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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

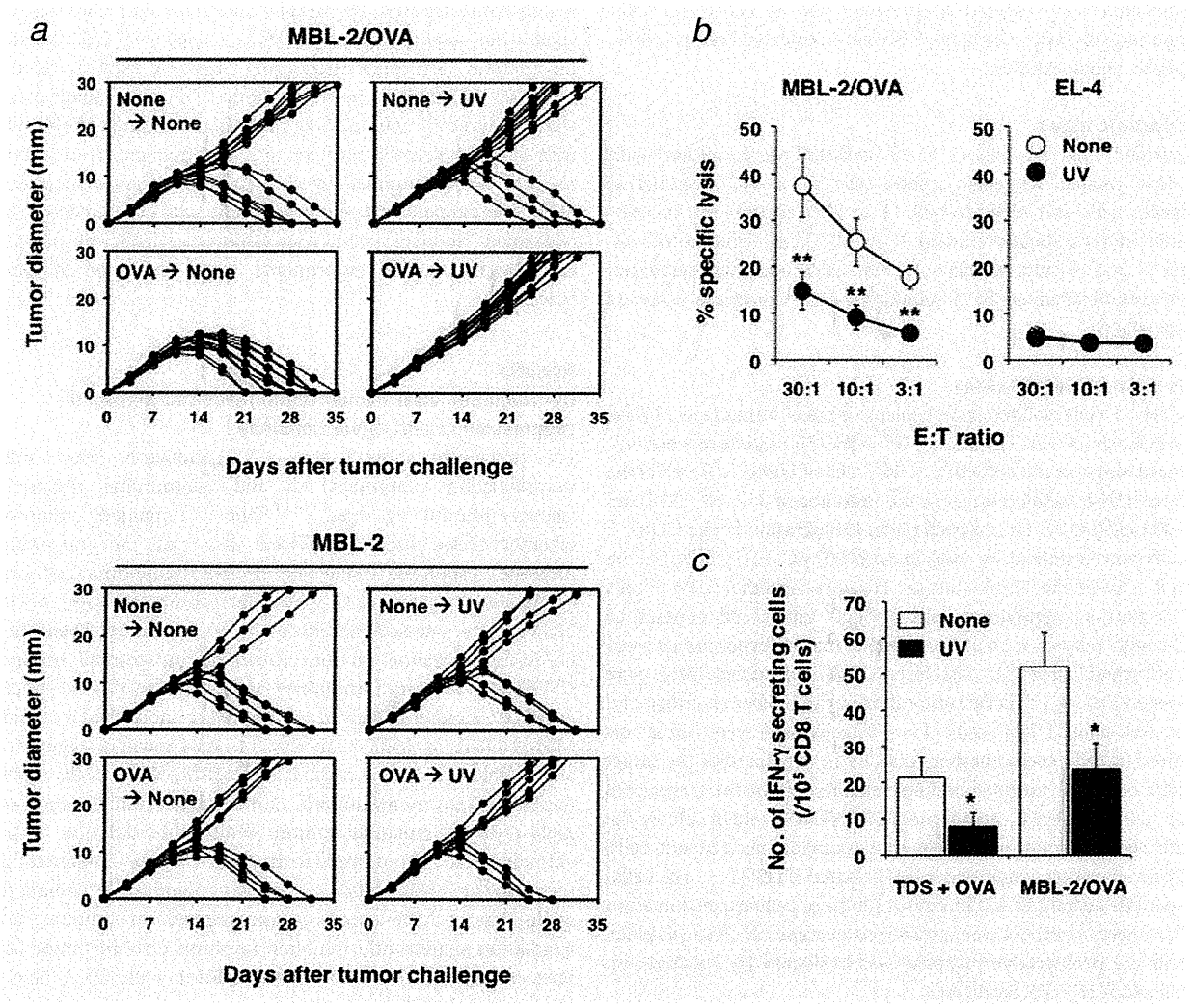


Figure 1. UV irradiation after immunization impairs the Ag-specific anti-tumor response. (*a*) Groups of mice ($n = 10$) were immunized with OVA (OVA) or PBS (None) in IFA on day 0, UV (UV) or mock (None) irradiated on day 7 and inoculated with 2×10^6 MBL-2/OVA (upper panels) or 1×10^6 MBL-2 cells (lower 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]

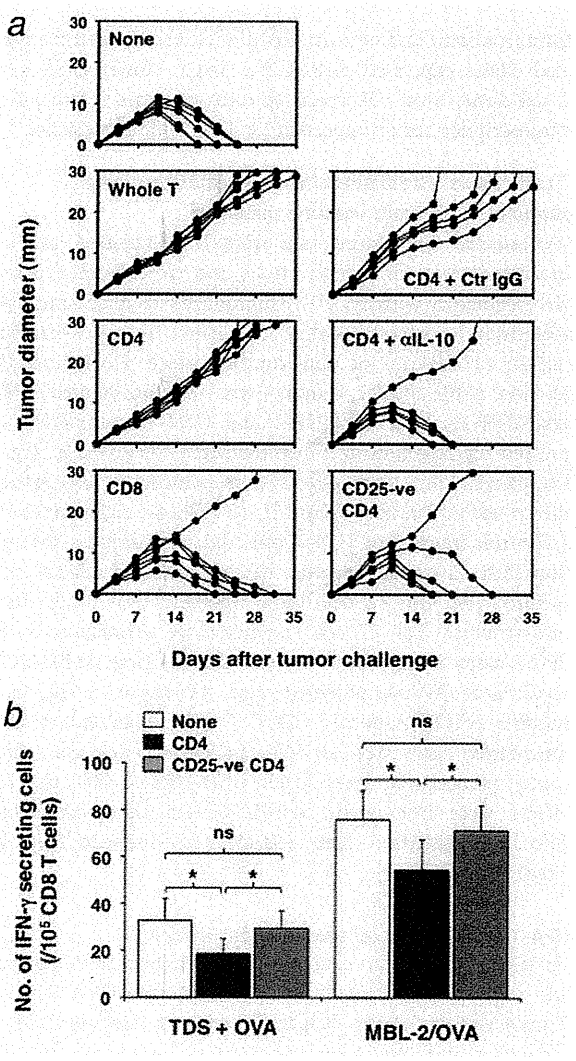
T cell clones established from UV-irradiated mice suppress anti-tumor immune response

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; $^*p < 0.05$; $^{**}p < 0.01$.

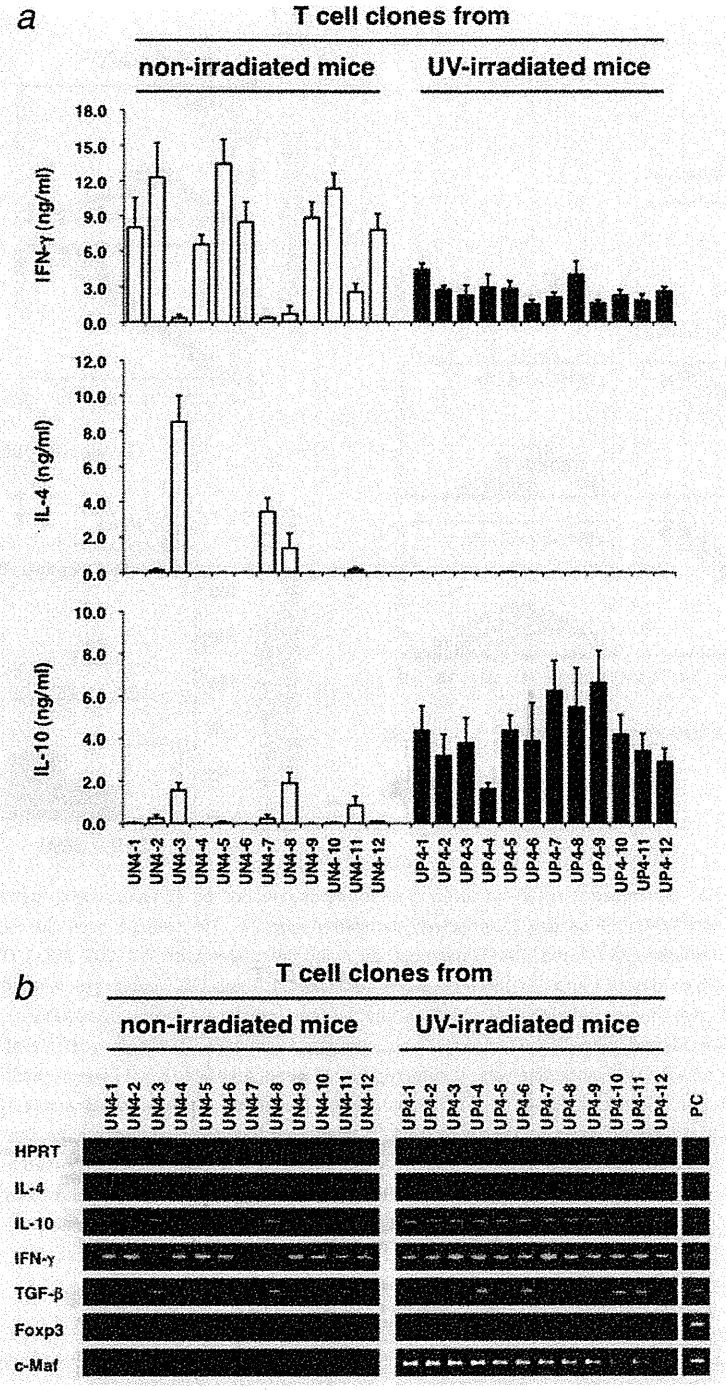


Figure 3. T cell clones established from UV-irradiated mice express IL-10, IFN-γ and c-Maf. (*a*) T cell clones from UV-irradiated (closed bars) or non-irradiated (open bars) mice were cultured with splenic APC in the presence of 100 μg/ml OVA. Culture supernatants were collected at 24 hr and assayed 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

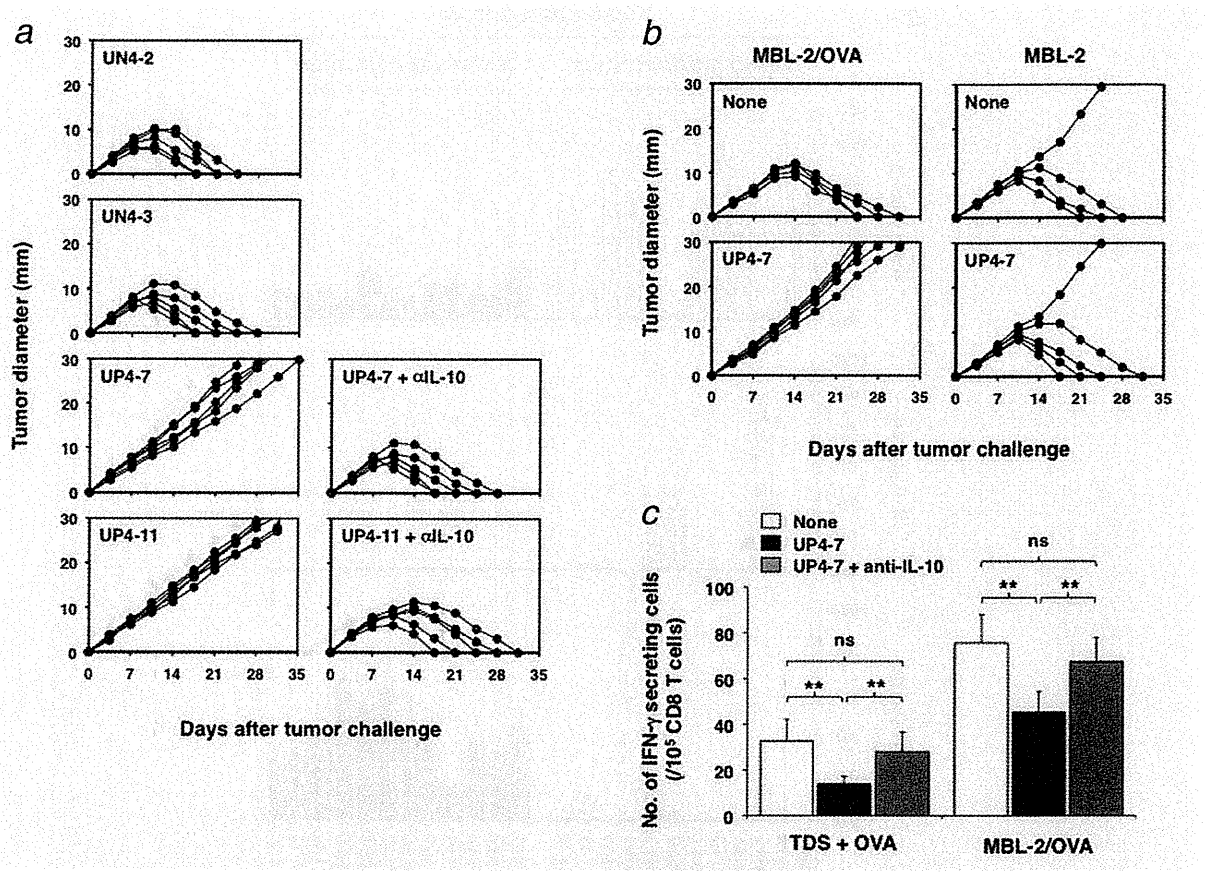


Figure 4. T cell clones from OVA-immunized and UV-irradiated mice suppress rejection of OVA-expressing tumor. (*a*) Recipient mice ($n = 4–5$) were immunized with OVA in IFA on day 0, adoptively transferred with 2×10^6 cells of T cell clones from UV-irradiated (UP4-7 and UP4-11) or non-irradiated (UN4-2 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

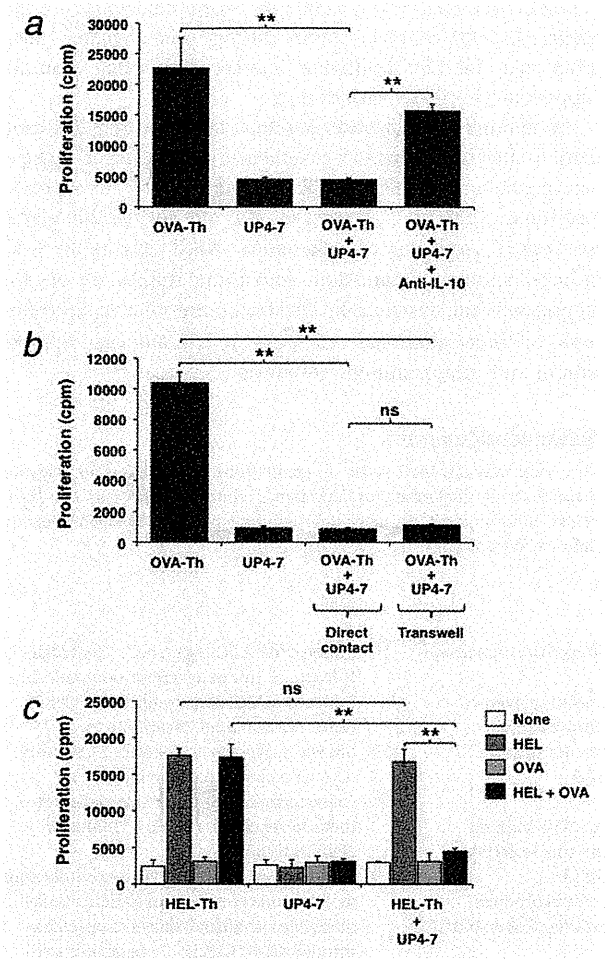


Figure 5. The T cell clone UP4-7 suppresses proliferation of Th cells. (*a*) $CD4^+$ T cells from OVA immunized mice (OVA-Th, 1×10^6 cells/ml) were cultured with TDS and OVA in the presence or absence of UP4-7 (0.5×10^6 cells/ml) together with or without 2 μg/ml of anti-IL-10 in the wells of a 96-well 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.

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ORIGINAL ARTICLE

Gemcitabine enhances Wilms' tumor gene WT1 expression and sensitizes human pancreatic cancer cells with WT1-specific T-cell-mediated antitumor immune response

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Abstract Wilms' tumor gene (WT1), which is expressed in human pancreatic cancer (PC), is a unique tumor antigen recognized by T-cell-mediated antitumor immune response. Gemcitabine (GEM), a standard therapeutic drug for PC, was examined for the regulation of WT1 expression and the sensitizing effect on PC cells with WT1-specific antitumor immune response. Expression of WT1 was examined by quantitative PCR, immunoblot analysis, and confocal microscopy. Antigenic peptide of WT1 presented on HLA class I molecules was detected by mass spectrometry. WT1-specific T-cell receptor gene–transduced human T cells were used as effecter T cells for the analysis of cytotoxic activity. GEM treatment of human MIAPaCa2 PC cells enhanced WT1 mRNA levels, and this increase is associated with nuclear factor kappa B activation. Tumor tissue from GEM-treated MIAPaCa2-bearing SCID mice also showed an increase in WT1 mRNA. Some human PC cell lines other than MIAPaCa2 showed up-regulation of WT1 mRNA levels following GEM treatment. GEM treatment shifted WT1 protein from the nucleus to the cytoplasm, which may promote proteasomal processing of WT1 protein and generation of antigenic peptide. In fact, presentation of HLA-A*2402-restricted antigenic peptide of WT1 (CMTWNQMNL) increased in GEM-treated MIAPaCa2 cells relative to untreated cells. WT1-specific cytotoxic T cells killed MIAPaCa2 cells treated with an optimal dose of GEM more efficiently than untreated MIAPaCa2 cells. GEM enhanced WT1 expression in human PC cells and sensitized PC cells with WT1-specific T-cell-mediated antitumor immune response.

Keywords Pancreatic cancer · WT1 · Gemcitabine · NF kappa B · T-cell response

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Introduction

Pancreatic cancer (PC) is a devastating disease with a 5% overall 5-year survival rate [1, 2]. This high mortality rate is due to a combination of factors that include a high incidence of metastatic disease at initial diagnosis, an aggressive clinical course, and the failure of systemic therapies used for treatment. Despite the fact that advanced loco-regional disease is found in 40% of patients [3], only 5–25% of patients with pancreatic cancer are treated surgically [4]. Even in cases where pancreatic cancer is discovered at a resectable stage, only 10–20% of patients are expected to survive for more than 5 years after curative resection [5].

Gemcitabine (GEM) is currently the most commonly used therapeutic drug prescribed in cases of advanced PC [6, 7]. Numerous phase III trials testing gemcitabine in combination with other cytotoxic drugs have failed to reveal any additional benefit compared with gemcitabine alone [8]. Erlotinib, a small molecule inhibitor of the epidermal growth factor receptor tyrosine kinase, is a notable exception in that it is the only drug reported to confer a significant improvement in survival over gemcitabine alone [9]. Recently, Folfirinox was reported to be a more efficient, but more toxic, regimen for pancreatic cancer and might be promising for the patients with good performance status [10]. Ultimately, improved treatment of advanced PC will likely require additional selected and targeted agents that provide the benefit of prolonged survival with minimum risk.

The Wilms' tumor gene WT1 encodes a zinc finger transcription factor. Although the WT1 gene was originally defined as a tumor suppressor gene [11–13], additional reports demonstrate that it is highly expressed in leukemia and various types of malignant tumors [14] and can confer oncogenic functions [15]. WT1-specific cytotoxic T lymphocytes (CTLs) and WT1 antibodies have both been shown to be induced spontaneously in tumor-bearing leukemia patients [16]. These results indicate that WT1 protein is highly immunogenic and establish it as a promising tumor antigen for recognition by specific CTLs [17]. The safety and clinical efficacy of major histocompatibility complex (MHC) class I-restricted WT1 epitope peptides against various malignancies have been confirmed in clinical immunotherapy trials [14, 15].

Reports indicate that WT1 is frequently overexpressed in human pancreatic cancer cells [18]. Recent clinical reports on treatments combining GEM drug therapy with peptide vaccine immunotherapy have demonstrated safe and promising results in cases of advanced PC [19, 20]. In our recent phase I clinical trial that tested a combination of WT1 peptide vaccine and GEM in treatment of advanced PC, several cases showed marked tumor regression (manuscript in preparation). These results suggest that the actions of WT1-targeted antitumor immunity and GEM can function synergistically against PC cells. In the present study, we demonstrate that GEM treatment up-regulates WT1 expression in PC cell lines, and that antitumor immune activity against PC cells via a WT1-specific T-cell response is augmented by GEM treatment.

Materials and methods

Cell lines, antibodies, and mice

Human pancreatic cancer cell lines (MIAPaCa2, PANC-1, AsPC-1, BxPC-3, Capan-1 and Capan-2) were obtained from the American Type Culture Collection (Manassas, VA, USA) [21]. A rabbit polyclonal antibody against WT1 (C-19) and a goat polyclonal antibody against Lamin B (C-20) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Eight- to ten-week-old SCID mice were supplied by Nihon SCL Co., Ltd. (Hamamatsu, Japan) and were maintained in our specific pathogen-free facilities. Mice received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institute of Health (NIH publication 86-23 revised 1985).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Tissue or cell samples were lysed directly in Buffer RLT Plus (Qiagen, Hilden, Germany) and homogenized. Reverse transcription (RT) was performed using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). TaqMan primers and non-fluorescent quencher probes complementary to WT1 (Assay ID:Hs00240913_m1) and 18S ribosomal RNA (rRNA, Assay ID:Hs99999901_s1) genes were purchased from Applied Biosystems. qRT-PCR was performed using the 7300 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). WT1 expression levels were normalized relative to those of 18S rRNA.

Inhibition of nuclear factor kappa B (NF-kB)

Inhibition of NF-kB activity in human PC cells was achieved using an NF-kB p65 (Ser276) inhibitory peptide kit (IMGENEX, San Diego, CA, USA). Briefly, MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates and incubated for 24 h. Growth medium was then changed to medium containing GEM (0 or 30 ng/ml) with NF-kB blocking peptide (50 μM) or control peptide (50 μM). After 24-h incubation, cellular expression of NK-kB was determined using qRT-PCR.

Immunoblot analysis

The nuclear fraction of MIAPaCa2 cells used for the detection of WT1 protein was isolated using an Active Motif extraction kit (Carlsbad, CA, USA). Protein samples (30 μg/well) separated by electrophoresis in 15% sodium dodecyl sulfate-polyacrylamide gels were then transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA). After blocking in 5% nonfat milk for 1 h, membranes were exposed to antibodies specific to WT1 (1:100) and beta-actin (1:10,000; Sigma–Aldrich, St. Louis, MO, USA) and then to horseradish peroxidase-conjugated secondary antibodies. The ECL-PLUS Detection System (GE Healthcare, Buckinghamshire, UK) was used for chemiluminescent detection of secondary antibodies.

Confocal microscopy

MIAPaCa2 cells cultured on glass coverslips were incubated with or without GEM (30 ng/ml) for 24 h. Cells were then washed and fixed in 4% paraformaldehyde. Immunofluorescent visualization of cells expressing WT-1 was achieved by incubating slides in rabbit anti-WT1 antibody (1/200), followed by Amaxa488-conjugated donkey anti-rabbit IgG antibody (Molecular probes, Eugene, OR, USA). Cell nuclei were stained with TO-PRO-3 iodide (Molecular Probes), and a laser scanning confocal microscope (LSM510, CarlZeiss, Thornwood, NY, USA) was used to obtain fluorescence images.

Positive ion ESI LC–MS/MS analysis of MHC class I binding peptides from MIAPaCa2 cells

MIAPaCa2-bearing mice were injected intraperitoneally with PBS or GEM (3.75 mg/mouse). After 48 h, tumors were resected and digested using collagenase to obtain single cells. MHC class I binding peptides were isolated from 10^8 cells using the method described by Storkus et al. [22]. Isolated peptides were dissolved in 50% methanol and analyzed via electrospray ionization (ESI) liquid chromatography (LC)-tandem mass spectrometry (MS/MS) using a triple quadrupole mass spectrometer (Q TRAP) (Applied Biosystems, Foster City, CA, USA). The mass spectrometer interfaced with an Agilent 1100 liquid chromatography (Agilent Technologies, Wilmington, DE, USA) was employed. The WT1 antigenic peptide (aa 235–243 CMTWNQMNL; MW = 1,139.5 Da) in 50% methanol was easily produced m/z 1171.5 as a methanol adduct ion $(M + MeOH)^+$. The multiple reaction monitoring (MRM) transition monitored for the detection of this peptide was m/z 1,171.5/1,154.5. This peptide was eluted at a flow rate 0.2 mL/min from an Intersil C8-3 column [50 × 2.1 mm, 3 μm particle size] (GL Science Inc., Tokyo Japan) using a linear gradient of 9.5% min^{-1} of 5–100% acetonitrile containing 1% formic acid. To estimate cellular peptide concentrations, a standard curve was prepared by increasing concentrations (0–1,000 pmol) with chemically synthesized WT-1 antigenic peptide. The response was considered to be linear if the correlation coefficient (r^2) was greater than 0.99, calculated by least-squares linear regression analysis.

Cytotoxicity assay

WT1-specific cytotoxic effector cells were generated as described below. Full-length WT1-specific T-cell receptor (TCR) a/b genes (Va20/J33/Ca for TCR-a and Vb5.1/J2.1/Cb2 for TCR-b, respectively) isolated from the HLA-A*2402-restricted $WT1_{235-243}$-specific $CD8^+$ CTL clone TAK-1 [23] were cloned into a pMEI-5 retroviral vector (Takara Bio, Shiga, Japan). WT1-specific TCR genes were then transduced into normal $CD8^+$ lymphocytes as described previously [24]. Cytotoxicity assays were performed using a standard 4-h culture ^{51}chromium (Cr) release assay described elsewhere [25].

Statistical analysis

The significance of differences between groups was analyzed using Student's *t* test for two independent groups and with Tukey's test for multiple-group comparisons. Values that did not fit a Gaussian distribution were analyzed with the Bonferroni method for multiple-group comparisons.

Results

Up-regulation of WT1 mRNA in human PC cells by in vitro treatment with GEM

Proliferation of MIAPaCa2 cells was inhibited for 48 h with stable numbers of viable cells following treatment with 30 and 100 ng/ml of GEM (Fig. 1a). Growth of MIAPaCa2 cells was also impaired by treatment with 10 ng/ml of GEM for 72 h. Levels of WT1 mRNA were enhanced significantly by treatment of MIAPaCa2 cells with 10, 30, and 100 ng/ml of GEM for 24, 48 and, 72 h, respectively (Fig. 1b). Enhancement of WT1 mRNA was also observed after 2-h treatment with GEM (100 ng/ml) in following 72 h (Fig. 1c). This GEM-mediated enhancement was suppressed by the addition of NF-kB blocking peptide in the culture (Fig. 1d).

GEM-mediated up-regulation of WT1 mRNA expression was examined in various human pancreatic cancer cell lines. GEM-treated Capan-2 cells showed a significant enhancement of WT1 mRNA expression (Fig. 2a). Low steady-state levels of WT1 mRNA expression in AsPC-1 and BxPC-3 cells were also enhanced by GEM treatment (Fig. 2b). In contrast, expression of WT1 mRNA in Capan-1 and PANC-1 cells was not up-regulated by GEM treatment (Fig. 2b, c).

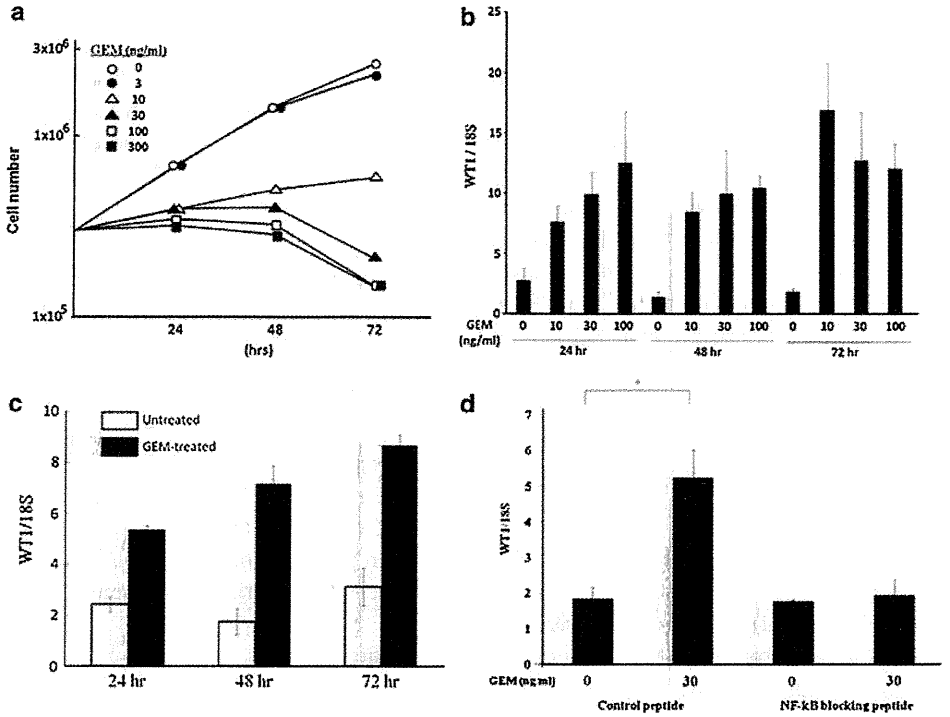

Fig. 1 **a** Proliferation of MIAPaCa2 cells in medium containing various concentrations of GEM. MIAPaCa2 cells (3×10^5/well) were seeded in 6-well culture plates in regular culture medium, which was then exchanged for GEM-containing medium after 24 h. At 24-h intervals, cells were detached using trypsin, and cell numbers were counted using a hemocytometer ($n = 3$). **b** Up-regulation of WT1 mRNA in MIAPaCa2 cells by GEM treatment. Twenty-four hours after plating, culture medium was exchanged to media containing GEM at indicated concentrations (0, 10, 30 and 100 ng/ml). MIAPaCa2 cells were harvested at 24-h intervals, and WT1 mRNA in cell homogenates was analyzed using qRT-PCR. WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **c** Up-regulation of WT1 mRNA in MIAPaCa2 cells after short treatment with GEM. Twenty-four hours after plating, MIAPaCa2 cells were untreated or treated with 100 ng/ml of GEM for 2 h. MIAPaCa2 cells did not proliferate but kept alive for following 72 h by this treatment with GEM. After GEM treatment, cells were washed well, cultured in regular culture medium, and harvested at 24-h intervals. WT1 mRNA in cell homogenates was analyzed using qRT-PCR, and WT1mRNA levels were normalized relative to those of 18S ribosomal RNA (18S). **d** NF-kB suppresses GEM-induced up-regulation of WT1 mRNA. MIAPaCa2 cells (6×10^4/well) were seeded in 24-well culture plates. After 24 h, medium was exchanged for media containing GEM (0 or 30 ng/ml) and/or NF-kB blocking peptide (50 μM) or control peptide (50 μM). WT1 mRNA levels were quantified after 24-h incubation using qRT-PCR. $^*P < 0.01$

Changes in WT1 mRNA expression levels were also examined in MIAPaCa2 cells following in vitro treatment with various other chemotherapeutic agents. Oxaliplatin, Doxorubicin, and five-fluorouracil showed significant enhancement of WT1 mRNA expression, but cisplatin and irinotecan did not (Suppl. 1). Because GEM is the standard drug used to treat human PC, its effect on human PC cells was studied thereafter.

In vivo up-regulation of WT1 mRNA in tumor tissue by treatment of MIAPaCa2-bearing SCID mice with GEM

In order to clarify whether in vivo treatment of tumor cells with GEM induces an enhancement of WT1 mRNA expression, SCID mice implanted subcutaneously with MIAPaCa2 cells were treated with a clinical dosage of GEM. We observed a significant increase in the levels of WT1 mRNA 48 h after injection of GEM (Fig. 3).

GEM treatment shifts localization of WT1 from the nucleus to the cytoplasm

We used immunoblot analysis to examine the levels of WT1 protein in MIAPaCa2 cells cultured in the absence or presence of GEM. Relative to untreated cells, WT1 protein levels in GEM-treated MIAPaCa2 cells were augmented; however, after 36 h of cell culture, levels of WT1 protein diminished in both untreated and GEM-treated cells (Fig. 4a). This decline in WT1 protein levels was rescued by treatment with the proteasome inhibitor MG-132, indicating that WT1 protein is susceptible to proteasomal degradation (Fig. 4b).

Confocal microscopy images demonstrate that WT1 protein is primarily located in nuclei of untreated cells (Fig. 5a). However, in MIAPaCA2 cells treated with GEM, localization of WT1 protein shifted to the cytoplasm and the intensity of WT1 immunofluorescence in the nucleus decreased

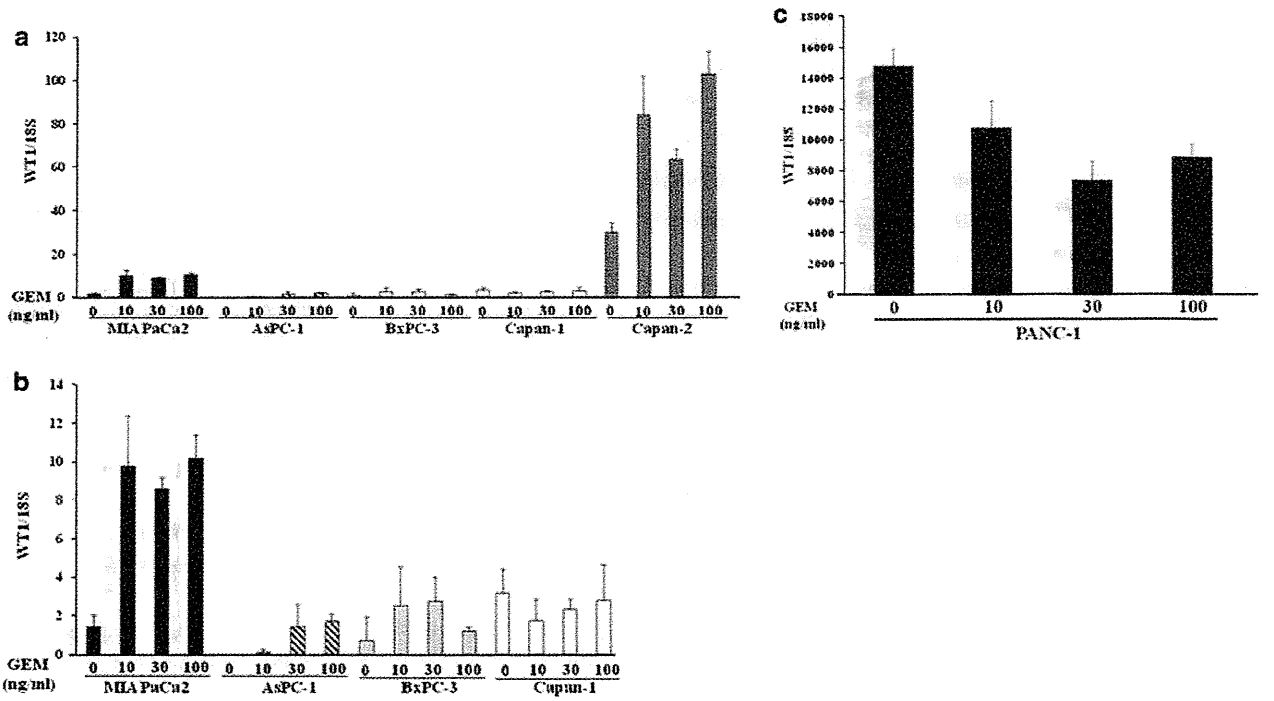

Fig. 2 **a** Up-regulation of WT1 mRNA levels in various human PC cell lines following GEM treatment. Human PC cells (1×10^6 MIAPaCa2, AsPC-1, BxPC-3, Capan-1 or Capan-2) were seeded in 10-cm culture plates. After 24-h incubation, medium was changed to media containing GEM (10, 30 or 100 ng/ml). After 48 h, we used qRT-PCR to quantify the relative ratio of WT1 to 18S mRNA levels in each cell line ($n = 3$). **b** GEM-induced up-regulation of WT1 mRNA in human PC cells with low basal levels of WT1 mRNA (MIAPaCa2, AsPC-1, BxPC-3 and Capan-1). To illustrate these results, we replotted data from (**a**) to represent a considerably narrower range of mRNA level ratios (0–14) on the *y*-axis. (**c**) Expression of WT1 mRNA in human PC cells with high basal levels of WT1 mRNA (PANC-1). To illustrate the results, we plotted data to represent a considerably wider range of mRNA level ratios (0–18,000) on the *y*-axis

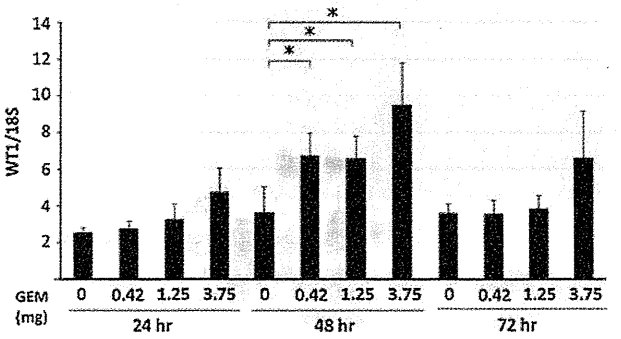

Fig. 3 Tumors in PC-bearing SCID mice treated with GEM show increased WT1 mRNA levels. Ten days after subcutaneous inoculation of SCID mice with 5×10^6 MIAPaCa2 cells (formation of approximately 1-cm diameter tumors), mice were injected intraperitoneally with GEM (0, 0.42, 1.25 and 3.75 mg/mouse). Tumors were resected every 24 h thereafter, and relative levels of WT1 mRNA were quantified using qRT-PCR ($n = 3$). Duplicate trials of the same protocol showed similar results. $^*P < 0.01$

(Fig. 5a). Decline in WT1 protein levels following GEM treatment was also observed in immunoblot analyses of the nuclear fraction of treated MIAPaCa2 cells (Fig. 5b).

Enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide following GEM treatment

Figure 6a shows typical standard curve obtained with increasing quantities of WT1 antigenic peptide. The data indicate a linear relation over a wide range (0–1,000 pmol) of analyte amount with correlation coefficients greater than 0.99. The data in the Fig. 6b demonstrate the sensitivity as well as the noise background of the LC–MS/MS. The noise background is less than 1 cps. The signal from injection of 10 pmol of this peptide spiked to MIAPaCa2 cells is approximately 16 cps, giving an S/N ratio of approximately 16. The low noise background and signal of 10 pmol of this peptide indicated the extrapolated limit of detection is less than 0.8 pmol on column under S/N = 2.

The level of the WT1 antigenic peptide was estimated among MHC class I binding peptides from MIAPaCa2 cells treated with either PBS or GEM to 6.49 pmol/10^8cell or 8.78 pmol/10^8cell, respectively. GEM treatment increased the presentation of HLA-A*2402-restricted WT1 antigenic peptide on MIAPaCa2 cells.

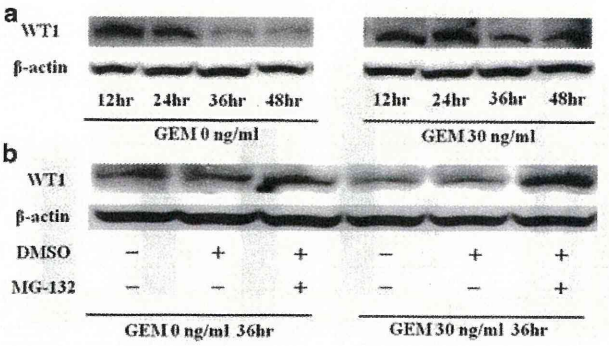

Fig. 4 **a** WT1 protein is degraded by proteasomal enzymes. Twenty-four hours after 3×10^5 MIAPaCa2 cells/well were seeded in 6-well culture plates, medium was exchanged from untreated to media containing GEM (0 or 30 ng/ml). Expression of WT1 protein in the cells was analyzed every 12 h thereafter from immunoblots described in Sect. "Materials and methods". **b** Protease inhibitors block WT1 degradation. Twenty-four hours after incubating MIAPaCa2 cells with GEM (0 or 30 ng/ml), MG-132 in DMSO or DMSO alone was added to each well at a concentration of 5 μM and 0.05%, respectively. Treated and control cells (in 0.05% DMSO alone) were incubated for 12 h before harvesting cells for immunoblot analysis of WT1 and beta-actin proteins

GEM-treated PC cells are killed efficiently by effector cells transduced with genes encoding a WT1-specific T-cell receptor

The susceptibilities of untreated and GEM-treated MIAPaCa2 cells to WT1-specific cytotoxic effector T cells were compared. The cytotoxic effect of WT1-specific effector cells on MIAPaCa2 cells was enhanced significantly when PC cells were treated with either 10 or 30 ng/ml of GEM for 48 h (Fig. 7). Notably, effector cell cytotoxicity was not enhanced by treatment of PC cells with 100 ng/ml of GEM, although this high dose of GEM was more toxic to PC cells than 10 or 30 ng/ml. Up-regulation of MHC class I in MIAPaCa2 cells by GEM treatment that possibly provides the similar results was not observed (data not shown).

Discussion

In the present study, we demonstrate that expression of WT1 mRNA in human PC cells is enhanced by treatment

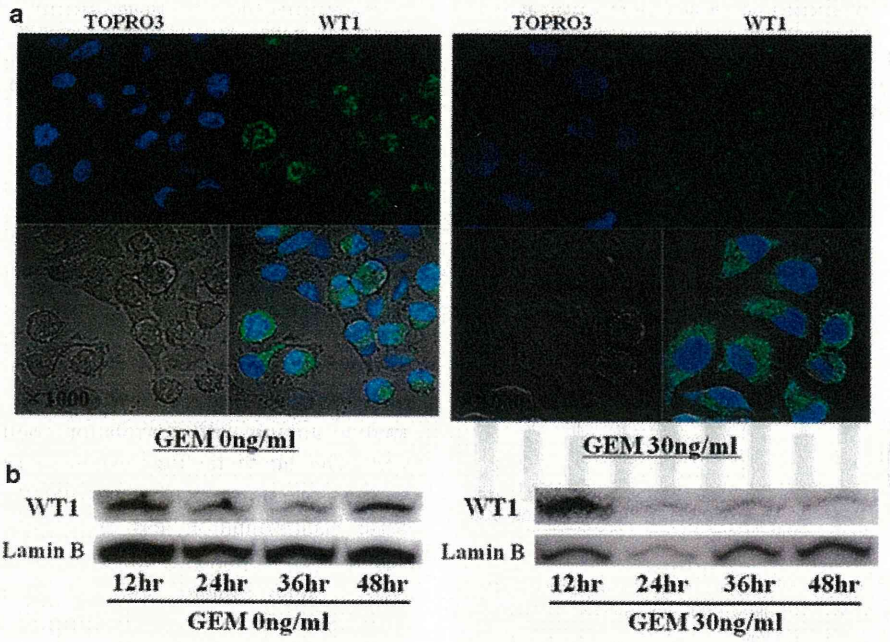

Fig. 5 **a** GEM treatment shifts WT1 protein localization from nucleus to cytoplasm. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, untreated medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). After 24-h incubation, cells were fixed with paraformaldehyde, followed by nuclear staining with TO-PRO-3 iodide (*blue color*) and detection of WT1 with rabbit anti-WT1 polyclonal antibody and anti-rabbit IgG conjugated with fluorescein isothiocyanate (*green color*). Stained cells were observed using confocal microscopy (original magnification ×1,000). **b** GEM treatment diminishes nuclear localization of WT1 protein. Twenty-four hours after seeding 3×10^5 MIAPaCa2 cells/well in 6-well culture plates, medium was exchanged for fresh medium with or without GEM (0 or 30 ng/ml). At 12-hour intervals thereafter, nuclei were isolated and WT1 protein levels of nuclear extracts were analyzed on immunoblots as described in Sect. "Materials and methods"

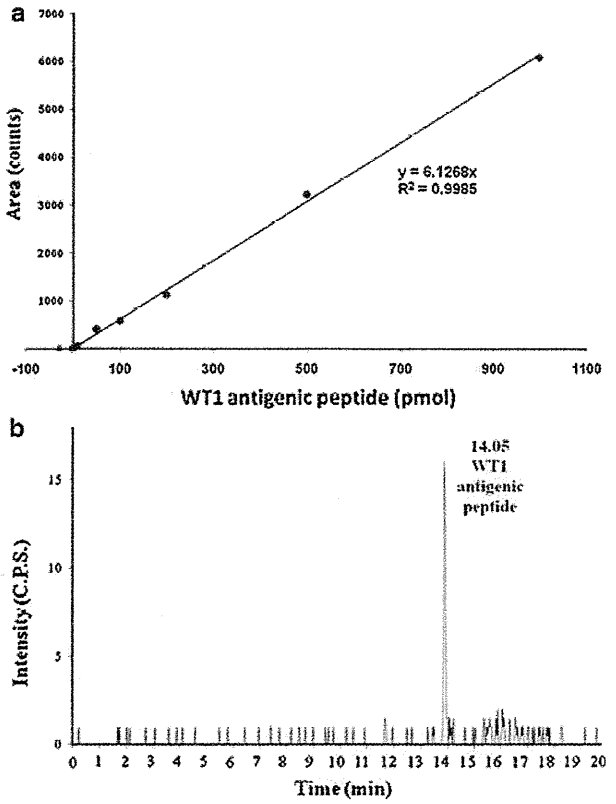

Fig. 6 **a** Standard curve for HLA-A*2402 restricted WT1 antigenic peptide. **b** Trace of MRM signal during LC–MS/MS analysis of spiked HLA-A*2402-restricted WT-1 antigenic standard peptide (10 pmol) in MIAPaCa2 cells

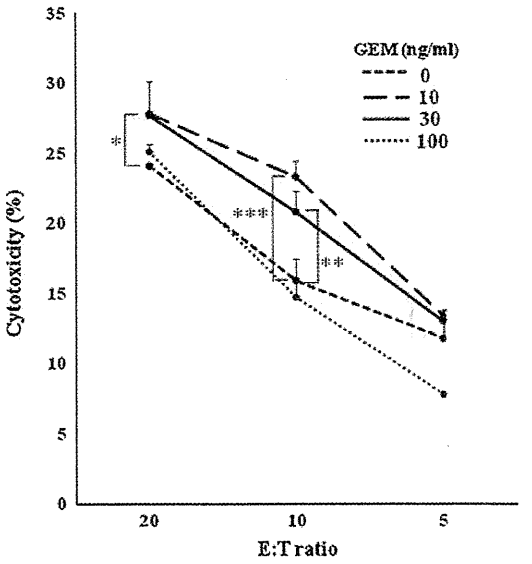

Fig. 7 WT1-specific CTLs kill GEM-treated MIAPaCa2 cells efficiently. MIAPaCa2 cells pretreated with 0, 10, 30, or 100 ng/ml GEM for 48 h were labeled with ^{51}Cr. ^{51}Cr release assays were used to measure the cytotoxic activity of WT1-specific effector cells against untreated or GEM-pretreated MIAPaCa2 cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

with GEM. MIAPaCa2 cells demonstrating GEM-mediated enhancement of WT1 mRNA levels did not proliferate but maintained stable numbers of viable cells with impaired growth by continuous treatment with low-dose GEM as well as short treatment with high-dose GEM. WT1 is a transcription factor with oncogenic potential, in that it can induce malignant cellular phenotypes, suppress apoptosis, and promote cell proliferation [15]. We hypothesize that up-regulation of WT1 levels in PC cells aids cell survival by conferring chemoresistance against GEM's toxic effects.

Based on the fact that GEM-mediated augmentation of WT1 mRNA expression was attenuated by addition of an NF-kB blocking peptide in the culture, activation of NF-kB also appears to play a significant role in WT1 enhancement. NF-kB is known to be active in many malignant tumors and has been implicated in cellular resistance to cytotoxic agents and escape from apoptosis [26]. Previous reports demonstrate that GEM activates NF-kB [27] and that the ensuing regulatory cascade activates the WT1 gene downstream [28]. Human PC cell lines with high NF-kB activity are resistant to GEM [27], and that silencing or suppression of NF-kB increases the sensitivity of PC cells to GEM and induces apoptosis [29–31].

It is of note and interest that some chemotherapeutic agents other than GEM showed capability on up-regulation of WT1mRNA expression. Especially, treatment with oxaliplatin (L-OHP) induced marked enhancement of WT1mRNA expression. Folfirinox including L-OHP was recently reported to be a more efficient regimen for metastatic pancreatic cancer (10). However, combined treatment with Folfirinox and WT1 targeting immunotherapy might be unsuccessful because of severe leukopenia by Folfirinox. GEM has relatively low hematologic toxicity and thus seems to be preferable for combination therapy with WT1 targeting immunotherapy.

We also observed up-regulation of WT1 mRNA by GEM treatment in vivo. Within 48 h of treating MIAPaCa2-bearing SCID mice with a clinical dose of GEM, steady-state levels of WT1 mRNA in the tumor increased. Despite its rapid disappearance after intraperitoneal injection, the enhancement of WT1 mRNA expression in tumor tissue was significant. Enhancement of WT1 mRNA expression was also observed after in vitro short treatment with GEM. These results suggest strongly that GEM treatment of human PC in a clinical setting might induce up-regulation of WT1 in PC cells.

In the present study, we found that the localization of WT1 protein shifted from nucleus to cytoplasm following GEM treatment. WT1 protein has been shown to undergo nucleocytoplasmic shuttling [32], and the function of WT1 has been suggested to correlate with its cellular location: Siberstein et al. [33] described that WT1 was localized to

the cytoplasm and not to nuclei in some human breast cancers and suggested that such localization may be regulated by alternative splicing of WT1 mRNA. On the other hand, immunohistochemical studies of Nakatsuka et al. [34] demonstrate a majority of WT1-positive tumors with diffuse or granular staining in the cytoplasm. Ye et al. [35] report that phosphorylation of WT1 protein resulted in cytoplasmic retention of WT1, thereby inhibiting DNA binding and altering transcriptional activity. Through the activation of NF-kB, GEM treatment may mediate a similar phosphorylation and translocation of WT1 protein from nucleus to cytoplasm.

In order for MHC class I-restricted antigen to be presented and recognized by antigen-specific CTLs, tumor antigen must be degraded by proteasomal enzymes located in the cytoplasm [36]. Retention of an intra-nuclear tumor antigen such as WT1 in the cytoplasm should favor tumor antigen processing, and in fact, we observed enhanced presentation of HLA-A*2402-restricted WT1 antigenic peptide using ESI LC–MS/MS analyses. GEM-treated MIAPaCa2 cells showed greater susceptibility than untreated cells to the cytotoxic effects of WT1-specific CTLs generated by transduction of a gene encoding a WT1-specific T-cell receptor. Importantly, treatment with 10–30 ng/ml of GEM enhanced the susceptibility of MIAPaCa2 cells to CTL, but treatment with 100 ng/ml did not. This phenomenon indicates that the enhanced susceptibility of GEM-treated MIAPaCa2 cells to CTLs is not due to GEM toxicity, but to augmented expression of the WT1 target antigen.

GEM is a nucleoside analog with clinical relevance to the treatment of several solid tumors, including PC; nonetheless, its antitumor effect is limited. We observed significant clinical response in a phase I clinical study of combined treatment against advanced PC using a WT1 peptide vaccine and GEM (manuscript in preparation). The presumed actions of GEM up-regulating WT1 expression in vivo and WT1-specific CTLs killing GEM-treated tumor cells efficiently may prove valuable for the treatment of human PC. It has been reported that GEM may suppress the activity of myeloid-derived suppressor cells that inhibit antitumor immunity [37]. In addition, GEM has been shown to increase the number of dendritic cells in blood without affecting T-cell activity in patients with PC [38]. We propose that combining GEM's proven role as an immunopotentiator with its ability to up-regulate target WT1 expression of PC cells will enhance the susceptibility of PC cells to WT1-specific CTLs. Furthermore, PC cells already acquired GEM resistance by the activation of NF-kB might be injured by WT1-specific CTLs. Assessment of the clinical response to combined therapy with WT1 peptide vaccine and GEM is presently underway.

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Conflict of interest There are no financial disclosures of any of the authors to declare.

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