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Regulatory CD8$^+$ T cells induced by exposure to all-trans retinoic acid and TGF-β suppress autoimmune diabetes

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ABSTRACT

Antigen-specific regulatory CD4$^+$ T cells have been described but there are few reports on regulatory CD8$^+$ T cells. We generated islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)-specific regulatory CD8$^+$ T cells from 8.3-NOD transgenic mice. CD8$^+$ T cells from 8.3-NOD splenocytes were cultured with IGRP, splenic dendritic cells (SpDCs), TGF-β, and all-trans retinoic acid (ATRA) for 5 days. CD8$^+$ T cells cultured with either IGRP alone or IGRP and SpDCs in the absence of TGF-β and ATRA had low Foxp3$^+$ expression (1.7 ± 0.9% and 3.2 ± 4.5%, respectively). In contrast, CD8$^+$ T cells induced by exposure to IGRP, SpDCs, TGF-β, and ATRA showed the highest expression of Foxp3$^+$ in IGRP-reactive CD8$^+$ T cells (36.1 ± 10.6%), which was approximately 40-fold increase compared with that before induction culture. CD25 expression on CD8$^+$ T cells cultured with IGRP, SpDCs, TGF-β, and ATRA was only 7.42%, whereas CD103 expression was greater than 90%. These CD8$^+$ T cells suppressed the proliferation of diabetogenic CD8$^+$ T cells from 8.3-NOD splenocytes *in vitro* and completely prevented diabetes onset in NOD-scid mice in cotransfer experiments with diabetogenic splenocytes from NOD mice *in vivo*. Here we show that exposure to ATRA and TGF-β induces CD8$^+$Foxp3$^+$ T cells *ex vivo*, which suppress diabetogenic T cells *in vitro* and *in vivo*.

1. Introduction

The adaptive immune response is initiated by T cells that express receptors for diverse antigens. The repertoire of T cell receptors is regulated by positive selection and negative selection. Autoreactive T cells are usually deleted by negative selection in the thymus [1]. However this process is not exhaustive: T cells expressing receptors with low affinities for self-antigens are usually present in peripheral organs and may cause autoimmune disease if activated. Suppressor mechanisms for self-reactive T cells in peripheral organs have been presumed. The concept of T cell suppression of the immune response was first proposed by Gershon and Kondo for regulatory CD8$^+$ T cells [2]. There are few studies on regulatory CD8$^+$ T cells because of the difficulty of identifying regulatory CD8$^+$ T cell populations and their mechanisms of action. The CD4$^+$ population of regulatory T cells (Tregs) was first described by Sakaguchi et al. as CD4$^+$CD25$^+$ T cells [3]. Although CD25 expression has been used as a marker to identify Tregs, it is not specific for Tregs because it is expressed by all activated T cells. Recent studies revealed that the forkhead/winged helix family (Foxp3) transcription factor is a master switch specific to CD4$^+$CD25$^+$ Tregs that induces differentiation of naive T cells into the Treg lineage and maintains their suppressive function [4,5].

The CD4$^+$CD25$^+$ T cells described by Sakaguchi et al. originated in the thymus and developed their suppressive function in peripheral organs. On the other hand, Luo et al. revealed that transforming growth factor-β (TGF-β) and β-cell peptide-pulsed dendritic cells from NOD mice induced CD4$^+$CD25$^+$Foxp3$^+$ T cells from naïve T cells in BDC2.5-NOD splenocytes, which suppressed diabetes onset in cotransfer experiments [6]. In contrast to the regulatory cells in the thymus, BDC2.5 CD4$^+$CD25$^-$ T cells can be switched to CD4$^+$CD25$^+$Foxp3$^+$ T cells by regulatory cytokines such as TGF-β.

Vitamin A (retinol) and its metabolites (retinoids) are a group of potent natural or synthetic molecules which act as modulators for a variety of inflammatory and immunological events in immune system. The mechanism of this molecule has been reported as suppression of inflammatory immune cells, modulation of the function of immune cells and production of several cytokines [7]. All-trans retinoic acid (ATRA), a potent retinoids, has been clinically used to treat acute leukaemia and acne vulgaris [8,9]. Now it has also been reported as having a potential of generating CD4$^+$ Tregs [10,11]. In addition, dendritic cells purified from the small intestine were found to undergo a high level of CD4$^+$ Treg conversion when exposed to TGF-β and ATRA that was highly expressed in GALT [12].

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Recent publications have shown that the regulatory T cell family consists not only of $CD4^+$ T cells but also $CD8^+$ T cells [13–17], presuming that regulatory $CD8^+$ T cells can be induced from these naïve $CD8^+$ T cells in 8.3-NOD mice in an adequate milieu. In this study, we generated regulatory $CD8^+$ T cells from transgenic 8.3-NOD mice expressing CD8-TCR specific for islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP) in β-cells [18]. $CD8^+$ T cells exposed to ATRA and TGF-β exhibited regulatory functions *in vitro* and *in vivo*.

2. Materials and methods

2.1. Mice

NOD/Shi/Kbe mice were maintained at the Institute for Experimental Animals, Kobe University School of Medicine, Kobe, Japan. The cumulative incidence of diabetes is 85% in females and 30% in males at 40 weeks of age. NOD-scid mice were purchased from Clea Japan (Osaka, Japan). 8.3-NOD mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were treated according to the Guidelines for Animal Experimentation of Kobe University School of Medicine.

2.2. Antibodies and reagents

FITC-conjugated anti-mouse monoclonal antibodies, CD8 (53–6.7) and CD103 (M290), were purchased from Pharmingen (San Diego, CA), as were the phycoerythrin (PE)-conjugated anti-mouse monoclonal antibodies, CD4 (L3T4), CD8 (53–6.7), and B220 (RA3–6B2), peridinin chlorophyll protein complex (PerCP)-conjugated anti-mouse CD4 monoclonal antibody (L3T4), and the APC-conjugated anti-mouse CD25 monoclonal antibody (3C7). The PE-conjugated anti-mouse Foxp3 monoclonal antibody (FJK-16s) was purchased from eBioscience (San Diego, CA). Anti-CD11c (N418) microbeads and the CD8 isolation kit were purchased from Miltenyi Biotec (Bergisch–Gladbach, Germany). Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Dojindo (Kumamoto, Japan).

2.3. Cell purification and culture

Splenic dendritic cells (SpDCs) were purified from 8- to 12-week-old 8.3-NOD mice splenocytes using CD11c-magnetic beads. After selection using an autoMACS magnetic cell sorter (Miltenyi Biotec), $CD8^+$ T cells were purified by depletion of CD4-, B220-, CD49b-, CD11b-, and Ter-119-positive cells. To study the proliferation of $CD8^+$ T cells, some cells were stained with 10 μM CFSE for 15 min at 37 °C followed by two washes with HBSS at 4 °C. All cells were cultured in an atmosphere containing 5% CO_2 at 37 °C in 96-well tissue culture plates in 200 μl of RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM sodium pyruvate, 10 mM Hepes buffer, 50 IU/ml penicillin, 50 μg/ml streptomycin, 40 μg/ml gentamycin and 5×10^{-5} M 2-mercaptoethanol. $CD8^+$ T cells (6.0×10^4) were cultured with 0.1 μM of IGRP alone or with IGRP and 2.0×10^4 of SpDCs as controls. In addition to IGRP and SpDCs, $CD8^+$ T cells in other groups were cultured with 2 ng/ml of TGF-β or with TGF-β and 10 nM of ATRA to induce the regulatory cells. Five-days later, cells were harvested and stained with several antibodies and propidium iodide (PI); only PI-negative cells were analyzed using the FACS 440 flow cytometer (Becton Dickinson, San Jose, CA). For each group, the $CD8^+$ T cells were cultured as described previously and harvested; only $CD8^+$ T cells were selected using the magnetic beads. $CD8^+$ T cells cultured with IGRP alone were designated I cells, those cultured with IGRP and SpDCs were designated ID cells, those cultured with IGRP, SpDCs, and TGF-β were designated IDT cells, and those cultured with IGRP, SpDCs, TGF-β, and ATRA were designated IDTA cells.

2.4. Proliferation assays

$CD8^+$ T cells from 8.3-NOD splenocytes that were selected using magnetic beads were stained with 10 μM CFSE for 15 min at 37 °C and were washed twice. Induced $CD8^+$ T cells (1.0×10^5) (I cells, ID cells, IDT cells, or IDTA cells) and CFSE-stained fresh $CD8^+$ T cells (1.0×10^5) were cultured together with 0.1 μM of IGRP. After 3 days, all cells were harvested and only $CFSE^+$ cells were analyzed using flow cytometry. To further examine the suppressor activity of induced $CD8^+$ T cells *in vitro*, freshly-isolated effector $CD8^+$ T cells (1.0×10^5) (E) were cultured with induced $CD8^+$ T cells (IDT cells or IDTA cells) (R) at the indicated ratios in the presence of 0.1 μM of IGRP.

2.5. Adoptive transfer

$CD8^+$ T cells (6.0×10^6) from 8.3-NOD splenocytes cultured with IGRP alone or IGRP, SpDCs, TGF-β, and ATRA were intravenously cotransferred into 8-week-old NOD-scid mice with 1×10^7 diabetogenic NOD splenocytes. The recipients were monitored for diabetes onset by testing urine glucose level twice weekly. Diabetes was defined as a blood glucose concentration greater than 250 mg/dl (13.9 mmol/l) on two consecutive days.

2.6. Statistical analysis

Statistical analysis of the incidence of diabetes was performed using the log-rank test. Statistical analyses of flow cytometric data were performed using the Mann–Whitney *U* test. A p value less than 0.05 was considered significant. All data are presented as the means ± SD.

3. Results

3.1. Induction of $CD8^+Foxp3^+$ T cells from splenocytes in 8.3-NOD mice

The prevalence of $CD8^+Foxp3^+$ T cells among splenocytes in 7-week-old 8.3-NOD mice was first analyzed using flow cytometry to evaluate "natural" $CD8^+Foxp3^+$ T cells. The population of $CD8^+$ T cells in the spleen from 8.3-NOD mice exceeded 50% of splenocytes, which was higher than that of littermate NOD mice (Fig. 1A). The population of $CD8^+Foxp3^+$ T cells in the spleen from 8.3-NOD mice was no more than 1.0% of splenocytes, which was equal to that in littermate NOD mice (Fig. 1B).

SpDCs and $CD8^+$ T cells were cultured for inducing regulatory $CD8^+$ T cells *ex vivo*. SpDCs were selected from the splenocytes of 8.3-NOD mice using CD11c-magnetic beads. $CD8^+$ T cells were negatively selected using magnetic beads followed by labeling with CFSE. CFSE-labeled $CD8^+$ T cells (6.0×10^4) were cultured for 5 days with 0.1 μM of IGRP alone (I cells), IGRP and 2.0×10^4 SpDCs (ID cells), IGRP, SpDCs, and 2 ng/ml of TGF-β (IDT cells) or with IGRP, SpDCs, TGF-β, and 10 nM of ATRA (IDTA cells). Fig. 1C and D show the *in vitro* proliferation of $Foxp3^+$ cells in cultured $CD8^+$ T cells from 8.3-NOD splenocytes. Only 1.7 ± 0.9% of I cells and 3.2 ± 4.5% of ID cells expressed Foxp3, whereas 8.6 ± 6.7% of IDT cells and 21.4 ± 4.2% of IDTA cells expressed Foxp3. IDTA cells showed the highest expression of Foxp3 and significantly higher expression than I cells ($^*p = 0.028$). To further examine whether the efficiency of conversion to Tregs can be increased, we also cultured $CD8^+$ T cells from 8.3-NOD mice with titrating concentrations of ATRA (0, 1, 5, 10, and 20 nM) in the presence of constant IGRP, splenic DC, and TGF-β concentrations. The percentage of $Foxp3^+$

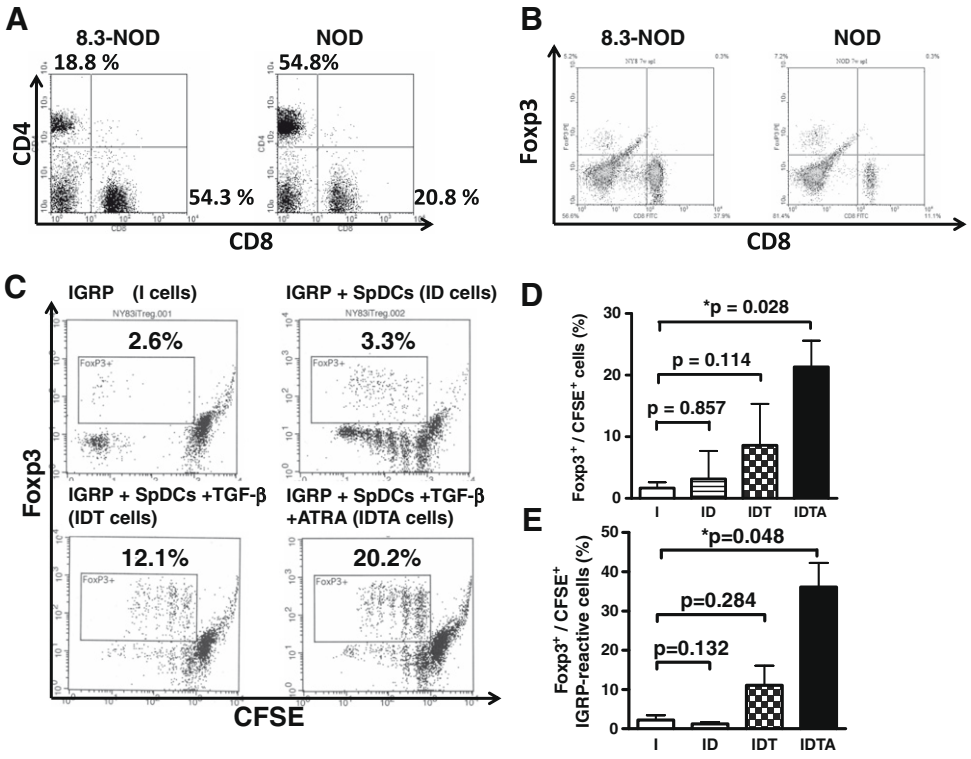

Fig. 1. (A, B) Splenocytes from 7-week-old 8.3-NOD or NOD mice were stained with anti-CD4 and CD8 antibodies (A) or anti-FoxP3 and CD8 antibodies (B). A representative example of four separate experiments is shown. (C, D, E) CFSE-labeled $CD8^+$ T cells (6.0×10^4) selected from the splenocytes of 7-week-old 8.3-NOD mice were cultured for 5 days with 0.1 μM of IGRP alone (I cells), IGRP and 2.0×10^4 SpDCs (ID cells), IGRP, SpDCs, and 2 ng/ml of TGF-β (IDT cells), or IGRP, SpDCs, TGF-β, and 10 nM of ATRA (IDTA cells). Five days later, all cells were harvested and gated on PI-negative and CFSE positive cells. A representative example (C), percentage of $Foxp3^+$ cells in $CFSE^+$ cells ($^*p = 0.028$, I vs. IDTA) (D) and percentage of $Foxp3^+$ cells in IGRP-reactive $CFSE^+$ cells ($^*p = 0.048$, I vs. IDTA) (E) are shown.

cells in 10 nM of ATRA was the highest among these titrating concentrations (data not shown). Among IGRP-responded $CD8^+$ T cells, 36.1 ± 10.6% of IDTA cells showed Foxp3 expression, which was approximately 40-fold increase compared with that before induction culture and significantly higher than that of I cells ($^*p = 0.048$) (Fig. 1E).

3.2. Cell surface marker of $CD8^+Foxp3^+$ T cells

To determine which surface markers were expressed on $CD8^+Foxp3^+$ T cells, IDT cells or IDTA cells were stained with CD25 and CD103 antibodies (Fig. 2). In contrast to $CD4^+Foxp3^+$ T cells, only some of the $CD8^+Foxp3^+$ T cells were CD25-positive, and most were CD103-positive.

3.3. In vitro suppression assay

To examine the suppressor activity of $CD8^+$ T cells *in vitro*, 1.0×10^5 of the CFSE-labeled effector $CD8^+$ T cells were cultured with IGRP and 1.0×10^5 of I, ID, IDT, or IDTA cells (Fig. 3A). Proliferation of $CD8^+$ T cells in the presence of I, ID, IDT, or IDTA cells with IGRP was compared with that induced by IGRP alone. % Suppression of each proliferation was −1.8 ± 7.6%, 2.9 ± 9.0%, 1.0 ± 8.5%, and 12.9 ± 8.9%, respectively, and the presence of I, ID, or IDT cells did not markedly suppress proliferation of IGRP-reactive $CD8^+$ T cells. In addition, there was no significant suppression in the presence of ID cells or IDT cells compared with the presence of I cells ($p > 0.05$). However, only the presence of IDTA cells significantly suppressed proliferation of IGRP-reactive effector $CD8^+$ T cells than the presence of I cells ($^*p = 0.009$) (Fig. 3B). Furthermore, the suppressive activity seems to be strengthened in a E:R ratio-dependent manner, though not all of IDTA cells express Foxp3 (Fig. 3C).

3.4. In vivo suppression assay

Because IDTA cells alone have suppressor activity for diabetogenic antigen-specific $CD8^+$ T cells *in vitro*, *in vivo* suppressor activity was examined using a diabetes-transfer model. IDTA cells or I cells were intravenously transferred into NOD-scid mice with diabetogenic splenocytes from NOD mice. All of five mice injected with diabetogenic NOD splenocytes alone and six mice injected with diabetogenic splenocytes and I cells became diabetic by 44 days after transfer. In contrast, none of the five mice injected with diabetogenic splenocytes and IDTA cells became diabetic in this cotransfer experiment ($^*p = 0.01$, IDTA vs. control) (Fig. 4). In another set of experiment, none of mice injected with diabetogenic splenocytes and IDTA cells became diabetic even at 150 days after transfer which was more than 30 days after last positive control became diabetic (data not shown). These findings suggest that $CD8^+$ T cells induced by exposure to SpDCs, TGF-β, and ATRA have suppressor activity against the autoimmune response *in vivo* and that the disease should be suppressed only by the $Foxp3^+$ population.

4. Discussion

This study demonstrates that regulatory $CD8^+$ T cells can be induced from diabetogenic 8.3 transgenic NOD mice, which express the TCR-α and TCR-β of a diabetogenic H-$2K_d$-restricted β-cell cytotoxic $CD8^+$ T cell clone and promote diabetes [19]. Original NY8.3 $CD8^+$ cloned T cells cause severe insulitis and diabetes when cotransferred with NOD $CD4^+$ T cells [20]. The 8.3-NOD mice in our colony develop diabetes more rapidly than the original NOD mice, but the incidence of diabetes does not differ from that of NOD mice (75% among females aged 20 weeks and 30 weeks, respectively). Santamaria et al. reported that high avidity of 8.3 TCR T cells devel-

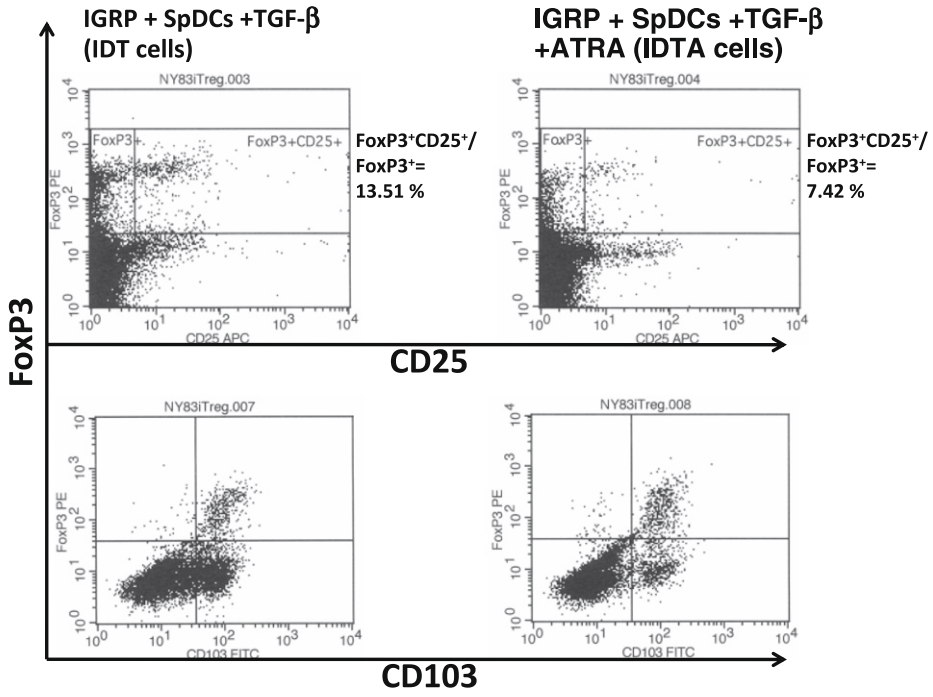

Fig. 2. IDT cells and IDTA cells were stained with antibodies against Foxp3, CD25, and CD103. Only some of the $CD8^+Foxp3^+$ T cells were CD25-positive, and most were CD103-positive. A representative example of four separate experiments is shown.

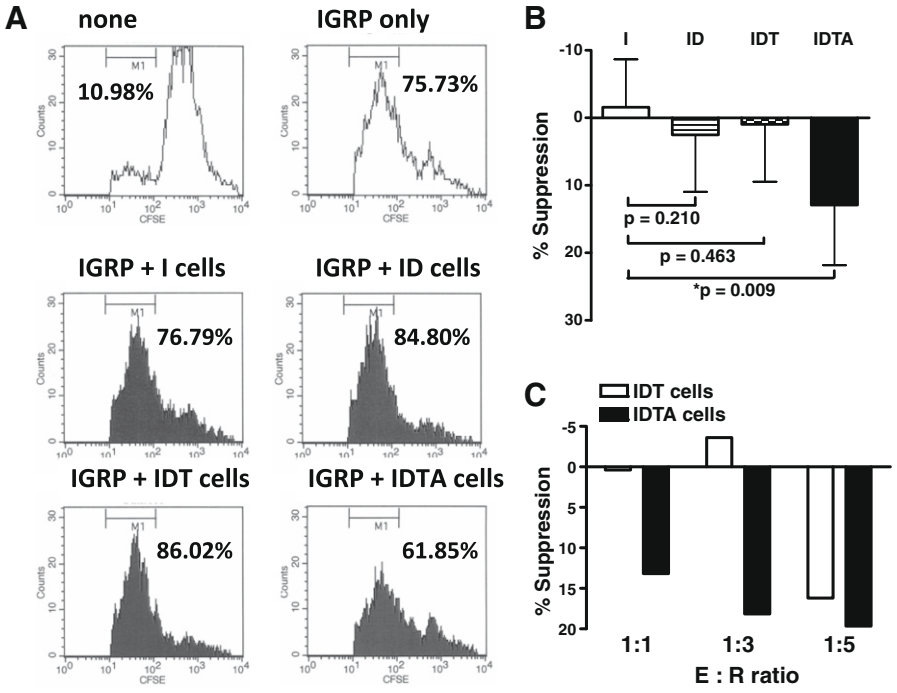

Fig. 3. CFSE-labeled $CD8^+$ T cells (1.0×10^5) purified from 8.3-NOD splenocytes were cultured for 5 days with IGRP alone or with IGRP and 1.0×10^5 induced $CD8^+$ T cells in each group shown in Fig. 1. Three days later, all cells were harvested and gated on $CFSE^+$ cells. (A) Proliferation of $CD8^+$ T cells induced by IGRP alone or in the presence of I, ID, IDT, or IDTA cells with IGRP was examined. A representative example of eight separate experiments is shown. (B) Proliferation of effector $CD8^+$ T cells in the presence of I, ID, IDT, or IDTA cells with IGRP was shown as % suppression, in comparison with that induced by IGRP alone ($^*p = 0.009$, I vs. IDTA). (C) Proliferation of effector $CD8^+$ T cells (E) in the presence of IDT, or IDTA cells (R) with IGRP in comparison with that induced by IGRP alone was shown as % suppression at the indicated E:R ratios.

ops with aging in 8.3-NOD mice [21]. Because 10–20% of the mice did not develop diabetes, we presumed that regulatory T cells were present in 8.3-NOD mice. We generated Tregs in $CD8^+$ T cells for this reason.

Induced regulatory $CD8^+$ T cells have been used in some studies [14–17], two of which showed that Foxp3 is an important marker of regulatory $CD8^+$ T cells [14,17]. Regulatory $CD8^+$ T cells cultured with IGRP, SpDCs, TGF-β, and ATRA showed suppressor activity and the highest expression of Foxp3, which indicates that $CD8^+Foxp3^+$ T cells played a key role in the generation of regulatory $CD8^+$ T cells. Although the role of the expression of Foxp3 in these $CD8^+$ T cells is not well understood, Foxp3 expression is known to induce regulatory T cells. The Foxp3-transduced 6426 $CD8^+$ T cell clone, which recognizes insulin B chain peptide 15–23, delayed the

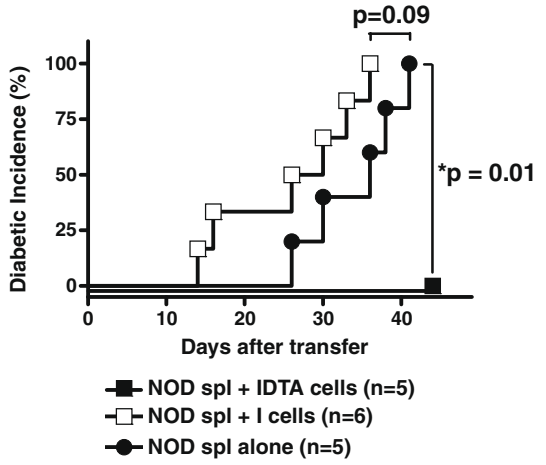

Fig. 4. Diabetogenic NOD splenocytes (1×10^7) alone (closed circle, $n = 5$) or with 6.0×10^6 I cells (open square, $n = 6$) or IDTA cells (closed square, $n = 5$) were transferred into 7- to 8-week-old NOD-scid mice. I cells had no effect on suppression of diabetes transfer, whereas IDTA cells completely suppressed diabetes transfer ($^*p = 0.01$, IDTA vs. control).

onset of diabetes compared with control 6426 clone when transferred into NOD-scid or young NOD mice [22]. This study indicated that expression of Foxp3 changes effector $CD8^+$ T cells into regulatory $CD8^+$ T cells.

Rigorous purification of regulatory $CD8^+Foxp3^+$ T cells in cellular transfusion material would prevent autoimmune diabetes. Because Foxp3 is not a surface marker, we examined whether the $CD8^+Foxp3^+$ T cells express specific surface markers that would enable purification of these cells *in vitro*. Unlike the case with $CD4^+CD25^+Foxp3^+$ T cells, CD25 is not a marker of regulatory $CD8^+$ T cells. More than 90% of the $CD8^+Foxp3^+$ T cells induced in our study also expressed CD103. CD103, the αEβ7 integrin, is a marker for alloantigen-induced regulatory $CD8^+$ T cells [15,23,24]. As $CD8^+Foxp3^-$ T cells also expressed CD103, it is not completely specific for $CD8^+Foxp3^+$ T cells. Purification of regulatory T cells using a CD103 antibody may isolate $CD8^+Foxp3^+$ T cells more efficiently.

ATRA has been reported to play an important role in immune system so far. It has been currently used to treat acute leukaemia and acne vulgaris [8,9]. The role of ATRA in the generation of $CD4^+$ regulatory T cells has also been reported recently [10–12]. Belkaid et al. reported that naïve $CD4^+Foxp3^-$ T cells converted $CD4^+Foxp3^+$ T cells in the gut and that gut-resident DCs produced ATRA. This conversion of Tregs occurred in a TGF-β and ATRA-dependent fashion [12]. Powrie et al. also reported that ATRA-dependent naïve T cells converted to Tregs after oral administration of antigen [11]. $CD4^+Foxp3^-$ T cells cultured with TGF-β, IL-2, and ATRA can convert into a $CD4^+Foxp3^+\alpha E\beta7^+CCR9^+$ phenotype *in vitro* and ATRA enhances the expression of Foxp3 and increases their suppressor activity [10]. ATRA can induce regulatory $CD4^+$ T cells from naïve $CD4^+$ T cells more efficiently in a Foxp3-dependent way. In this study, we first showed that ATRA and TGF-β was used to generate regulatory $CD8^+$ T cells *ex vivo*.

5. Conclusions

ATRA and TGF-β induce antigen-specific regulatory $CD8^+$ T cells in autoimmune diabetic mice. Regulatory $CD8^+$ T cells induced *ex vivo* would be useful as a therapeutic tool for autoimmune diabetes.

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