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Human CD4+ CD25+ Regulatory T Cells Suppress NKT Cell Functions

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ABSTRACT

$CD4^+CD25^+$ regulatory T cells play an important role in peripheral tolerance. These cells have been reported to be capable of suppressing the response of $CD4^+CD25^-$ T cells *in vitro*. The depletion of these cells evokes effective immune responses to tumor cells *in vivo*. In this study, we demonstrate that $CD4^+CD25^+$ T cells also suppress all subsets of $V\alpha24^+$NKT cells ($V\alpha24^+CD4^-CD8^-$ double negative, $V\alpha24^+CD4^+$, and $V\alpha24^+CD8^+$) in both proliferation and cytokine production [IFN-γ, interleukin-4 (IL-4), IL-13, and IL-10]. This suppression is mediated by cell-to-cell contact but not by a humoral factor or the inhibition of antigen-presenting cells. Moreover, the cytotoxic activity of $V\alpha24^+$NKT cells against some tumor cell lines is suppressed by $CD4^+CD25^+$ T cells. This finding is important in developing an effective immunotherapy for cancer.

INTRODUCTION

Immunotherapy is a promising treatment for cancer. However, vaccination with a tumor antigen or the adoptive transfer of *ex vivo* propagated lymphocytes may lead to the development of autoimmune diseases because there are antigenic cross-reactions between a tumor antigen and normal tissue antigens (1–3). These findings suggest the possibility that the mechanisms maintaining immunological tolerance to self-constituents impede the generation of effective tumor immunity.

$CD4^+CD25^+$ T cells have been shown to represent a unique population of immunoregulatory cells. These cells constitute 5–10% of peripheral $CD4^+$ T cells and resemble anergic cells *in vitro* (4–8). After T-cell receptor-mediated stimulation, $CD4^+CD25^+$ T cells suppress the activation and proliferation of $CD4^+CD25^-$ T cells in an antigen-nonspecific manner (4–8). Their inhibitory capacity is mediated by direct cell-to-cell contact but not by cytokines, such as IL-10[2] and TGF-β (4–8). It has been reported that the depletion of $CD4^+CD25^+$ T cells can disrupt immunological unresponsiveness to autologous tumors *in vitro* and *in vivo,* leading to the spontaneous development of tumor-specific and tumor-nonspecific effector cells (9, 10).

$V\alpha24^+$ NKT cells have been recently defined as a novel lymphocyte lineage, characterized by the expression of an invariant T-cell receptor encoded by $V\alpha24$-JαQ and an NK receptor (11, 12). These cells recognize a glycolipid antigen, such as α-galactosylceramide in a CD1d-dependent fashion (13–16). Recently, three subsets of $V\alpha24^+$ NKT cells have been identified, which had different cytokine production patterns (17). $V\alpha24^+$ NKT cells had cytotoxicity against some tumor cells (13, 18). Studies using Jα281 knockout mice, which lack $V\alpha14^+$ NKT cells, indicate that $V\alpha24^+$ NKT cells contribute to antitumor immunity (13). Moreover, the *in vivo* stimulation of invariant NKT cells with α-GalCer stimulates IL-12 production and NK cell activation and augments antitumor responses (13, 19–21). Thus, $V\alpha24^+$ NKT cells are promising for antitumor immunotherapy.

Although it has been evident for years that $CD4^+CD25^+$ T cells are a unique population of immunoregulatory cells, the potential inhibitory activity of $CD4^+CD25^+$ T cells on non-$CD4^+$ T cells has not been studied in detail. In this study, we demonstrate that $CD4^+CD25^+$ T cells can suppress proliferation, cytokine secretion, and cytotoxic activity in each subset of $V\alpha24^+$ NKT cells. This inhibitory effect is dependent on cell-to-cell contact. This finding is important in developing an effective immunotherapy for cancer.

MATERIALS AND METHODS

Abs and Reagents

The following Abs were purchased from Immunotech (Marseilles, France): IgG1 (679.1 Mc7), IgG2b (MOPC-195), anti-CD3 (UCHT-1), anti-CD4 [SFCI12T4D11(T4)], anti-CD8β (2ST8.5H7), anti-CD161 (191B8), anti-$V\alpha24$ (C15), anti-$V\beta11$ (C21), anti-CD25 (m-A251), anti-CD45RA (HI 100), anti-CD45RO (UCHL1), and anti-CD152 (BNI3.1). Anti-CD1d (55.3.1) Ab (22) was a gift from Dr. S. Porcelli (Albert Einstein College of Medicine, Bronx, NY). α-GalCer was obtained from Kirin (Gunma, Japan). rhGM-colony-stimulating factor and rhIL-4 were purchased from CellGenix (Freiburg, Germany), and rhIL-2 was obtained from Shionogi (Osaka, Japan). Anti-TGF-β (R&D Systems, Abingdon, United Kingdom) and anti-IL-10 (JES3–19F1) Abs (BD PharMingen, San Diego, CA) were used for neutralization experiments.

Cell Populations

$CD4^+CD25^-$ and $CD4^+CD25^+$ T Cells. PBMCs were isolated from peripheral blood samples by density gradient centrifugation using Ficoll-Hypaque (Pharmacia, Upsala, Sweden). PBMCs were incubated in the AIM medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS at 37°C on tissue culture dishes (IWAKI, Tokyo, Japan) for 1 h to remove adherent cells. Nonadherent cells were collected and washed. $CD4^+$ T cells were purified by negative selection with a cocktail of antibodies [anti-CD8 (OKT8), anti-CD14 (ME2), anti-CD56 (N901), anti-CD19 (BU12), and anti-class II (L243)] followed by magnetic bead sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany). The isolated $CD4^+$ T cells were incubated with FITC-conjugated anti-CD25 Ab and then with anti-FITC magnetic beads. $CD4^+CD25^+$ T cells were obtained by positive selection and $CD4^+CD25^-$ T cells by negative selection.

Establishment of $V\alpha24^+$NKT Cells. Monocytes from healthy human donors were cultured in the AIM medium supplemented with 10% FCS, 500 units/ml rhIL-4, and 500 units/ml rhGM-colony-stimulating factor for 5 days and used as Mo-DCs. Lymphocytes from the same donor were cultured with irradiated (50 Gy) Mo-DCs, which were pulsed for 12 h with α-GalCer (100 ng/ml). After 7 days, $V\alpha24^+$ cells were established by positive ($V\alpha24$) magnetic bead sorting and maintained in the presence of 40 units/ml rhIL-2. The cells were restimulated every 7 days. Allogeneic Mo-DCs pulsed with α-GalCer were used in restimulation. $V\alpha24^+$NKT cells recognized monomorphic CD1d expressed in allogeneic Mo-DCs; thus, they could be stimulated by this method. When the cells were expanded, $V\alpha24^+V\beta11^+CD4^-CD8^-$, $V\alpha24^+V\beta11^+CD4^+$, and $V\alpha24^+V\beta11^+CD8^+$ subsets were sorted using the FACSVantage apparatus (Becton Dickinson, San Jose, CA) and maintained in the presence of 40 units/ml rhIL-2 and by restimulation every 7–10 days.

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[2] The abbreviations used are: IL, interleukin; TGF, transforming growth factor; DN, double-negative; rh, recombinant human; Mo-DC, monocyte-derived dendritic cell; NK, natural killer; Ab, antibody; CTLA-4, CTL-associated antigen-4; ICAM, intercellular adhesion molecule; FACS, fluorescence-activated cell sorter; dThd, thymidine; PBMC, peripheral blood mononuclear cell.

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Flowcytometry and Sorting

Cells were analyzed by flowcytometry (FACScalibur; Becton Dickinson). Immunofluorescence staining using specific monoclonal antibody was performed according to a standard procedure. $V\alpha24^+V\beta11^+CD4^-CD8^-$, $V\alpha24^+V\beta11^+CD4^+$, and $V\alpha24^+V\beta11^+CD8^+$ subsets were sorted using the FACSVantage apparatus (Becton Dickinson).

Proliferation of NKT Cells in Response to α-GalCer-pulsed Mo-DCs

The bulk of 1×10^5 NKT or $CD4^+CD25^-$ T cells as responders and 5×10^4 irradiated allogeneic Mo-DCs (all allogeneic monocytes were derived from the same donor) as stimulators in 0.2 ml of the culture medium was seeded onto roundbottomed microtiter wells with or without 100 ng/ml α-GalCer. For the assessment of their suppression properties, $CD4^+CD25^+$ T cells were added at different concentrations. The culture was incubated for 72 h. At the final 12 h of incubation, 1 μCi of [^{3}H]dThd was added to each well, and the incorporation of [^{3}H]dThd was determined by liquid scintillation counting.

Cytokine Production Assay

NKT cells (1×10^5) and 5×10^4 irradiated−α-GalCer-pulsed Mo-DCs (all allogeneic monocytes were derived from the same donor) were suspended in 200 μl of the culture medium in 96-well plates. After 18 h, the supernatant was collected from each well and subjected to ELISA of the concentrations of IFN-γ, IL-4, IL-10, and IL-13 by ELISA according to the manufacturer's protocol (AN′ALYZA; Genzyme, Cambridge, MA).

Transwell Experiments

Transwell experiments were performed in 24-well plates. NKT cells (1×10^5) were stimulated with 5×10^4 irradiated allogeneic Mo-DCs pulsed with α-GalCer. In addition, 1×10^5 $CD4^+CD25^-$ T cells were either added directly to the culture or placed in transwell chambers (Millicell, 0.4μ; Millipore). After 72 h of coculture, NKT cells were transferred to 96-well plates in triplicates. Proliferation was measured by liquid scintillation counting after pulsing with [^{3}H]dThd for 12 h.

Cytotoxic Assay

^{51}Cr-labeled ($Na_2{}^{51}CrO_3$; Amersham, Arlington Heights, IL) MOLT-4 or Jurkat cells (5×10^3) as the target and effector cells of various numbers in 200 μl of the culture medium were seeded onto roundbottomed microtiter wells. The culture was incubated for 4 h, and 100 μl of the supernatant were collected from each well. The percentage of specific ^{51}Cr release was calculated using [(cpm experimental release − cpm spontaneous release)/(cpm maximal release − cpm spontaneous release)] × 100.

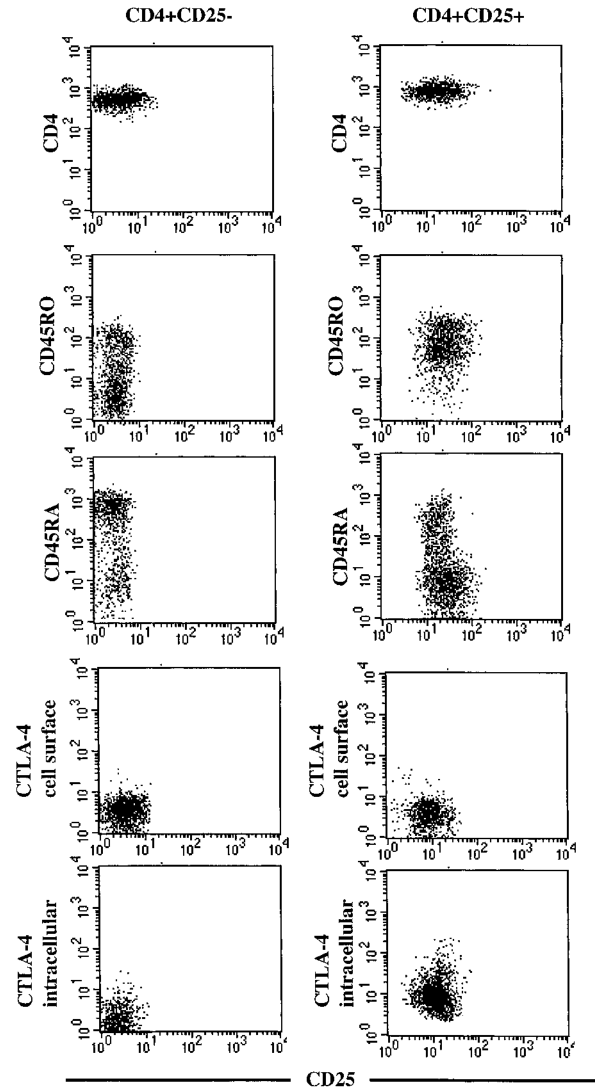

Fig. 1. Isolation and cell surface phenotype of $CD4^+CD25^+$ T cells. $CD4^+$ T cells were isolated from PBMCs and separated into $CD25^+$ and $CD25^-$ fractions. The phenotypes of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were analyzed by two-color flow cytometry using the indicated monoclonal antibodies. In addition, intracellular CTLA-4 expression was analyzed. Representative results of three independent experiments are shown.

RESULTS

Phenotypic Characterization of $CD4^+CD25^+$ Regulatory T and $V\alpha24^+$NKT Cells. $CD4^+CD25^+$ T cells represented 5–10% of $CD4^+$ T cells. Both $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were isolated with purities of >90%. The expressions of surface antigens on these populations were compared by FACS analysis (Fig. 1). $CD4^+CD25^+$ T cells expressed CD45RO but not CTLA-4. No surface expression of CTLA-4 was observed in both populations. However, $CD4^+CD25^+$ T cells expressed CTLA-4 intracellularly. Furthermore, CTLA-4 expression was detectable after activation by allogeneic DCs (data not shown). Two subsets ($CD45RA^-$ and $CD45RA^+$) of $CD4^+CD25^+$ T cells were identified. $CD45RA^+$ had a lower CD25 expression level than $CD45RA^-$. We considered that the $CD45RA^+$ comprised $CD4^+CD25^{+low}$ T cells, which were isolated by Baecher-Allan *et al.* (7).

We generated $V\alpha24^+$NKT cells by the stimulation of α-GalCer-pulsed Mo-DCs, and three cell populations were identified: (*a*) $V\alpha24^+V\beta11^+$ $CD4^-CD8^-$ NKT ($V\alpha24^+$DN NKT); (*b*) $V\alpha24^+V\beta11^+CD4^+$ NKT ($V\alpha24^+CD4^+$ NKT); and (*c*) $V\alpha24^+V\beta11^+CD8^+$ NKT ($V\alpha24^+CD8^+$ NKT; data not shown; Ref. 17). Each subset expressed the NK receptor, CD161 (NKR-P1A; data not shown; Ref. 17).

$CD4^+CD25^+$ Regulatory T Cells Suppress the Proliferation and Cytokine Secretion of $V\alpha24^+$NKT Cells in Response to α-GalCer-pulsed Mo-DCs. We confirmed that $CD4^+CD25^+$ T cells had regulatory properties (4–6). These cells suppressed the proliferative response of alloreactive $CD4^+CD25^-$ T cells (data not shown). Then, to investigate the ability of $CD4^+CD25^+$ T cells to suppress the proliferation of $V\alpha24^+$NKT cells, each subset of $V\alpha24^+$NKT cells was stimulated with allogeneic Mo-DCs using α-GalCer, and variable numbers of autologous $CD4^+CD25^+$ T cells were added. $CD4^+CD25^+$ T cells inhibited the proliferation of all subsets of $V\alpha24^+$NKT cells in a dose-dependent manner. $CD4^+CD25^+$ T cells themselves failed to proliferate in response to alloantigens (Fig. 2*A*).

Next, the effect of $CD4^+CD25^+$ T cells on cytokine secretion of $V\alpha24^+$NKT cells under the coculture conditions was examined. We analyzed the culture supernatants for IFN-γ, IL-4, IL-10, and IL-13 concentrations (Fig. 2*B*). The cytokine production pattern of each

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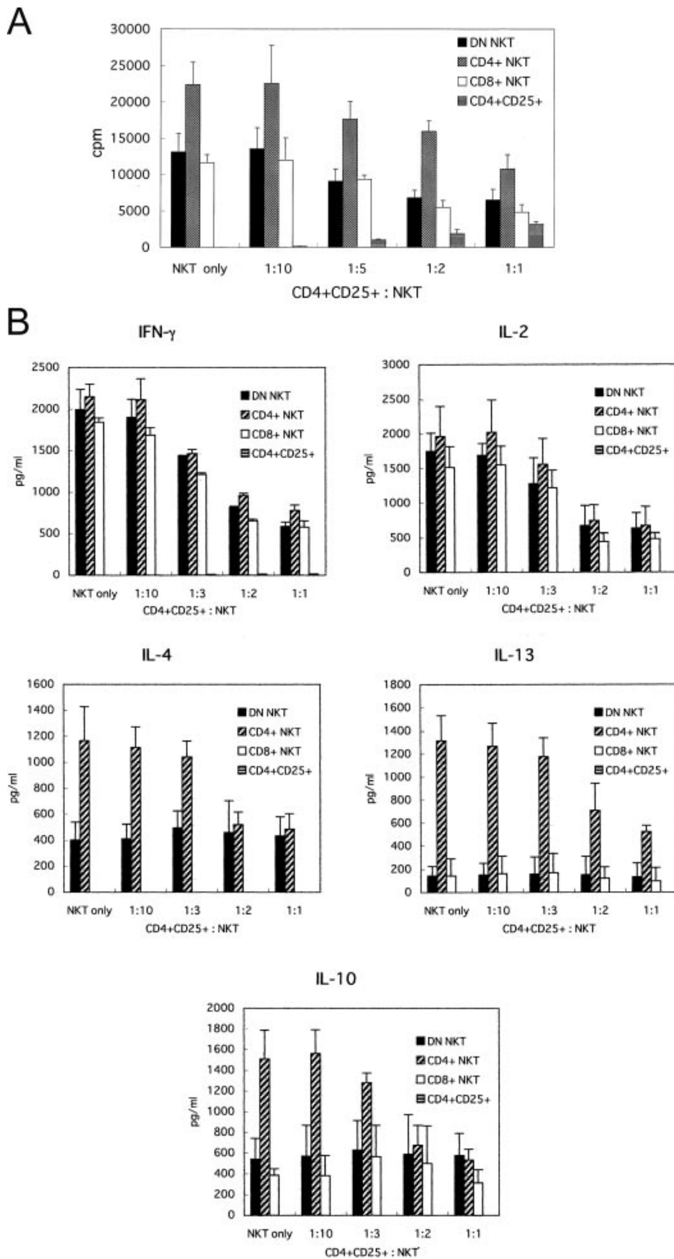

Fig. 2. In *A,* CD4$^+$CD25$^+$ T cells suppress the proliferation of Vα24$^+$ DN, Vα24$^+$CD4$^+$, and Vα24$^+$CD8$^+$ NKT cells in a dose-dependent manner. The proliferative response of each NKT population (1×10^5 cells/well) was stimulated with irradiated allogeneic Mo-DCs (5×10^4 cells/well) in the presence of CD4$^+$CD25$^+$ T cells of various numbers from the same donor. Proliferation was determined after 72 h of culture by the addition of [^{3}H]dThd at 12 h. All data represent the means $\pm$ SD of triplicate assays. Representative results of three independent experiments. In *B,* CD4$^+$CD25$^+$ T cells do not secrete cytokines when stimulated with allogeneic Mo-DCs but suppress the secretion of IFN-γ, IL-4, IL-10, and IL-13 by NKT cells in a dose-dependent manner. The supernatants were collected from the proliferation cultures incubated for 18 h. Concentrations of IFN-γ, IL-4, IL-10, and IL-13 were determined by ELISA. All data represent the means $\pm$ SD of triplicate assays. Representative results of three independent experiments are shown.

subset differs. All subpopulations produced a large amount of IFN-γ. Both Vα24$^+$DN and Vα24$^+$CD4$^+$ NKT cells produced a large amount of IL-4. In contrast, Vα24$^+$CD8$^+$ NKT cells produced a small amount of IL-4. Vα24$^+$CD4$^+$ NKT cells produced large amounts of IL-10 and IL-13, whereas Vα24$^+$DN and Vα24$^+$CD8$^+$ NKT cells produced small amounts of these cytokines. In contrast, CD4$^+$CD25$^+$ T cells produced only a marginal amount of all cytokines when stimulated with allogeneic Mo-DCs. CD4$^+$CD25$^+$ T cells suppressed the secretion of IFN-γ in all subsets as well as that of IL-4, IL-10, and IL-13 from Vα24$^+$ CD4$^+$ NKT cells in a dose-dependent manner.

We investigated the cytotoxicity of CD4$^+$CD25$^+$ T cells to analyze mechanisms underlying the above suppression. The cytotoxicity of CD4$^+$CD25$^+$ T cells against Vα24$^+$NKT cells was not observed (data not shown).

The Inhibitory Effect of CD4$^+$CD25$^+$ Regulatory T Cells on Vα24$^+$NKT Cells Is Direct and Contact Dependent but Cytokine Independent. We investigated whether cell-to-cell contact or soluble factors mediate the inhibitory effect of CD4$^+$CD25$^+$ T cells. As shown in Fig. 3, the addition of neutralizing antibodies against IL-10 or TGF-β could not restore the proliferation of all Vα24$^+$NKT subsets. ICAM-1 is an adhesion molecule that plays important roles in cell-to-cell contact. The blockade of ICAM-1 using anti-ICAM-1 Abs could reverse the inhibitory effect. These observations suggest that direct cell contact rather than humoral factors is responsible for the inhibitory effect of CD4$^+$CD25$^+$ T cells.

To confirm these observations, transwell chamber experiments were performed. CD4$^+$CD25$^+$ T cells suppressed the proliferation of Vα24$^+$NKT cells under coculture conditions. In contrast, the separation of the two populations in transwell chambers virtually abolished their inhibitory effect. These observations suggest that the initial direct cell contact is essential for the inhibitory effect of CD4$^+$CD25$^+$ T cells, as the semipermeable membrane of transwell chambers allows only free passage of soluble factors but not direct cell contact.

CD4$^+$CD25$^+$ Regulatory T Cells Inhibit the Cytotoxic Activity of Vα24$^+$NKT Cells against Tumor Cell Lines. Vα24$^+$NKT cells have cytotoxic activity against some tumor cell lines. We investigated whether CD4$^+$CD25$^+$ T cells regulate the cytotoxic activity of Vα24$^+$NKT cells. The cytotoxicity of Vα24$^+$NKT cells against MOLT-4 and Jurkat cells was examined after coculture *in vitro* with or without CD4$^+$CD25$^+$ T cells for 8 h. These T-cell leukemia cell lines were chosen as the target cells because these cell lines were the most sensitive to human Vα24$^+$NKT cells among the cell lines tested (data not shown). CD4$^+$CD25$^+$ T cells inhibited the cytotoxic activity of all subsets of Vα24$^+$NKT cells against these tumor cell lines. At a

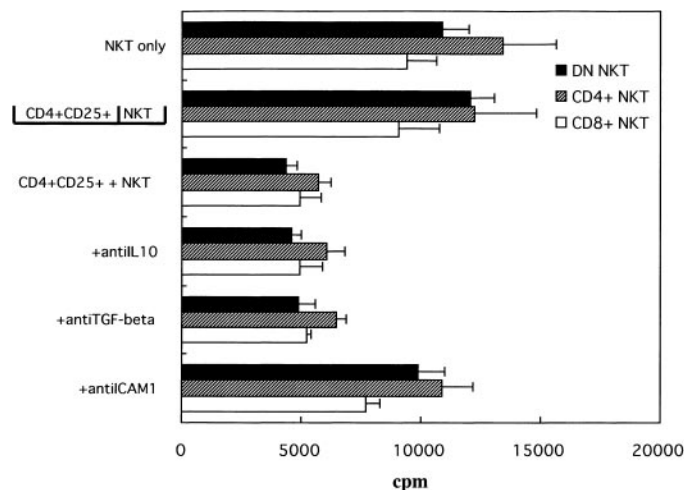

Fig. 3. CD4$^+$CD25$^+$ T-cell inhibitory function is direct cell contact dependent but cytokine independent. NKT cells (1×10^5 cells/well) were stimulated with irradiated allogeneic Mo-DCs in the bottom chamber of the transwell plate; CD4$^+$CD25$^+$ T cells (1×10^5 cells/well) were then added and activated with irradiated allogeneic Mo-DCs separately in the top chamber. Alternatively, CD4$^+$CD25$^+$ T cells (1×10^5 cells/well) were cocultured with NKT cells at a ratio of 1:1 (1×10^5 cells/well) and stimulated with allogeneic Mo-DCs (5×10^4 cells/well) with or without 10 μg/ml anti-IL-10, 10 μg/ml anti-TGF-β, and 10 μg/ml anti-ICAM-1 Abs. All data represent the means $\pm$ SD of triplicate assays. Representative results of three independent experiments are shown.

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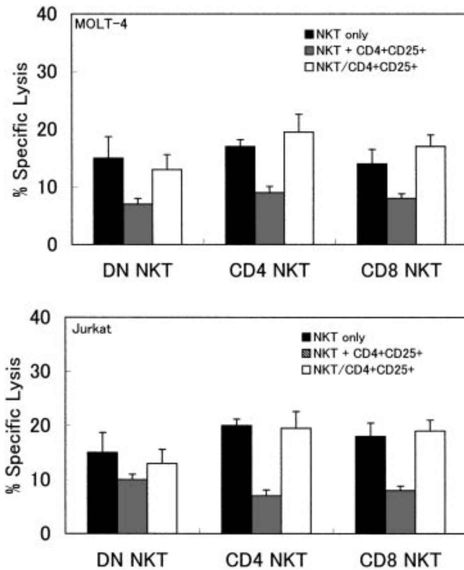

Fig. 4. $CD4^+CD25^+$ T cells inhibit the cytotoxicity of NKT cells against MOLT-4 and Jurkat cells. ^{51}Cr-labeled MOLT-4 (5×10^3) or Jurkat cells and effector cells were incubated with or without $CD4^+CD25^+$ T cells for 4 h. The percentage of specific ^{51}Cr release was calculated. All data represent the means ± SD of triplicate assays. Representative results of three independent experiments are shown.

ratio of 1:1, cytotoxicity was inhibited by 50%, and the separation of the two populations in transwell chambers abolished this inhibitory effect (Fig. 4).

DISCUSSION

It has been suggested that one of the elements impeding tumor immunity in tumor-bearing hosts is the concomitant development of a T-cell population suppressing the generation and function of effector cells (23–25). It was demonstrated that these suppressor cells could be $CD4^+CD25^+$ T cells, in part, because the depletion of $CD4^+CD25^+$ T cells before tumor implantation is effective in evoking specific tumor immunity and generating cytotoxic cells (9, 10). In a mouse model without $CD4^+CD25^+$ T cells, non-$CD4^+$ cells were suggested to play a role in tumor rejection, and the generation of two more types of effector cell ($CD8^+$ T and $CD4^-CD8^-$ NK-like cells) was observed (9, 10). The potential inhibitory activity of $CD4^+CD25^+$ T cells against $CD4^+$ T cells has been studied in detail (4–6, 8, 26, 27). However, the regulatory action of $CD4^+CD25^+$ T cells on non-$CD4^+$ responder cells in humans has not been evaluated. This is the first report that $CD4^+CD25^+$ T cells can suppress the proliferation and function of $V\alpha24^+$NKT cells in humans.

Many reports have clarified the phenotypic and functional characteristics of human $CD4^+CD25^+$ T cells (4–6, 8). Our isolated cells had a distinctive phenotype, expressing a mixture of markers of memory and naïve T cells, and inhibited the proliferation of $CD4^+CD25^-$ T cells (data not shown). Our $CD4^+CD25^+$ T cells were suggested to be of the same population as in previous reports (4–6, 8).

We showed that $CD4^+CD25^+$ T cells could suppress proliferation, cytokine secretion, and the cytotoxic activity of $V\alpha24^+$NKT cells. Fifty percent suppression was observed in the proliferation assay at a cell ratio of 1:1. Previous studies on the inhibitory activity of human $CD4^+CD25^+$ T cells against $CD4^+CD25^-$ T cells showed 80–50% inhibition at a cell ratio of 1:1 (4–8). It is important to note that this inhibition is in a dose-dependent manner.

$V\alpha24^+$NKT cells can produce large amounts of IL-4 and IFN-γ (18), which can potentially influence immune regulation, such as the determination of the Th1/Th2 profile. In several mouse models, it has been indicated that $V\alpha24^+$NKT cells augment protective cellular Th1-like immune responses (28–32). Moreover, previous studies showed the decreased numbers of $V\alpha24^+$NKT cells in patients with melanoma and prostate cancer (33, 34). These data suggest that the loss of the $V\alpha24^+$NKT cell function is a general finding in advanced cancers. Our data may support the idea that $CD4^+CD25^+$ T cells are associated with the prevention of the increase in the number of $V\alpha24^+$NKT cells and can suppress the secretion of both Th1 and Th2 cytokines. Recently, it has been reported that there are three subsets of $V\alpha24^+$NKT cells ($V\alpha24^+$DN, $V\alpha24^+CD4^+$, and $V\alpha24^+CD8^+$ NKT cells), which show different cytokine production patterns (17). $V\alpha24^+CD4^+$ NKT cells can produce a larger amount of Th2 cytokines (17). However, there was no difference in the degree of suppression among the three subsets.

$V\alpha24^+$NKT cells have also been considered to be promising for cancer immunotherapy for several reasons (13, 18, 33, 35). These cells exhibit a high cytotoxic activity against some tumor cells, and normal tissue cells are not susceptible to them. α-GalCer can be applied to all patients, irrespective of MHC haplotype, because the activation of $V\alpha24^+$NKT cells is restricted to CD1d molecules, which are monomorphic among individuals. The initial number of $V\alpha24^+$NKT cells in cancer patients is significantly lower than that in healthy volunteers. Recently, it has been reported that α-GalCer can effectively induce antitumor cytotoxic activity through the specific activation of NKT cells in mouse models (19, 33, 35). It is unclear whether $CD4^+CD25^+$ T cells directly suppress the cytotoxicity of effector cells. We showed that $CD4^+CD25^+$ T cells suppress the cytotoxicity of $V\alpha24^+$NKT cells against tumor cells. It has also not been reported that $CD4^+CD25^+$ T cells suppress the cytotoxicity of $CD4^+CD25^-$ T cells. This is the first report on the suppression of cytotoxicity of $CD4^+CD25^+$ T cells. Our results suggest that the combination of the depletion of $CD4^+CD25^+$ T cells and immunotherapy using the NKT cell system might result in the maximal efficacy.

The mechanism underlying $CD4^+CD25^+$ T-cell suppression remains to be fully elucidated. Our results demonstrate that the addition of anti-IL-10 or anti-TGF-β Ab has no effect on the activity of $CD4^+CD25^+$ T cells, whereas the addition of anti-ICAM-1 Ab or transwell analysis abolishes the inhibitory effect of $CD4^+CD25^+$ T cells. Thus, it is not dependent on inhibitory cytokines, IL-10 or TGF-β, but requires cell-to-cell contact initially. There remains a possibility that the suppression mechanism might be associated with a soluble factor after initial cell-to-cell contact. Next, we evaluated the possibility that the inhibitory function of $CD4^+CD25^+$ T cells is mediated by adenomatous polyposis colis. Recently, it has been reported that mouse $CD4^+CD25^+$ T cells down-regulate the expressions of CD80 and CD86 in Mo-DCs (36). However, our results showed that they have no effect on the development of Mo-DCs. We also confirmed that there is no significant down-regulation of CD1d molecule expression, which is necessary for $V\alpha24^+$NKT cell activation (13–16). Moreover, we confirmed that $CD4^+CD25^+$ T cells do not inhibit Mo-DC function by the mixed leukocyte reaction. These results suggest that $CD4^+CD25^+$ T cells may directly regulate the function of $V\alpha24^+$NKT cells in a cell-to-cell contact manner.

In conclusion, we demonstrated that $CD4^+CD25^+$ T cells suppress the proliferation and function of $V\alpha24^+$NKT cells. Most of the effects of $CD4^+CD25^+$ T cells on $V\alpha24^+$NKT cells were similar to those observed in $CD4^+$ T cells (4–6, 8, 26, 27). Two important findings should be noted: (*a*) in addition to cell proliferation, secretion of various cytokines by all subsets of $V\alpha24^+$NKT cells is inhibited by $CD4^+CD25^+$ T cells; and (*b*) the cytotoxicity of $V\alpha24^+$NKT cells against tumor cell lines is suppressed by $CD4^+CD25^+$ T cells. These

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data are important in developing an effective immunotherapy for human cancers.

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