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Epitope diversification driven by non-tumor epitope-specific Th1 and Th17 mediates potent antitumor reactivity

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ABSTRACT

MHC class I-restricted peptide-based vaccination therapies have been conducted to treat cancer patients, because $CD8^+$ CTL can efficiently induce apoptosis of tumor cells in an MHC class I-restricted epitope-specific manner. Interestingly, clinical responders are known to demonstrate reactivity to epitopes other than those used for vaccination; however, the mechanism underlying how antitumor T cells with diverse specificity are induced is unclear. In this study, we demonstrated that dendritic cells (DCs) that engulfed apoptotic tumor cells in the presence of non-tumor MHC class II-restricted epitope peptides, $OVA_{323–339}$, efficiently presented tumor-associated antigens upon effector-dominant $CD4^+$ T cell balance against regulatory T cells (Treg) for the $OVA_{323–339}$ epitope. Th1 and Th17 induced tumor-associated antigens presentation of DC, while Th2 ameliorated tumor-antigen presentation for $CD8^+$ T cells. Blocking experiments with anti-IL-23p19 antibody and anti-IL-23 receptor indicated that an autocrine mechanism of IL-23 likely mediated the diverted tumor-associated antigens presentation of DC. Tumor-associated antigens presentation of DC induced by $OVA_{323–339}$ epitope-specific $CD4^+$ T cells resulted in facilitated antitumor immunity in both priming and effector phase *in vivo*. Notably, this immunotherapy did not require pre-treatment to reduce Treg induced by tumor. This strategy may have clinical implications for designing effective antitumor immunotherapies.

1. Introduction

Epitope spreading is a process in which the dominant or primary epitope-specific T cell response induces immune responses against subdominant or cryptic antigens and results in the diversification of epitope specificity. Epitope diversification has been demonstrated in anti-virus immunity, graft rejection, and as an important component to enhance immune response efficiency [1,2]. During epitope diversification, self-reactive T and/or B cells can be primed when the immune response spreads to include self-epitopes regardless of the type of primary epitope [3]. This type of epitope spreading is thought to play a critical role in the pathogenesis of autoimmune diseases [4–7]. Notably, the epitope-spreading mechanism can break peripheral tolerance to evoke immunological responses against cryptic self-epitopes.

MHC class I-restricted peptide-based vaccination therapies have been conducted to treat cancer patients. Although frequencies of vaccinated peptide-specific $CD8^+$ CTL increased in many patients, few patients achieved objective clinical responses. In several recent immunotherapy trials, clinical responders were shown to display reactivity to epitopes other than those used for vaccination [8–12]. Epitope diversification is an attractive strategy for evoking effective antitumor immunity, however, the procedures that preferentially induce T cell responses to diverse determinants of tumor-associated antigens are unknown. Interaction between antigen-presenting cells (APCs) and $CD4^+$ effector T cells mediated by CD40/CD40L co-stimulation is required to present widespread epitopes for T cell priming [13]. In this scenario, certain MHC class II epitope-specific $CD4^+$ T cells and APCs that possess both the desired antigens and the MHC class II epitope peptides are indispensable to enhance the required immune response.

In this study, we examined whether $CD4^+$ T cells specific for a non-tumor MHC class II epitope can promote tumor-associated antigen presentation by dendritic cells (DCs) that concurrently acquired tumor-associated antigens and a non-tumor MHC class II peptide. $CD4^+$ T cells specific for $OVA_{323–339}$ were used to drive epitope diversification. OT-II effector $CD4^+$ T cells, especially, Th1 and Th17 promoted priming of antitumor T cells in LN draining

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DC that possessed tumor-associated antigens and $OVA_{323-339}$. Tumor-associated antigen presentation of DC induced by non-tumor epitope-specific $CD4^+$ T cells resulted in potent antitumor reactivity in both priming and effector phase.

2. Materials and methods

2.1. Mice

Female C57BL/6J (B6) and OT-II mice with a transgenic TCR to recognize MHC class II ($I\text{-}A^b$)-restricted $OVA_{323-339}$ epitope peptide on CD57BL/6 background were purchased from the CLEA Laboratory (Tokyo, Japan). Mice were maintained in a specific pathogen-free environment and used for experiments at the age of 8–10 weeks. All animal experiments were conducted with the permission of the Niigata University Ethics Committee for Animal Experiments (permission number 204107).

2.2. Tumors

MCA205 is a fibrosarcoma of B6 origin induced by intramuscular injection of 3-methylcholanthrene [14]. An MCA205 tumor cell line was established and maintained *in vitro*.

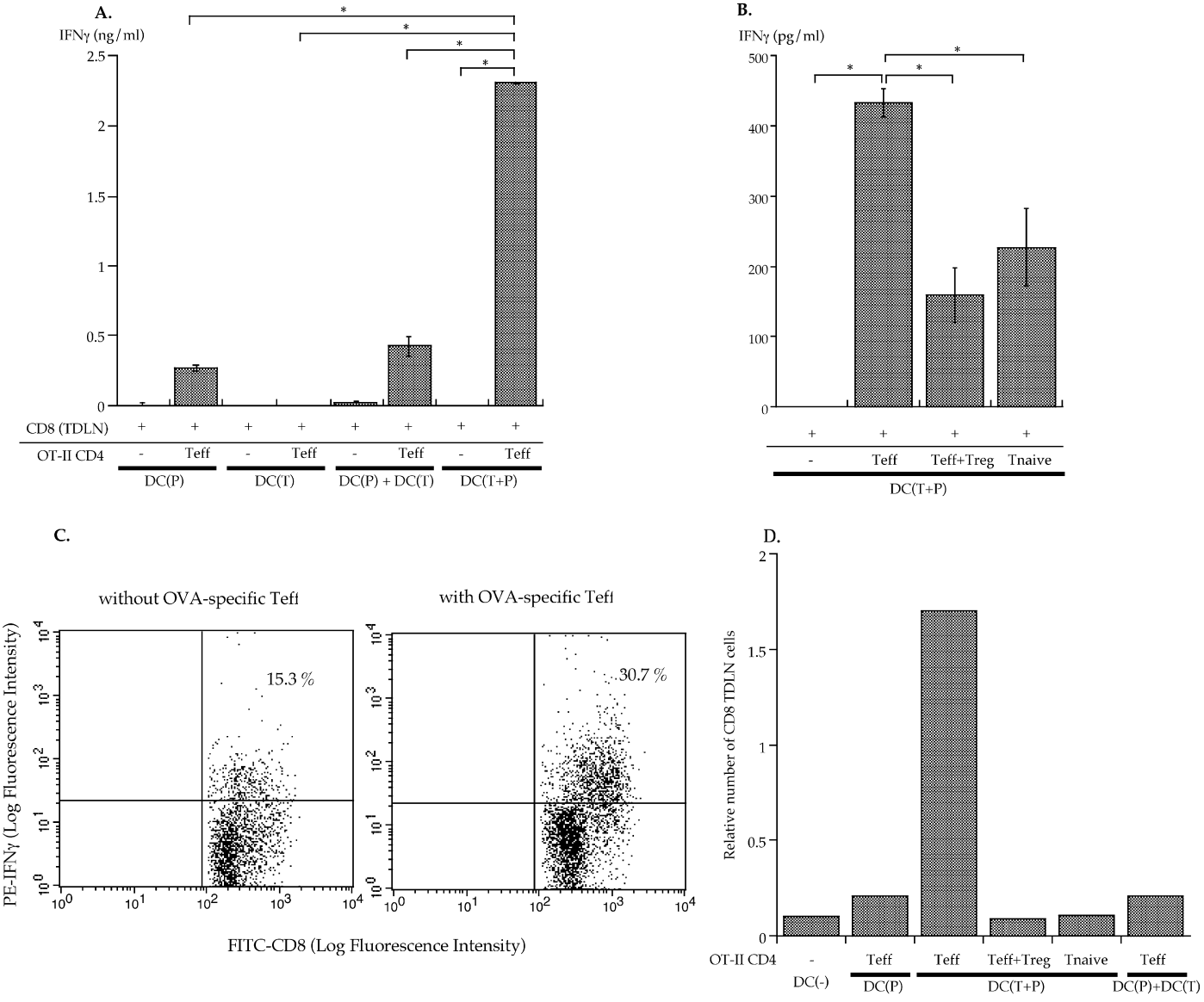

Fig. 1. DC (1×10^6) were conditioned with 100 μg/ml $OVA_{323-339}$ peptide and/or 1×10^6 irradiated MCA205 tumor cells (5000 cGy) overnight in 2 ml CM on 24-well plates. Prior to co-culture with T cells, DC were washed and purified as $CD11c^+$ cells with magnetic beads. As the responder cells, $CD62L^{low}$ $CD8^+$ T cells (1×10^5) derived from LNs draining growing MCA205 tumors for 12 days (TDLN) were cocultured with 1×10^4 DC in the presence or absence of 1×10^4 $CD4^+$ T cells derived from OT-II mice in 200 μl CM on 96-well plates for 48 h. Effector $CD4^+$ T cells (Teff), regulatory $CD4^+$ T cells (Treg), and naïve $CD4^+$ T cells (Tnaïve) were obtained as described in Materials and Methods. $^*p$ value < 0.01. (A) Supernatants were harvested and measured for IFNγ with enzyme linked immunosorbent assay (ELISA). DC(P), DC(T), and DC(T + P) indicate DC that were pulsed with $OVA_{323-339}$ peptide, DC that were cocultured with lethally irradiated MCA205 tumor cells, and DC that were cocultured with lethally irradiated MCA205 tumor cells in the presence of OVA peptide. DC(P) + DC(T) indicates a 1:1 mixture of DC(P) and DC(T). Responder $CD8^+$ tumor-draining LN T cells were cocultured with the conditioned DC in the presence or absence of OT-II Teff. (B) Supernatants were harvested and the IFNγ levels in them were determined. The responder $CD8^+$ tumor-draining LN T cells were cocultured with DC(T + P), in the presence or absence of OT-II Teff, Treg, or Tnaïve cells. (C) These panels indicate cytosolic IFNγ staining of $CD8^+$ T cells. $CD62L^{low}$ $CD8^+$ TDLN T cells (TA-$CD8^+$ T cells) were cocultured with DC that were pulsed with both OVA peptide and irradiated MCA205 tumor cells in the presence or absence of $CD62L^{low}$ $CD4^+$ T cells from OT-II mice (OT-II $CD4^+$ Teff). More $IFN\gamma^+$ $CD8^+$ T cells were detected when TA-$CD8^+$ T cells were co-cultured with DC(T + P) and OT-II $CD4^+$ Teff. (D) TA-$CD8^+$ T cell numbers were determined after 48-h co-culture with DC and OT-II $CD4^+$ T cells. *Y*-Axis indicates relative number of $CD8^+$ T cells based on the $CD8^+$ T cell number before co-culture.

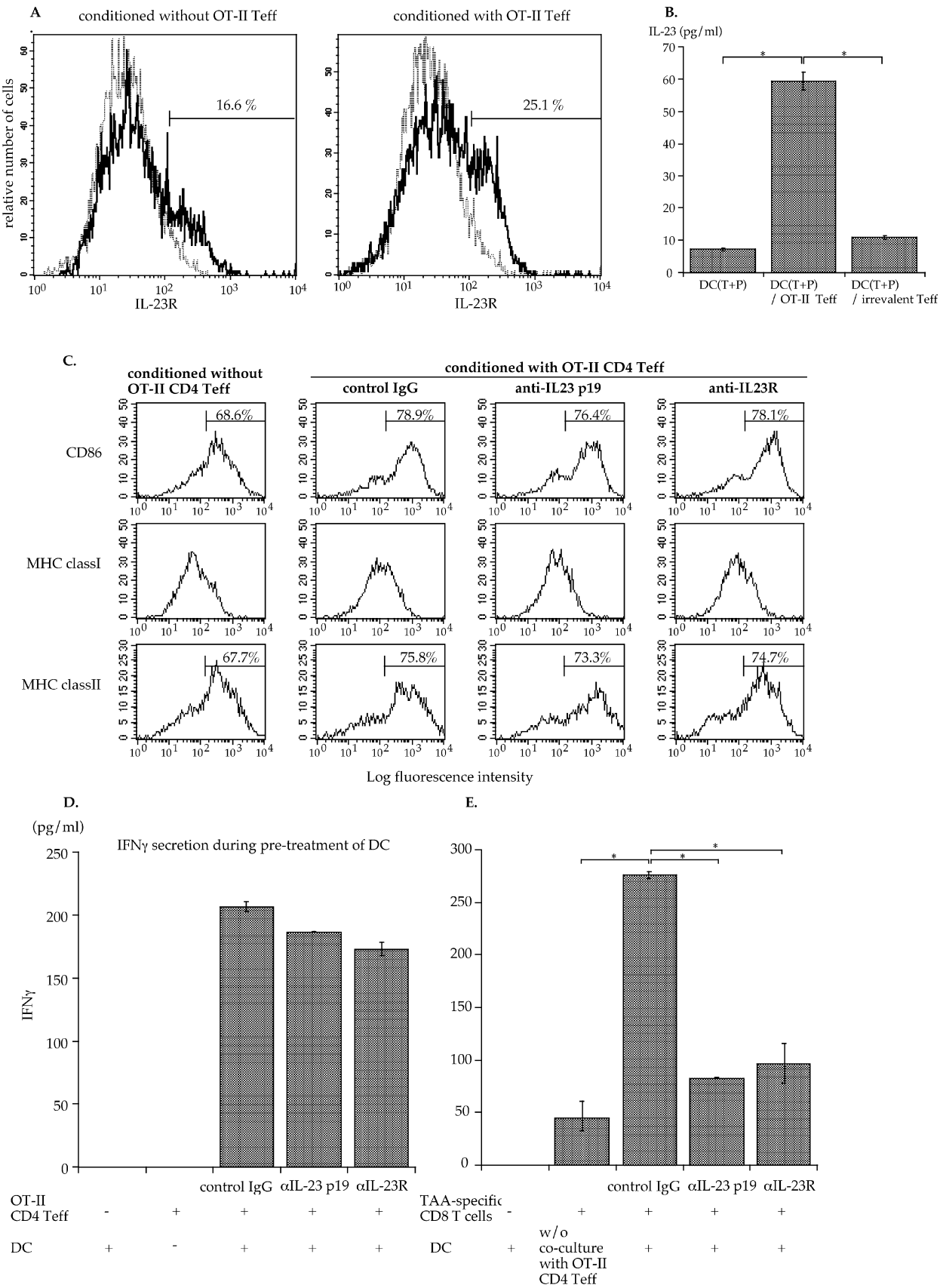

Fig. 2. (A and B) Purified DC (1×10^6) that concurrently pulsed with $OVA_{323–339}$ peptide and MCA205 tumor-associated antigens were cultured for 24 h in the presence or absence of 1×10^5 OT-II $CD4^+$ Teff in 2 ml CM supplemented with GM-CSF on 24-well plates. Cells were stained with FITC-conjugated anti-CD11c mAb and PE-conjugated anti-IL-23 receptor (IL-23R) mAb in the presence of anti-CD16/CD32 mAb. Supernatants were measured for IL-23 with a murine IL-23 ELISA kit according to the manufacturer's instructions. (A) IL-23R expression on gated $CD11c^+$ cells. Dotted lines indicate staining of PE-conjugated isotype control mAb. (B) Content of IL-23. Statistical analyses were performed with Student's *t*-test. **p* value < 0.01. (C–E) Purified DC(T + P) (1×10^6) were conditioned with 1×10^5 OT-II $CD4^+$ Teff in 2 ml CM in the presence of 50 μg/ml

2.3. Peptides

$OVA_{323–339}$ peptide ISQAVHAAHAEINEAGR was purchased from Funakoshi Co. Ltd. (Tokyo, Japan).

2.4. Monoclonal antibodies (mAbs)

Hybridomas producing mAbs against murine CD3 (2C11), and CD62L (MEL14) were obtained from the American Type Culture Collection (Rockville, MD). Anti-CD62L mAb were produced as ascites fluid from sublethally irradiated (500 cGy) DBA/2 mice. PE-conjugated anti-CD86 (GL1), anti-CD62L (MEL14), anti-IL23 receptor (3C9) and anti-CD8 (2.43); fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (2.43); and anti-CD11c (HL3) mAbs were purchased from BD PharMingen (San Diego, CA). For DC staining to avoid non-specific binding with the Fc receptor, anti-CD16/CD32 mAb (2.4G2, Fc block™, BD PharMingen) was used according to the manufacturer's instructions.

2.5. Tumor-draining LN cells

B6 mice were inoculated subcutaneously (s.c.) with 1.5×10^6 MCA205 tumor cells in both flanks. Inguinal LNs draining tumors were harvested 10–12 days after tumor inoculation. Single-cell suspensions were prepared mechanically as described previously [14].

2.6. Bone marrow-derived DCs

DCs were generated from bone marrow cells as described previously [14]. DC were purified as $CD11c^+$ cells with anti-CD11c mAb-coated microbeads (Miltenyi Biotec) and autoMACS™ according to the manufacturer's instructions.

2.7. Fractionation of T cells

T cells in LN cell suspension were concentrated by passing through nylon wool columns (Wako Pure Chemical Industries, Osaka, Japan). To yield highly purified (>90%) cells with down-regulated CD62L expression ($CD62L^{low}$), LN T cells were isolated by sheep anti-rat Ig Ab/anti-CD62L mAb-coated DynaBeads (M-450; Dynal, Oslo, Norway). T cells with high CD62L expression ($CD62L^{high}$) were obtained as cells attached to flasks coated with goat anti-rat Ig Ab (Jackson ImmunoResearch Laboratories, West Grove, PA)/anti-CD62L mAb (MEL14). In some experiments, cells were further separated into $CD4^+$ and $CD8^+$ cells using magnetic beads as described previously [15]. $CD25^+$ cells were isolated using PE-conjugated anti-CD25 mAb and anti-PE microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cell purity was >90%.

2.8. Adoptive immunotherapy

B6 mice were injected s.c. with 1.5×10^6 MCA205 tumor cells in 100 μl HBSS to establish subcutaneous tumors on the abdomen midline. Mice were infused intravenously (i.v.) with T cells. LN T cells were stimulated with immobilized anti-CD3 mAb (2C11) for 2 days and expanded with 20 U/ml recombinant human (rh) interleukin 2 (IL-2; gift from Shionogi, Osaka, Japan) for 3 days before infusion as described previously [14]. The perpendicular diameters of subcutaneous tumors were measured with calipers.

2.9. Cytokine ELISA

T cells (1×10^5) were co-cultured with 2×10^4 DC in 200 μl complete medium (CM) on 96-well plates for 24 h in triplicate. Supernatants were harvested and assayed for mouse (m)IFNγ content by a quantitative "sandwich" enzyme immunoassay using a mIFNγ ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer's instructions. CM consists of RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 0.1 mM nonessential amino acids, 1 μM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (all from Life Technologies, Inc.), and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO).

2.10. Proliferation assay

$CD8^+$ T cells (4×10^5) were co-cultured with 1×10^5 conditioned DC for 48 h in the presence or absence of 1×10^5 $CD4^+$ T cells in 2 ml CM supplemented with 10 U/ml rhIL-2 on 24-well plates. $CD62L^{low}$ $CD8^+$ tumor-draining lymph node (TDLN) T cells were labeled with 5 μM 5-(6)-carboxyfluorescein diacetate succinimidyl diester (CFSE) (Molecular Probes, Inc., Eugene, OR) in HBSS at 37 °C for 15 min and washed twice before co-culture. After co-culture, cell numbers were counted, and percentages of $CFSE^+$ cells were determined with a microfluorometer.

2.11. Generation of OVA-specific Th1, Th2, and Th17 cells from OT-II T-cell receptor-transgenic mice

$CD62L^{high}CD25^-$ naïve $CD4^+$ T cells isolated from OT-II mice were cultured for 6 days with 10 μg/ml OVA peptide and irradiated spleen cells (2500 cGy) under specific conditions: Th1 condition, IL-2 (20 U/ml), IL-12 (10 μg/ml), IFNγ (1 μg/ml) and anti-IL-4 mAb (10 μg/ml); Th2 condition, IL-2 (20 U/ml), IL-4 (1 ng/ml), anti-IL-12 mAb (10 μg/ml), and anti-IFNγ mAb (10 μg/ml); and Th17 condition, IL-2 (20 U/ml), TGFβ (3 ng/ml), and IL-6 (20 ng/ml).

2.12. Statistical analysis

Statistical analyses of were performed with ANOVA and *post hoc* test using Prism™ (GraphPad Software). A two-tailed p value less than 0.05 was considered significant.

3. Results

3.1. Tumor-associated antigen presentation of DC was induced by interaction with the non-tumor epitope-specific effector $CD4^+$ T cells

Diversed determinant presentation to induce autoimmune diseases requires APC that interact with $CD4^+$ effector T cells [1,13]. We examined whether DC presentation of tumor-associated antigen was induced by the non-tumor epitope-specific $CD4^+$ T cells. DC that were pulsed with 100 μg/ml $OVA_{323–339}$ peptide and/or 1×10^6 irradiated MCA205 tumor cells (5000 cGy) overnight were conditioned with $OVA_{323–339}$-specific OT-II $CD4^+$ T cells in 2 ml CM on

anti-IL-23p19 Ab (R&D Systems Inc., Minneapolis, MN), anti-IL-23R mAb, or isotype control mAb on 24-well plates for 24 h. Supernatants in which DC were conditioned were measured for IFNγ content. After conditioning, DC (1×10^4) were isolated as $CD11c^+$ cells and were co-cultured with 1×10^5 TA-$CD8^+$ T cells in 200 μl CM on 96-well plates for next 24 h. Supernatants in which TA-$CD8^+$ T cells and conditioned DC were co-cultured were measured for IFNγ content. (C) CD86, MHC class I, or MHC class II expression on gated $CD11c^+$ cells after the first 24-h conditioning with OT-II $CD4^+$ Teff. (D) IFNγ produced by OT-II $CD4^+$ Teff during the first 24-h conditioning. (E) IFNγ secreted by TA-$CD8^+$ T cells in the presence of conditioned DC during the next 24-h co-culture. *p value < 0.01.

Table 1
DC (2×10^6) that acquired both tumor-associated antigen and $OVA_{323-339}$ peptide were cultured in the presence or absence of 1×10^6 OT-II CD4$^+$ Teff for 24 h in 2 ml CM on 24-well plates. CD4$^+$ T cells were depleted from conditioned DC with anti-CD4 mAb-coated magnetic beads. CD62L^{low} CD4$^+$ T cells derived from TDLN were stained with CFSE and co-cultured with conditioned DC for 24 h in the presence of breferdin A. Cytosolic IFNγ was stained with an intracellular mIFNγ staining kit (BD Pharmingen^{TM}) according to the manufacturer's instructions. Cells were analyzed with a microfluorometer and percentages of IFNγ$^+$ cells based on gated CFSE$^+$ cells were indicated.

	OT-II CD4+	IFNγ$^+$ cells/CAFÉ$^+$ cells (%)
DC(T + P)	–	15.7
DC(T + P)	Teff	28.9
DC(T + P)	Teff + Treg	19.0
DC(T) + DC(P)	Teff	12.9

24-well plates. Prior to co-culture with tumor-associated antigens-specific responder T cells, DC were washed and purified as CD11c$^+$ cells with magnetic beads. CD62L^{low} T cells in LN draining growing MCA205 tumor were used as tumor-associated antigen-specific responder T cells [16].

Tumor antigen-primed CD62L^{low} CD8$^+$ T cells (TA-CD8$^+$) produced significantly more IFNγ and percentages of IFNγ-producing CD8$^+$ T cells increased upon antigen stimulation by DC that had engulfed lethally irradiated MCA205 tumor cells in the presence of $OVA_{323-339}$ peptide (DC(T + P)) and were conditioned with $OVA_{323-339}$-specific effector CD4$^+$ T cells (OT-II CD4$^+$ Teff, Fig. 1A–C). In contrast, a mixture of DC that acquired tumor-associated antigens (DC(T)) and DC that were pulsed with $OVA_{323-339}$ peptide (DC(P)) failed to induce IFNγ production by TA-CD8$^+$ T cells even though the DC were conditioned with OT-II CD4$^+$ Teff. The augmented capacity of DC(T + P) to induce IFNγ production by TA-CD8$^+$ T cells was not due to merely DC maturation, because no differences of CD80, CD86, MHC class I, and class II expression were observed between the conditioned DC(T + P) and the conditioned DC(P) (data not shown). To test if effector type CD4$^+$ T cells are required to promote tumor-associated antigen presentation, naïve CD62L^{high}CD25^{low} CD4$^+$ T cells purified from OT-II mice (OT-II CD4$^+$ Tnaïve) were used (Fig. 1B). OT-II CD4$^+$ Tnaïve cells showed significantly inferior capacity to promote tumor-associated antigen presentation by DC. Further, we tested if CD25^{high} CD4$^+$ Treg affected tumor-associated antigen presentation that was induced by OT-II CD4$^+$ Teff. In the presence of OT-II Treg, OT-II CD4$^+$ Teff failed to promote tumor-associated antigen presentation of DC(T + P), thereby IFNγ production of TA-CD8$^+$ T cells was inhibited. Percentages of IFNγ-producing TA-CD4$^+$ T cells were also increased by DC(T + P) that were co-cultured with OT-II CD4$^+$ Teff (Table 1).

Consistent with the IFNγ production assay, proliferation assay demonstrated that TA-CD8$^+$ T cells proliferated only when they were co-cultured with DC(T + P) that were conditioned with OT-II CD4$^+$ Teff (Fig. 1D). DC(T + P) that were treated with OT-II CD4$^+$ Tnaïve or a mixture of DC(T) and DC(P) that were conditioned with OT-II CD4$^+$ Teff showed no effect. In the presence of OT-II Treg, OT-II CD4$^+$ Teff could not give DC the capacity to promote TA-CD8$^+$ T cell proliferation. Collectively, effector-dominant CD4$^+$ T cell balance on $OVA_{323-339}$, a non-tumor epitope, could give DC licenses to present tumor-associated antigens to facilitate antitumor CD8$^+$ T cell activation and proliferation when DC concurrently possessed tumor-associated antigens and the non-tumor MHC class II epitope.

3.2. Augmented tumor-associated antigen presentation by DC depended on IL-23 autocrine mechanism

IL-23 was reported to play a critical role in epitope spreading in experimental autoimmune encephalomyelitis (EAE) [17]. Therefore, we examined if IL-23 participated in augmented tumor-associated antigen presentation by DC. IL-23 receptor was upregulated on DC, and IL-23 production were detected when DC were conditioned with OT-II CD4$^+$ Teff (Fig. 2A and B). Next, DC(T + P) and OT-II CD4$^+$ Teff were cocultured overnight in the presence of anti-IL-23p19 Ab, anti-IL-23 receptor mAb, or isotype control mAb. Then, the CD11c$^+$ DC were purified and co-cultured with TA-CD8$^+$ T cells in fresh CM for 24 h. In the presence of anti-IL-23p19 or anti-IL-23 receptor antibodies, MHC class I, II antigen and CD86 on DC, or IFNγ production by OT-II CD4$^+$ Teff during overnight conditioning (Fig. 2C and D) were not disturbed, thus, it seems that recognition of $OVA_{323-339}$ peptide by OT-II CD4$^+$ Teff was not affected. In contrast, anti-IL-23p19 or anti-IL-23 receptor antibody treatment abrogated tumor-associated antigen presentation by DC thereby inhibited IFNγ production by TA-CD8$^+$ T cells (Fig. 2E). Thus, an IL-23 autocrine mechanism is likely required for diverse tumor-associated antigen presentation on DC induced by OT-II CD4$^+$ Teff.

OT-II CD4$^+$ Teff promoted priming of antitumor T cells in LNs draining subcutaneously injected DC that were concurrently pulsed with tumor-associated antigen and $OVA_{323-339}$ peptide.

To test whether upregulated presentation of tumor-associated antigen on DC by OT-II CD4$^+$ Teff could induce priming of antitumor T cells, DC(T + P) were inoculated s.c. at both flanks while mice were infused i.v. with OT-II CD4$^+$ T cells. As shown in Fig. 3A, the number of T cells in DC-draining LN increased when the mice were infused with OT-II CD4$^+$ Teff. OT-II CD4$^+$ Treg or effector type CD62L^{low} CD4$^+$ T cells with irrevalent antigen specificity failed to facilitate T-cell priming in DC-draining LN. We then examined the antitumor therapeutic efficacy of LN T cells. LN T cells were infused i.v. into mice bearing 3-day established skin tumor after sublethal whole body irradiation (500 cGy). T cells derived from DC-draining LNs of the mice infused with OT-II CD4$^+$ Teff exhibited significantly higher therapeutic efficacy on a per-cell basis, resulting in regression of skin tumors (Fig. 3B).

Next, to examine that protective antitumor immunity can be mounted with this strategy, mice were i.v. infused with 1×10^6 OT-II CD4$^+$ Teff and vaccinated with s.c. injection of 1×10^6 DC(T + P) or a mixture of 1×10^6 DC(T) and 1×10^6 DC(P). Twenty-one days after vaccination, the mice were inoculated s.c. with 2×10^6 MCA205 tumor cells (Fig. 3C). Co-infusion of OT-II CD4$^+$ Teff resulted in potent protective immunity in the mice vaccinated with DC(T + P), but not those vaccinated with the DC(T) and DC(P) mixture.

3.3. OT-II CD4$^+$ Teff augmented antitumor therapeutic efficacy of TA-CD8$^+$ T cells in vivo

To examine whether tumor-associated antigen presentation of DC induced by OT-II CD4$^+$ Teff could augment antitumor reactivity of TA-CD8$^+$ T cells in the effector phase. Co-infusion of OT-II CD4$^+$ Teff facilitated the antitumor therapeutic efficacy of TA-CD8$^+$ T cells in the presence of DC(T + P), resulting in regression of skin tumor in all mice (Fig. 3D).

Additionally, we treated 5-day established skin tumor without pretreatment to deplete host lymphocytes. As shown in Fig. 3E, TA-CD8$^+$ T cells mediated potent antitumor reactivity in the presence of DC(T + P) and OT-II CD4$^+$ Teff, resulting in regression of 5-day established tumor without whole body irradiation.

3.4. Th1 and Th17 but not Th2 induced tumor antigen presentation by DC resulting in priming of antitumor T cells in draining LN

To elucidate the Th subsets that augment TAA presentation on DC, DC(T + P) were co-cultured overnight with highly differentiated

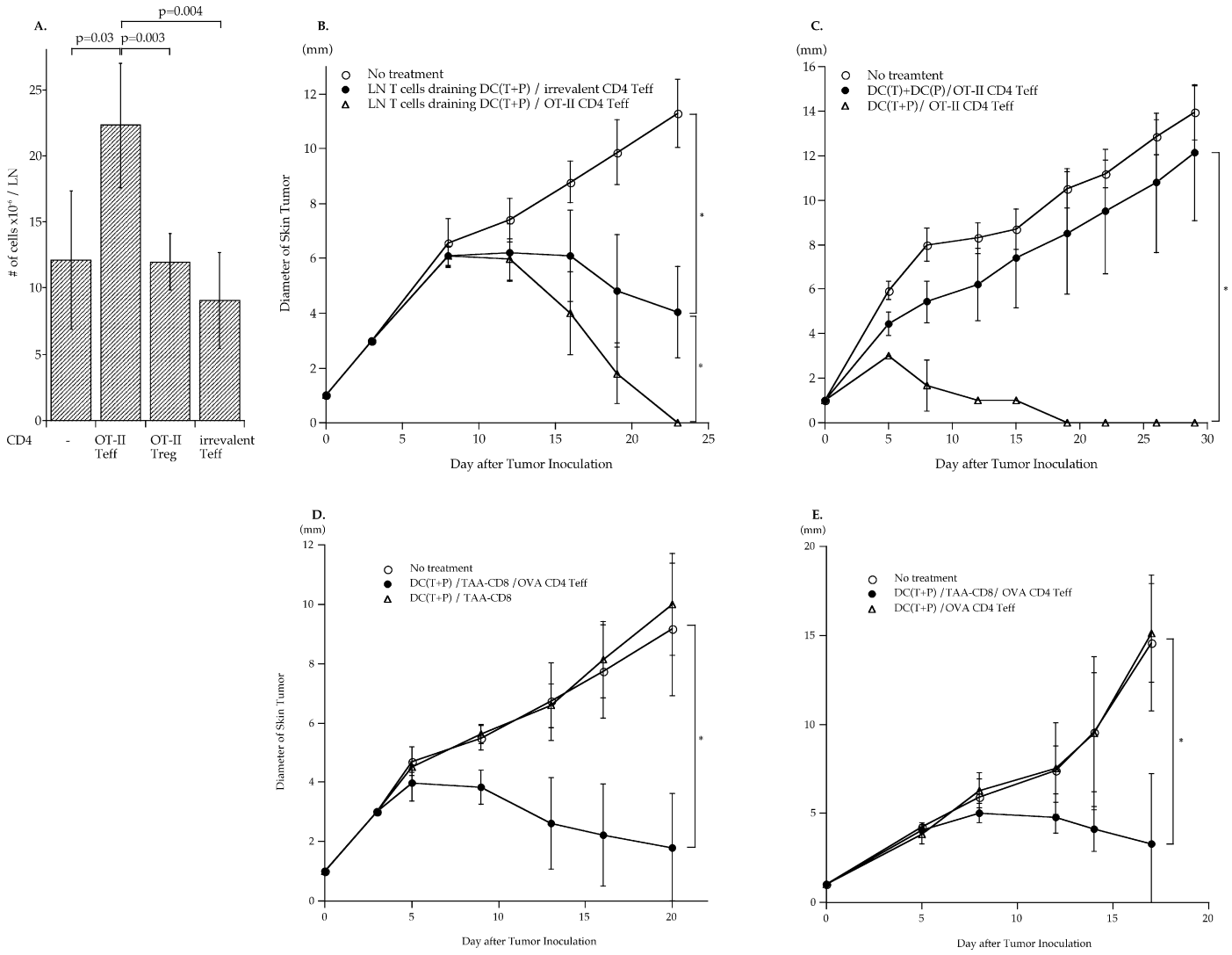

Fig. 3. (A and B) One million DC(T + P) were inoculated s.c. at both flanks. On the same day, 1×10^6 OT-II CD4$^+$ Teff or CD62L^{low} CD4$^+$ T cells isolated from splenocytes of B6 mice were infused intravenously. Inguinal LNs were harvested as draining LNs 7 days after DC inoculation. (A) Number of cells per lymph node. (B) DC-draining LN T cells (2×10^7) that were stimulated with immobilized anti-CD3 mAb for 2 days and expanded with 20 U/ml IL-2 for 3 days were infused i.v. into mice bearing 3-day established skin tumors after sublethal whole body irradiation (500 cGy). *p value < 0.01. (C) DC(T + P) or a mixture of DC(P) and DC(T) were inoculated s.c. at the right flank. On the same day, 1×10^6 OT-II CD4$^+$ Teff were infused intravenously. Two million MCA 205 tumor cells were inoculated on midline, 21 days after DC vaccination. Averages of 5 mice per treatment group ± standard deviations, representative of 3 experiments. *p value < 0.01. (D and E) MCA 205 tumor cells (1.5×10^6) were inoculated s.c. on midline to establish skin tumor. Purified DC(T + P) (1×10^6) were inoculated s.c. at the right flank of mice bearing 3-day established skin tumor (D) or 5-day established skin tumor (E). TA-CD8$^+$ T cells (1×10^7) and OT-II CD4$^+$ Teff (1×10^7) were infused i.v. on the same day of DC inoculation. (D and E) Indicate growth curves of skin tumors. Each group contained 5 mice. *p value < 0.01.

Th1, Th2, or Th17 cells derived from OT-II mice (Fig. 4A). After conditioning with the Th cells, CD11c$^+$ DC were isolated with magnetic beads. TA-CD8$^+$ T cells exhibited significantly more IFNγ production when co-cultured with DC(T + P) that were conditioned with $OVA_{323-339}$ peptide-specific Th1 or Th17 but not with DC conditioned with Th2 (Fig. 4B), although CD80, CD86, and MHC class I and II antigen expression on DC showed no differences across groups (data not shown).

Next, OT-II Th1, Th2, or Th17 were infused i.v. into mice that were s.c. inoculated with DC(T + P), and DC-draining LN were obtained 7 days later. The number of cells increased in DC-draining LN from mice infused with OT-II Th1 or Th17 (Fig. 4C). In contrast, OT-II Th2 cell infusion showed no effect on the number of DC-draining LN cells. All CD62L^{low} T cells isolated from LN draining DC(T + P) demonstrated the same per-cell antitumor therapeutic efficacy except the DC-draining LN T cells derived from mice infused with Th2 cells, which showed ameliorated antitumor reactivity (Fig. 4D). The LN T cells showed significantly delayed antitumor therapeutic efficacy in draining the DC(T + P) of the mice infused with Th2. Collectively, tumor-associated antigen presentation of DC induced by OT-II Th1 and Th17 resulted in augmented priming of antitumor T cells in draining LN, while OT-II Th2 had a negative effect on DC to prime antitumor T cells.

4. Discussion

The epitope-spreading mechanism is known to be associated with chronic relapsing episodes of multiple sclerosis, and *in vivo* anti-IL-23p19 antibody treatment has been established to inhibit epitope spreading in EAE, a murine model of multiple sclerosis [17]. However, how anti-IL-23 treatment inhibits epitope spreading has remained unclear, since activated DC were thought to produce IL-23, and IL-23 receptor is expressed on T cells. This study elucidated that upon interaction with effector CD4$^+$ T cells, DC transiently

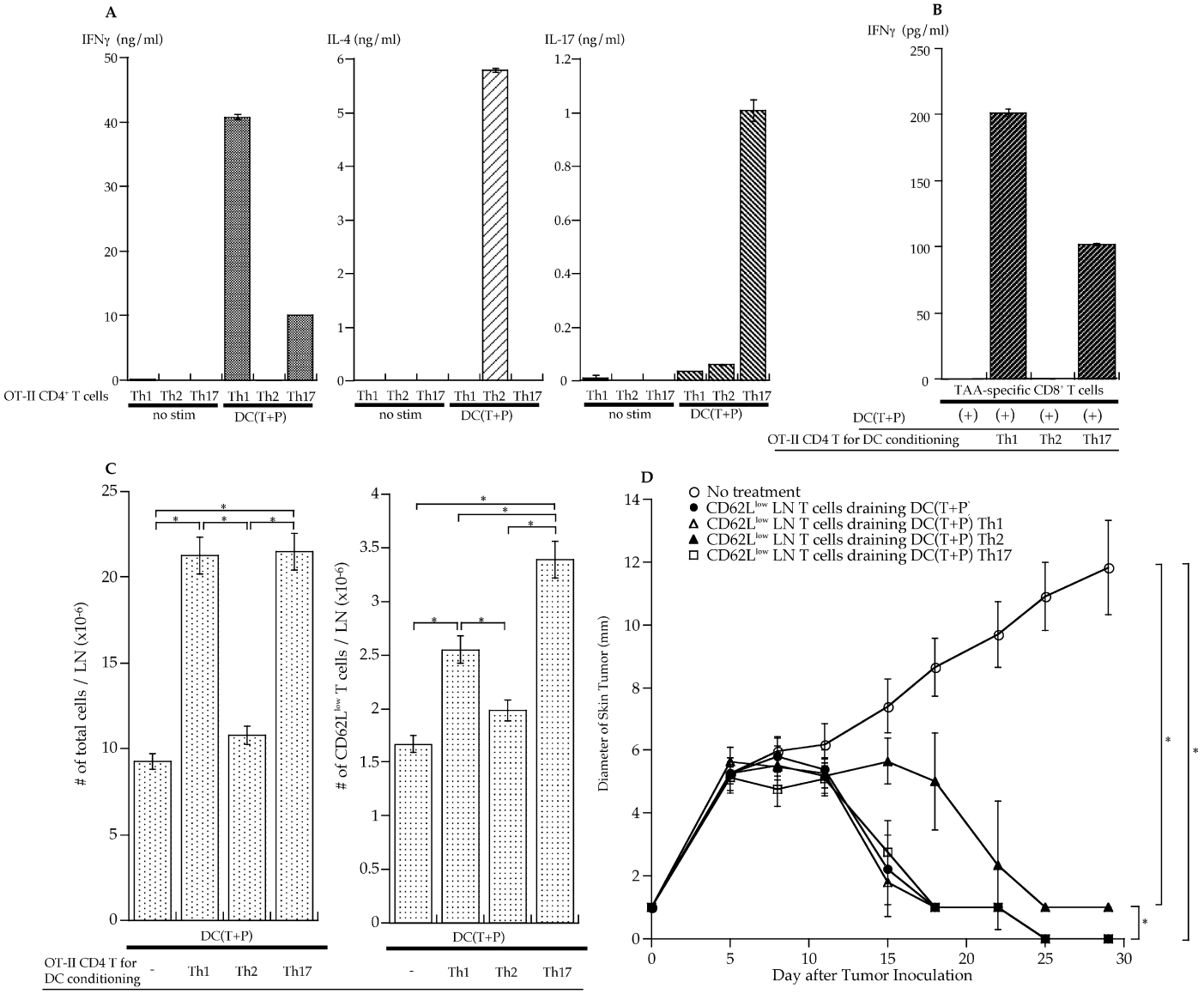

Fig. 4. (A and B) IFNγ, IL-4, and IL-17 production by differentiated Th1, Th2, and Th17 cells derived from OT-II mice during overnight co-culture with DC. Isolated CD11c$^+$ DC (2×10^4) was then co-cultured with 1×10^5 TA-CD8$^+$ T cells in 200 μl CM on 96-well plates. (B) Indicates IFNγ content in each well. Data shown are averages ± standard deviations. *p value < 0.01. (C and D) The mice vaccinated by s.c. injection of 1×10^6 DC(T + P) were i.v. infused with 2×10^6 Th1, Th2, or Th17 derived from OT-II mice on the same day. Bilateral inguinal LN were harvested and analyzed 7 days after vaccination. Each group contained 3 mice. The left panel of (C) indicates the number of total cells in each LN, and the right panel indicates the number of CD62L^{low} T cells per LN. CD62L^{low} LN T cells (2×10^6) that were isolated from DC-draining LN as primed T cells were infused i.v. into mice bearing 3-day established MCA205 skin tumors after sublethal whole body irradiation (500 cGy). Data shown in (D) are averages of 5 mice per treatment group ± standard deviations and representative of 2 independent experiments. *p value < 0.01.

upregulated expression of IL-23 receptor while also secreting IL-23 (Fig. 2). Since DC that were treated with anti-IL-23p19 or anti-IL-23 receptor antibody during overnight co-culture with OT-II CD4$^+$ Teff failed to present tumor-associated antigens for TA-CD8$^+$ T cells, an autocrine mechanism of IL-23 by DC likely plays a critical role in diversified antigen presentation to induce intermolecular epitope spreading. Interestingly, upregulated expression of CD86 and MHC class II antigen induced by CD4$^+$ T cells was not disturbed by the anti-IL-23 treatment. Thus, DC maturation induced by CD4$^+$ T cells and diverse antigen presentation seems to be controlled separately. Anti-IL-23 treatment may be used to prevent undesired epitope spreading that induces autoimmune responses.

Clinical studies using MHC class I-restricted tumor antigen peptides have been conducted; however, CD8$^+$ T cell-mediated antitumor therapeutic responses based on a single peptide are usually weak and transient. The magnitude of the T cell pool specific for the immunizing epitope was reported not to predict clinical responses. In contrast, a high frequency of epitope spreading was observed in clinical responders, while non-responders did not show epitope spreading [18–20]. Antitumor CD4$^+$ T cells have been shown capable of mediating long-lasting potent antitumor reactivity and recruitment of APC in tumor tissues, resulting in priming of antitumor T cells with widespread epitope specificity [15,21,22]. These findings are consistent with our study, which indicates that effector CD4$^+$ T cells and DC that possess tumor-associated antigens are key machinery to induce tumor-associated epitope diversification. However, difficulties in identifying effective MHC class II-restricted tumor-associated antigens make it impossible to design antitumor immunotherapy that evokes both CD8$^+$ and CD4$^+$ T cell immunity to achieve clinical responses [19].

In this study, we used OVA-derived MHC class II-restricted antigen peptides as an epitope through which tumor-associated

antigen-carrying DC and CD4+ T cells interacted, since even tumor-bearing mice are naïve to these antigens, and no specific inducible Treg are thought to exist. In the presence of effector CD4+ T cells that recognized the $OVA_{323–339}$ peptide and DC that possessed both the $OVA_{323–339}$ peptide and tumor-associated antigens, TA-CD8+ T cells mediated potent antitumor therapeutic efficacy resulting in regression of 5-day established tumor *in vivo* without any pretreatment such as whole body irradiation to reduce Treg number. This strategy does not require identification of MHC class II-restricted tumor-associated antigens to design an immunotherapy. Th1 and Th17 facilitated the antitumor activity of DC in the priming and effector phases. The antitumor therapeutic efficacy of the LN T cells of the mice infused with Th2 was significantly ameliorated, and the tumor shrinking effect was delayed. According to our recent study, CD8+ antitumor T cells alone mediate early but transient therapeutic efficacy [22]. In contrast, the CD4+ antitumor T cells mediate relatively delayed but long-lasting antitumor activity. Because DC treated with Th2 cells could not stimulate the antitumor CD8+ T cells (Fig. 4B), probably the deficit of DC in priming the CD8+ T cells along with the interaction of DC with the Th2 cells resulted in a delayed antitumor effect. Collectively, antitumor immunotherapy utilizing DC and non-tumor MHC class II epitope-specific Th1 and Th17 to induce tumor-associated epitope diversification may have clinical implications.

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