co-incubated with 1×10^5 WT1 peptide-pulsed (1 μM) or unpulsed C1R-A24 cells for 24 hours in various concentrations of CCL2. IFN-γ in the culture supernatant was similarly measured using an ELISA kit (R&D Systems).

11. CD107a assay

CD107a expression mediated by double-transfected $CD8^+$ T cells in response to stimulation with WT1 peptide was examined as described previously [26]. Briefly, 1×10^5 C1R-A24 cells were seeded into a 96-well round-bottom plate and incubated with or without WT1 peptide for 2 hours in various concentrations of CCL2. Then, 2×10^5 of these double-transfected $CD8^+$ T cells were seeded into each well with FITC-conjugated CD107a mAb (BioLegend), and incubated for 3 hours. The cells were then additionally labeled with anti-CD3, anti-CD8 mAbs (BD Biosciences) and PE-conjugated HLA-A*2402/$WT1_{235-243}$ tetramer, and subjected to flow cytometric analysis.

12. Transwell experiments

Functional validation of transduced CCR2 was conducted in vitro employing transwell experiments. In brief, 2×10^5/well *CCR2*-transfected Jurkat cells were placed in the upper well, and 500 μL RPMI 1640 with 10% FCS culture medium containing various concentrations of CCL2 was added to the bottom well of a 3-μm pore-size 24-well transwell plate (Coster). After 4 hours of incubation, the cells in the bottom well were counted using the trypan blue dye exclusion method. All experiments were conducted in triplicate and independently three times. When human lung cancer cell lines were seeded into the bottom wells, transwell experiments were similarly conducted after a 24-hour pre-incubation. A blocking experiment was conducted using ant-CCR2 mAb (R&D Systems). Finally, both tumor trafficking and cytocidal activities mediated by double gene-modified $CD8^+$ T cells were examined in the same transwell experiment. Briefly, using a 3-μm pore-size 6-well transwell plate, LK79 cells were seeded into the bottom well at 10^6/well and incubated for 24 hours. Then, double-transfected $CD8^+$ T cells were loaded onto the upper well at 2×10^6/well. WT1-specific *TCR* single-transfected $CD8^+$ T cells were used as a control. After 12 hours of incubation, each supernatant in the bottom well was harvested. After centrifugation at 1200 rpm for 5 min for removal of residual cells, 100 μL of each supernatant was subjected to ELISA assay (Roche) for lactate dehydrogenase (LDH) having leaked from the LK79 cells disrupted by migrated WT1-specific effector T cells. Experiments were conducted in triplicate. Since the retroviral *CCR2* gene expression vector simultaneously delivered a red-colored fluorescent protein (Ds-Red), double-transfected effector cells having migrated to the bottom well were also detected by confocal microscopy (A-1R, Nikon, Japan).

13. In vivo WT1 antigen-independent migration

In a xenografted mouse model, CCL2-dependent tumor trafficking activity mediated by *CCR2* and *luciferase* double-transfected normal $CD3^+$ T cells was assessed using bioluminescence imaging assay. Briefly, 1×10^7 LK79 cells were subcutaneously inoculated into the abdominal wall of 1 Gy-irradiated 9-week-old NOG mice (n = 6). One cohort (n = 3) received intravenous administration of single *luciferase*-transfected human $CD3^+$ T cells (5×10^6 cells/mouse) as a control, and the other cohort (n = 3) received double-transfected $CD3^+$ T cells on day 0. Thereafter all mice received serial intraperitoneal administrations of 500 IU recombinant human IL-2 (Roche) on days 0, 2, 4, and 6. Serial acquisition of luciferase photon counts using luciferin (Promega Corporation) was carried out on days 1, 3, 5, and 7 using AEQUORIA (Hamamatsu Photonics, Japan), and analyzed using AQUACOSMOS software (Hamamatsu Photonics).

14. In vivo tumor-suppressive activity mediated by infused double-transfected effector T cells in a therapeutic mouse model

For therapeutic adoptive transfer experiments, we prepared *luciferase*-transfected LK79 cells (LK79/luc) whose CCL2 production was equal to that of the parental LK79 cells. All 9-week-old NOG mice (n = 18) were 1 Gy-irradiated and then inoculated subcutaneously with 5×10^6 LK79/luc cells into the abdominal wall on day 0. These mice were divided into three cohorts: i) those intravenously administered 5×10^6 OKT-3/IL-2-activated $CD8^+$ T cells without any gene modification (n = 6), ii) those treated with 5×10^6 single WT1-specific *TCR*-transfected $CD8^+$ T cells from an identical donor (n = 6), and iii) those treated with 5×10^6 double-transfected $CD8^+$ T cells also from the same donor (n = 6). The mice in each cohort received the same cell therapy weekly three times in total (on days 4, 11, and 18). Serial acquisition of luciferase photon counts for inoculated LK79/luc cells was carried out. The photon counts relative to that on the first day of cell therapy, which indicated the residual tumor mass burden, were calculated in each mouse.

15. Statistical analysis

The paired *t* test was used to assess differences between two groups, and a one-way factorial analysis of variance followed by a Tukey's post-hoc test was used for comparisons among three groups; differences at $p<.05$ were considered significant.

Results

1. Production of various amounts of CCL2 by human lung cancer cell lines, and rare expression of the corresponding receptor CCR2 on T cells activated using OKT-3 and IL-2

The expression profiles of the 12 chosen chemokine mRNAs produced by each of the human lung cancer cell lines assessed by qRT-PCR are summarized in Table 2. As shown in Figure 1A, various amounts of CCL2 protein were produced in all of the lung cancer cell lines assessed, among which LK79, a SCLC cell line, produced notably high amounts of CCL2. The corresponding receptor, CCR2, was rarely expressed on the surface of both resting T cells or those activated using OKT-3/IL-2 (n = 5). Activated T cells were gated with CD69 positivity. A representative example among 5 cases is shown in Figure 1B. The levels of expression for resting and activated cells were: CD8, 0.83±0.72% and 0.75±0.51%, and CD4, 0.66±0.45% and 0.5±0.26% (mean± SD), respectively. Additionally, qRT-PCR revealed that the expression levels of *CCR2* mRNA in activated and resting T cells (n = 5) did not differ in either $CD4^+$ or $CD8^+$ T cells (data not shown). On the other hand, effector $CD8^+$ T cells double-transfected to express HLA-A*2402-restricted and $WT1_{235-243}$-specific-TCR successfully killed candidate human lung cancer cell lines in an HLA-A*2402-restricted manner (Fig. 1C and 1D). Taken together, the data suggested that the choice of the CCR2-CCL2 axis and the pairing of CTLs comprising effector cells gene-modified to express WT1-specific TCR with LK79 target cells that were sensitive to the cytocidal activity mediated by the transfected CTL was reasonably suitable for demonstrating the proof-of-concept of this study. Accordingly, these double-transfected effector $CD8^+$ T cells paired with target LK79 cells were employed in the subsequent experiments.

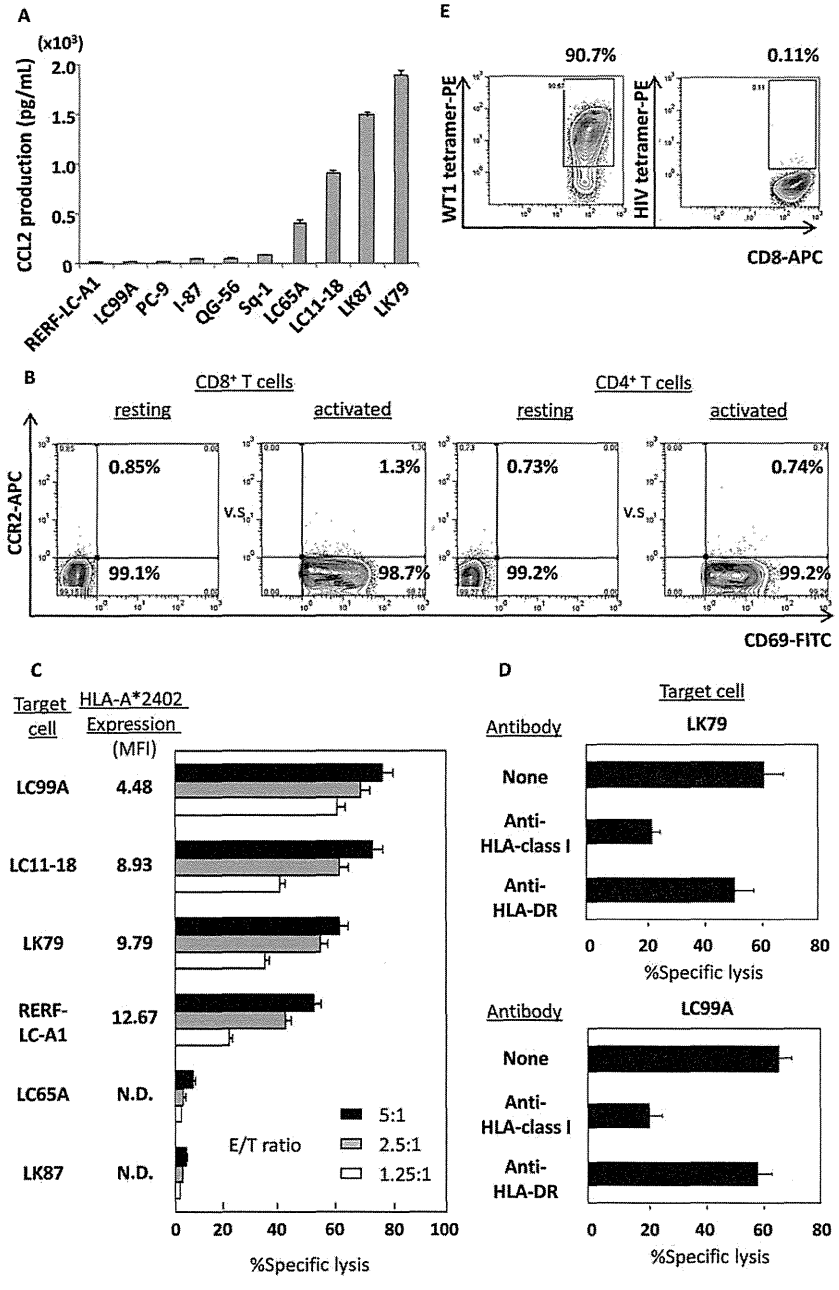

Figure 1. Human lung cancer cells produce variable amounts of CCL2 and show sensitivity to cytocidal activity mediated by CD8⁺ T cells genetically engineered to express WT1-specific TCR. (A) ELISA assay revealed that 10 human lung cancer cell lines examined produced various amounts of CCL2 in the culture supernatant. Error bars represent SDs. (B) Similarly to transduction of the WT1-specific *TCR* gene, CD69-positive $CD8^+$ and $CD4^+$ T cells activated using IL-2 and OKT-3 rarely displayed cell-surface CCR2. A representative example of 5 cases is shown. (C) $CD8^+$ T cells gene-modified to express HLA-A*2402-restricted and $WT1_{235-243}$ nonamer-specific TCR successfully displayed cytocidal activity against lung cancer cell line cells in a HLA-A*2402-restricted manner, as determined by standard ^{51}Cr release assay. Error bars represent SDs. MFI indicates mean fluorescence intensity, N.D indicates less than detectable. (D) Such anti-lung cancer activity (vs. LK79 and LC99A) was inhibited by anti-HLA class I framework mAb (clone w6/32), but not by anti-HLA-DR mAb (clone L243), again illustrating that the introduced WT1-specific TCR-mediated tumoricidal activity was HLA-class I-restricted. Error bars represent SDs. (E) Tetramer assay showed that effector cells used in these experiments were positive for WT1/HLA-A*2402-tetramer. HIV/HLA-A*2402 tetramer was employed as a negative control.
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2. Functional validation of introduced CCR2

Firstly, functional validation of introduced CCR2 was conducted using *CCR2*-transfected Jurkat cells (Jurkat/CCR2) in transwell experiments. Jurkat/CCR2, but not parental Jurkat cells, successfully displayed CCL2-mediated migration activity in a dose-dependent manner. Figure 2 shows the numbers of cells migrating relative to that at a CCL2 concentration of 12.5 ng/mL. Jurkat/CCR2 cells similarly displayed migration activity towards LK79, LK87, LC11-18, LC65A and LC99A cells seeded in the bottom wells according to the level of CCL2 produced by each cell line

Table 2. *Production of chemokine mRNA by human lung cancer cell line cells.*

Demographics of examined human lung cancer cell line										
	LC99A	**LK79**	**RERF-LC-A1**	**LC11-18**	**PC-9**	**Sq-1**	**QG-56**	**LC65A**	**1–87**	**LK87**
HLA-A*2402	+	+	+	+	+	−	−	−	−	−
#WT1mRNA	+	+	+	+	−	+	+	+	+	+
Production of chemokine mRNA by examined human lung cancer cell line										
Chemokine	**LC99A**	**LK79**	**RERF-LC-A1**	**LC11-18**	**PC-9**	**Sq-1**	**QG-56**	**LC65A**	**1–87**	**LK87**
CCL2	0.001	0.937	0.0004	0.505	0.001	0.002	0.005	0.330	0.021	0.413
CCL3	0	0	0	0	ND	ND	ND	ND	ND	ND
CCL4	0	0.001	0	0	ND	ND	ND	ND	ND	ND
CCL5	0.001	0.003	0.001	0.002	ND	ND	ND	ND	ND	ND
CCL8	0	0	0	0	ND	ND	ND	ND	ND	ND
CCL22	0	0	0	0	ND	ND	ND	ND	ND	ND
CXCL9	0	0	0	0	ND	ND	ND	ND	ND	ND
CXCL10	0	0.02	0	0	ND	ND	ND	ND	ND	ND
CXCL11	0	0.002	0	0	ND	ND	ND	ND	ND	ND
CXCL12	0	0	0	0.001	ND	ND	ND	ND	ND	ND
CXCL16	0.032	0.09	0.02	0.006	ND	ND	ND	ND	ND	ND
CX3CL1	0	0.11	0.006	0.034	ND	ND	ND	ND	ND	ND
*hHPRT1	1	1	1	1	1	1	1	1	1	1

#WT1mRNA positivity was reported in our previous paper (ref. No. 12).

*The expression level of each chemokine mRNA was corrected by reference to that of hHPRT1 mRNA.

ND indicates "not done".

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(Fig. 1A). Finally this chemotaxis mediated by Jurkat/CCR2 towards LK79 was completely inhibited by anti-CCR2 mAb (Fig. 2B). Next, using a xenografted NOG mouse model, tumor antigen-independent in vivo tumor trafficking mediated by *CCR2* and *luciferase* double-transfected normal $CD3^+$ T cells was examined using bioluminescence imaging. One day after intravenous infusion, these luciferase-labeled CCR2-expressing $CD3^+$ T cells started to accumulate at the site of inoculated LK79 cells in the anterior abdominal wall, whereas luciferase-labeled $CD3^+$ T cells lacking CCR2 were dispersed throughout the entire body during the observation period (Fig. 2C). Next, validation of the function of double-transfected normal $CD8^+$ T cells to express both WT1-specific TCR and CCR2 was carried out. First, we assessed whether the co-introduction of CCR2 impaired the WT1-specific cytocidal activity against lung cancer cells mediated by double-transfected effector $CD8^+$ T cells. As shown in Figure 3A and 3B, WT1 peptide-responsive cytocidal activity and anti-lung cancer activity against HLA-A*2402$^+$ LK79 cells (but not that against HLA-A*2402$^-$ LK87 cells as negative control) mediated by these effector cells, being double-positive for Vβ5.1 and CCR2 (Fig. 3C), was not compromised relative to that mediated by WT1-specific *TCR* single-transfected $CD8^+$ T cells (Fig. 1C). Next, the combined antitumor functionality against LK79 cells mediated by double-transfected $CD8^+$ T cells, comprising both increased tumor trafficking activity and WT1-specific cytocidal activity, was examined using a modified transwell experiment. These double-transfected $CD8^+$ T cells (n = 3), but not WT1-si*TCR* single-transfected $CD8^+$ T cells (n = 3), in the upper well efficiently migrated into the bottom well where LK79 cells had been seeded and pre-incubated for 24 hours. Both double- and WT1-si*TCR* single-transfected effector cells loaded into the upper wells were equally 90–92% positive for Vβ5.1, and 70–72% of the double-transfectants expressed CCR2 (data not shown). Consequently, significantly ($p<0.05$) more LK79 cells were destroyed by migrated double-transfected $CD8^+$ T cells than by WT1-si*TCR* single-transfectants, as represented by elevated levels of LDH in the culture supernatants (Fig. 4A). At the end of these transwell experiments, flow cytometric analysis revealed that 3.4 times more Vβ5.1-positive effector cells remained in the bottom well treated with double-transfected $CD8^+$ T cells $7.91\pm0.51(\times10^4)$ for double-transfectants, and $2.35\pm0.13(\times10^4)$ for single-transfectants, respectively), supporting the results of the LDH assay. Microscopic examination confirmed a greater degree of destruction of LK79 cells in the bottom well after treatment with double-transfected effector cells, than with single-transfected cells. Furthermore, confocal microscopic examination demonstrated red-labeled migrated CCR2-expressing effector cells in the bottom well only after treatment with double gene-modified effector cells. A representative example among three experiments is shown in Figure 4B.

3. Co-introduced CCR2 augments in vivo anti-lung cancer reactivity mediated by double-transfected $CD8^+$ T cells

Next, using a therapeutic xenografted mouse model, we examined in vivo anti-lung cancer reactivity mediated by infused effector cells double-transfected to express both WT1-specific TCR and CCR2. For this assay, we prepared *luciferase* gene-transduced LK79 cells (LK79/luc) that produced similar amounts of CCL2 to the parental LK79 cells (Fig. 5A). After 1 Gy of irradiation, cohorts of NOG mice (n = 6) were subcutaneously inoculated with 5×10^6 LK79/luc cells in the anterior abdominal wall. Four days later, when the tumor mass had become palpable, these mice started to receive weekly intravenous administration of OKT-3/IL-2-activated, but not gene-modified, $CD8^+$ T cells (cohort i), WT1-*siTCR* single-transfected $CD8^+$ T cells (cohort ii) or *CCR2* and WT1-si*TCR* double-transfected $CD8^+$ T cells (cohort iii), in a total of 3 infusions. As shown in Figure 5B, mice in cohort iii immediately displayed significant tumor suppression on day 3 after the first therapeutic infusion ($p<0.01$ for cohort iii vs. cohort i, $p<0.05$ for cohort iii vs. cohort ii), and thereafter the growth of LK79/luc was continuously suppressed until day 28, also with statistical significance ($p<0.01$ for both vs. cohort i and cohort ii). In contrast, WT1-si*TCR* single-transfected effector cells in cohort ii gradually suppressed the growth of LK79/luc cells. On day 14, the tumors in cohort ii mice first began to show a significant reduction in size relative to the mice in cohort i ($p<0.01$). Effector cells activated with only OKT-3/IL-2 in cohort i displayed a marginal tumor-suppressive effect, which was probably attributable to xenoreactivity. Serial bioluminescence images of the mice in each cohort are shown in Figure 5C. Co-introduction of functional CCR2 successfully enhanced in vivo anti-lung cancer reactivity mediated by infused double-transfected effector T cells, notably in the phase immediately after the start of therapeutic infusion, reflecting the increased tumor trafficking activity in response to CCL2.

4. Positive impact of the co-introduced CCR2-CCL2 axis on WT1-responsive TCR signaling in double-transfected effector cells

Besides enhanced tumor trafficking activity, we attempted to examine the impact of the co-introduced CCR2-CCL2 axis on WT1-responsiveness itself in double-transfected effector cells. First, we generated double-transfected Jurkat/MA/CD8α/luc cells that expressed both WT1-specific TCR and CCR2. The magnitude of WT1-responsive TCR signaling in these cells became measurable using WT1 peptide-responsive luciferase production. WT1-si*TCR* and *CCR2* double-transfected Vβ5.1 and CCR2 double-positive Jurkat/MA/CD8α/luc cells were incubated with 20 μM heteroclitic WT1 peptide-loaded or unloaded C1R-A24 cells in several concentrations of CCL2, then subjected to luciferase assay. Experiments were carried out in triplicate and independently three times. It was found that CCL2 dose-dependently augmented the WT1 peptide-responsive luciferase production mediated by the double-transfected Jurkat/MA/CD8α/luc cells with statistical significance (Fig. 6A). Next, using similarly double-transfected normal $CD8^+$ T cells obtained from 3 different individuals, being double-positive for HLA-A*2402/$WT1_{235-243}$ tetramer and CCR2, we examined whether WT1 epitope-responsiveness could also be augmented in the presence of CCL2. All experiments were carried out in triplicate. It was found that the WT1 epitope-responsive IFN-γ production mediated by double-transfected $CD8^+$ T cells was significantly upregulated in accordance with the concentration of CCL2 (Fig. 6B), while the WT1 epitope-responsive cytotoxic degranulation, as assessed in terms of increased cell-surface CD107a expression, also tended to increase, but not to a significant degree (Fig. 6C). Representative data for CD107a expression in three independent experiments are shown in Figure 6D. Collectively, the co-introduced CCR2-CCL2 axis potentiated not only tumor trafficking activity, but also WT1 epitope-responsiveness mediated by these double-transfected CTLs, resulting in enhanced anti-lung cancer functionality in vivo, notably in the phase immediately after therapeutic infusion.

Discussion

In this study, using LK79 – a SCLC cell line – as a target, which produces high amounts of CCL2 (Table 2 and Fig. 1A), and effector CTLs double-transfected to express WT1-specific TCR

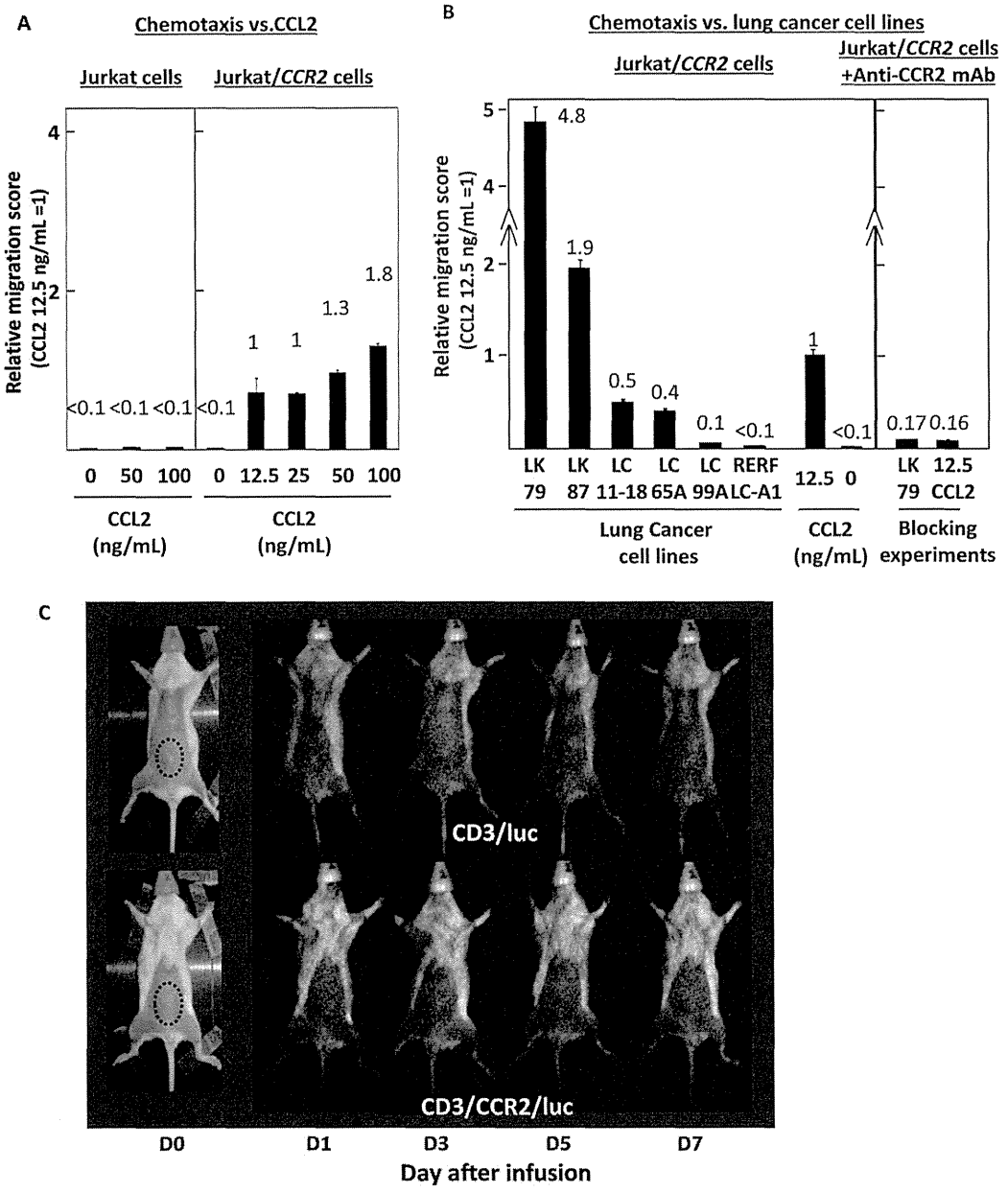

Figure 2. In vitro and in vivo functionality of introduced CCR2. (A) *CCR2*-transduced Jurkat cells, but not parental Jurkat cells, displayed dose-dependent transwell CCL2-chemotaxis. The numbers of cells migrating are shown relative to that at a CCL2 concentration of 12.5 ng/mL. Error bars represent SDs. (B) Similarly, the numbers of migrating *CCR2*-transduced Jurkat cells for each cancer cell line are shown. The migration of *CCR2*-transduced Jurkat cells was obviously inhibited by anti-CCR2 mAb, suggesting that the number of migrating cells was dependent on the level of CCL2 produced by each cell line (in Fig. 1A) and mediated by the introduced CCR2 on Jurkat cells. Error bars represent SDs. (C) CCL2-based target antigen-independent in vivo tumor trafficking towards inoculated LK79 cells mediated by *CCR2*-transfected $CD3^+$ T cells was successfully demonstrated in a xenografted mouse model. Intravenously infused luciferase-labeled $CD3^+$ T cells expressing CCR2 (CD3/CCR2/luc) (lower), but not those lacking CCR2 (CD3/luc) (upper), started to migrate towards LK79 cells immediately on day 1 after infusion, and their targeted migration was maintained throughout the observation period.
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and CCR2, we successfully demonstrated both the feasibility and advantages of targeting an optimal chemokine produced by tumor cells, to achieve successful adoptive therapy.

Targeting of an optimal chemokine produced by tumor cells or tumor-infiltrating immune cells to improve the antitumor efficacy mediated by tumor-reactive T cells was first demonstrated in vitro by Kershaw et al. [15]. Subsequently, a series of preclinical studies showed that the increased antitumor functionality mediated by these effector cells was mainly attributable to enhanced tumor trafficking activity caused by the introduced chemokine receptor [16–19]. An early clinical trial using ex vivo-expanded and radiolabeled TILs for treatment of patients with advanced melanoma had already demonstrated that localization of infused TILs was an important determinant of clinical efficacy [27]. More

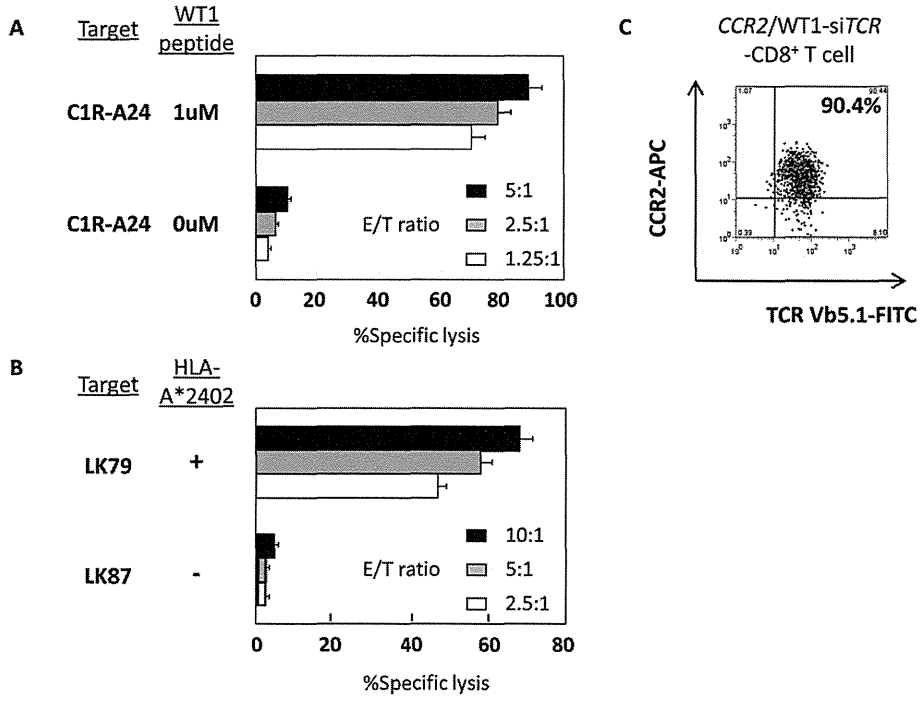

Figure 3. WT1-specific TCR-mediated cytocidal activity is not impaired by additional gene modification for expression of CCR2. (A) Using standard ^{51}Cr release assay, these double-transfected effector cells sufficiently lysed 1 μM WT1 peptide-loaded, but not unloaded, C1R-A24 cells that expressed HLA-A*2402. E/T ratio indicates effector:target ratio. Error bar indicates SDs. (B) These double-transfected effector cells also sufficiently lysed HLA-A*2402$^+$ LK79 cells, but not LK87 cells lacking the HLA-A*2402 molecule used as a negative control. (C) Double-transfected CD8$^+$ T cells successfully expressed both cell-surface CCR2 and Vβ5.1, the germ-line component of the WT1-specific TCR β chain. *CCR2*/WT1-si*TCR*-CD8$^+$ T cells indicates double-transfected CD8$^+$ T cells.
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recently, using biopsied tissues from melanoma patients, Harlin et al. confirmed the clinical importance of the chemokine production pattern in tumor tissues as a critical determinant of the effectiveness of antitumor immunity [28]. Although targeting of appropriate chemokines produced by tumor cells or infiltrating elements within the tumor microenvironment has become an attractive option for increasing the numbers of tumor-infiltrating effector cells, this approach is still at the preclinical stage. The fundamental issue of which chosen chemokine would be optimal for use against a wide range of tumors still remains to be explored.

CCL2, a CC chemokine that stimulates CCR2, its corresponding receptor expressed on T cells [29], NK cells [30] and γδ T cells [31], is reported to be expressed by various types of cancer cells including those of glioma [17], melanoma [28], and cancers of the breast [32], prostate [33], colon [34] and lung [35]. Our method used for retroviral transduction of the WT1-si*TCR* gene into normal CD8$^+$ T cells involves stimulation of T cells using OKT-3/IL-2 in a RetroNectin-coated plate [6]. Whenever we transduced the WT1-si*TCR* gene, CCR2 was rarely expressed on activated CD8$^+$ T cells (Fig. 1B). Moreover, positivity for cell-surface CCR2 could only be achieved by *CCR2* gene-transfection. Consequently, the *CCR2*-transfectants acquired sufficient CCL2-responsiveness both in vitro and in vivo (Figs. 2 and 4), thus confirming the therapeutic efficacy and feasibility of introducing the CCR2-CCL2 axis into tumoricidal T cells. On the other hand, a previous report has indicated that CCR2 expression in activated T cells was functionally positive [36]. This discrepancy was likely due to the difference in the method used for stimulating T cells, as Craddock et al. have previously discussed [17].

On the other hand, the suppressive role of CCL2 in antitumor immunity has also been intensively studied. CCL2 produced in the tumor microenvironment is known to recruit tumor-associated macrophages (TAMs) [37], which are known to support the growth of neuroblastoma cells via production of IL-6 [38], to suppress antitumor immunity in cancer-bearing hosts via IL-10 production [39] and recruitment of regulatory T cells [40], and to promote tumor-supportive angiogenesis [41]. Furthermore, TAM itself produces CCL2 and thus amplifies this circuit [37]. However, even though CCL2 in the tumor microenvironment is diversely implicated in the suppression of host antitumor immunity, abundant production of CCL2 by tumor cells or infiltrating immune cells might be advantageous for efficient localized recruitment of therapeutically infused tumoricidal CCR2-expressing T cells into tumor tissues. As well as increased tumor trafficking activity, we demonstrated that the co-introduced CCR2-CCL2 axis might also be advantageous for potentiating target-responsive cytocidal activity, i.e., WT1-specific TCR-mediated anti-lung cancer reactivity. Double-transfected Jurkat/MA/CD8α/luc cells displayed increased production of luciferase triggered by WT1 peptide-responsive TCR signaling, and this effect was dependent on the concentration of CCL2. Even without WT1 peptide stimulation, CCL2 stimulated the cells to produce a small amount of luciferase, suggesting that influx of calcium into the double-transfected Jurkat/MA/CD8α/luc cells triggered by CCL2 ligation might, to some extent, directly stimulate the NFAT pathway shared by WT1-responsive TCR signaling (Fig. 6A). Finally, in the presence of CCL2, augmentation of both WT1 epitope-responsive IFN-γ production and CD107a expression was mediated by double-transfected normal CD8$^+$ T cells stimulated

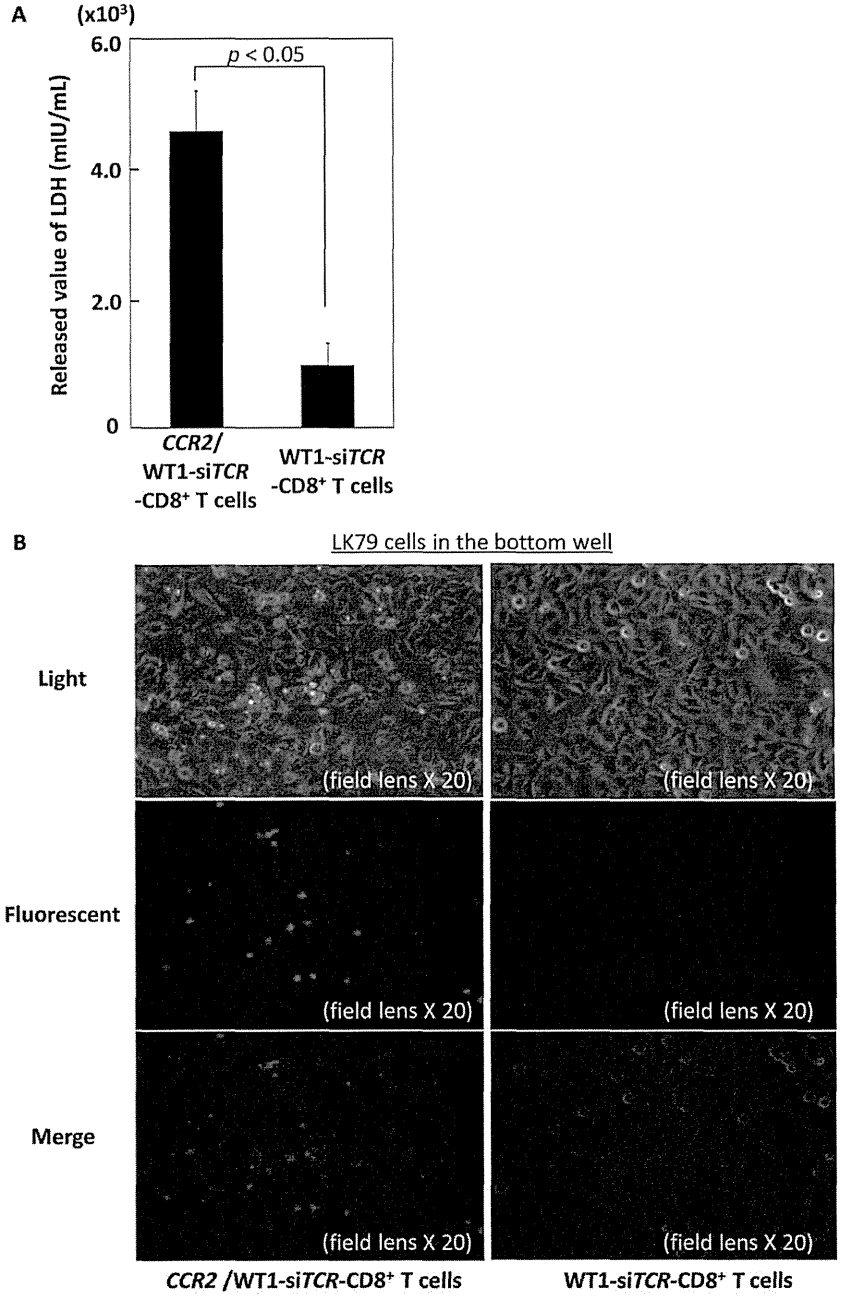

Figure 4. Transwell experiments for functional assessment of CD8$^+$ T cells double-transfected to express both WT1-specific TCR and CCR2. (A) Double-transfected CD8$^+$ effector cells (n = 3), but not WT1-si*TCR* single-transfected effector cells (n = 3), seeded in the upper well migrated to the bottom well more effectively, where they lysed seeded LK79 cells, as assessed in terms of the increase in the amount of LDH released from the disrupted LK79 cells. Error bars represent SDs. LDH indicates lactate dehydrogenase. (B) A representative set of light micrographs of three experiments, demonstrating a greater degree of LK79 cell destruction after treatment with double-transfected effector cells (top left), than with WT1-si*TCR* single-transfected effector cells (top right). Because CCR2 was genetically introduced along with a red fluorescent protein (DS red), only double gene-modified CCR2$^+$ effector cells were detectable by red-colored labeling in the bottom well after migration (middle and bottom). In Figure 4A and 4B, *CCR2*/WT1-si*TCR*-CD8$^+$ T cells indicates double-transfected CD8$^+$ T cells, and WT1-si*TCR*-CD8$^+$ T cells indicates WT1-si*TCR* single-transfected CD8$^+$T cells.
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using WT1 peptide-loaded C1R-A24 cells (Fig. 6B, 6C and 6D). With regard to the interplay between chemokine receptors and TCR in the same effector T cell, the role of the CXCR4-SDF-α axis in T cell activation [42,43,44] and that of the CCR7-CCL19/CCL21 axis in T-cell homing to lymph nodes [45] have been studied in detail. In our system, although details of the mechanism still remain undetermined, it can be suggested that after CCR2-CCL2 axis-mediated migration into tumor tissues, CCL2 in the tumor microenvironment may strengthen WT1 epitope-responsive

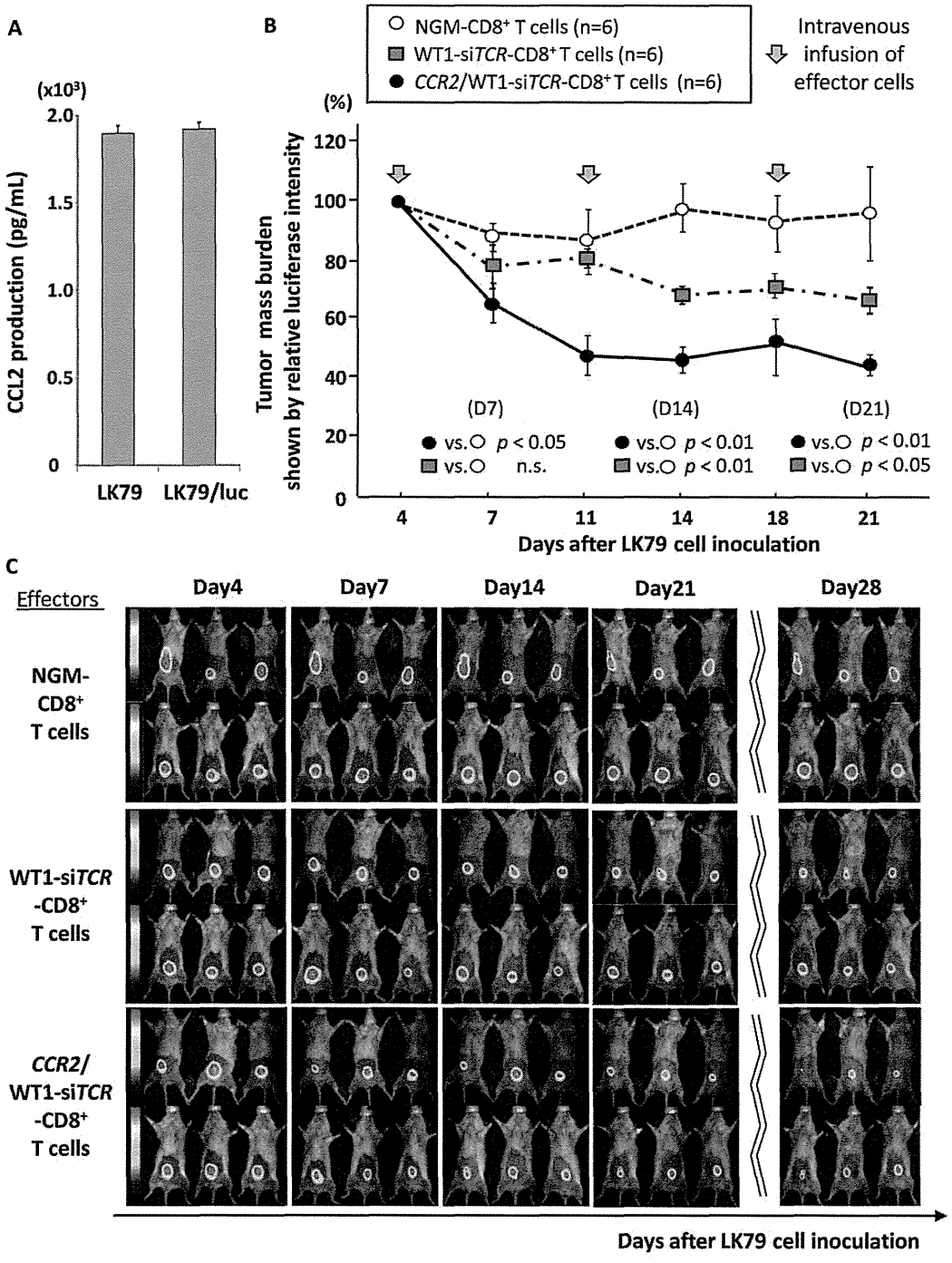

Figure 5. In vivo anti-lung cancer activity mediated by double-transfected effector cells in therapeutic xenografted mouse models. (A) The amount of CCL2 produced by *luciferase*-transfected LK79 cells (LK79/luc) was similar to that produced by the parent LK79 cells. (B) Nine-week-old NOG mice (n = 18) inoculated into the abdominal wall with 5×10^6 LK79/luc cells were divided into 3 cohorts. On day 4, when each tumor mass had become palpable, three mice in each cohort started to receive weekly intravenous administration of 5×10^6 double-transfected effector cells (cohort iii; black circles), WT1-si*TCR* single-transfected effector cells (cohort ii; gray square), or $CD8^+$ T cells simply activated using OKT-3/IL-2 as a negative control (cohort i; clear circles), the effector cells all being generated from an identical donor. Intravenous administration was performed three times in total, and the relative mass burden was serially monitored on the basis of luciferase photon counts relative to those on day 4, before the start of therapeutic infusion. Double-transfected effector cells in cohort iii mice most effectively suppressed the growth of LK79/luc cells, notably in the immediate phase after therapeutic infusion (on day 7). In contrast, WT1-si*TCR* single-transfected effector cells gradually suppressed the growth of LK79/luc cells, being apparently dependent on time and the total number of effector cells infused. Effector cells that had been simply activated also displayed a marginal degree of tumor suppression, probably because of xenoreactivity. Error bars represent SDs. NGM-$CD8^+$ T cells indicate $CD8^+$ T cells simply activated using OKT-3/IL-2, expressing neither CCR2 nor WT1-specific TCR. (C) Serial bioluminescence images of mice in each cohort are shown. On day 28, 10 days after the last therapeutic infusion, durable growth suppression of LK79/luc cells was most evident in cohort iii mice that had received double-transfected effector cells. NGM-$CD8^+$ T cells represent $CD8^+$ T cells simply activated using OKT-3/IL-2, expressing neither CCR2 nor WT1-specific TCR.

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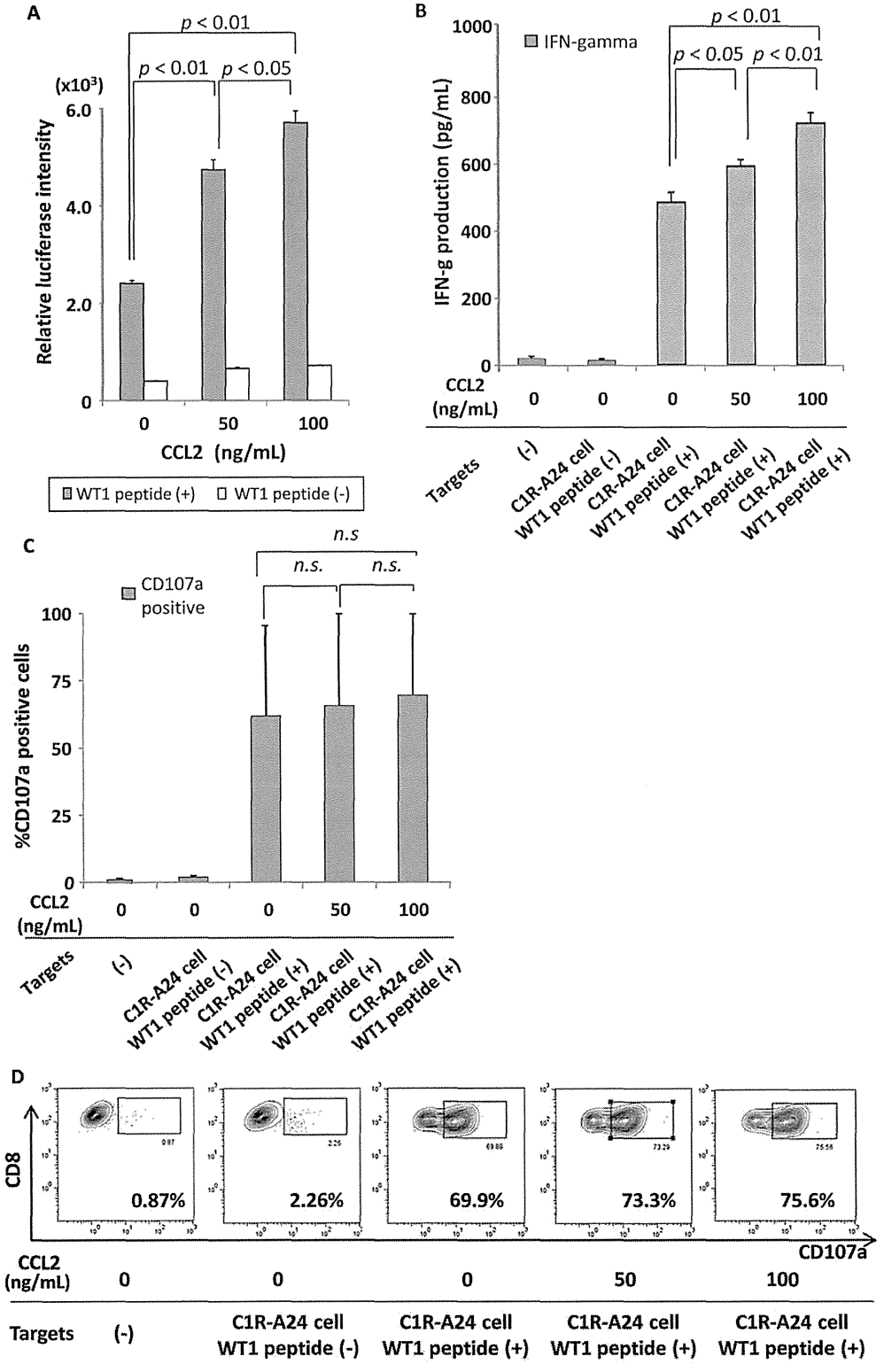

Figure 6. Positive cross-talk between CCR2 and WT1 epitope-responsive TCR signaling in double-transfected T cells. (A) In double-transfected Jurkat/MA/CD8α/luc cells, CCL2 ligation to introduced CCR2 significantly augmented WT1-responsive luciferase production triggered by stimulation with 20 μM WT1 peptide-loaded C1R-A24 cells in a dose-dependent manner. In the absence of WT1 peptide, CCL2 ligation induced a low level of luciferase production. (B) In normal peripheral $CD8^+$ T cells (n = 3) double-transduced to express both CCR2 and WT1-specific TCR, CCL2 ligation significantly enhanced WT1-responsive IFN-γ production in response to stimulation with 1 μM WT1 peptide-loaded C1R-A24 cells in a dose-dependent manner. (C) Similarly treated double-transfected normal effector cells (n = 3) showed a trend to increase WT1-responsive cytotoxic degranulation, but not to a significant degree. (D) Representative data for CD107a expression from 3 cases examined are shown. (B), (C) and (D)

illustrate that cellular outputs triggered by TCR ligation with the WT1 epitope/HLA complex were augmented in the presence of CCL2. n.s. indicates not significant.
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cytocidal activity against LK79 cells mediated by infused double-transfected effector $CD8^+$ T cells in vivo.

In our previous study, we demonstrated the therapeutic potential of intravenously infused WT1-specific CTL clone cells (TAK-1) against human lung cancer cells in vivo [12]. In the present study, we demonstrated the WT1-specific anti-lung cancer effect mediated by $CD8^+$ T cells genetically engineered to express WT1-specific TCR originating from TAK-1 [13]. Furthermore, we successfully provided experimental evidence that the suppression of tumor growth mediated by *CCR2* and WT1-si*TCR* double-transfected effector T cells was more effective than that mediated by WT1-si*TCR* single-transfected cells (Fig. 5B and 5C). In human lung cancers, expression of both *WT1* mRNA and WT1 protein has been demonstrated in tumor cells, suggesting the therapeutic potential of WT1 as a target of anti-lung cancer immunotherapy [10,46]. Indeed Oka et al. have reported clinical responses in lung cancer patients after WT1 peptide vaccination [47].

Non-malignant cells including fibroblasts, endothelial cells, smooth muscle and microglial and astrocytic cells, also produce CCL2 under physiological or inflammatory conditions [48]. These cells might cause CCR2-introduced effector cells to become dispersed into those tissues, thus blunting any therapeutic effect or causing unintended adverse events. However, because the lung is the first-pass organ for intravenously infused effector cells, when intending to treat lung cancer, CCL2 at tumor sites in the lung field would preferentially attract such infused effector cells to a much greater extent than CCL2 expressed physiologically in other peripheral tissues.

In the present study, we have demonstrated both the feasibility and advantages of $CD8^+$ T cells double-transfected to express WT1-specific TCR and CCR2 for the treatment of at least some human lung cancers. Similar studies using T cells genetically redirected to express CCR2 and CARs for the treatment of neuroblastoma [17] and malignant mesothelioma [19] also appear to support the idea that the CCR2-CCL2 axis is a rational choice for use in redirected T cell-based anticancer adoptive immunotherapy. Further studies are needed to assess the clinical potential of this strategy.

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Author Contributions

Conceived and designed the experiments: HF MY. Performed the experiments: HA HF JA TO YM KN. Analyzed the data: HA HF HS MY. Contributed reagents/materials/analysis tools: SO JM KK HI. Wrote the paper: HA HF MY.

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Overcoming regulatory T-cell suppression by a lyophilized preparation of *Streptococcus pyogenes*

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Cancer vaccines have yet to yield clinical benefit, despite the measurable induction of humoral and cellular immune responses. As immunosuppression by $CD4^+CD25^+$ regulatory T (Treg) cells has been linked to the failure of cancer immunotherapy, blocking suppression is therefore critical for successful clinical strategies. Here, we addressed whether a lyophilized preparation of *Streptococcus pyogenes* (OK-432), which stimulates Toll-like receptors, could overcome Treg-cell suppression of $CD4^+$ T-cell responses in vitro and in vivo. OK-432 significantly enhanced in vitro proliferation of $CD4^+$ effector T cells by blocking Treg-cell suppression and this blocking effect depended on IL-12 derived from antigen-presenting cells. Direct administration of OK-432 into tumor-associated exudate fluids resulted in a reduction of the frequency and suppressive function of $CD4^+CD25^+Foxp3^+$ Treg cells. Furthermore, when OK-432 was used as an adjuvant of vaccination with HER2 and NY-ESO-1 for esophageal cancer patients, NY-ESO-1–specific $CD4^+$ T-cell precursors were activated, and NY-ESO-1–specific $CD4^+$ T cells were detected within the effector/memory T-cell population. $CD4^+$ T-cell clones from these patients had high-affinity TCRs and recognized naturally processed NY-ESO-1 protein presented by dendritic cells. OK-432 therefore inhibits Treg-cell function and contributes to the activation of high-avidity tumor antigen-specific naive T-cell precursors.

Keywords: Cancer · Treg cells · Tumor immunology · Vaccination

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Introduction

Many tumor-associated antigens recognized by the immune system are normal self-constituents, and tumor immunity is considered to be in part an autoimmune response [1–3]. Therefore, mechanisms for maintaining immunological self-tolerance hamper effective anticancer immunity. $CD4^+CD25^+$ Treg cells are one of the major components in maintaining immunological self-tolerance in hosts by suppressing a wide range of immune responses [4–7]. Indeed, depletion of Treg-cell populations enhances spontaneous and vaccine-induced antitumor immune responses [6, 8, 9], and the stimulation of $CD4^+CD25^+$ Treg cells by immunization with self-antigens induces enhanced chemically induced primary tumor development and increased numbers of pulmonary metastasis following injection of transplantable tumor cells [10–12]. In human cancers, the presence of high numbers of $CD4^+CD25^+$ Treg cells or low ratio of $CD8^+$ T cells to $CD4^+CD25^+$ Treg cells in tumors is correlated with unfavorable prognosis [13, 14]. In addition, the depletion of $CD4^+CD25^+$ Treg cells in patients receiving a DC vaccine enhances the stimulation of tumor-specific T-cell responses, indicating a crucial role for Treg cells in the regulation of antitumor immune responses in humans [15].

NY-ESO-1, a germ cell protein, was found by SEREX (serological identification of antigens by recombinant expression cloning) using the serum of an esophageal cancer patient [16, 17]. We have previously shown that NY-ESO-1–specific $CD4^+$ T cells are detectable in cancer patients with spontaneous NY-ESO-1 serum Ab responses [17, 18]. In addition, NY-ESO-1–specific $CD4^+$ T-cell precursors can expand and become detectable in healthy individuals after in vitro antigenic stimulation of peripheral $CD4^+$ T cells, but only following depletion of $CD4^+CD25^+$ T cells [19, 20]. These results suggested that NY-ESO-1–specific $CD4^+$ T-cell precursors are actually present at relatively high frequencies in healthy individuals, and that the activation/expansion of NY-ESO-1–specific naive $CD4^+$ T cells is suppressed by $CD4^+CD25^+$ Treg cells. In healthy donors and in cancer patients with NY-ESO-1–expressing tumors but without spontaneous anti-NY-ESO-1 Ab (seronegative), naturally arising NY-ESO-1–specific T-cell responses are susceptible to Treg-cell suppression and are exclusively detected from naive populations ($CD4^+CD25^-CD45RA^+$). In contrast, most NY-ESO-1–specific $CD4^+$ T cells in cancer patients with spontaneous anti-NY-ESO-1 Ab (seropositive) are derived from memory populations ($CD4^+CD25^-CD45RO^+$) and are detectable even in the presence of $CD4^+CD25^+$ Treg cells [20, 21]. After vaccination with HLA-DPB1*0401/0402-restricted NY-ESO-$1_{157-170}$ peptide in incomplete Freund's adjuvant, ovarian cancer patients develop NY-ESO-1–specific $CD4^+$ T cells with only low avidity to antigen and low sensitivity to Treg cells, even though they have an effector/memory phenotype ($CD4^+CD25^-CD45RO^+$) [21]. Still, high-avidity naive NY-ESO-1–specific T-cell precursors are present in the peripheral blood of vaccinated patients, but they are subjected to continuous $CD4^+CD25^+$ Treg-cell suppression throughout vaccination [21]. Thus, a strategy to overcome Treg-cell suppression on preexisting high-avidity naive T-cell precursors is an essential component for effective cancer vaccines.

Accumulating data shed light on recognition of pathogen-associated molecular patterns through TLRs to break the suppressive environment in tumors [22]. It has been reported that TLR stimulants, such as lipopolysaccharide or CpG, block the suppressive activity of $CD4^+CD25^+$ Treg cells partially by an IL-6–dependent mechanism [23]. TLR2 signaling was reported to stimulate the proliferation of $CD4^+CD25^+$ Treg cells and to induce temporal loss of suppressive activity of $CD4^+CD25^+$ Treg cells [24]. TLR2 signaling has also been shown to increase IL-2 secretion by effector T cells, thereby rendering them resistant to $CD4^+CD25^+$ Treg-cell–mediated suppression [25]. We and others have recently reported that vaccination of tumor antigens by TLR stimulating viral or bacterial vectors was able to not only inhibit the suppressive function of $CD4^+CD25^+$ Treg cells but also break tolerance or hyporesponsiveness of effector T cells to tumor antigens even in the presence of Treg cells [26–28].

OK-432 is a lyophilized preparation of *Streptococcus pyogenes* that binds TLR-2, TLR-4, and/or TLR-9 and activates APCs, making it attractive for potential use as an adjuvant of cancer vaccine [29–33]. OK-432–matured DCs effectively prime antigen-specific T cells in vitro [29, 34]. Importantly, OK-432 has already been used for many years as a direct anticancer agent, particularly in Japan, and has a well-established clinical safety profile. However, while it is considered that OK-432 may inhibit Treg-cell suppressive activity by stimulating several TLR signaling pathways, its influence on Treg cells has not yet been shown. In this study, we addressed whether OK-432 inhibits Treg-cell suppressive function and could be a promising adjuvant of cancer vaccines.

Results

OK-432 inhibits the suppressive activity of $CD4^+CD25^+$ Treg cells

To address whether OK-432 inhibited $CD4^+CD25^+$ Treg-cell suppression, we employed the standard in vitro suppression system. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells (highest 3% of $CD4^+CD25^+$ cells) were isolated from PBMCs of healthy individuals. $CD4^+CD25^-$ T cells were cultured with irradiated autologous APCs (CD4-depleted PBMCs) and anti-CD3 Ab in the presence or absence of $CD4^+CD25^{high}$ Treg cells. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and methods*. In accordance with previous reports [7], $CD4^+CD25^{high}$ Treg cells markedly suppressed the proliferation of $CD4^+CD25^-$ T cells (Fig. 1A and B). In sharp contrast, when OK-432 was added in the culture, suppressive activity of $CD4^+CD25^{high}$ T cells was significantly inhibited (Fig. 1A and B). In addition, OK-432 did not induce death of $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of OK-432 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells exhibited marginal proliferation in the presence of

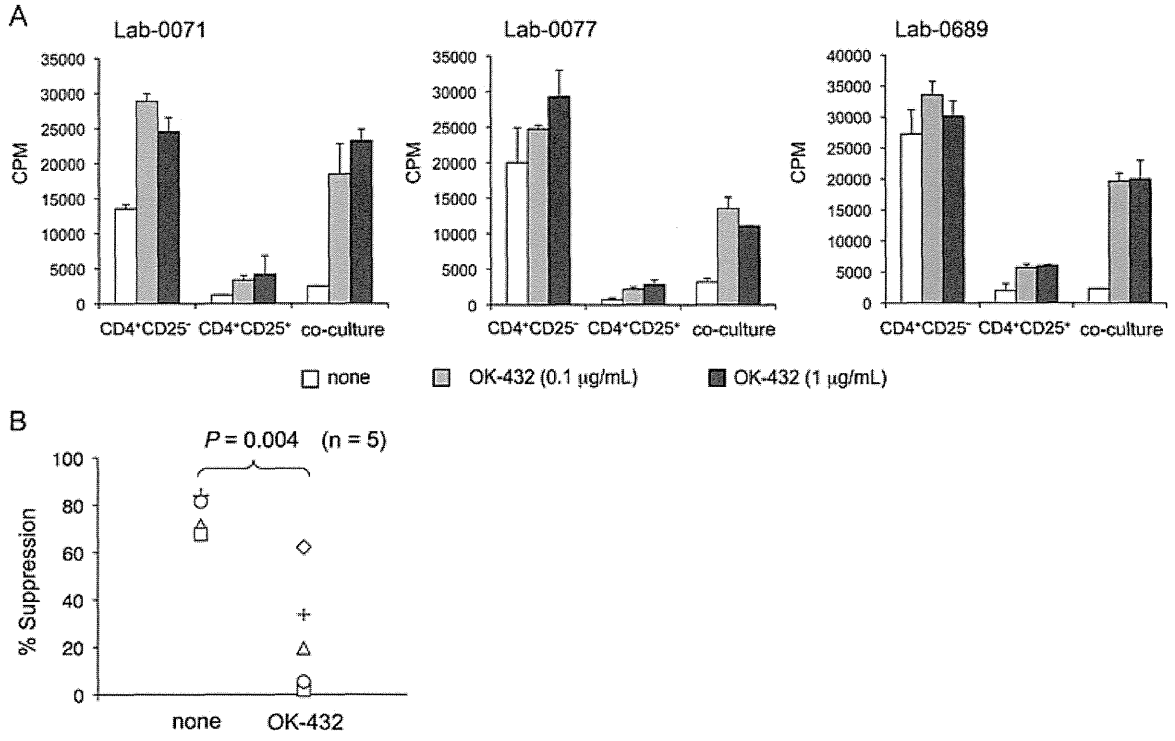

Figure 1. OK-432 overcomes Treg-cell suppression in vitro. (A, B) $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals as described in the *Materials and Methods*. 1×10^4 $CD4^+CD25^-$ T cells were cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 5×10^3 $CD4^+CD25^+$ Treg cells with/without OK-432. Proliferation was analyzed by ^3H-thymidine incorporation. Data of three donors are shown as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. (B) Summary of percent suppression in five healthy individuals. Percent suppression was calculated as: (1 – (cpm in coculture)/(cpm in $CD4^+CD25^-$ T cells)) × 100. Data shown are from one experiment representative of at least two performed.

OK-432 (Fig. 1A). These data indicate that addition of OK-432 impairs the suppressive activity of $CD4^+CD25^{high}$ Treg cells and partially reverses anergy status of Treg cells.

Inhibition of the suppressive activity of $CD4^+CD25^+$ Treg cells by OK-432 is dependent on IL-12

Since OK-432 reportedly induces TLR-2, TLR-4, and/or TLR-9 activation and subsequent production of proinflammatory cytokines [29–33], we examined the involvement of cytokines in this inhibition of Treg-cell suppression. To this end, Abs against several candidate cytokines were added to cultures. Among cytokines tested, only blocking Ab against IL-12 significantly abrogated the inhibition of Treg-cell suppression by OK-432 (Fig. 2A).

To confirm the importance of IL-12, we next analyzed whether the addition of IL-12 could inhibit Treg-cell suppression as observed by OK-432. $CD4^+CD25^-$ T cells were cultured with $CD4^+CD25^{high}$ Treg cells, irradiated autologous APCs and anti-CD3 Ab in the presence of IL-12. Treg-cell suppressive activity was significantly inhibited by the addition of IL-12, but not IL-6 or IFN-γ (Fig. 2B). Again, IL-12 did not kill $CD4^+CD25^{high}$ Treg cells as the frequency of Annexin V^+ and 7-AAD^+ cells was not significantly increased in the presence of IL-12 (data not shown). Instead, $CD4^+CD25^{high}$ Treg cells slightly proliferated in the presence of OK-432 (Fig. 2B). These data suggest a critical role for IL-12 in the inhibition of Treg-cell suppression by OK-432.

OK-432 induces higher amounts of IL-12 but not IL-10 from APCs compared with other stimuli

To gain insight into the cellular target(s) of OK-432, we explored the origin of IL-12 after OK-432 treatment based on the essential role of IL-12 in the inhibition of Treg-cell suppression by OK-432. We then analyzed whether OK-432 stimulation indeed induced IL-12 production from APCs, such as CD3-depleted PBMCs used in the standard Treg-cell suppression assays. CD3-depleted PBMCs from healthy donors were stimulated with OK-432, LPS, or TNF-α, and cytokine production was examined. OK-432 induced significantly higher amounts of IL-12 from CD3-depleted PBMCs than LPS or TNF-α (Fig. 3A). In addition, CD3-depleted PBMCs stimulated with OK-432 induced much less IL-10 production than LPS (Fig. 3A). Similar results, i.e. IL-12 rather than IL-10 was dominantly produced by CD3-depleted PBMCs stimulated with OK-432, were obtained from four esophageal cancer patients (Fig. 3B).

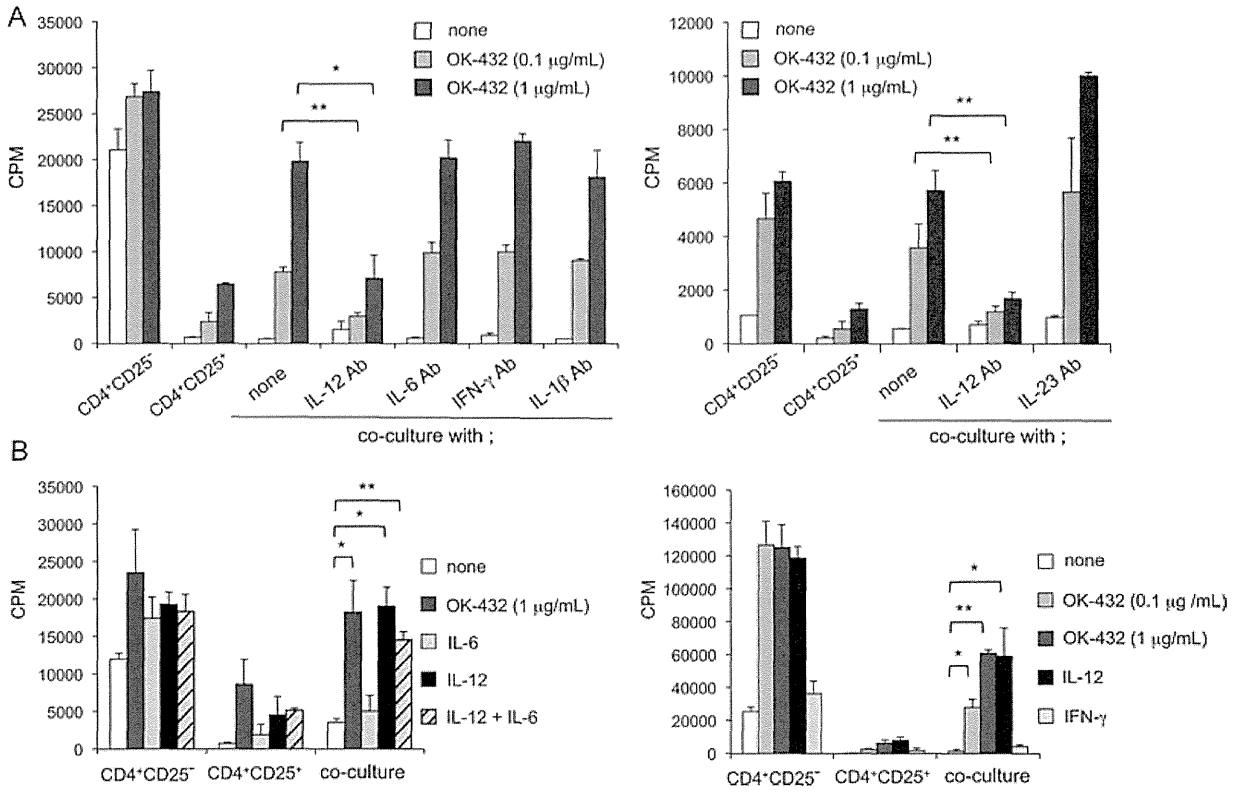

Figure 2. IL-12 is a critical cytokine to overcome Treg-cell suppression by OK-432. $CD4^+CD25^-$ T cells and $CD4^+CD25^{high}$ Treg cells were collected from PBMCs of healthy individuals. 1×10^4 $CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 2.5×10^3 $CD4^+CD25^{high}$ Treg cells with/without OK-432. (A) Blocking Abs (10 μg/mL) against several cytokines as indicated were added in the culture and proliferation was measured. (B) Recombinant cytokines (IL-12; 5 ng/mL, IL-6; 5 ng/mL, IFN-γ; 100 U/mL) were added into the cultures and proliferation was analyzed. Data for one representative donor among three donors are expressed as mean + SD of two replicates/samples and are from one experiment representative of at least two performed. $^*p < 0.05$ and $^{**}p < 0.01$ as compared with control, Student's *t*-test.

We next examined which cell types in PBMCs produced IL-12 after OK-432 stimulation. The major sources of IL-12 in PBMCs after OK-432 stimulation were $CD11c^+$ and $CD14^+$ cells, and neither NK cells nor T cells produced IL-12 (Fig. 3C). Taken together, APCs, such as monocytes, macrophages, and DCs are considered to be the cellular targets of OK-432 to induce IL-12 which is a crucial component for the inhibition of Treg-cell suppression by OK-432.

OK-432 administration to tumor-associated exudates reduces local Treg-cell accumulation and function

As OK-432 is available as an anticancer agent in Japan and has been used for controlling tumor-associated exudate fluids by direct injection to the cavity, we next investigated its influence on Treg cells following in vivo treatment of OK-432. We analyzed the local Treg-cell accumulation and function of tumor-associated sites before and 2–3 days after local OK-432 administration. Cells were isolated from tumor-associated exudate fluids, such as pleural effusions and ascites. The frequency of Treg cells before and after treatment with OK-432 was examined by staining with Abs for CD4, CD25, and Foxp3. The $Foxp3^+$ T-cell population in $CD4^+$ T cells was markedly reduced (Fig. 4A). Furthermore, the proportion of $Foxp3^+$ T cells in $CD4^+CD25^+$ T cells was also significantly reduced after OK-432 administration (Fig. 4A and B), indicating that the balance of helper T cells to Treg cells had changed.

We next addressed the suppressive activity of $CD4^+CD25^{high}$ T cells in tumor-associated exudate fluids. $CD4^+CD25^{high}$ T cells (highest 3% gate of $CD4^+CD25^+$ cells defined with peripheral blood was applied) were isolated from tumor-associated exudate fluids and cultured with $CD4^+CD25^-$ T cells from PBMCs with irradiated autologous APCs and anti-CD3 Ab. After OK-432 administration, as the volume of tumor-associated exudate fluids decreased, sufficient amounts of $CD4^+CD25^{high}$ T cells for proliferation assays were available only from two patients. $CD4^+CD25^-$ T-cell proliferation was analyzed as described in the *Materials and Methods*. There was a trend, albeit not significant, toward a decrease in Treg-cell function after OK-432 administration (Fig. 4C). In contrast, we did not observe any differences in frequency and function of Treg cells in PBMCs

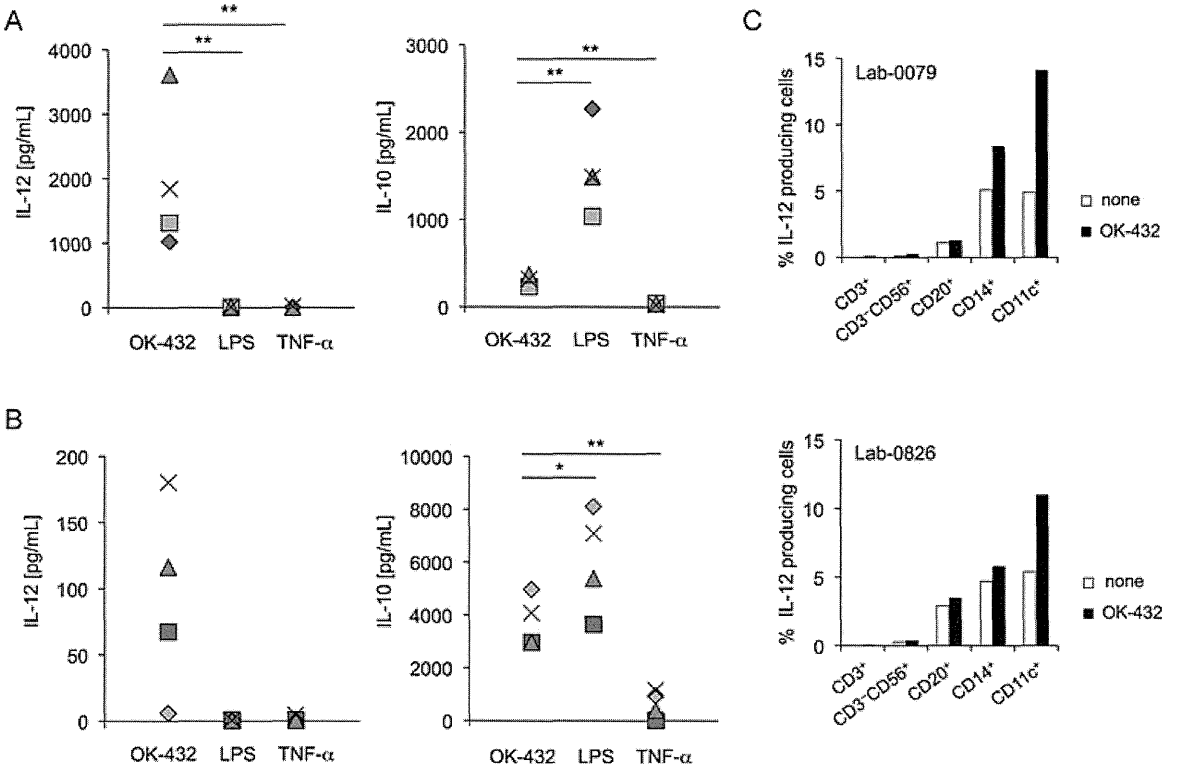

Figure 3. APCs stimulated with OK-432 produce significantly higher amounts of IL-12. (A) 1×10^5 CD3-depleted PBMCs from four healthy individuals were cultured with TNF-α (100 ng/mL), LPS (1 mg/mL), or OK-432 (1 μg/mL) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (B) 1×10^5 CD3-depleted PBMCs from four esophageal cancer patients were cultured as in (A) and supernatant was collected 48 h later. IL-12p70 and IL-10 production was analyzed with ELISA. (A, B) Each symbol represents an individual donor; data shown are from one experiment representative of at least two performed. (C) PBMCs from two healthy individuals were cultured with/without OK-432. Cells were subjected to staining with the indicated surface markers and then intracellular IL-12, and were analyzed by flow cytometry. Data of two donors from one experiment representative of at least two performed. *$p < 0.05$ and **$p < 0.01$ as compared with control, Student's *t*-test.

before and after OK-432 administration (data not shown). These data propose that in vivo injection of OK-432 decreases the local Treg-cell accumulation and function.

Origin of the repertoire of CD4$^+$ T-cell effectors elicited by vaccination with NY-ESO-1 and OK-432

To further explore the effect of OK-432 on the inhibition of in vivo Treg-cell activity, we also examined the potential of OK-432 as an adjuvant in a cancer vaccine. We have reported that high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors are present in naive CD45RA$^+$ populations and that their activation is rigorously suppressed by CD4$^+$CD25$^+$ Treg cells [20, 21]. We also found that synthetic peptide vaccination with incomplete Freund's adjuvant induces only peptide-specific CD4$^+$ T cells with low-avidity TCRs (recognition of >1 μM peptide but not naturally processed NY-ESO-1 protein), but not high-avidity CD4$^+$ T cells (recognition of naturally processed NY-ESO-1 protein or <0.1 μM peptide) that are susceptible to Treg-cell suppression [21]. Together, these data highlight the importance of blocking Treg-cell activity to allow activation/expansion of high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors. For this reason, we investigated whether high-avidity NY-ESO-1–specific CD4$^+$ T-cell precursors were activated by NY-ESO-1 protein vaccination with OK-432 as an adjuvant and were present in memory CD45RO$^+$ populations.

Samples from two patients who received vaccination with cholesteryl hydrophobized pullulan (CHP)-HER2 and NY-ESO-1 with OK-432 (Supporting Information Fig. 1) were available for this analysis. Whole CD4$^+$ T cells or CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T cells before and after vaccination were presensitized with NY-ESO-1–overlapping peptides covering the entire sequence of NY-ESO-1 and specific CD4$^+$ T-cell induction was analyzed with ELISPOT assays. As the sample size was not sufficient to analyze specific CD4$^+$ T-cell induction within CD4$^+$CD25$^-$CD45RA$^+$ (naive) T cells, we analyzed whether NY-ESO-1–specific high-avidity CD4$^+$ T cells were induced from the CD4$^+$CD25$^-$CD45RO$^+$ (effector/memory) T-cell population after vaccination in Pt #1 (HLA-DR 4, 12 and HLA -DQ 4, 8) and #2 (HLA-DR 9, 15 and HLA-DQ 6, 9). Pt #1 exhibited spontaneously induced CD4$^+$ T-cell responses against NY-ESO-1_{91-110} before vaccination and the responses were maintained after extensive vaccination (Fig. 5A). These spontaneously

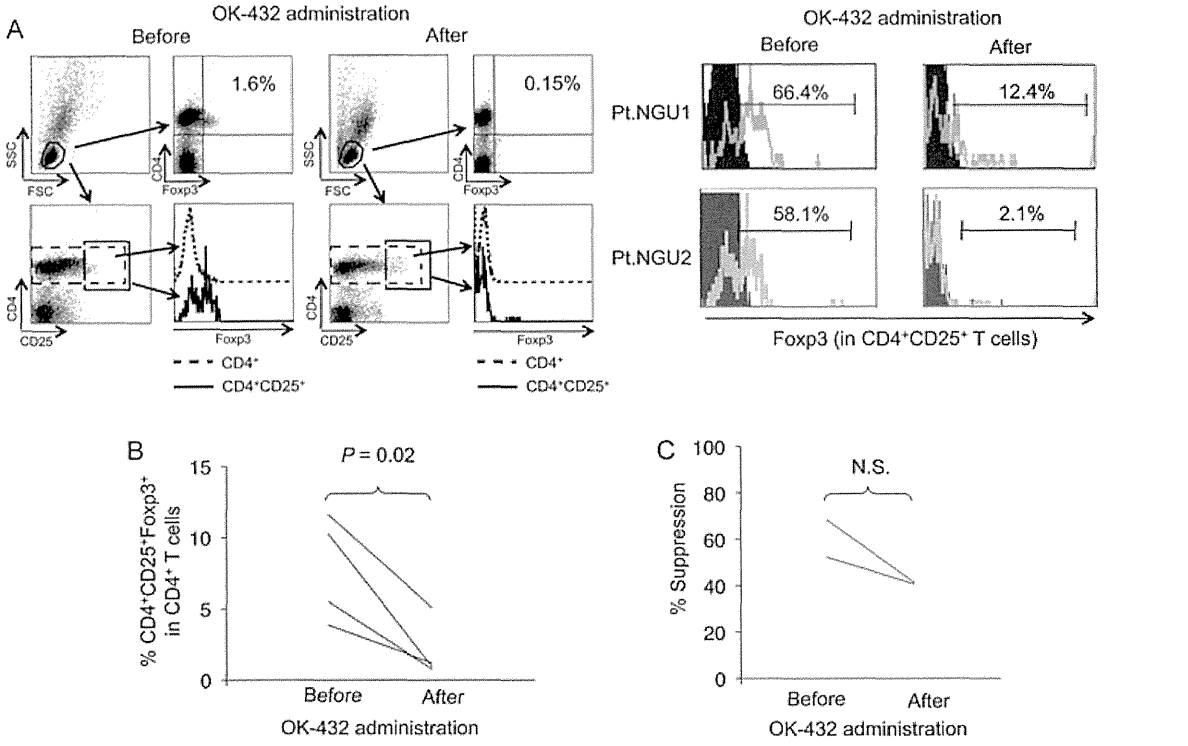

Figure 4. OK-432 administration into tumor-associated exudate fluids elicits reduction of local Treg-cell accumulation and function. Cells were isolated from tumor-associated exudate fluids (two pleural effusions or two ascites) and peripheral blood before and 2 days after OK-432 administration. (A) Cells were stained with anti-CD4, anti-CD25, and anti-Foxp3 Abs and were analyzed with flow cytometry. The staining pattern and gating method of a representative patient is shown (left); solid line histogram, Foxp3 expression in $CD4^+$ $CD25^+$ T cells; dotted line histogram, Foxp3 expression in $CD4^+$ T cells. Foxp3 expression in $CD4^+CD25^+$ T cells of two representative patients is shown (right); filled histogram, control staining; gray line histogram, Foxp3. (B) The percentage of $CD4^+CD25^+Foxp3^+$ cells in $CD4^+$ T cells is shown for $n = 4$ patients' samples. Statistical significance determined by Student's *t*-test. (C) 1×10^5 $CD4^+CD25^-$ T cells were isolated from PBMCs before OK-432 administration and cultured with irradiated autologous CD4-depleted PBMCs and anti-CD3 Ab in the presence or absence of 1×10^5 $CD4^+CD25^{high}$ T cells isolated from tumor-associated exudate fluids before and after OK-432 administration ($n = 2$). Data shown are from one experiment representative of two independent experiments.

induced NY-ESO-1_{91-110}–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population before and after vaccination. Following vaccination with NY-ESO-1 protein in the presence of OK-432, $CD4^+$ T-cell immune responses against NY-ESO-$1_{111-130}$ were newly elicited (Fig. 5A). These vaccine-induced NY-ESO-$1_{111-130}$–specific $CD4^+$ T cells were detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population only after vaccination (Fig. 5A). In Pt #2, while specific $CD4^+$ T cells were not observed before vaccination, NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were elicited after vaccination. The vaccine-induced NY-ESO-$1_{119-141}$–specific $CD4^+$ T cells were also detected in the $CD4^+CD25^-CD45RO^+$ (effector/memory) T-cell population, as observed in Pt #1 (Fig. 5B).

NY-ESO-1 vaccination with OK-432 activates high-avidity preexisting NY-ESO-1–specific $CD4^+$ T-cells

We then asked whether vaccine-induced T cells had a high-affinity TCR that recognized naturally processed antigens [21, 28]. We established NY-ESO-1–specific $CD4^+$ T-cell clones. Four clones and a single clone that recognized different epitopes were generated from Pt #1 and Pt #2, respectively. Four minimal epitopes (NY-ESO-$1_{83-96,\ 94-109,\ 119-130,121-134}$) were defined from $CD4^+$ T-cell clones derived from Pt #1 (Fig. 6A and data not shown). Both spontaneously induced (#2–11) and vaccine-induced (#3–1) $CD4^+$ T-cell clones recognized naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6A). One minimal epitope defined from Pt #2 was NY-ESO-$1_{122-133}$ and the vaccine-induced $CD4^+$ T-cell clone (#1–1) again recognized both the naturally processed NY-ESO-1 protein and as little as 0.1 nM of peptide (Fig. 6B), indicating that these T-cell clones had high-affinity TCRs against NY-ESO-1. Together, OK-432 as an adjuvant could overcome Treg-cell suppression and activate high-affinity preexisting NY-ESO-1–specific $CD4^+$ T-cell precursors.

Discussion

While a subset of patients treated with immunotherapy has been shown to experience objective and durable clinical responses, it is becoming increasingly clear that several mechanisms

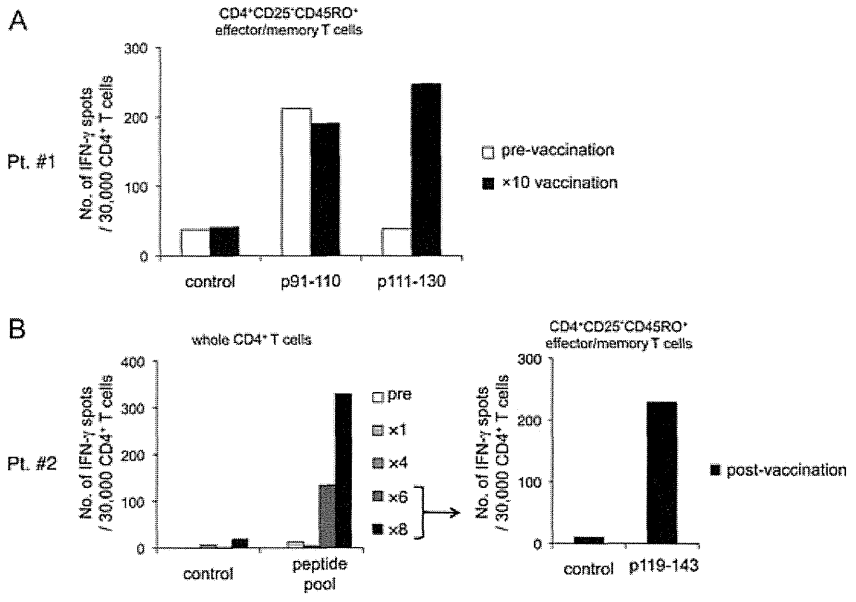

Figure 5. Vaccination with OK-432 elicits NY-ESO-1–specific effector/memory $CD4^+$ T cells $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were prepared from PBMCs of (A) Pt #1 (HLA-DR 4, 12 -DQ 4, 8) and (B) Pt #2 (HLA-DR 9, 15 -DQ 6, 9) before and after vaccination as described in the *Materials and Methods*; 3–5 × 10^5 $CD4^+CD25^-CD45RO^+$ T cells or unfractionated $CD4^+$ T cells were cultured with 3–5 × 10^5 CD4-depleted PBMCs pulsed with 10 μM pooled peptides covering the entire sequence of NY-ESO-1 for 3 weeks. Induction of NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data shown are from one experiment representative of at least two experiments performed.

downregulate antitumor immunity during the course of the immune response and play a major role in limiting the effectiveness of cancer immunity [6, 35, 36]. A plethora of cell types, cell surface molecules, and soluble factors mediate this suppressive activity [3, 6, 35, 36]. Among them, $CD4^+CD25^+Foxp3^+$ Treg cells play a crucial role by suppressing a wide variety of immune responses, and finding ways to control Treg-cell suppression is a major priority in this field [6, 7]. In this study, we showed the potential of OK-432 (a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*) which stimulates TLR signals [30, 33, 34] to control Treg-cell suppression, supporting the idea that OK-432 may be a promising adjuvant for cancer vaccines by inhibiting Treg-cell suppression and by augmenting induction of tumor-specific T cells against coadministered protein antigens.

Appropriate adjuvant combinations, such as those that are MyD88-dependent or MyD88-independent, or those that are TRIF-coupled and include endosomal signals, are known to synergistically activate DCs with regard to the production of inflammatory cytokines [37, 38]. As OK-432 is derived from bacterial components, its capacity to bind a combination of various TLRs makes it attractive. It has been shown that OK-432 exhibits antitumor effects through TLR-2, TLR-4, and TLR-9 using knockout mice for each TLR [30, 33, 34]. Alternatively, OK-432 reportedly stimulates DCs through the β_2-integrin system rather than via TLR signals [29]. In the presence of OK-432, Treg cells slightly proliferated with TCR stimulation. TLR2 triggering results in a temporary loss of the anergic status of Treg cells and is associated with loss of Treg-cell suppressive function [24, 25]. The perturbation of Treg-cell anergy by OK-432 through TLR2 stimulation may play a role, at least in part, in the inhibition of Treg-cell suppressive function.

In accordance with previous reports [29, 34], we showed that APCs, including $CD11c^+$ and $CD14^+$ cells (monocytes, macrophage, and DCs), stimulated with OK-432 exhibited significantly higher production of IL-12 as compared with that of LPS- or TNF-α–matured APCs, and that OK-432–induced IL-12 from these APCs was a critical component for abrogating Treg-cell activity. Additionally, we found that monocyte-derived DCs stimulated with OK-432 produced significantly higher amounts of IL-12 compared with DCs stimulated with LPS or TNF-α (Supporting Information Fig. 2). It has been reported that IL-12 receptor expressed on effector T cells, but not on Treg cells has a critical role for abrogating Treg-cell suppression by IL-12 in mice [39, 40]. In accordance with this, downregulation of IL-12 receptors by siRNA on effector cells partially abrogated the OK-432–induced inhibition of Treg-cell suppressive activity (Supporting Information Fig. 3). IL-12 receptor was induced in both effector T cells and Treg cells after activation (Supporting Information Fig. 3). We attempted to downregulate the IL-12 receptor on Treg cells with siRNA to explore the exact target(s) of IL-12, however, the limitation in the availability of human materials hampered these analyses. Thus, IL-12 produced by APCs on the OK-432 stimulation could have two (or more) mutually compatible activities, (i) rendering effector cells resistant to Treg-cell suppression and (ii) inhibiting Treg-cell suppressive function directly, though the in vivo data argue against direct inhibition of Treg-cell suppression [39, 40].

Local administration of OK-432 reduced the number of $CD4^+CD25^+Foxp3^+$ Treg cells in tumor-associated exudate fluids. After administration of OK-432, local chemokine gradient may be changed and infiltration of Treg cells may be blocked [6, 13]. Alternatively, the inflammatory environment after OK-432 administration may be suitable for effector T-cell activation and IL-2, that is critical for Treg-cell survival and function [41], may not be adequately provided, as observed during severe *Toxoplasma gondii* infection [42]. In addition, suppressive function of $CD4^+CD25^{high}$ T cells in tumor-associated

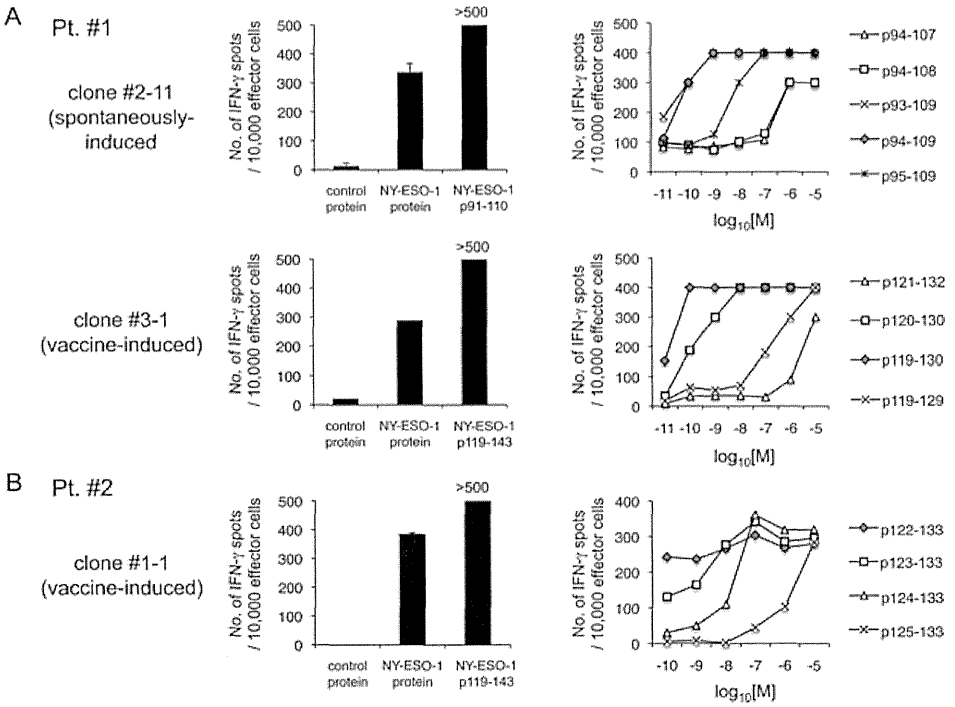

Figure 6. Vaccination with OK-432 activates high-avidity NY-ESO-1–specific CD4$^+$ T cells. NY-ESO-1–specific CD4$^+$ T-cell clones were generated from PBMCs of patients harboring NY-ESO-1–specific CD4$^+$ T cells. CD4$^+$ T-cell clones from (A) Pt. #1 and (B) Pt. #2 were stimulated and expanded by 30 ng/mL anti-CD3 Ab in the presence of 20 U/mL IL-2 and irradiated PBMCs and EBV-transformed human B lymphocytes as feeder cells. A couple of weeks later, these NY-ESO-1–specific CD4$^+$ T cell clones (1×10^4/well) were cultured with 1×10^4 NY-ESO-1 protein-pulsed DCs or 2×10^4 EBV-B cells pulsed with graded amounts of peptides and NY-ESO-1–specific IFN-γ production was analyzed by ELISPOT. Data are shown as mean ± SD of two replicates and are from one experiments representative of at least two experiments performed.

exudate fluids was reduced after OK-432 treatment in accordance with decreased expression of Foxp3 [43]. Considering the fact that IL-12, a main effector molecule induced by OK-432, renders effector cells resistant to Treg-cell suppression, direct administration of OK-432 may change the immunological balance in the local microenvironment from suppression by Treg cells to activation of helper T cells by augmenting helper T-cell activity. However, the sample size of patients analyzed in this study was relatively small and warrants cautious interpretation.

We have previously shown that while naive NY-ESO-1–specific CD4$^+$ T-cell precursors are present in wide range of healthy individuals and cancer patients, their activation is kept under stringent CD4$^+$CD25$^+$ Treg-cell control [20, 21, 28]. Using OK-432 as an adjuvant, we detected high-affinity NY-ESO-1–specific CD4$^+$ T cells in effector/memory population after vaccination in two esophageal cancer patients. In Pt #1, we found two responses; spontaneous and vaccine-induced NY-ESO-1–specific CD4$^+$ T cells. Both of them exhibited a similar efficiency to recognize titrated peptide, indicating that these NY-ESO-1–specific CD4$^+$ T cells had TCRs with similar affinity and were likely activated from naive high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors. Vaccination with minimal peptide in incomplete Freund's adjuvant fails to activate high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, rather it dominantly expands low-avidity effector/memory CD4$^+$ T cells that cannot recognize naturally processed antigens [21]. In addition, following DNA vaccination covering the entire sequence of NY-ESO-1, high-avidity NY-ESO-1–specific CD4$^+$ T cells were not detected persistently because of rapid suppression by Treg cells [44]. While these data suggest a critical role for the inhibition of Treg-cell suppression by OK-432 in the activation of high-affinity NY-ESO-1–specific CD4$^+$ T-cell precursors, it is still difficult to obtain conclusive evidence without direct in vivo Treg-cell inhibition/depletion. To formally address this issue, clinical trials using Treg-cell depletion reagents and another clinical trial having two arms of patients receiving NY-ESO-1 with/without OK-432 would be required.

Certain types of immunization methods or DC stimulations elicit/augment CD4$^+$CD25$^+$ Treg cells in vivo [10–12, 45]. As many tumor-associated antigens recognized by autologous tumor-reactive lymphocytes are antigenically normal self-constituents [1–3], they also could be recognized with CD4$^+$CD25$^+$ Treg cells. Given that a proportion of cancer/testis antigens are targets of Treg cells [46], it is necessary to avoid unwanted activation of cancer/testis antigen-specific CD4$^+$CD25$^+$ Treg cells. Though the sample size of patients analyzed in this study was small and warrants cautious interpretation, including OK-432 in vaccine components as an adjuvant would be a promising strategy to establish favorable circumstances for stimulating effector T cells by inhibiting Treg-cell activation. Furthermore, since this agent has a long history and is widely applied as an anticancer drug, particularly

in Japan, its clinical safety profile has been already established. Our data provide a critical cue for effective cancer vaccines and immunotherapy during antigen priming through modulation of $CD4^+CD25^+$ Treg-cell function.

Materials and methods

Blood samples

All healthy donors were subjects with no history of autoimmune disease. PBMCs, pleural effusions, or ascites from cancer patients were collected before and after local administration of OK-432 based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Nagasaki University Graduate School of Medicine. PBMCs from esophageal cancer patients enrolled in a clinical trial of CHP-NY-ESO-1 and CHP-HER2 vaccination with OK-432 [47] (Supporting Information Fig. 1) were collected based on the protocol approved by the Human Ethics Committees of Mie University Graduate School of Medicine and Kitano Hospital. The clinical trial was conducted in full conformity with the current version of the Declaration of Helsinki and was registered as NCT00291473 of Clinical Trial.gov, and 000001081 of UMIN Clinical Trial Registry. All samples were collected after written informed consent.

Abs and reagents

Synthetic peptides of NY-ESO-1_{1-20} (MQAEGRGTGGSTGDADGPGG), NY-ESO-1_{11-30} (STGDADGPGGPGIPDGPGGN), NY-ESO-1_{21-40} (PGIPDGPGGNAGGPGEAGAT), NY-ESO-1_{31-50} (AGGPGEAGATGGRGPRGAGA), NY-ESO-1_{41-60} (GGRGPRGAGAARASGPGGGA), NY-ESO-1_{51-70} (ARASGPGGGAPRGPHGGAAS), NY-ESO-1_{61-80} (PRGPHGGAASGLNGCCRCGA), NY-ESO-1_{71-90} (GLNGCCRCGARGPESRLLEF), NY-ESO-1_{81-100} (RGPESRLLEFYLAMPFATPM), NY-ESO-1_{91-110} (YLAMPFATPMEAELARRSLA), NY-ESO-$1_{101-120}$ (EAELARRSLAQDAPPLPVPG), NY-ESO-$1_{111-130}$ (QDAPPLPVPGVLLKEFTVSG), NY-ESO-$1_{119-143}$ (PGVLLKEFTVSGNILTIRLTAADHR), NY-ESO-$1_{131-150}$ (NILTIRLTAADHRQLQLSIS), NY-ESO-$1_{139-160}$ (AADHRQLQLSISSCLQQLSLLM), NY-ESO-$1_{151-170}$ (SCLQQLSLLMWITQCFLPVF), NY-ESO-$1_{161-180}$ (WITQCFLPVFLAQPPSGQRR), and HIV $P17_{37-51}$ (ASRELERFAVNPGLL) [48] were obtained from Invitrogen (Carlsbad, CA, USA). Recombinant NY-ESO-1 protein was prepared using similar procedures as described previously [49]. OK-432 was purchased from Chugai Pharmaceutical (Tokyo, Japan). LPS (*Escherichia coli* 055:B5) was obtained from Sigma (St. Louis, MO, USA). Purified and FITC-conjugated anti-IL-12 (C8.6; mouse IgG1), purified anti-IL-6 (MQ2–13A5; rat IgG1), purified anti-IFN-γ (NIB42; mouse IgG1), purified anti-IL-23 (HNU2319; mouse IgG1), PE-conjugated anti-CD20 (2H7; mouse IgG2b) and PE-conjugated anti-CD56 (MEM188; mouse IgG2a) Abs were purchased from eBioscience (San Diego, CA, USA). Purified anti-IL-1β Ab (8516; mouse IgG1) was purchased from R&D Systems (Minneapolis, MN, USA). PE-conjugated anti-CD14 (MφP9; mouse IgG2b), PE-conjugated anti-CD45RA (HI100; mouse IgG2b), PerCP-conjugated anti-CD4 (RPA-T4; mouse IgG1), and FITC-conjugated anti-CD4 (RPA-T4; mouse IgG1), Foxp3 (259D; mouse IgG1), and CD45RO (UCHL1; mouse IgG2a) Abs were purchased from BD Biosciences (Franklin Lakes, NJ, USA). PerCP-Cy5.5-conjugated anti-CD11c Ab (3.9; mouse IgG1) was obtained from Biolegend (San Diego CA, USA). PE-conjugated anti-CD25 Ab (4E3; mouse IgG2b) was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). Recombinant IL-6, IL-12, and TNF-α were purchased from PeproTech (Rocky Hill, NJ, USA).

Intracellular cytokine staining

PBMCs were cultured with/without OK-432 and GolgiStop reagent (BD Biosciences) for 20 h. Cells were stained for cell surface markers and then for intracellular cytokine (IL-12) after permeabilization. Results were analyzed by flow cytometry (FACSCanto; BD Biosciences).

Generation of NY-ESO-1–specific $CD4^+$ T cells

NY-ESO-1–specific $CD4^+$ T cells were elicited as described previously [20]. Briefly, $CD4^+$ T cells and $CD4^+CD25^-$ T cells were isolated from PBMCs using a $CD4^+CD25^+$ Treg Isolation Kit (Miltenyi Biotec). $CD4^+CD25^-$ T cells were further separated into $CD45RO^+$ T cells or $CD45RA^+$ T cells by FACSAria (BD Bioscience) after staining with anti-CD45RO and CD45RA Abs. $CD4^-$ PBMCs pulsed with 10 μM of peptide overnight were used as APCs. After irradiation, 5×10^5 APCs were added to round-bottom 96-well plates (Nunc, Roskilde, Denmark) containing 1–5 $\times 10^5$ unfractionated $CD4^+$ or $CD4^+CD25^-CD45RO^+$ T cells and were fed with 10 U/mL IL-2 (Kindly provided by Takeda Pharmaceutical, Osaka, Japan) and 20 ng/mL IL-7 (R&D Systems). Subsequently, one-half of medium was replaced by fresh medium containing IL-2 (20 U/ml) and IL-7 (40 ng/mL) twice per week.

Generation of NY-ESO-1–specific $CD4^+$ T-cell clones

Cloning was performed by limited dilution as described previously [50]. Briefly, NY-ESO-1–specific $CD4^+$ T cell lines (0.3 cells/well) were stimulated and expanded in the presence of irradiated 5×10^4 cells/well PBMCs and 1×10^4 cells/well irradiated EBV-transformed human B lymphocytes with 10% AB serum, 20 U/ml IL-2, and 30 ng/mL anti-CD3 Ab (OKT3; eBioscience) in 96-well round-bottom plates.

Proliferation assay

$CD4^+CD25^-$ T cells were cultured with 1×10^5 irradiated CD4-depleted PBMCs and stimulated with 0.5 μg/mL anti-CD3 Ab

