Depletion of IL-10- and TGF-β-Producing Regulatory γδ T Cells by Administering a Daunomycin-Conjugated Specific Monoclonal Antibody in Early Tumor Lesions Augments the Activity of CTLs and NK Cells

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Depletion of IL-10- and TGF-β-Producing Regulatory γδ T Cells by Administering a Daunomycin-Conjugated Specific Monoclonal Antibody in Early Tumor Lesions Augments the Activity of CTLs and NK Cells

Naohiro Seo,2* Yoshiki Tokura, † Masahiro Takigawa, † and Kohji Egawa3*  

It has been demonstrated that γδ T cells accumulating in early tumor lesions and those purified from spleen cells of tumor-bearing mice attenuate the activity of CTLs and NK cells. We, therefore, investigated whether depletion of γδ T cells from early lesions of tumors results in restoration of CTL and NK cell activities and subsequent regression of tumors. A daunomycin-conjugated anti-γδ TCR mAb UC7-13D5 (Dau-UC7) was prepared to efficiently deplete γδ T cells. An in vitro study revealed that Dau-UC7 specifically lysed γδ TCR+ cells and effectively inhibited splenic γδ T cells from tumor-bearing mice to produce cytotoxic cell-suppressive factors. Furthermore, intralesional injections of Dau-UC7 at an early stage of tumor development led to augmentation of tumor-specific CTL as well as NK cell activities and to the resultant regression or growth inhibition of the tumors. On analysis of cytokine profile, γδ T cells transcribed mRNAs for IL-10 and TGF-β, but not IL-4 or IFN-γ, suggesting the T regulatory 1-like phenotype. Finally, a blocking study with mAbs showed that the inhibitory action of γδ T cells on CTLs and NK cells was at least partly mediated by IL-10 and TGF-β. These results clearly demonstrated the novel mechanism by which T regulatory 1-like γδ T cells suppress anti-tumor CTL and NK cell activities by their regulatory cytokines in early tumor formation. The Journal of Immunology, 1999, 163: 242–249.

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these cells at the tumor sites may result in tumor regression by relaxing CTLs and NK cells. The purpose of this study was to investigate whether daunomycin-conjugated anti-γδ TCR mAb can effectively damage the suppressor functions of γδ T cells, whether intradimensional treatment with this conjugate at an early stage can lead to tumor regression by restoring tumor-specific CTL and NK cell functions, and whether these γδ T cells produce regulatory cytokines such as IL-10 and TGF-β. The results show that successful elimination of γδ T cells producing Tr1-type cytokines results in tumor regression by the actions of CTLs and NK cells.

Materials and Methods

Mice and tumor cells

Seven to nine-week-old male C3H/He and C57BL/6 (B6) mice were obtained from Japan SLC (Hamamatsu, Japan), MM2, MH134, B16, and YAC-1 tumor cells were used in this study. MM2, MH134, and B16 were mammary tumor cell lines of C3H/He, a hepatoma cell line of C3H/He, and a melanoma cell line of B6, respectively. MM2 cells were maintained i.p. in C3H/He. MH134 and B16 cells were maintained by culturing them in DMEM (Nissui, Tokyo, Japan) supplemented with 10% FCS. In vivo experiments, MM2 and MH134 cells (2 × 10^5 cells/mouse) were inoculated into C3H/He mice i.p. and s.c., respectively. The same number of B16 cells were injected s.c. into B6 mice. YAC-1 cells are an NK-sensitive cell line that was cultured in DMEM supplemented with 10% FCS and used in in vitro assays.

mAbs and chemical substances

Anti-γδ TCR mAb (UC7-13D5)-producing hybridoma was a gift from Dr. Bluestone (Chicago University, Chicago, IL). UC7-13D5 and anti-γδ TCR mAb (H57-597) were purified from hybridoma culture supernatants by affinity column chromatography with anti-hamster IgG-Sepharose after ammonium sulfate precipitation. Anti-hamster IgG-Sepharose was prepared by covalent coupling of anti-hamster IgG sheep polyclonal Abs (Organan Teknika, Westchester, PA) with cyanogen bromide-activated Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden). Purified forms of anti-CD4 mAb (GL.1), anti-CD8 mAb (53-6-7), and hamster IgG were obtained from Pharmingen (San Diego, CA). Anti-IL-10 and anti-TGF-β1, -2, and -3 neutralizing mAbs, rIL-10, and rTGF-β1 were purchased from Genzyme (Cambridge, MA). Daunomycin (Sigma, St. Louis, MO), daunorubicin, and daunomycin hydrogen were purchased from Sigma (St. Louis, MO). A solution was mixed and incubated at 20°C for 2 h. After centrifugation, the supernatant obtained after centrifugation proceeded at 20°C for 30 min. Daunomycin-conjugated Ab was cultured in RPMI 1640 supplemented with 10% FCS and rIL-2 (5 U/ml) at 37°C for 3 days. The expanded cells were recultured in RPMI 1640 supplemented with 10% FCS in a 24-well plate (Corning, Corning, NY; 1 × 10^5 cells/well) for 24 h. After centrifugation, each culture supernatant was obtained and added to the anti-H-2b CTL assays at a 50% volume. Otherwise, to examine the effect of daunomycin conjugates on the ability of γδ T cells to produce cytokines, γδ T cells isolated from MM2 tumor-bearing mice were cultured in RPMI 1640 supplemented with 10% FCS in 24-well plates (1 × 10^5 cells/well). Culture supernatants obtained by this manipulation were also added to the anti-H-2b CTL assays at a 50% volume.

Preparation of lymphocyte culture supernatants

Splenic γδ T cells purified from spleens of normal or MM2-bearing mice and from MM2 tumor-infiltrating lymphocytes were cultured in RPMI 1640 supplemented with 10% FCS and rIL-2 (5 U/ml) at 37°C for 3 days. The expanded cells were recultured in RPMI 1640 supplemented with 10% FCS in a 24-well plate (Corning, Corning, NY; 1 × 10^5 cells/well) for 24 h. After centrifugation, each culture supernatant was collected and added to the anti-H-2b CTL assays at a 50% volume. To test the cytotoxicity of daunomycin-conjugated Ab, purified γδ and γδ T cells (1 × 10^5 cells/well) were cultured and expanded in an anti-CD3 mAb-immobilized 24-well culture dish with RPMI 1640 supplemented with 10% FCS and 50 U/ml rIL-2. The propagating cells (5 × 10^5 cells/ml) were radiolabeled with RPMI 1640 containing 10% FCS and 200 μCi Na^219Cr (DuPont-New England Nuclear, Boston, MA) for 1 h at 37°C. After washing four times, 11C-labeled γδ and γδ T cells were used as target cells for drug-conjugated Abs. Daunomycin-conjugated H57-597, UC7-13D5, or hamster IgG was added at varying concentrations to each well containing target cells (1 × 10^5 cells/200 μl). The mixtures were incubated at 37°C for 12 h. To examine in vitro CTL induction in TILs, MM2 cells (1 × 10^5), and B16, YAC-1 TILs were treated with daunomycin-conjugated mAbs (100 ng/ml daunomycin-5 μg/ml Abs) at 37°C for 4 h and washed three times with DME. Dau-Ab-treated TILs were cultured with rIL-2 (100 U/ml)-containing medium at 37°C for 5 days. The expanded cells were subjected to the CTL assays against 11C-labeled tumor target cells. For cytotoxicity test of CTLs against tumor cells, MM2, MH134, and YAC-1 cells (5 × 10^5 cells/ml) were radiolabeled with RPMI 1640 containing 10% FCS and 200 μCi Na^219Cr (DuPont-New England Nuclear, Boston, MA) for 1 h at 37°C. After washing three times, the cells were incubated at 37°C for 4 h on a rocking shaker. Anti-hamster IgG-conjugated beads were prepared by coupling the anti-hamster IgG (Organan Teknika) with tosyl-activated magnetic beads (Dynal) according to the Dynal manual. Cells bearing beads with a magnet and cultured overnight in RPMI 1640 medium supplemented with 10% FCS to separate cells from beads. γδ and γδ T cells purified by this manipulation were confirmed to be >95% pure by flow cytometric analysis using FITC-conjugated αβTCR- or γδTCR-specific mAb (PharMingen, San Diego, CA).

Tumor-infiltrating lymphocytes (TILs) were separated from B16 and MH134 tumor-bearing mice. B16 and MH134 tumor suspensions on day 7 after s.c. inoculation (2 × 10^5/mouse) were collected into C3H/He mice. Seven days after inoculation, ascites containing MM2 and tumor-infiltrating lymphocytes were collected and then diluted with DME containing 10% FCS. After washing three times, the cells were suspended in DMEM and subjected to weak centrifugation (500 × g, 10 s, five times). Approximately 90% of large MM2 cells were precipitated by this manipulation. The remaining cells in culture supernatants were used as MM2-tumor infiltrating lymphocytes using magnetic beads as described above. Anti-H-2b and anti-MM2 CTLs were prepared from T cell-enriched splenocytes of C3H/He mice immunized with B6 lymphocytes and MM2 regressor mice, respectively, as described previously (18).

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were mixed with $^{51}$Cr-labeled B6 lymphoblasts ($1 \times 10^6$ cells) for 6 h at 37°C. B6 lymphoblasts were prepared by culturing B6 splenocytes with RPMI 1640 supplemented with 10% FCS and 5 µg/ml Con A (Pharmacia Biotech) at 37°C for 3 days. In all cytotoxicity tests, the radio activities of medium and cells were counted by gamma counter, and the percent specific lysis was calculated as follows: % specific lysis = (cpm experimental release – cpm spontaneous release)/cpm maximum release – cpm spontaneous release) × 100.

**Proliferation assay**

H-2b- or MM2-specific CTLs ($2 \times 10^5$ cells/well) were incubated in triplicate for 24 h in 96-well plates (Corning Glass Works, Corning, NY) in 100 µl of complete medium. Methyl-titiated thymidine ($^{3}H$Tdr; Amersham, Arlington, IL; 1 µCi/ml) was added to the culture 8 h before harvest. The cells were harvested on glass-fiber filters using a cell harvester (Cambridge Technologies, Watertown, MA), and their radio uptake was measured in a scintillation counter. Culture supernatants from γδ T cells, rIL-10, and rTGF-β1 were added to the H-2b- or MM2-specific CTL proliferation assay at varying concentrations.

**Administration of daunomycin-conjugated Abs in vivo**

MM2 or MH134 cells ($2 \times 10^5$) were inoculated i.p. or s.c. into C3H/He mice, respectively. On either 3 consecutive days, days 4–6 or days 15–17 after tumor inoculation, 100 µg of daunomycin-conjugated Abs containing 5 ng of daunomycin were injected at a tumor site, and subsequent tumor progression was observed. As a negative control, 2 µg of daunomycin alone was injected at a tumor site.

**RT-PCR of cytokine mRNAs**

Tumor-infiltrating γδ T cells accumulating on day 7 after MM2 i.p. inoculation and splenocytes from MM2-bearing C3H/He were prepared as described above. Cells containing PBMC were prepared from blood of MM2-bearing and regressor mice by treatment with 0.17 M ammonium chloride. Total RNAs of these cells were extracted with an RNA extraction kit (RNeasy, Qiagen, Hilden, Germany). First-strand cDNA was reverse transcribed using each RNA sample and was amplified by PCR with a RNA PCR kit (GeneAmp RNA PCR Kit, Takara Biomedicals, Osaka, Japan) according to the manufacturer’s directions. All pairs of primers for β-actin, IL-4, IFN-γ (20), IL-10 (21), and TGF-β (22) were used in this PCR study. PCR was run for 35 cycles with a thermal cycler (DNA amplifier, Sanyo Co., Osaka, Japan) as follows: 1 min at 94°C, 1 min at 55°C, and 1.5 s at 72°C. The PCR products and DNA m.w. marker VI (Boehringer Mannheim, Mannheim, Germany) were loaded in 2% agarose gels and visualized with UV exposure of 1 µg/ml ethidium bromide-staining agarose gel.

**Results**

**Depletion of γδ T cells and abrogation of their functions by Dau-UC7**

To prepare the daunomycin-conjugated Abs, the sugar moiety of daunomycin was cleaved with periodate and then coupled with an anti-TCR mAb (UC7-13D5), anti-αβ TCR mAb (H57-597), or hamster IgG. Following reduction with sodium borohydride, the conjugates were separated from the unbound daunomycin by Bio-Gel P-100 column chromatography. Fractionated eluates were collected, and their absorbances at 280 nm (Ab; ——) and 495 nm (daunomycin; zzzz) of each fraction were measured. A mixture of hamster IgG and daunomycin without coupling manipulation (D) was used as control.

**Discussion**

To test the effect of daunomycin-conjugated Abs, γδ T cells were purified from PBMC of MM2-bearing mice. The conjugates of UC7-13D5, H57-597, and hamster IgG contained daunomycin at a ratio of approximately six daunomycin moieties to one Ab. The conjugates were intraperitoneally injected into syngeneic C3H/He mice, and their radio activities were measured in a scintillation counter. The conjugates of UC7-13D5, H57-597, and hamster IgG contained daunomycin at a ratio of approximately six daunomycin moieties to one Ab.

To investigate whether these daunomycin-conjugated Abs can specifically destroy corresponding cells, splenic αβ TCR$^+$ and γδ TCR$^+$ cell populations were separated from normal C3H/He mice and were cultured over a short term under CD3 stimulation. The expanded cells were $^{51}$Cr labeled and incubated with daunomycin-conjugated UC7-13D5 (Dau-UC7), H57-597 (Dau-H57), or hamster IgG (Dau-IgG), unconjugated UC7-13D5 (UC7), H57-597 (H57) or hamster IgG (IgG), or daunomycin alone. Following 12-h incubation, αβ TCR$^+$ and γδ TCR$^+$ cells were killed by Dau-H57 and Dau-UC7, respectively, while neither conjugate exhibited substantial cytotoxicity against the irrelevant T cell population (Fig. 2A). Dau-IgG, any of the unconjugated Abs (data not shown), or daunomycin did not lyse αβ TCR$^+$ or γδ TCR$^+$ cell target.

In tumor-bearing mice, γδ T cells suppress NK lineage cells and/or CTLs at least partly by releasing soluble factor(s) (18, 19). To confirm the functional abolishment of γδ T cells by Dau-UC7, we used culture supernatants from γδ T cells by testing their immunosuppressive activity against CTLs. γδ T cells purified from MM2-bearing mice were treated with Dau-UC7, and their culture supernatant was added in assays of cytotoxicity and proliferation of anti-H-2b CTL. γδ T cells separated from splenocytes or tumor-infiltrating lymphocytes of MM2-bearing mice produced suppressive factors against anti-H-2b CTL activity (Fig. 2, B and C). The treatment of these γδ T cells with Dau-UC7 totally abrogated their ability to secrete suppressive factors. In contrast, the γδ T cells exposed to Dau-H57, Dau-IgG, any of the unconjugated Abs, or daunomycin produced these factors unchanged. Interestingly, the γδ T cells treated with UC7 produced these suppressors more vigorously than untreated cells, suggesting that intact UC7 mAb transmits activation signals into the γδ T cells. These results further demonstrated that daunomycin-conjugated mAbs were able to attenuate the development of specific lymphocytes.

**Tumor regression by intrallesional injections of Dau-UC7 at an early stage of tumor development**

The above finding raised the possibility that intrallesional injections of Dau-UC7 restore the function of tumor-specific CTLs and NK cells by depressing γδ T cells and result in subsequent inhibition of tumor development. When Dau-UC7 was intrallesionally given on days 4, 5, and 6 after i.p. inoculation of MM2, the MM2 tumor regresss completely (Figs. 3A and 4), and the MM2 regressor mice subsequently survived for over 10 mo. On the other hand, the growth of MH134 tumor was also suppressed by the administration of Dau-UC7 on days 4, 5, and 6 after s.c. inoculation of tumor cells, although the MH134 tumor gradually developed thereafter.

**FIGURE 1.** Preparation of daunomycin-conjugated Abs. Daunomycin conjugates were prepared by coupling anti-αβ TCR mAb (A), anti-γδ TCR mAb (B), or hamster IgG (C) with oxidized daunomycin. After reduction by sodium borohydride, daunomycin-conjugated Ab in the reaction mixture was separated from unbound daunomycin by Bio-Gel P-100 column chromatography. Absorbances at 280 nm (Ab; ——) and 495 nm (daunomycin; zzzz) of each fraction were measured. A mixture of hamster IgG and daunomycin without coupling manipulation (D) was used as control.
and all of the mice died within 2 mo after the tumor inoculation (Fig. 3B). In contrast, there were no therapeutic effects when Dau-UC7 was administered intraleesionally on days 15, 16, and 17 after MM2 or MH134 inoculation (data not shown). This is consistent with our previous finding that γδ T cells function as suppressor cells in an early tumor formation (19). No attenuation of tumors was found when Dau-IgG or daunomycin alone was used at the same concentration as Dau-UC7 (Figs. 3 and 4). A weak inhibition of MM2 tumor progression was found in mice treated with Dau-H57 on days 4, 5, and 6 after i.p. MM2 inoculation, while such an inhibition of tumor growth was not observed in MH134-bearing mice (Fig. 3B). Subsequently, the mice died within 25 days following vigorous progression of MM2 (Fig. 3A). These results raise the possibility that a portion of αβ T cell populations modestly

FIGURE 2. Specific deletion of γδ T cells by Dau-UC7 as assessed by the cytolysis of γδ T cells and the suppressive function of their supernatants. A, 11C-labeled αβTCR⁺ or γδTCR⁺ cells were incubated with Dau-H57 (○), Dau-UC7 (□), Dau-hamster IgG (□), or daunomycin (○) at the indicated concentrations of daunomycin or daunomycin in conjugates. In conjugates, 20, 100, and 200 ng of daunomycin correspond to 1.5, and 10 μg of Abs, respectively. Data are expressed as means of duplicate assays. B, MM2 tumor-infiltrating γδ T cells were treated with Dau-UC7, Dau-H57, Dau-IgG, UC7, H57, IgG, or Dau. After 1 day of cultivation, culture supernatants were collected and added to anti-H-2b CTL assays at a 50% volume. Culture supernatants from untreated MM2 tumor-infiltrating γδ T cells and those from untreated γδ T cells of spleen of MM2-bearing or normal mice were used as controls. Data are expressed as the mean ± SE of duplicate experiments. C, Culture supernatants from MM2-infiltrating γδ T cells with (○) or without (□) Dau-UC7 treatment were added to the proliferation assay of anti-H-2b CTL assay at varying volumes.

FIGURE 3. Therapeutic effectiveness of Dau-UC7. A, Dau-UC7, Dau-H57, Dau-IgG, or Dau was injected at tumor sites on days 4, 5, and 6 after i.p. inoculation of MM2. Untreated MM2-bearing mice were used as a control. B, Dau-UC7, Dau-H57, or Dau-IgG was injected at tumor sites on days 4, 5, and 6 after s.c. inoculation of MH134. Untreated MH134-bearing mice were used as a control. Data are representative of two independent experiments.

MM2 or MH134 inoculation (data not shown). This is consistent with our previous finding that γδ T cells function as suppressor cells in an early tumor formation (19). No attenuation of tumors was found when Dau-IgG or daunomycin alone was used at the same concentration as Dau-UC7 (Figs. 3 and 4). A weak inhibition of MM2 tumor progression was found in mice treated with Dau-H57 on days 4, 5, and 6 after i.p. MM2 inoculation, while such an inhibition of tumor growth was not observed in MH134-bearing mice (Fig. 3B). Subsequently, the mice died within 25 days following vigorous progression of MM2 (Fig. 3A). These results raise the possibility that a portion of αβ T cell populations modestly

FIGURE 4. Disappearance of MM2 ascites tumor by treatment with Dau-UC7. Dau-UC7 or Dau-hamster IgG was injected at tumor sites on days 4, 5, and 6 after i.p. inoculation of MM2. Photographs show two representative mice in each group on day 16 after MM2 inoculation.
Suppressive cytokines produced by MM2-infiltrating γδ T cells

To investigate the cytokine expression pattern of γδ T cells accumulating in tumor lesions, γδ T cells were freshly isolated from 7-day ascites fluid of i.p. inoculated MM2 using γδTCR-specific mAb (UC7-13D5)-conjugated magnetic beads. Their cytokine profile was examined by PCR of cDNA with primers specific for IL-4, IL-10, IFN-γ, and TGF-β. Fig. 7 shows that freshly isolated MM2-infiltrating γδ T cells transcribed IL-10 and TGF-β mRNAs, whereas neither amplified product of IL-4 nor IFN-γ was detected. In addition, when cultured over a short term in the presence of IL-2, these γδ T cells secreted IFN-γ but not IL-4 (data not shown), suggesting the IFN-γ-producing capacity of the γδ T cells. Since it has recently been reported that CD4+ T lymphocytes producing IL-10, TGF-β, and IFN-γ are a novel population, termed Tr1 cells (5), these results suggested that γδ T cells accumulating in MM2 tumor lesions are of the Tr1 type.

To elucidate the participation of these Tr1 cytokines in γδ T cell suppression of CTL and NK activities, culture supernatants of MM2-infiltrating γδ T cells were mixed with IL-10- and TGF-β-specific neutralizing mAbs and added to the culture of the anti-MM2 CTL proliferation assay. The inhibitory effect of the γδ T cell culture supernatant was reduced by the addition of either anti-

FIGURE 5. Induction of antitumor cytotoxic cells from TILs. MM2 (A), MH134 (B), and B16 (C) TILs prepared from 7-day tumor cell suspensions were treated with Dau-UC7 or Dau-hamster IgG and cultured with IL-2-supplemented medium for 5 days. The expanded cells (effectors) were subjected to CTL assays against 51Cr-labeled MM2, MH137, and B16 target cells, respectively, at an E:T cell ratio of 1. Data are expressed as means of duplicate experiments. Untreated TILs were used as a control.

FIGURE 6. Induction of MM2-specific CTLs in MM2 regressor mice. A, MM2 regressor mice were prepared by injecting Dau-UC7 at tumor sites on days 4, 5, and 6 after MM2 inoculation. Splenocytes taken from MM2 regressor mice 4 days after Dau-UC7 treatment were assayed with MM2, MH134, or YAC-1 target cells at the indicated E:T cell ratios (○). Splenocytes from normal mice (□) and from Dau-H57-treated (■) or daunomycin-treated (△) mice were used as controls. Data are expressed as means of duplicate experiments. B, Anti-CD4, anti-CD8, anti-αβTCR, or anti-γδTCR mAb was added to the cytotoxicity assay of MM2 regressor splenocytes against MM2 target cells at the indicated Ab concentrations. The CTL assay was performed at an E:T cell ratio of 20.

FIGURE 7. Cytokine profile of MM2-infiltrating γδ T cells. Freshly isolated γδ T cells from lesions on day 7 after MM2 i.p. inoculation (left panel) and con A-stimulated splenocytes of MM2-bearing mice (right panel) were subjected to RT-PCR with primers specific for IL-4, IL-10, IFN-γ, and TGF-β. The product size was 762 bp for β-actin, 401 bp for IL-4, 210 bp for IL-10, 307 bp for IFN-γ, and 361 bp for TGF-β.
mAbs were used as controls. Proliferation assay of anti-MM2 CTLs at an 80% volume. Isotype control the indicated concentrations. These blocked supernatants were added to the medium with or without a combination of IL-10- and TGF-b. Dau-UC7-treated MM2-bearing mice (Fig. 8 for inhibitory effect of TGF-b). However, only weak inhibition was obtained with rIL-10, and the number subsequently decreases thereafter (19). Thus, γδ T cells in these two systems function as immunosuppressors against effector T cells by producing Tr1 or Th2 cytokines in innate immune responses (23). It is well known that Th2 cytokines down-regulate Th1 cell functions, resulting in abolishment of effective induction of CTL and NK cells (24). Likewise, Tr1 cells directly attenuate Ag-specific immune responses mediated by Th1 cells (5, 25). In our preliminary study, Th2-type γδ T cells in the B16 system also secrete a great amount of TGF-β (unpublished data), suggesting a similarity between these two types of T cells. Since TGF-β is an autocrine and paracrine inhibitor of CTLs (26, 27), this cytokine seems to be a key factor in the γδ T cell-mediated suppression of antitumor activities. In fact, blocking of TGF-β with mAb abrogated the activity of γδ T cell supernatants, and the function of γδ T cells was replaced by exogenously added TGF-β.

On the other hand, the effect of IL-10 on suppression of CTL generation and NK activities remains controversial. IL-10 suppresses the cytotoxicity of and IFN-γ production by NK cells, and the induction of tumor-specific CTLs (28, 29), whereas IL-10 augments CTL and NK activities synergistically with IL-2 (30–32). In our study neutralization of IL-10 in γδ T cell culture supernatants resulted in elevation of CTL and NK activities, indicating the down-regulatory role of IL-10. However, the exogenous addition of IL-10 did not suppress the proliferative response of CTLs as did TGF-β. This suggests that as yet unelucidated factors that synergize with IL-10 are required for the suppression. Our previous study has demonstrated that γδ T cells release soluble suppressant(s) acting on the cytolytic effector phase of CTLs, and this soluble factor seems to be different from TGF-β and IL-10 (18). This unidentified factor may participate in the synergistic inhibition with IL-10. Taken together, Tr1 cytokines IL-10 and TGF-β are involved in the mechanism underlying the γδ T cell-mediated inhibition of CTL and NK activities.

Many reports, however, have provided in vitro evidence for a cytotoxic effector role for γδ T cells against tumor cells (33–35). The vast majority of these observations were obtained from experiments using culture conditions with high doses of IL-2. Since the amounts of Th2 cytokines, including IL-4 and IL-10, are frequently elevated in tumor-bearing mice (1, 36), γδ T cells may overt their suppressive immunoregulatory capacity under such Th2-predominant conditions. In this situation, cultivation of γδ T cells under artificial IL-2-rich conditions possibly converts their function to cytotoxic cells. Our observation that fresh MM2-infiltrating γδ T cells acquire the ability to secrete IFN-γ after short term culture with IL-2 suggests the conversion of immunosuppressive γδ T cells to cytotoxic cells by Th1 cytokines. Alternatively, it is possible that some γδ T cells originally distributed in certain organs, such as skin, liver, intestine, periphery, and reproductive organs, such as skin, liver, intestine, periphery, and reproductive.

**Discussion**

This study demonstrated that TGF-β and IL-10 produced by Tr1-type γδ T cells inhibit CTL generation and NK activity, and that elimination of this type of γδ T cells from tumor-bearing mice with a daunomycin-conjugated specific mAb augments the activity of CTLs and NK cells and subsequent tumor regression. The in vitro study further confirmed the accumulation of γδ T cells functioning as suppressors against tumoridal lymphocytes in early tumor lesions, as cytotoxic cells were rapidly induced in γδ T cell-depleted TILs of melanoma as well as hepatoma and mammary tumors. Our previous study has shown that Th2-type γδ T cells present at an early stage of B16 melanoma development also exert an inhibitory action on CTLs and NK cells. Those γδ T cells dominantly infiltrate in early tumor lesions, and their number subsequently decreases thereafter (19). Thus, γδ T cells in these two systems function as immunosuppressors against effector T cells by producing Tr1 or Th2 cytokines in innate immune responses (23). It is well known that Th2 cytokines down-regulate Th1 cell functions, resulting in abolishment of effective induction of CTL and NK cells (24). Likewise, Tr1 cells directly attenuate Ag-specific immune responses mediated by Th1 cells (5, 25). In our preliminary study, Th2-type γδ T cells in the B16 system also secrete a great amount of TGF-β (unpublished data), suggesting a similarity between these two types of T cells. Since TGF-β is an autocrine and paracrine inhibitor of CTLs (26, 27), this cytokine seems to be a key factor in the γδ T cell-mediated suppression of antitumor activities. In fact, blocking of TGF-β with mAb abrogated the activity of γδ T cell supernatants, and the function of γδ T cells was replaced by exogenously added TGF-β.

On the other hand, the effect of IL-10 on suppression of CTL generation and NK activities remains controversial. IL-10 suppresses the cytotoxicity of and IFN-γ production by NK cells, and the induction of tumor-specific CTLs (28, 29), whereas IL-10 augments CTL and NK activities synergistically with IL-2 (30–32). In our study neutralization of IL-10 in γδ T cell culture supernatants resulted in elevation of CTL and NK activities, indicating the down-regulatory role of IL-10. However, the exogenous addition of IL-10 did not suppress the proliferative response of CTLs as did TGF-β. This suggests that as yet unelucidated factors that synergize with IL-10 are required for the suppression. Our previous study has demonstrated that γδ T cells release soluble suppressant(s) acting on the cytolytic effector phase of CTLs, and this soluble factor seems to be different from TGF-β and IL-10 (18). This unidentified factor may participate in the synergistic inhibition with IL-10. Taken together, Tr1 cytokines IL-10 and TGF-β are involved in the mechanism underlying the γδ T cell-mediated inhibition of CTL and NK activities.

Many reports, however, have provided in vitro evidence for a cytotoxic effector role for γδ T cells against tumor cells (33–35). The vast majority of these observations were obtained from experiments using culture conditions with high doses of IL-2. Since the amounts of Th2 cytokines, including IL-4 and IL-10, are frequently elevated in tumor-bearing mice (1, 36), γδ T cells may overt their suppressive immunoregulatory capacity under such Th2-predominant conditions. In this situation, cultivation of γδ T cells under artificial IL-2-rich conditions possibly converts their function to cytotoxic cells. Our observation that fresh MM2-infiltrating γδ T cells acquire the ability to secrete IFN-γ after short term culture with IL-2 suggests the conversion of immunosuppressive γδ T cells to cytotoxic cells by Th1 cytokines. Alternatively, it is possible that some γδ T cells originally distributed in certain organs, such as skin, liver, intestine, periphery, and reproductive.
tracts (37) acquire immunosuppressive activity when they accumulate in tumor lesions. Interestingly, a study from another group has shown that extrathymically differentiated γδ T cells may negatively regulate immune reactions, as administration of hepatic γδ T cells leads to unresponsiveness to skin allograft (38). Our preliminary study showed that γδ T cells accumulate markedly in early tumor lesions of athymic nude mice (N. Seo et al., unpublished observation), further providing evidence for the participation of extrathymic γδ T cells in the suppression of tumor immunity. In addition, studies from experimental pregnancy have revealed that the appearance of extrathymic γδ T cells in early decidua of pregnant mice is a crucial event for the maintenance of pregnancy (39, 40). These different lines of studies suggest the role for extrathymic γδ T cells in suppression of cytotoxic cell-mediated immune reactions.

It should be noted that the intrallesional administration of Dau-UC7 at an early stage of tumor development also augmented NK activity. The NK-suppressive role of IL-10 and TGF-β has been demonstrated by several groups (28, 29, 41). However, it has also been reported that IL-10 and TGF-β demonstrate by several groups (28, 29, 41). However, it has also been reported that IL-10 and TGF-β are involved in the suppression of tumor immunity. In addition, studies from experimental pregnancy have revealed that the appearance of extrathymic γδ T cells in early decidua of pregnant mice is a crucial event for the maintenance of pregnancy (39, 40). These different lines of studies suggest the role for extrathymic γδ T cells in suppression of cytotoxic cell-mediated immune reactions.

We have previously demonstrated the modulation of NK activity for extrathymic γδ T cells in suppression of tumor immunity. In addition, studies from experimental pregnancy have revealed that the appearance of extrathymic γδ T cells in early decidua of pregnant mice is a crucial event for the maintenance of pregnancy (39, 40). These different lines of studies suggest the role for extrathymic γδ T cells in suppression of cytotoxic cell-mediated immune reactions.

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**References**


20. Maraskovsky, E., A. B. Troutt, and A. Kelso. 1992. Co-engagement of CD3 with TCR Ab conjugates synthesized via the dextran bridge may be a more powerful tool than conjugates constructed by the classical method. On the other hand, since diphtheria toxin-conjugated (48, 49) and ricin-conjugated (50, 51) tumor cell-specific Abs have been used for targeting of tumors, we suggest that Ts cell-specific Abs with diphtheria toxin or ricin may also be useful as an effective eliminator of T cells compared with daunomycin conjugates.

In conclusion, elimination of T cells that down-regulate CTLs and/or NK lineage cells is one strategy for tumor immunotherapy. Depletion of the down-regulator such as Th2- and Tr1-type γδ T cells, when the treatment is efficacious but not harmful, may be of clinical importance for the development of an alternative way to enhance immunity against tumor cells.


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