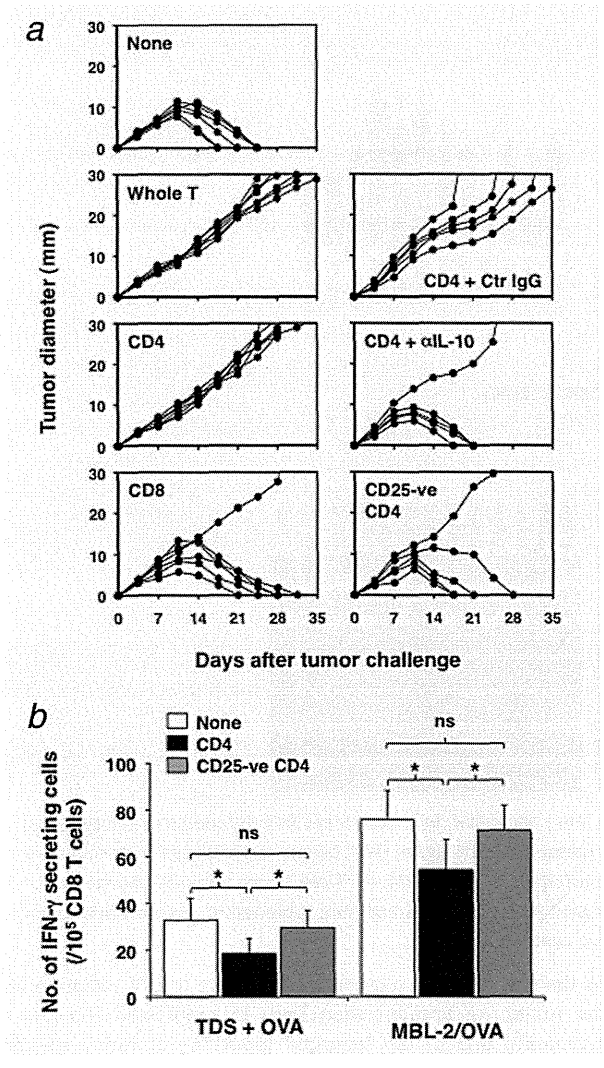
T cell clones established from UV-irradiated mice suppress anti-tumor immune response

We selected two clones each from UV-irradiated and non-irradiated mice for further study, and we examined their *in vivo* regulatory activity. OVA-immunized recipient mice were adoptively transferred with T cell clones from UV-irradiated (UP4-7 or UP4-11) or non-irradiated mice (UN4-2 or UN4-3), and MBL-2/OVA tumors were then inoculated. UP4-7 and UP4-11, but not UN4-2 and UN4-3, completely suppressed the rejection of implanted MBL-2/OVA cells (Fig. 4*a*, left panels), but these effects were completely abrogated by the co-administration of anti-IL-10 (Fig. 4*a*, right panels). As a control, transfer of UP4-7 cells did not suppress the rejection MBL-2 tumors, arguing that the observed effects are Ag specific (Fig. 4*b*). To determine whether UP4-7 suppress tumor-specific $CD8^+$ T cell responses, we performed an ELISPOT assay using $CD8^+$ T cells isolated from MBL-2/OVA challenged, OVA-immunized mice. As shown in Fig. 4*c*, the number of OVA-specific $CD8^+$ T cells generated in OVA-immunized and MBL-2/OVA challenged mice was significantly reduced by the transfer with UP4-7 cells, but these effects were lost when anti-IL-10 was also administered. Thus, we considered these cells to be UV-induced Treg (UV-Treg) clones.

UP4-7 impairs Th cell proliferation in vitro

Finally, we wished to examine the mechanism of suppression and regulatory activity of the UV-Treg clone UV4-7 *in vitro*. Tumor-specific $CD4^+$ helper T cells are important for the induction and long-term maintenance of an effective anti-

tumor CTL response,[33,34] and we hypothesized that $CD4^+$ T cells were the targets of the regulatory activity of UP4-7 *in vivo*. Consistent with this, $CD4^+$ T cells from OVA immunized mice (OVA-Th) proliferated well when cultured in the presence OVA and TDS, but UP4-7 proliferated poorly (Fig. 5*a*). However, when OVA-Th were co-cultured with UP4-7 in the presence of OVA and TDS, cell proliferation was significantly suppressed, but proliferation was partially restored by the inclusion of anti-IL-10 in the culture media (Fig.5*a*). Identical effects were seen when UP4-7 cells were incubated with OVA-Th across a transwell system, confirming that a soluble factor (*e.g.*, IL-10) is responsible for inhibiting cell proliferation (Fig. 5*b*). Furthermore, co-culture of $CD4^+$ T cells from HEL immunized mice (HEL-Th) with UP4-7 inhibited the proliferation of HEL-Th in the presence of HEL plus OVA, but not HEL alone (Fig. 5*c*), indicating that UP4-7 can also exert regulatory activities in a bystander fashion in the presence of appropriate antigenic stimuli.



Discussion

In our study, we showed that UV irradiation of immunized mice suppresses anti-tumor immune response in an Ag dependent manner. This suppression was mediated by the release of IL-10 from $CD4^+CD25^+$ T cells, leading to impaired induction and/or expansion of anti-tumor $CD8^+$ CTL. Furthermore, we established a panel of T cell clones derived from UV-irradiated and non-irradiated mice, and, using these cells, we unequivocally demonstrated that Tr1-type regulatory T cells expressing IL-10 and the transcription factor c-Maf, but not Foxp3, are responsible for the suppression of the anti-tumor CTL response in mice irradiated UV after immunization. The Tr1-type regulatory T cells induced by UV-irradiation suppress Th cell activation essential for CTL induction in a cell-contact independent, but IL-10 dependent manner.

The ability of UV radiation to promote the generation of regulatory T cells and associated immunosuppression have been demonstrated in models of tumor immunity and contact hypersensitivity, wherein $CD8^+$ T cells play a critically role. However, the lack of defined Ags in these systems has limited the detailed analysis of the effects of UV radiation on the induction and/or expansion of Ag-specific $CD8^+$ T cells, and a limited study suggested that UV exposure can suppress $CD8^+$ T cell responses.[35–37] In our study, we used a tumor

Figure 2. $CD4^+$ T cells mediate UV-induced Ag-specific immune suppression. (*a*) Donor mice were immunized with OVA in IFA on day 0 and then subjected to UV radiation on day 7. On day 14, T cells were obtained from these mice and further fractionated into $CD4^+$, $CD8^+$ and $CD4^+CD25^-$ T cells. Recipient mice ($n = 5–6$) were immunized with OVA in IFA on day 0, adoptively transferred with 2×10^6 T cell subpopulations together with or without antibodies as indicated on day 7, and inoculated with 2×10^6 MBL-2/OVA on day 14. Tumor size was monitored twice a week. Tumor growth of mice without transfer (none) or recipient mice transferred with total T cells (whole T), $CD4^+$ T cells (CD4), $CD8^+$ T cells (CD8), $CD4^+$ T cells together with anti-IL-10 (CD4 + αIL-10) or control IgG (CD4 + Ctr IgG), or $CD4^+$ T cells depleted of $CD25^+$ cells (CD25-ve CD4) was measured twice a week. (*b*) Recipient mice ($n = 5$) were immunized with OVA in IFA on day 0, adoptively transferred with 2×10^6 $CD4^+$ T cells or $CD4^+$ T cells depleted of $CD25^+$ cells from OVA-immunized and UV-irradiated mice on day 7, and inoculated with 2×10^6 MMC-treated MBL-2/OVA on day 14. On day 21, $CD8^+$ T cells were isolated from these mice and assayed for IFN-γ production after cultivation with T cell-depleted spleen (TDS) cells plus OVA or MBL-2/OVA by ELISPOT (*c*) in triplicate cultures. The results are expressed as mean ± standard deviation (SD) between animals ($n = 5$/group). Open bars, $CD8^+$ T cells from mice without transfer; black bars, $CD8^+$ T cells from recipient mice transferred with $CD4^+$ T cells; gray bars, $CD8^+$ T cells from recipient mice transferred with $CD25^-CD4^+$ T cells. Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test. n.s., not significant; $*p < 0.05$; $**p < 0.01$.

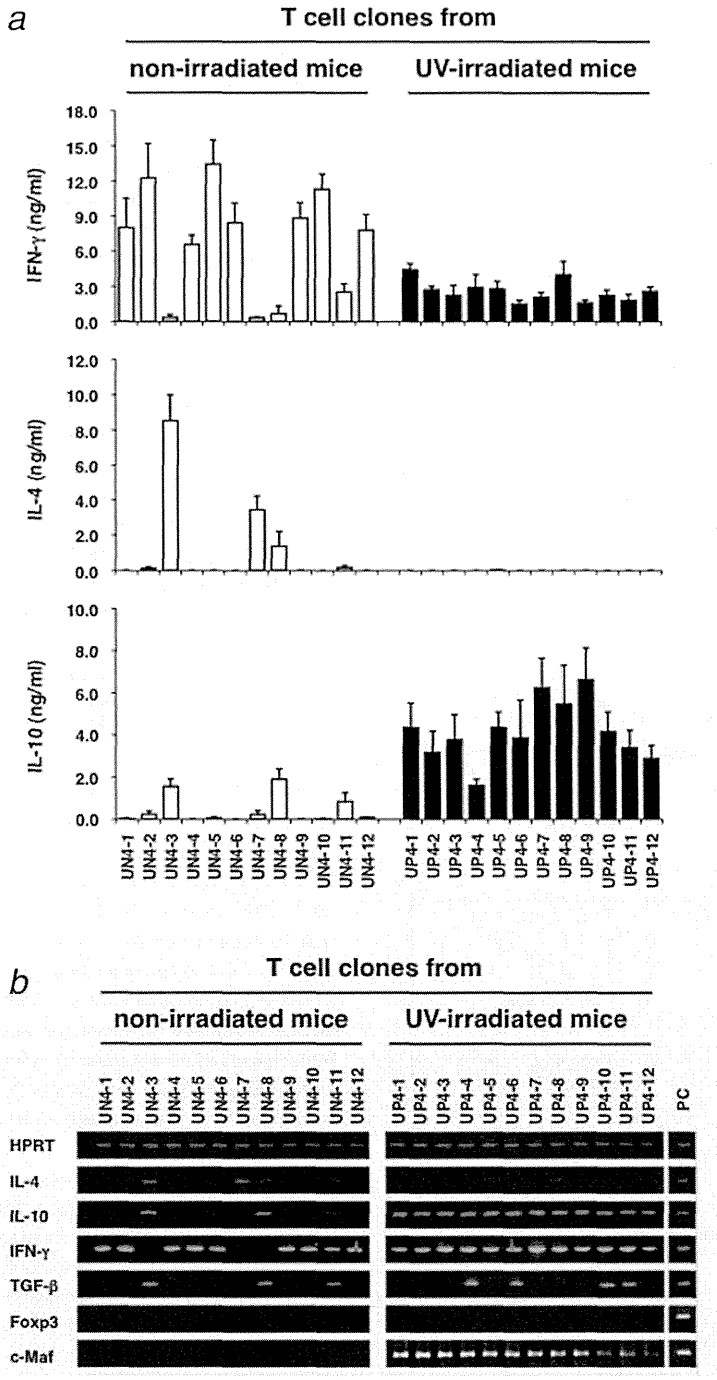


Figure 3. T cell clones established from UV-irradiated mice express IL-10, IFN-γ and c-Maf. (*a*) T cell clones from UV-irradiated (closed bars) or non-irradiated (open bars) mice were cultured with splenic APC in the presence of 100 μg/ml OVA. Culture supernatants were collected at 24 hr and assayed for the indicated cytokines by enzyme-linked immunosorbent assay (ELISA). (*b*) T cell clones from UV-irradiated or non-irradiated mice were cultured as above for 16 hr. Total RNA was extracted from these cells and then subjected to reverse transcription polymerase chain reaction (RT-PCR) with indicated primer sets. PC, positive control.

model in which OVA acted as a surrogate tumor Ag to gain better insight into the effects of UV irradiation on the $CD8^+$ T cell responses of immunized mice. To our knowledge, this is the first study to suggest that exposure to UV radiation after tumor Ag immunization leads to suppressed anti-tumor immunity due to the impaired induction of tumor-specific

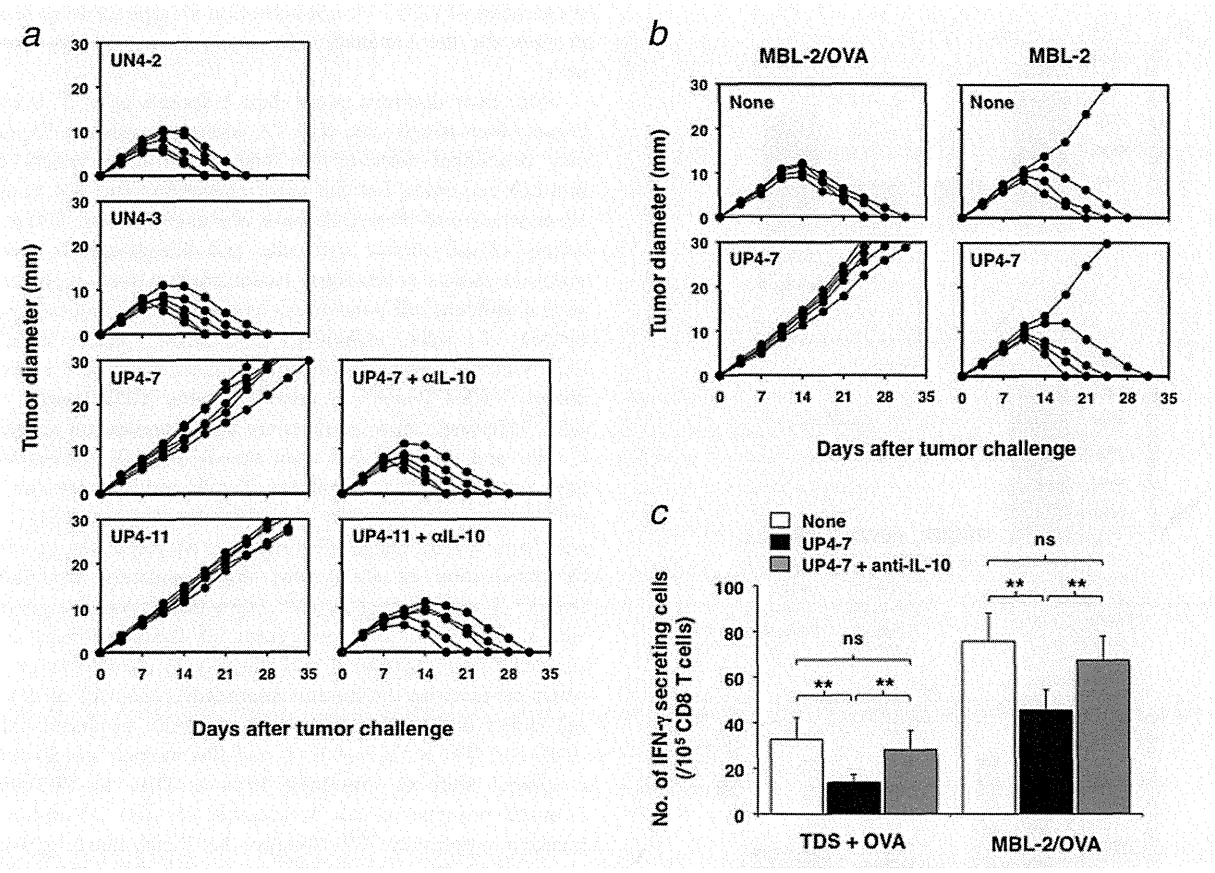


Figure 4. T cell clones from OVA-immunized and UV-irradiated mice suppress rejection of OVA-expressing tumor. (*a*) Recipient mice ($n = 4–5$) were immunized with OVA in IFA on day 0, adoptively transferred with 2×10^6 cells of T cell clones from UV-irradiated (UP4-7 and UP4-11) or non-irradiated (UN4-2 and UN4-3) mice on day 7 and inoculated with 2×10^6 MBL-2/OVA on day 14. A group of mice also received anti-IL-10 on days 13 and 15 (UP4-7 + αIL-10 and UP4-11 + αIL-10). Tumor size was monitored twice a week. (*b*) Recipient mice ($n = 4–5$) were immunized with OVA in IFA on day 0, mock transferred (none) or adoptively transferred with 2×10^6 cells of T cell clones from UV-irradiated mice (UP4-7) on day 7 and inoculated with MBL-2/OVA (2×10^6 cells) (left panels) or MBL-2 (1×10^6 cells) (right panels) on day 14. Tumor size was monitored twice a week. (*c*) Mice ($n = 5$) were immunized with OVA in IFA on day 0, adoptively transferred with the T cell clone UP4-7 (2×10^6 cells) on day 7 together with or without anti-IL-10 (100 μg), and inoculated with 2×10^6 MMC-treated MBL-2/OVA on day 14. On day 21, $CD8^+$ T cells were isolated from these mice and individually assayed for IFN-γ production by ELISPOT in triplicate cultures as described in legend for Fig. 2. The results are expressed as mean ± standard deviation (SD) between animals ($n = 5$/group). Open bars, $CD8^+$ T cells from mice without transfer; black bars, $CD8^+$ T cells from recipient mice transferred with UP4-7; gray bars, $CD8^+$ T cells from recipient mice transferred with UP4-7 and treated with anti-IL-10. Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test. n.s., not significant; $*p < 0.05$; $**p < 0.01$. The data shown are representative of two to three experiments with similar results.

CTL responses. To formally prove this possibility, however, CTL responses to endogenous tumor Ags in mice immunized with tumors and exposed to UV using tumors with defined tumor Ags such as CT26 with AH1, CMS with mERK2, and/or B16 melanoma with TRP-2/gp100, need to be tested.

UV irradiation of immunized mice led to the generation of Ag-specific $CD4^+CD25^+$ T cells capable of indirectly suppressing $CD8^+$ CTL responses. Furthermore, $CD4^+$ T cell clones established from OVA-immunized and UV-irradiated mice did not express Foxp3, but upon adoptive transfer they efficiently suppressed CTL induction/expansion in an IL-10 dependent manner. A previous study showed that $CD4^+$ T cells in mice UV-irradiated before sensitization with hapten were able to suppress priming/expansion of $CD8^+$ T cells reactive to hapten-modified self-Ag.[35] In addition, IL-10 negatively regulates $CD8^+$ T cell activation in UV-irradiated tumor-bearing mice, and this was hypothesized to be related to enhanced tumor growth.[36] Although these previous studies examined the ability of regulatory $CD4^+$ T cells and/or IL-10 in UV-irradiated mice to suppress the activation of $CD8^+$ T

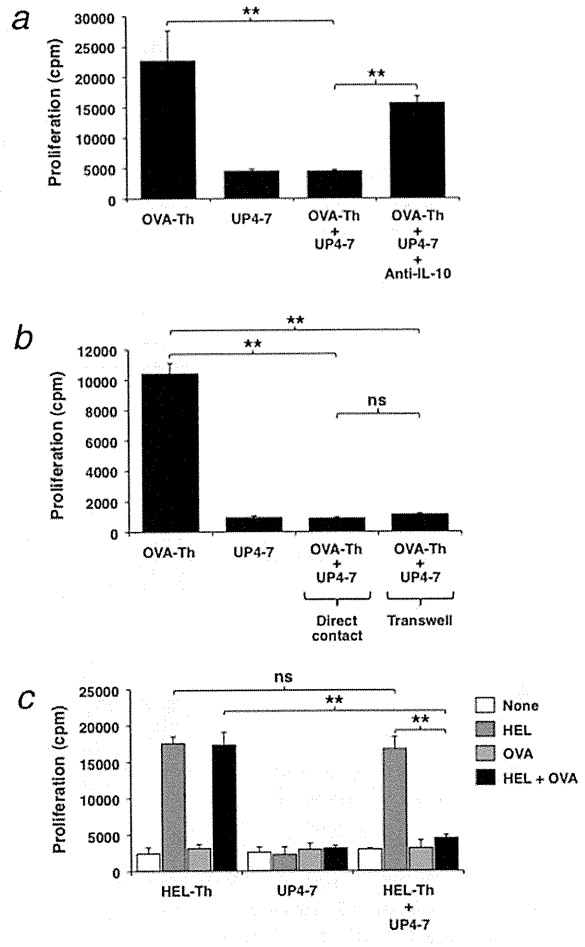


Figure 5. The T cell clone UP4-7 suppresses proliferation of Th cells. (*a*) $CD4^+$ T cells from OVA immunized mice (OVA-Th, 1×10^6 cells/ml) were cultured with TDS and OVA in the presence or absence of UP4-7 (0.5×10^6 cells/ml) together with or without 2 μg/ml of anti-IL-10 in the wells of a 96-well plate. (*b*) UP4-7 were added at a ratio of 1:2 to OVA-Th seeded in the lower chambers of a 24-well plate. UP4-7 were added either directly (direct contact) or separated by a Transwell semipermeable membrane (Transwell). (*c*) $CD4^+$ T cells from HEL immunized mice (HEL-Th) were cultured at 1×10^6 cells/ml with TDS together with or without UP4-7 (0.5×10^6 cells/ml) in the presence or absence of the indicated Ags. Proliferations were measured as described in the Materials and Methods. The results are expressed as mean ± standard deviation (SD) of triplicate cultures. Statistical significance was determined by one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test. n.s., not significant; $*p < 0.05$; $**p < 0.01$. The data shown are representative of two to three experiments with similar results.

cells, the mechanism(s) underlying this suppression are not well understood. $CD4^+$ Th cells are essential for the generation and long-term maintenance of $CD8^+$ CTL.[33,34] Consistent with this, our past[16] and current data indicate that the suppression of $CD4^+$ Th cell activation is responsible, at least in part, for the impaired CTL responses in UV-irradiated mice.

Since their discovery more than 2 decades ago,[38,39] it has become increasingly clear that UV-induced regulatory T cells play prominent roles in the suppression of a variety of immune responses, but the exact phenotypic and functional characteristics of these cells have remained unclear. Using a variety of cell surface molecules and lineage-specific transcription factors as markers, recent studies have implicated several different cell populations as putative UV-induced regulatory T cells including $CD3^+DX5^+$ NKT cells,[17] $CD4^+CD25^+$ T cells coexpressing CTLA-4, GITR and neuropilin-1,[18] $CD4^+Foxp3^+$ T cells[19,20] and/or $CD4^+Foxp3^-$ T cells.[21] However, these markers are also expressed on effector T cells, and indeed it has been shown that UV tolerization does not only induce regulatory T cells but also effector T cells.[18] Our previous study also demonstrated that $CD4^+$ T cells from UV-irradiated mice suppress Ab responses through the production of IL-10, but this population contained $Foxp3^+$ T cells lacking IL-10.[21] With this caveat, we generated a panel of T cell clones from UV-irradiated mice and examined their functional and phenotypic characteristics to better characterize the immunosuppressive cells. All of the T cell clones derived from UV-irradiated mice produced both IL-10 and IFN-γ, but not IL-4, and this strongly argues that a general shift of immunity from a Th1- to Th2-type immune response is not responsible for the UV-induced immune suppression.[40,41] Notably, the T cell clones derived from UV-irradiated mice lacked expression of Foxp3 mRNA, but they uniformly expressed c-Maf mRNA. c-Maf is originally described for Th2-specific transcription factor, but subsequent studies revealed that c-Maf transactivates IL-10 gene transcription independently of Th2 differentiation.[42,43] More recent studies indicate that c-Maf transactivates IL-21, which acts as an autocrine growth factor for the expansion and/or maintenance of Tr1 cells.[32,44] Although Th2 cells express c-Maf, it also has been shown that the expression levels of c-Maf mRNA are ~ 500-fold higher in Tr1 cells than Th2 cells.[32] Therefore, c-Maf now can be regarded as a critical transcription factor for Tr1 cells. In addition, T cell clones from UV-irradiated mice exerted Ag specific and bystander suppression of Th activation in an IL-10 dependent but a contact independent fashion. These phenotypic and functional features are essentially identical to those of Tr1 cells originally described by Groux *et al.*[45–47] Importantly, the adoptive transfer of these cells suppressed the rejection of OVA-expressing tumor cells. These results are consistent with the experiments showing that adoptive transfer of OVA-specific Tr1 cells abrogates the rejection of OVA-expressing tumors in mice immunized with OVA-pulsed DC.[48] In addition, our data indicates that Tr1 cells suppress the anti-tumor $CD8^+$ CTL responses *in vivo*. Although the mechanism(s) by which Tr1 cells suppress $CD8^+$ T cell activation remains elusive, it is possible that Tr1 cells indirectly suppress $CD8^+$

activation by inhibiting Th cell function.[33,34] It is also possible that Tr1 cells directly suppress CTL activation, because Tr1 cells have been reported to acquire Ag-loaded MHC class I molecules from APCs, interact with congnate Ag-specific $CD8^+$ T cells and suppress their activation via IL-10.[49]

Together with our previous studies,[15,16,21] our study indicates that UV-irradiation after immunization induces Tr1 cells specific to immunizing Ag and dominantly suppress variety of immune responses that control tumor development. Accumulating evidence indicates that precancerous and malignant cell can induce specific immune response which resulted in the elimination of malignant and/or transformed cell before they developed detectable tumors (cancer immunosurveillance[25]). Furthermore, recent multivariate analysis of a multicounty ecological study and population based, case–control study have shown a significant positive association between exposure to UV radiation and increase in the risks of non-Hodgkin's lymphoma and colon cancer, in addition to skin melanoma.[8,9] In this regard, our findings have potential relevance that UV irradiation might contribute to the progression of various tumors during premalignant stage, given that the UV irradiation induces Ag-specific immune suppression in Ag-sensitized mice.

In summary, we provide definitive evidence that UV irradiation after immunization generates a population of Ag-specific regulatory T cells with Tr1 phenotype able to suppress anti-tumor $CD8^+$ CTL responses. We did not exclude a role for $Foxp3^+$ regulatory T cells and/or NKT cells in the UV-induced immune suppression, and future studies are needed to precisely and systemically determine the relative contributions of these additional cell types to UV-induced suppression of anti-tumor immune responses.

Acknowledgements

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Novel adoptive T-cell immunotherapy using a WT1-specific TCR vector encoding silencers for endogenous TCRs shows marked antileukemia reactivity and safety

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Adoptive T-cell therapy for malignancies using redirected T cells genetically engineered by tumor antigen-specific *T-cell receptor* (*TCR*) gene transfer is associated with mispairing between introduced and endogenous TCR chains with unknown specificity. Therefore, deterioration of antitumor reactivity and serious autoimmune reactivity are major concerns. To address this problem, we have recently established a novel retroviral vector system encoding siRNAs for endogenous TCR genes (*siTCR* vector). In this study, to test the clinical application of *siTCR* gene therapy for human leukemia, we examined in detail the efficacy and safety of WT1-*siTCR*–transduced T cells. Compared with conventional WT1-*TCR* (WT1-*coTCR*) gene-transduced T cells, these cells showed significant enhancement of antileukemia reactivity resulting from stronger expression of the introduced WT1-specific TCR with inhibition of endogenous TCRs. Notably, WT1-*siTCR* gene-transduced T cells were remarkably expandable after repetitive stimulation with WT1 peptide in vitro, without any deterioration of antigen specificity. WT1-*siTCR* gene–transduced T cells from leukemia patients successfully lysed autologous leukemia cells, but not normal hematopoietic progenitor cells. In a mouse xenograft model, adoptively transferred WT1-*siTCR* gene-transduced T cells exerted distinct antileukemia efficacy but did not inhibit human hematopoiesis. Our results suggest that gene-immunotherapy for leukemia using this WT1-*siTCR* system holds considerable promise. (*Blood.* 2011;118(6):1495-1503)

Introduction

Recent identification of various tumor-associated antigens has encouraged the clinical development of cell-mediated immunotherapy for leukemia targeting leukemia-associated antigens.[1,2] Among various kinds of immunotherapy, adoptive tumor-specific T-cell therapy using ex vivo expansion of autologous tumor-responsive T cells seems to be an attractive option. Indeed, patients with advanced metastatic melanoma have been treated successfully with melanoma-specific T cells obtained from tumor-infiltrating T cells.[3-5] Although adoptive transfer of tumor-specific tumor-infiltrating T cells is a promising strategy, its general application for therapy would appear to be unlikely because of the complex procedures and difficulties involved in the timely preparation of sufficient numbers of tumor-specific cytotoxic T lymphocytes (CTLs) with adequate therapeutic quality.[6,7] To address these problems, an innovative approach involving substituting redirected T cells using predefined tumor antigen-specific *T-cell receptor* (*TCR*) gene transfer has been developed. In recent clinical trials, melanoma antigen-specific *TCR* gene-transferred T cells have been used for treatment of patients with advanced melanoma.[8,9] However, the clinical efficacy of *TCR* gene-engineered T cells is still not satisfactory, and serious autoimmune responses have been observed in some melanoma patients. In addition, adoptive immunotherapy using tumor antigen-specific *TCR* gene-transferred T cells targeting malignancies other than melanoma still remains in its infancy. Therefore, the development of *TCR* gene-immunotherapy targeting universal tumor-associated antigens is essential to popularize this strategy for cancer treatment.

Wilms tumor gene product 1 (WT1) is one of the zinc-finger transcriptional regulators that is abundantly expressed in the vast majority of acute leukemias, but not in normal cells.[10,11] In addition, the expression level of WT1 in tumor cells is clinically correlated with disease aggressiveness and prognosis.[12,13] Furthermore, a study using a model involving immunodeficient mice engrafted with human acute myelogenous leukemia has recently obtained important evidence that WT1 is expressed abundantly in chemotherapy-resistant acute myelogenous leukemia stem cells.[14] These data have prompted us and other groups to develop adoptive T-cell immunotherapy targeting leukemia stem cells using WT1-specific *TCR* gene transfer.[15-17]

To facilitate the clinical application of adoptive immunotherapy using genetically engineered WT1-specific CTLs, some important issues need to be addressed. First, there is the problem of mispairing between endogenous and introduced TCR chains that would reduce the expression of introduced TCR on the surface of gene-modified T cells, resulting in lower functionality.[18] Mispairing of TCR could also carry a risk of evoking severe autoimmunity.[19,20] Therefore, it is essential to clarify both the on- and off-target adverse effects mediated by WT1-*TCR* gene-engineered T cells using in vivo as well as in vitro systems. The other issue of concern is bone marrow suppression mediated by WT1-specific

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T cells because it has been reported that hematopoietic progenitor cells express *WT1* mRNA.[21,22] In previous trials of WT1 peptide vaccine, suppression of normal hematopoiesis has not been reported in most cases, even though WT1-specific CTLs were generated following vaccination.[23,24] However, long-term adverse effects on hematopoietic progenitors mediated by adoptively transferred WT1-*TCR* gene-engineered T cells should be considered because, in this therapy, a larger number of WT1-specific T cells are infused at one time into patients.

To overcome the aforementioned problems, we have recently developed a novel retroviral vector system for *TCR* gene transfer that can selectively express target antigen-specific TCR, whereas expression of intrinsic TCRs is suppressed by built-in siRNAs (*siTCR* vector).[25] MAGE-A4–specific *TCR* gene-engineered T cells prepared by this vector system successfully showed both up-regulated expression of the introduced TCR and enhanced anti-MAGE-A4 reactivity. We also constructed a novel WT1-*siTCR* retroviral vector encoding human leukocyte antigen (HLA)–A*24:02-restricted and WT1-specific *TCR* genes cloned from a CTL clone, TAK-1.[26] This WT1-*siTCR* vector similarly appeared capable of increasing the expression of the introduced WT1-specific TCR; however, the usefulness of WT1-*siTCR* for clinical application remains to be clarified before clinical trials can begin. In the present study, with the aim of clinically applying this WT1-*siTCR* gene transfer system for treatment of leukemia, we assessed in detail the efficacy and safety of adoptive immunotherapy using WT1-specific *TCR* gene-modified CTLs using both in vivo as well as in vitro experimental systems. On the basis of the data we have obtained, we discuss the feasibility of this novel gene immunotherapy for leukemia using a WT1-*siTCR* retroviral vector.

Methods

Cloning of WT1-specific *TCR* gene and construction of WT1-*TCR* retroviral vectors

The HLA-A*24:02-restricted and $WT1_{235-243}$-specific *TCR-α* and *TCR-β* genes were cloned from our originally established CTL clone, TAK-1, using the 5′ RACE method (Clontech).[27] The *TCR-α* and *TCR-β* genes of TAK-1 appeared to be Vα20/J33/Cα and Vβ5.1/J2.1/Cβ2, respectively. Retrovirus vectors expressing TAK-1-derived *TCR* (WT1-*TCR*) genes were constructed as reported previously.[25] Briefly, the WT1-*TCR-α* and *TCR-β* genes were bicistronically integrated into a conventional MS-bPa retroviral vector (WT1-*coTCR* vector).[28] Partially codon-optimized *TCR-α* and *TCR-β* genes were similarly integrated into a novel MS-bPa–based retroviral vector encoding shRNAs that complementarily bind to the constant regions of the endogenous *TCR-α* and *TCR-β* genes (WT1-*siTCR* vector) (supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Ecotropic retroviral vectors were obtained by transient cotransfection of WT1-TCR-expression retroviral vector and other components (Takara Bio) to HEK293 cell line; subsequently, GaLV-pseudotyped retroviral vectors were obtained by sequential infection of ecotropic retroviral vectors to PG13 cell line. The Jurkat/MA cell line, lacking endogenous TCR and engineered with the hCD8α and NFAT-luciferase construct,[29] was transduced with the WT1-*TCR* vector to confirm that our retroviral vector was actually able to express functional WT1-specific and HLA-A*24:02-restricted TCR molecules on the cell surface (supplemental Figure 2).

Cell lines, freshly isolated leukemia cells, and normal cells

Approval for this study was obtained from the Institutional Review Board of Ehime University Hospital. Written informed consent was given by all patients, healthy volunteers, and the parents of the cord blood donors in accordance with the Declaration of Helsinki. All cell lines and freshly isolated cells were cultured as described previously.[30] B-lymphoblastoid cell lines (B-LCLs) were established by transformation of peripheral blood B lymphocytes with Epstein-Barr virus. An HBZ_{26-34} peptide-specific and HLA-A*02:01-restricted CTL clone, designated HBZ-1, was established as reported previously.[31] The *HLA-A*24:02* gene-transduced C1R cell line (C1R-A*24:02) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 0.5 mg/mL hygromycin B (Invitrogen) and the *HLA-A*24:02* gene-transduced K562 cell line (K562-A*24:02) was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and 1.0 μg/mL puromycin (Sigma-Aldrich). Peripheral blood mononuclear cells and bone marrow mononuclear cells from leukemia patients and healthy volunteers, and cord blood mononuclear cells from healthy donors were isolated and stored in liquid nitrogen until use. All leukemia samples contained more than 95% leukemia cells. In some experiments, $CD34^+$ cells from cord blood mononuclear cells were isolated using $CD34^+$ cell-isolating immunomagnetic beads (MACS beads; Miltenyi Biotec).

Establishment of WT1-*TCR* gene-transduced CTL lines

$CD8^+$ T cells were isolated from peripheral blood mononuclear cells of healthy volunteers and leukemia patients in complete remission, and cord blood mononuclear cells using $CD8^+$ cell-isolating MACS beads and stimulated with 1 μg/mL anti-CD3 monoclonal antibody (MoAb, OKT-3; BioLegend). $CD8^+$ T cells were cultured in GT-T503 medium (Takara Bio) supplemented with 5% human serum, 0.2% human albumin, 50 U/mL recombinant human IL-2 (R&D Systems), 5 ng/mL IL-7 (R&D Systems), 10 ng/mL IL-15 (PeproTech), and 10 ng/mL IL-21 (Shenandoah Biotechnology). Then, $CD8^+$ T cells were transfected with the WT1-*TCR* retrovirus vector using RetroNectin (Takara Bio)-coated plates as described previously.[25] In some experiments, Vβ5.1-positive cells among WT1-*TCR* gene-transduced $CD8^+$ T cells were further isolated using fluorescein isothiocyanate (FITC)-conjugated Vβ5.1 MoAb (Beckman Coulter) and an anti-FITC-conjugated MACS beads system. To measure the expression levels of introduced WT1-specific TCR in gene-engineered $CD8^+$ T cells, the cells were labeled with anti-CD8, anti-CD4, anti-CD3 (BD Biosciences), and anti-Vβ5.1 MoAbs and phycoerythrin-conjugated HLA-A*24:02/$WT1_{235-243}$-tetramer or HLA-A*24:02/HIV-1 $Env_{584-592}$-tetramer (supplemental Figure 3).[32] The labeled cells were analyzed using a Gallios flow cytometer (Beckman Coulter) and FlowJo Version 7.2.2 software (TreeStar). To compare the expandability of WT1-*coTCR*–transduced and WT1-*siTCR*-transduced $CD8^+$ T cells by stimulation with WT1 peptide, WT1-*TCR* gene-transduced $CD8^+$ T cells were weekly stimulated with mitomycin-C (Kyowa Hakko)-treated and heteroclitic $WT1_{235-243}$ peptide (CYTWNQMNL)-pulsed HLA-A*24:02-positive LCLs.

^{51}Cr-release assays

To determine the cytotoxic activity of WT1-*TCR* gene-transduced $CD8^+$ T cells, standard ^{51}Cr-release assays were performed as described previously.[30] Briefly, 5×10^3 ^{51}Cr ($Na_2{}^{51}CrO_4$; New England Nuclear)–labeled target cells and various numbers of effector cells in 200 μL of RPMI 1640 medium supplemented with 10% fetal calf serum were seeded into 96-well round-bottomed plates. The target cells were incubated with or without WT1 peptide for 2 hours before adding the effector cells. To assess the HLA class I-restricted cytotoxicity, target cells were incubated with an anti-HLA class I framework MoAb (w6/32; ATCC) or an anti-HLA-DR MoAb (L243; ATCC) at an optimal concentration (10 μg/mL) for 1 hour before adding the effector cells. After incubation with the effector cells for 5 hours, 100 μL of supernatant was collected from each well. The percentage of specific lysis was calculated as: (experimental release cpm − spontaneous release cpm)/(maximal release cpm − spontaneous release cpm) × 100 (%).

Detection of CD107a and intracellular IFN-γ expression in WT1-*TCR* gene-transduced $CD8^+$ T cells

CD107a expression in WT1-*TCR* gene-transduced $CD8^+$ T cells in response to stimulation with WT1 peptide was assessed as described previously.[33] Briefly, 1×10^5 C1R-A*24:02 cells were seeded into a

96-well round-bottom plate and incubated with or without WT1 peptide for 2 hours. Then, 2×10^5 WT1-*TCR* gene-transduced $CD8^+$ T cells were seeded into each well along with FITC-conjugated CD107a MoAb (BioLegend) for 3 hours. Similarly, CD107a expression in the HBZ-1 cell line and WT1-*TCR* gene-transduced HBZ-1 cells in response to stimulation with HBZ_{26-34} peptide-loaded HLA-A*02:01-positive T2 cells was analyzed. To investigate intracellular interferon-γ (IFN-γ) production, effector cells were incubated with target cells and 10 μg/mL brefeldin A for 4 hours. They were then collected, fixed, and permeabilized with fluorescence-activated cell sorter lysing solution and fluorescence-activated cell sorter permeabilizing solution (BD Biosciences).[34] After permeabilization, the washed cells were stained with FITC-conjugated IFN-γ MoAb (BD Biosciences). Finally, these effector cells were stained with anti-CD3, anti-CD8 MoAbs (BD Biosciences) and phycoerythrin-conjugated HLA-A*24:02/WT1-tetramer, and then analyzed using a Gallios flow cytometer and FlowJo Version 7.2.2 software.

Quantitative analysis of *WT1* mRNA expression

Total RNA was extracted from each sample with an RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. Quantitative real-time polymerase chain reaction of *WT1* mRNA was performed using the QuantiTect SYBR Green PCR Kit (QIAGEN) and primers as follows: forward; 5′-AGCACAGGGTACGAGAGCGATAAC-3′, reverse; 5′-TATTGCAGCCTGGGTAAGCACA-3′ (Takara Bio). *GAPDH* mRNA as an internal control was prepared as described previously.[30] These samples were analyzed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The expression level of *WT1* mRNA was corrected by reference to that of *GAPDH* mRNA, and the amount of *WT1* mRNA in each sample relative to that in the K562 leukemia cell line, which strongly expresses *WT1* mRNA (shown as 1.0), was calculated by the comparative ΔC_t method.

In vivo antileukemia effect of WT1-*siTCR* gene-transduced CTLs

Six-week-old NOD/scid/γc^{null} (NOG) female mice[35] were purchased from the Central Institute for Experimental Animals and maintained in the institutional animal facility at Ehime University. All in vivo experiments were approved by the Ehime University animal care committee. For xenografting of human leukemia cells, NOG mice were inoculated subcutaneously in the left flank with 5×10^6 K562-A*24:02 cells, which had been preincubated with 2.5×10^7 effector cells for 5 hours. Then, 1×10^7 effector cells were additionally administered intravenously via the tail vein every week for a total of 5 times. The mice were monitored for tumor growth and survival after inoculation; the tumors were measured at 5-day intervals, and the tumor area was determined.

In vivo differentiation of human hematopoietic stem cells in humanized mice

$CD34^+$ cells were isolated from cord blood mononuclear cells (hCB-$CD34^+$ cells), and then 5×10^4 of the cells were coincubated with 2.5×10^5 autologous WT1-*siTCR*–transduced or non–gene-modified CTLs generated from cord blood $CD8^+$ T cells for 5 hours. hCB-$CD34^+$ cells were then reisolated from the cell mixture and injected intravenously into 7-week-old NOG mice that had been irradiated with 1.5 Gy. Three months later, these mice were killed to study the engraftment and differentiation of human hematopoietic cells in both the bone marrow and spleen. Human leukocytes were discriminated from murine cells using anti-hCD45 MoAb (BD Biosciences). Human cells were further stained with MoAbs against cell lineage-related surface molecules, including CD3, CD8, CD4, CD19, CD33, CD34, CD38, CD41a, and GPA (BD Biosciences). The expression of HLA-A*24:02 in the engrafted human cells was also measured using FITC-conjugated anti-HLA-A24 MoAb (One Lambda), and the immunostained cells were analyzed using a Gallios flow cytometer and FlowJo Version 7.2.2 software.

Results

Comparison of WT1-TCR expression and WT1-specific cytotoxic reactivity of WT1-*siTCR*- and WT1-*coTCR*–transduced $CD8^+$ T cells

First, we confirmed the augmented and inhibitory efficacies of the WT1-*siTCR* vector for expression of the respectively introduced and endogenous TCRs. To do so, we transduced WT1-*siTCR* into HBZ-1, which is an HLA-A*02:01-restricted and HBZ_{26-34}-specific $CD8^+$ T-cell clone. As shown in Figure 1A, positivity for HLA-A*24:02/WT1-tetramer staining in nontreated, WT1-*coTCR*- and WT1-*siTCR*–transduced HBZ-1 was $< 1\%$, 29%, and 65%, respectively, whereas the corresponding values for HLA-A*02:01/HBZ-tetramer staining were 98%, 20%, and 4%, respectively. For functional assessment of the efficacy of the *siTCR* vector for suppression of endogenous HBZ-TCR, CD107a assays were performed. We evaluated the extent of the decreased responsiveness to the cognate HBZ peptide mediated by WT1-*coTCR*– and WT1-*siTCR*–transduced HBZ-1 compared with that mediated by the parent HBZ-1. As shown in Figure 1B, WT1-*siTCR*–transduced HBZ-1 exhibited an apparent loss of responsiveness to the HBZ peptide-loaded T2 cells, indicating that sufficient functional suppression of endogenous TCR is achievable using the WT1-*siTCR* vector. The reactivity of WT1-*coTCR*–transduced HBZ-1 to stimulation with HBZ peptide also appeared to decrease compared with that of the parental HBZ-1 clone; however, the inhibitory effect of the *siTCR* vector appeared to be higher than that of the *coTCR* vector at high concentrations of the HBZ peptide. On the basis of these data, we were able to confirm the efficacy of the WT1-*siTCR* vector for augmentation of introduced TCR expression and inhibition of endogenous TCR expression.

Next, we further investigated whether enhanced expression of the introduced TCR by the WT1-*siTCR* vector actually up-regulated the function of engineered $CD8^+$ T cells. To do this, we compared the amounts of intracellular IFN-γ production and degrees of granular exocytosis by WT1-*coTCR*– and WT1-*siTCR*–transduced $CD8^+$ T cells in response to stimulation with WT1 peptide. As shown in the upper column of Figure 1C, the proportion of tetramer-positive cells in WT1-*siTCR*–transduced $CD8^+$ T cells was higher than that in WT1-*coTCR*–transduced $CD8^+$ T cells. The data for intracellular IFN-γ production and CD107a expression in WT1 tetramer-positive cells are shown in the lower columns. The degrees of both IFN-γ production and CD107a expression in WT1-*siTCR*–transduced $CD8^+$ T cells in response to stimulation with WT1 peptide appeared to be higher than those in WT1-*coTCR*-transduced $CD8^+$ T cells.

Similarly, as shown in Figure 1D, up-regulation of cytotoxicity mediated by WT1-*siTCR*–transduced $CD8^+$ T cells against both WT1 peptide-loaded target cells and HLA-A*24:02-positive leukemia cells was detected compared with that mediated by WT1-*coTCR*–transduced $CD8^+$ T cells.

Enhanced expandability without deterioration of antigen-specific responsiveness of WT1-*siTCR*–transduced $CD8^+$ T cells

Efficient expansion of transferred T cells in vivo as well as in vitro is an important issue affecting the efficacy of adoptive T-cell