Protection from Type 1 Diabetes by Invariant NK T Cells Requires the Activity of CD4 + CD25+ Regulatory T Cells

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Protection from Type 1 Diabetes by Invariant NK T Cells

Dalam Ly,*† Qing-Sheng Mi,* Shabbir Hussain,* and Terry L. Delovitch3*†

Invariant NK T (iNKT) cells regulate immune responses, express NK cell markers and an invariant TCR, and recognize lipid Ags in a CD1d-restricted manner. Previously, we reported that activation of iNKT cells by α-galactosylceramide (α-GalCer) protects against type 1 diabetes (T1D) in NOD mice via an IL-4-dependent mechanism. To further investigate how iNKT cells protect from T1D, we analyzed whether iNKT cells require the presence of another subset(s) of regulatory T cells (Tregs), such as CD4+CD25+ Tregs, for this protection. We found that CD4+CD25+ T cells from NOD.CD1d−/− mice deficient in iNKT cell function similarly in vitro to CD4+CD25+ T cells from wild-type NOD mice and suppress the proliferation of NOD T responder cells upon α-GalCer stimulation. Cotransfer of NOD diabeticogenic T cells with CD4+CD25+ Tregs from NOD mice pretreated with α-GalCer demonstrated that activated iNKT cells do not influence the ability of Tregs to inhibit the transfer of T1D. In contrast, protection from T1D mediated by transfer of activated iNKT cells requires the activity of CD4+CD25+ T cells, because splenocytes pretreated with α-GalCer and then inactivated by anti-CD25 of CD25− mice did not protect from T1D. Similarly, mice inactivated of CD4+CD25+ T cells before α-GalCer treatment were also not protected from T1D. Our data suggest that CD4+CD25+ T cells retain their function during iNKT cell activation, and that the activity of CD4+CD25+ Tregs is required for iNKT cells to transfer protection from T1D. The Journal of Immunology, 2006, 177: 3695–3704.

Type 1 autoimmune diabetes (T1D) results from the T cell-mediated destruction of insulin-producing pancreatic islet β cells. Studies of the pathogenesis of T1D performed in female NOD mice that spontaneously develop T1D suggest that T1D develops in part from a deficiency in the function of regulatory T cells (Tregs) that fail to control the pathogenic mechanisms responsible for the disease (1). Of the various mouse Treg subsets, naturally occurring CD4+CD25+ Tregs and invariant NK T (iNKT) cells appear to play important roles in maintaining self-tolerance and autoimmune prevention in NOD mice (2–5).

CD4+CD25+ Tregs maintain self-tolerance by a mechanism of dominant tolerance that suppresses the response of other immune cells (2), because anti-CD25 Ab-mediated inactivation of these Tregs in non-autoimmune-prone mice leads to an autoimmune wasting disease (6). Functionally, Tregs are characterized by their ability to suppress the proliferation and effector function of CD4+ and CD8+ T cells, and to modulate the function of Ag-presenting dendritic cells (DC). Typically, Tregs comprise 5–10% of murine peripheral CD4+ T cells and express many surface markers including CTLA-4, CD62L, GITR, and CD45RB (7). To date, the most definitive lineage marker for naturally occurring CD4+CD25+ Tregs is the transcription factor FoxP3 (8–10). These Tregs are reduced in NOD mice deficient in CD80/86 or CD28 expression, which contributes to accelerated T1D in these strains (11). Furthermore, single-cell analyses of CD4+CD25+ T cells in NOD mice demonstrated that FoxP3 and TGF-β become functionally deficient in this subset as autoimmunity progresses (12), suggesting the overall importance of CD4+CD25+FoxP3+ T cells in the regulation of autoimmunity.

Another well-characterized Treg subset consists of CD1d-restricted iNKT cells, which are unique in that they share receptor structures with conventional T cells and NK cells. The majority of murine iNKT cells use an invariant Vα14Jα18 TCR chain paired preferentially with a Vβ8.2, Vβ2, or Vβ7 chain and recognize lipid Ags presented in the context of CD1d, an MHC class I-like molecule (13, 14). The distinctive feature of iNKT cells is their ability to secrete large amounts of cytokines upon activation. Importantly, activation of iNKT cells with a superagonist glycosphingolipid such as α-galactosylceramide (α-GalCer) can transactivate B cells, NK cells, conventional T cells, and DC, indicating that α-GalCer can act as an adjuvant to promote many other Ag-specific responses during innate and adaptive immunity (4, 15–19).

NOD diabetes-prone mice possess numerical and functional deficiencies in iNKT cells, and NOD.CD1d−/− mice that are deficient in CD1d expression and lack iNKT cells show an exacerbated T1D phenotype (20). In contrast, transgenic overexpression of the TCR Vα14Jα18 rearrangement protects against T1D (21). Furthermore, protection of NOD mice from T1D can be achieved by activation of iNKT cells upon treatment with a multi-low-dose protocol of α-GalCer, which seems to promote preferential IL-4 secretion by iNKT cells (22–26). In view of the ability of activated iNKT cells to transactivate several other immune cells, precedence has been given to determine the cellular subsets that iNKT cells...
may interact with and may be required to promote their regulatory properties. Because CD4\(^+\)CD25\(^+\) T\(_{reg}\) and iNKT cells both mediate protection of NOD mice from T1D and are self-reactive (27), we determined whether iNKT cells require the activity of CD4\(^+\)CD25\(^+\) T\(_{reg}\) for this protection. We found that upon activation of iNKT cells, CD4\(^+\)CD25\(^+\) T cells modulate their surface Ag phenotype and yet retain their suppressive capacity. In addition, we demonstrate that iNKT cell-mediated protection against T1D requires the activity of CD4\(^+\)CD25\(^+\) T cells. Our findings suggest that CD4\(^+\)CD25\(^+\) T\(_{reg}\) may be required to regulate the activity of previously activated iNKT cells.

**Materials and Methods**

**Mice**

NOD/Del and NOD.Scid mice were bred in a specific pathogen-free barrier facility at the Robarts Research Institute. The incidence of T1D in female NOD mice in our colony is 25–30% at 15 wk of age and ≥75% by 25 wk. All experimental mice were female and were maintained in a specific pathogen-free facility in the Animal Care and Veterinary Services at the University of Western Ontario according to institutional guidelines.

**Abs and reagents**

α-GalCer (KRN7000) was provided by Pharmaceutical Research Laboratories, Kirin Brewery (Gunma, Japan), and was reconstituted in MilliQ water to a final working concentration of 25 μg/ml. The vehicle control used was water supplemented with polysorbate-20. The anti-TCRβ-FITC (H57-597), anti-CD4-alkaline phosphatase (RM4-5), anti-CD8ε-alkaline phosphatase (53-2-1), anti-CD25-FITC (7D4), anti-CD3ε-PerCP (145-2C11), anti-CD45RB-P (C363.16A), anti-CD62L-PE (MEL-4), anti-CD69-PE (H1.2F3), anti-CD120a-PE (Rh166), anti-CD11c-FITC (N418), anti-CD80-PE (16-10A1), anti-CD86-PE (GL-1), anti-CD11b-PE (N418), and anti-CD14-PE (M4B14) mAbs were purchased from eBioscience or BD Pharmingen. Fluorescently labeled tetrameric CD1d molecules loaded with α-GalCer (α-GalCer/CD1d) were prepared in house, as previously described (28, 29). The anti-CD25 (PC61) mAb used to inactivate CD25 cells in vivo was prepared in house from hybridomas or BD Pharmingen. Fluorescently labeled tetrameric CD1d molecules loaded with α-GalCer (α-GalCer/CD1d) were prepared in house, as previously described (28, 29). The anti-CD25 (PC61) mAb used to inactivate CD25 T cells in vivo was prepared in house from hybridomas or BD Pharmingen.

**Monitoring of diabetes**

Mice were monitored beginning at 10 wk of age for hyperglycemia by measurement of blood glucose levels twice weekly using an Ascensia ELITE glucometer and strips (Bayer). Mice were considered diabetic when two consecutive blood glucose level readings of >11.1 mmol/L were obtained.

**Cell purification and flow cytometry**

Single-cell lymphocyte suspensions were prepared from the spleen and pancreatic draining lymph nodes (PLN) as described (26). Nonviable cells were excluded by electronic gating for all experiments. iNKT cells were stained with anti-CD25-PE (clone 7D4) and then with anti-PE magnetic beads. Magnetic labeled CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)CD25\(^-\) T cells were collected using MiniMACs columns following the manufacturer’s protocol. For suppression assays, CD4\(^+\)CD25\(^+\) T cells from vehicle-treated NOD mice were cultured (37°C, 5% CO\(_2\)) for 72 h in 96-well plates (0.2 ml) with vehicle-treated irradiated (3000 rad) T cell-depleted spleen cells as APC, anti-CD3 (1.5 mg/ml) with or without α-GalCer (100 ng/ml), and the indicated numbers of CD4\(^+\)CD25\(^+\) T cells. Cultures were pulsed with [\(^{3}H\)]thymidine (1 μCi/ml; Amersham Biosciences) for the last 18 h of culture. Percent inhibition was calculated as follows: [1 – (cocluture/average Tresp alone)] \times 100%.

**In vivo adoptive transfers and development of T1D**

NOD mice (7–8 wk old) were treated with α-GalCer (5 μg/dose) or vehicle (control) every other day for 3 wk, and spleen lymphocyte suspensions were prepared 1 wk after the last treatment. CD4\(^+\)CD25\(^-\) or CD4\(^+\)CD25\(^+\) T cells (2 \times 10\(^6\)) from α-GalCer- or vehicle-treated mice were transferred i.v. with T cells (2 \times 10\(^6\)) from recently diagnosed diabetic mice into 6-wk-old NOD.Scid mice. In some experiments, NOD mice (10 wk old) were treated with α-GalCer or vehicle as described above, injected (i.v.) once 1 wk later with anti-CD25 (PC61; 500 μg) to inactivate CD25\(^+\) T cells; then rested for 3 days. Splenocytes (10\(^6\)) from donor mice were then transferred into 6-wk-old NOD.Scid recipients, and their incidence of T1D was monitored. To assay the spontaneous development of T1D, NOD mice (4–5 wk old) were similarly treated with anti-CD25 to inactivate CD25\(^+\) T cells, rested for 3 days, administered α-GalCer or vehicle every other day for 2–3 wk, and monitored for the onset of T1D.

**Cytokine secretion assay**

Spleen lymphocytes isolated from mice treated with 500 mg of either anti-CD25 mAb or IgG and then α-GalCer (5 μg) or vehicle were cultured (5 \times 10\(^6\) cells/ml) for 48 h in the presence of α-GalCer (100 ng/ml) or vehicle. Cytokines secreted by the cultured spleen cells were detected by ELISA using paired Ab kits for IFN-γ and IL-2 (eBiosciences) or for IL-4 and IL-10 (BD Biosciences). For signal detection, a streptavidin-HRP conjugate and a development solution from BD OptiEIA Reagent Set A (BD Biosciences) were used.

**Results**

Activated iNKT cells do not express FoxP3 and do not regulate the expansion of CD4\(^+\)CD25\(^+\) FoxP3\(^+\) T\(_{reg}\) cells

Initially, we attempted to distinguish between iNKT cells and CD4\(^+\)CD25\(^+\) T\(_{reg}\) in a mixed spleen cell population by flow cytometry. Only a small subpopulation of unstimulated conventional CD4\(^+\) T cells was found to constitutively express the surface CD25 (4.7%) and intracellular FoxP3 (5.4%) markers of mouse T\(_{reg}\) cells (Fig. 1). In contrast, neither unstimulated iNKT cells nor α-GalCer activated iNKT cells constitutively express FoxP3. However, CD25 expression was detected on a subpopulation of iNKT cells at 3 days after activation. Thus, iNKT cells and CD4\(^+\)CD25\(^+\) T\(_{reg}\) can be easily distinguished in a mixed T cell population, consistent with their being members of distinct cell lineages.

Next, we evaluated whether iNKT cell activation by α-GalCer alters the frequency and/or phenotype of CD4\(^+\)CD25\(^+\) FoxP3\(^+\) T\(_{reg}\) population, consistent with their being members of distinct cell lineages.
Treg. NOD mice (8 wk old) were treated with a single dose of α-GalCer, and their spleen and PLN lymphocytes were analyzed by flow cytometry. iNKT cell activation resulted in a transient lymphocytosis in the spleen and PLN (Table I, Fig. 2), as reported (17). At day 3 posttreatment, a 5- to 6-fold increase was detected in the percentage of iNKT cells in the spleen and PLN relative to day 0. The absolute number of iNKT cells was also increased ~10-fold and 30-fold in the spleen and PLN, respectively. At day 7 posttreatment, smaller but significant increases in the percentages and numbers of iNKT cells were found in the PLN but not spleen (Table I). Note that although no differences in the numbers and percentages of Treg cells in the spleen were seen from days 0 to 7, the numbers of Treg cells in the PLN were elevated at days 3 and 7 due to significant increases in PLN cellularity at this time (Table I, Fig. 2). In contrast, at 1 wk after multi-low-dose α-GalCer treatment, the number but not percentage of CD4+ CD25+FoxP3+ T cells was elevated 2- to 3-fold in the spleen and

![Figure 1](http://www.jimmunol.org/)

**Figure 1.** Activated iNKT cells do not express FoxP3 and do not promote the expansion of CD4+ CD25+ FoxP3+ T cells. PLN cells from NOD mice treated 3 days earlier with either vehicle (control) or α-GalCer (5 mg/dose) were costained with anti-TCRβ-FITC and α-GalCer/CD1d tetramer-allophycocyanin. Cells were gated on TCRβ+ and α-GalCer/CD1d tetramer− subpopulations, and then analyzed for staining with anti-FoxP3-PE, anti-CD4-PerCP, or anti-CD25-FITC. Representative flow cytometric data from one of three independent and reproducible experiments are shown. Gates indicated are based on staining obtained with isotype controls analyzed in parallel in each experiment.

![Figure 2](http://www.jimmunol.org/)

**Figure 2.** CD4+ CD25+ FoxP3+ T cells are not expanded upon iNKT cell activation. Spleen and PLN cells from NOD mice administered single dose α-GalCer (5 μg) were costained with anti-TCRβ-FITC and α-GalCer/CD1d tetramer-allophycocyanin, and were then analyzed by flow cytometry for the presence of iNKT cells and CD4+ CD25+ FoxP3+ T cells at days 0, 3, and 7 posttreatment. Dot plots shown are representative of one of three independent and reproducible experiments from the α-GalCer-treated group. Day 0 plots represent cells obtained from mice on the day treatment was initiated. Adjacent histograms (panels on right) show the cumulative percentages of cells from both α-GalCer- and vehicle (control)-treated groups at the indicated time points. Percentages are presented as the cumulative means ± SD of nine individual mice from three independent experiments.

### Table 1. Tissue distribution of CD4+ CD25+ FoxP3+ T cells during iNKT cell activation

<table>
<thead>
<tr>
<th>Day</th>
<th>Spleen</th>
<th>PLN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cells (×10^6)</td>
<td>iNKT cells (×10^6)</td>
</tr>
<tr>
<td>Single doseα (α-GalCer)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>75.1 ± 23.9</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>3</td>
<td>143.7 ± 21.1*</td>
<td>5.3 ± 0.9*</td>
</tr>
<tr>
<td>7</td>
<td>71.7 ± 6.0</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Multidoseα (α-GalCer) Vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 wk</td>
<td>86.3 ± 5.12</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

α Mice (n = 9) were administered α-GalCer (5 μg) and sacrificed on day 0 before treatment and at days 3 and 7 posttreatment. Spleen and PLN lymphocytes were pooled, and their relative frequencies and absolute numbers of iNKT and Treg cells were determined by flow cytometry or enumerated, respectively. iNKT cells (TCRβ+ α-GalCer/CD1d tetramer−) and CD3+ CD4+ CD25+ FoxP3+ T cells were stained as indicated.

β Mice (n = 9 per treatment time point) were administered α-GalCer (5 μg) every other day for 2 wk. Spleen and PLN lymphocytes were collected 1 wk after the last dose and analyzed as above for their iNKT and Treg cell frequencies and numbers.

* *, Significant values relative to those obtained either at day 0 (control) or vehicle treatment alone (p < 0.01).
PLN. Thus, activation of iNKT cells results in an increase in the total number but not percentage of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells.

Activation of iNKT cells results in the transactivation of other immune cell types but does not alter the surface Ag phenotype of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells

CD69 surface expression was used as a cell activation marker to determine whether stimulation of iNKT cells transactivates other immune cell types and elicits a change in the surface Ag phenotype of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells. Indeed, α-GalCer stimulation of iNKT cells transactivated B cells, conventional T cells, NK cells, and mature CD11c<sup>+</sup> DC (Fig. 3A). Gating on CD4<sup>+</sup>CD25<sup>+</sup> T cells identified marked increases in the expression of surface GITR, CD62L<sup>+</sup>, CD45Rb<sup>+</sup>, and intracellular CTLA-4 (Fig. 3B). The mean fluorescent intensity (MFI) values of several of these T<sub>reg</sub> markers increased as much as 2-fold in activated PLN and spleen T<sub>reg</sub> at 24 h after a single-dose α-GalCer treatment (Fig. 3A) (our unpublished observations). Because a multi-low-dose α-GalCer treatment during 2 wk protects NOD mice from T1D (22–26), we determined the phenotype of CD4<sup>+</sup> cells, and mature CD11c<sup>+</sup> DC (Fig. 3 of iNKT cells transactivated B cells, conventional T cells, NK cells, and CD11c<sup>+</sup> DC. PLN-derived cells from NOD mice were prepared 24 h after treatment with single dose (5 mg) α-GalCer, and were then stained with combinations of anti-TCR<sub>β</sub>-FITC, α-GalCer/CD1d<sup>+</sup> tetramer-allophycocyanin, anti-CD3-PerCP, anti-B220-FITC, anti-DX5-FITC, and CD69-PE. CD69 expression was evaluated on B cells (B220<sup>+</sup>CD3<sup>+</sup>), conventional T cells (TCR<sub>β</sub> tetramer<sup>+</sup>), and NK cells (DX5<sup>+</sup>TCR<sub>β</sub>). In addition, cells were stained with anti-CD11c-FITC, anti-CD40-PE, anti-CD80-PE, and anti-CD86-PE. Expression of CD80, CD86, and CD40 were analyzed on gated CD11c<sup>+</sup> DC. B, PLN-derived cells were prepared from NOD mice either 24 h after treatment with single-dose (5 mg) α-GalCer or 2 wk after multi-low-dose (5 mg/dose) α-GalCer treatment and stained with anti-CD4-allophycocyanin, anti-CD25-FITC, anti-GITR-PE, anti-CD45RB-PE, anti-CD62L-PE, or anti-CTLA-4-PE. CD4<sup>+</sup>CD25<sup>+</sup> T cells were gated as indicated and analyzed for their surface expression of GITR, CD45RB<sup>+</sup>, CD62L<sup>+</sup>, and intracellular CTLA-4. MFI of selected peaks or whole channel in each fluorogram are shown, and data from one of three representative independent and reproducible experiments are presented.

FIGURE 3. iNKT cell activation modulates CD4<sup>+</sup>CD25<sup>+</sup> T cells marker expression. A, iNKT cells transactivate B cells, conventional T cells, NK cells, and CD11c<sup>+</sup> DC. PLN-derived cells from NOD mice were prepared 24 h after treatment with single dose (5 mg) α-GalCer, and were then stained with combinations of anti-TCR<sub>β</sub>-FITC, α-GalCer/CD1d tetramer-allophycocyanin, anti-CD3-PerCP, anti-B220-FITC, anti-DX5-FITC, and CD69-PE. CD69 expression was evaluated on B cells (B220<sup>+</sup>CD3<sup>+</sup>), conventional T cells (TCR<sub>β</sub> tetramer<sup>+</sup>), and NK cells (DX5<sup>+</sup>TCR<sub>β</sub>). In addition, cells were stained with anti-CD11c-FITC, anti-CD40-PE, anti-CD80-PE, and anti-CD86-PE. Expression of CD80, CD86, and CD40 were analyzed on gated CD11c<sup>+</sup> DC. B, PLN-derived cells were prepared from NOD mice either 24 h after treatment with single-dose (5 mg) α-GalCer or 2 wk after multi-low-dose (5 mg/dose) α-GalCer treatment and stained with anti-CD4-allophycocyanin, anti-CD25-FITC, anti-GITR-PE, anti-CD45RB-PE, anti-CD62L-PE, or anti-CTLA-4-PE. CD4<sup>+</sup>CD25<sup>+</sup> T cells were gated as indicated and analyzed for their surface expression of GITR, CD45RB<sup>+</sup>, CD62L<sup>+</sup>, and intracellular CTLA-4. MFI of selected peaks or whole channel in each fluorogram are shown, and data from one of three representative independent and reproducible experiments are presented.

Activation of iNKT cells requires T<sub>reg</sub> for protection from T1D

CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells can suppress the proliferation of responder T (T<sub>resp</sub>) cells in vitro and inhibit autoimmunity in vivo (2, 3). To evaluate the functional capacity of CD4<sup>+</sup>CD25<sup>+</sup> T cells after iNKT cell activation, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>resp</sub> cells (containing iNKT cells) and CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells from naïve NOD mice were cocultured in vitro in the presence or absence of α-GalCer. α-GalCer stimulated an increase in T cell proliferation at all T<sub>resp</sub>:T<sub>reg</sub> ratios assayed compared with that observed in control cultures (Fig. 4A, left panel). However, this increase was not seen when the data were normalized and plotted as a percent inhibition of proliferation (Fig. 4A, right panel). Hence, iNKT cell activation can occur in the presence of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells but does not change the suppressive function of these T<sub>reg</sub> cells.

To assay whether CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> suppression is maintained in the absence of iNKT cell activation, we analyzed the suppressive capacity of CD4<sup>+</sup>CD25<sup>+</sup> T cells from naïve NOD,CD1d<sup>−/−</sup> mice.
To analyze whether iNKT cell activation alters the ability of Treg cells to protect from T1D in vivo, CD4\(^+\)CD25\(^+\) T cells from multi-low-dose \(-\text{GalCer}\)-treated mice retain the ability to transfer protection against T1D, because they can prevent the transfer of T1D.

**iNKT cell-mediated transfer of protection from T1D requires the activity of Treg cells**

Because CD4\(^+\)CD25\(^+\) T\(_{reg}\) cells retain their regulatory activity after iNKT cell activation, we investigated whether the reverse is true, i.e., does iNKT cell-mediated transfer of protection against T1D require the activity of CD4\(^+\)CD25\(^+\) T\(_{reg}\) cells? Initially, we attempted to confirm the report by Kohm et al. (30) that anti-CD25 mAb treatment does not physically deplete CD4\(^+\)FoxP3\(^+\) T\(_{reg}\) cells but rather down-regulates and/or induces the shedding of CD25 from the surface resulting in the inactivation of T\(_{reg}\) cells. NOD mice were treated first with a single dose of anti-CD25 mAb (clone PC61) or control IgG and then with single dose \(\alpha\)-GalCer or vehicle. One week later, the PLN were assayed for the presence of CD4\(^+\)CD25\(^+\) T cells and CD4\(^+\)FoxP3\(^+\) T cells using a noncompetitive anti-CD25 mAb (clone 7D4) to stain the T cells. Importantly, the 7D4 and PC61 mAbs are known to bind different CD25 epitopes (31), and a lack of staining of CD4\(^+\)CD25\(^+\) T cells by 7D4 would not be expected to arise by the blocking of a CD25 epitope by PC61. We found that CD4\(^+\)CD25\(^+\) T cells from PC61-treated NOD mice were not detected by 7D4, but that CD4\(^+\)FoxP3\(^+\) T cells indeed remained after PC61 treatment (Fig. 6). These data are similar to those of Kohm et al. (30) and confirm that anti-CD25 mAb treatment in vivo may inactivate but not deplete CD4\(^+\)FoxP3\(^+\) T cells.

To assay whether T\(_{reg}\) cell activity is required for iNKT cell-mediated transfer of protection from T1D, we took advantage of our anti-CD25 treatment findings above as well as reports that multi-low-dose \(\alpha\)-GalCer treatment protects NOD mice from T1D and that splenocytes from these protected mice do not transfer T1D.
The activity of CD4+ T cells requires the regulation and/or shedding of CD25 expression by CD4+ Treg cells. Given the requirement of CD4+CD25+ Treg cells for α-GalCer-induced iNKT cell protection from T1D, we next investigated the role of these Treg cells in the activation of iNKT cells. Functional inactivation of CD4+CD25+ Treg cells did not inhibit the subsequent activation of iNKT cells, because NOD mice that received either anti-CD25 mAb or control isotype control as above, rested for 3 days, treated with multi-low-dose α-GalCer (5 μg/dose, every other day for 3 wk) or vehicle, and their incidence of T1D was monitored to 30 wk of age. α-GalCer inactivation of CD4+ T cells regulates the activity of iNKT cells (Fig. 9B). Because iNKT cell activation occurs in anti-CD25-treated mice, we assayed whether iNKT cells in such mice can transactivate different immune cells. Activated iNKT cells in anti-CD25-treated mice augmented and sustained the ability of iNKT cells to transactivate B cells, conventional T cells, and NK cells in the PLN at 6, 12, and 24 h postactivation, which then returned to basal levels at 48 h after activation (Fig. 9).
We next analyzed whether anti-CD25 treatment also augments α-GalCer-induced cytokine secretion by iNKT cells. CD4+ and CD4− iNKT cell subsets produce different cytokines, because CD4+ iNKT cells produce IFN-γ and IL-4 whereas CD4− iNKT cells produce mainly IFN-γ (4, 5). Accordingly, we assayed the effect of anti-CD25 treatment on the ability of CD4+ and CD4− iNKT cells to secrete IL-2, IFN-γ, and IL-4. Mice that received anti-CD25 mAb or control IgG were treated with α-GalCer, and CD4+ and CD4− iNKT cells were analyzed for their intracellular production of cytokines. Consistent with the observation that anti-CD25 mAb augmented the iNKT cell transactivation of lymphocytes, mice treated with anti-CD25 also enabled a greater proportion of iNKT cells to produce IL-2, IFN-γ, and IL-4 in response to α-GalCer. Interestingly, this was the case for both of the CD4+ and CD4− subsets of iNKT cells (Fig. 10A). Moreover, anti-CD25 treatment in vivo before culture resulted in an increase in IL-2, IFN-γ, IL-4, and IL-10 secretion by splenocytes as determined by ELISA, independent of whether iNKT cells were previously exposed in vivo to vehicle (Fig. 10B) or α-GalCer (C). Taken together, these findings suggest that iNKT cell activation and the resultant transactivation of B cells, T cells, and NK cells are regulated by CD4+CD25+ Treg cells.

**Discussion**

The role of CD4+CD25+ Treg cells in the prevention of T1D in NOD mice is well documented, with many therapeutic strategies attributing their effectiveness to the activation and expansion of these cells (32). Here, we demonstrate that the expansion of CD4+CD25+FoxP3+ T cells is not required during α-GalCer-induced iNKT cell-mediated protection against T1D NOD mice. This observation is consistent with previous reports that these Treg cells are not required for iNKT cell activation (22, 23). However, because the latter studies only evaluated the effect of α-GalCer stimulation on the frequencies of Treg cells, we determined whether α-GalCer treatment alters the phenotypic and/or functional properties of CD4+CD25+ Treg cells. Our data show that

**FIGURE 8.** Transient down-regulation of CD25 expression on CD4+CD25+ T cells during α-GalCer therapy. NOD mice (4–5 wk old) were injected i.v. once with anti-CD25 (PC61; 500 μg/dose) or IgG control, rested for 3 days, and treated with multi-low-dose α-GalCer (5 μg/dose, every other day for 3 wk). Peripheral blood was collected from mice of each treatment group at the indicated time after anti-CD25 treatment to monitor the down-regulation of CD25 expression on CD4+CD25+ T cells by staining with anti-CD25-FITC and flow cytometry. The numbers shown inside the boxed areas indicate the percentages of CD4+CD25+ T cells detected.

**FIGURE 9.** iNKT cell expansion and transactivation in anti-CD25-treated mice. NOD mice (8 wk old) were treated with anti-CD25 (PC61; 500 mg/dose, i.v.) or IgG isotype control, rested for 3 days, and then administered single-dose (5 mg) α-GalCer or vehicle. PLN cells were analyzed by flow cytometry at 6, 12, 24, and 48 h post treatment with α-GalCer for CD69 surface expression after staining with anti-CD69-PE. B cells (B220+CD3−), conventional T cells (TCRβ+α-GalCer/CD1d tetramer+), and NK cells (DX5+TCRβ+) were gated, and the CD69 expression values are displayed as MFI for the FL-2 channel of the various treatment groups at the indicated times. Data shown are representative of one of three independent and reproducible experiments.
iNKT cell activation indeed modulates the phenotype of CD4+CD25+ Treg cells during an early response to single dose α-GalCer, as revealed by an increase in the surface expression of the GITR, CD45RBlow, and CD62L-high markers of CD4+CD25high Treg phenotype and function. In contrast, the level of intracellular expression of CTLA-4 and FoxP3 did not change upon activation by single-dose α-GalCer treatment. It follows that subsequent iNKT cell responses to repeated exposure of α-GalCer may be required to regulate the latter vigorous responses that ensue upon iNKT cell activation by single-dose α-GalCer. To illustrate that CD4+CD25high Treg cells retain their functional properties in vitro and in vivo following iNKT cell activation, even in the presence of the robust cell transactivation and DC maturation observed. This is in contrast to DC activation mediated by direct TLR and costimulation agonists that block CD4+CD25high Treg cell function (33–35). Although α-GalCer-activated human iNKT cells can trigger the suppressor function of CD4+CD25high Treg cells (36), it remains to be determined whether activated iNKT cells interact directly or indirectly with CD4+CD25high Treg cells in vivo. Functional inactivation of CD4+CD25high Treg cells resulted in the development of TID independent of whether α-GalCer or vehicle was administered to NOD mice. These data identify a requirement for the activity of CD4+CD25high Treg cells in iNKT cell-mediated protection against TID. Recently, a role for cooperation between iNKT cells and CD4+CD25high Treg cells was also described for the prevention of autoimmune myasthenia (37). Both the latter report and our results for TID further underscore the importance of CD4+CD25high Treg cells in regulating the development of autoimmunity. It is also important to mention that the
requirement for two subsets of CD4+ TCRαβ+ T cells, distinguished by their expression of the DX5 cell surface marker found on all NK cells and a small fraction of iNKT cells, in the protection from T1D was first described by Gonzalez et al. (38). In the latter studies, collaboration between CD4+DX5+ T cells and CD4+DX5− T cells were required for optimal transfer of protection from T1D into young prediabetic mice before the establishment of invasive insulitis. Protection did not cause the deletion or anergy of islet β cell-autoreactive effector T cells, but rather modulated the severity of insulitis and extent of β cell destruction via a mechanism of damage control. It is possible that, with the current availability of α-GalCer/CD1d tetramers to purify and characterize iNKT cells and the extensive analyses of iNKT cell and CD4+CD25+ Treg cell activity performed since the studies of Gonzalez et al., the two subsets of CD4+ T cells characterized by these workers would now be considered to be iNKT cells and CD4+CD25+ Treg cells. Importantly, we found that collaboration between iNKT and CD4+CD25+ Treg cells is required to achieve complete protection from spontaneous T1D before the establishment of invasive insulitis in 4- to 5-wk-old NOD mice (Fig. 7A) as well as after the development of aggressive insulitis in 10-wk-old NOD recipients of diabeticogenic T cells (A). The latter result differs from that of Gonzalez et al. (38) who showed that the presence of protective T cells was essential before the initial β cell attack and could not reverse established aggressive insulitis, and may arise from the use of BDC2.5 RAG−/− mice in their studies and NOD/Scid mice in our studies.

Our findings may unravel a novel mechanism for these CD4+CD25+ Treg cells in iNKT cell-mediated protection against T1D. Previously, we and others suggested that protection against T1D is mediated mainly by the induced increase of IL-4 secretion by iNKT cells (22–24, 26), which may be in part due to the sustained ability to secrete IL-4 but not IFN-γ upon repeated exposure to α-GalCer (17–19). In the present study, we demonstrate that functional inactivation of CD4+CD25+ T cells from prediabetic NOD mice treated with anti-CD25 mAb and multi-low-dose α-GalCer can give rise to T1D in NOD/Scid recipient mice (Fig. 7A). This result raises the possibility that activation of iNKT cells in the presence of inactive CD4+CD25+ Treg cells may not be sufficient to prevent the onset of T1D, and that CD4+CD25+ Treg cell-mediated regulation of activated iNKT, B, T, and NK cells as well as DC may also be necessary. This possibility derives support from our studies on the spontaneous development of T1D in NOD mice in which transient inactivation of CD4+CD25+ T cells during iNKT cell activation also induced T1D (Fig. 7B). iNKT cells activated in mice treated with anti-CD25 mAb resulted in the increased secretion of proinflammatory (e.g., IFN-γ) as well as noninflammatory (e.g., IL-4) cytokines, which suggests that an unregulated activation of iNKT cells may contribute to the development of T1D. Furthermore, the ability of iNKT cells to transactivate other immune cells was amplified in mice whose Treg cells were inactivated, as measured by the increased activation of B cells, T cells, and NK cells, which may elicit T1D in these mice. The capacity of CD4+CD25+ T cells to regulate iNKT cells is consistent with a previous study demonstrating that CD4+CD25+ Treg cells can down-regulate the activation of iNKT cell clones (39).

Although multi-low-dose α-GalCer treatment did not elicit any detectable increases in the relative frequency of Treg cells, a 2-fold increase in cellularity in the spleen and a 5-fold increase in the PLN were found. This observation is reminiscent of the effect of CFA, another protective immunization strategy that results in increase lymphoid tissue cellularity (40–42). King et al. (41) recently proposed a model in which NOD lymphopoeia may be causal to autoimmunity, which may be reversed by an increase in T cell number that buffers the expansion of self-reactive T cells. In the case of α-GalCer activation of iNKT cells, the resultant transactivation by iNKT cells may correct NOD lymphopenia with this correction being under the control of CD4+CD25+ T cells, because functional inactivation of this population before and after α-GalCer activation results in T1D development (Fig. 7).

The onset of T1D was not exacerbated under conditions of CD4+CD25+ Treg inactivation plus repeated iNKT cell activation vs Treg inactivation alone. Several mechanisms may explain this observation. First, iNKT cells and CD4+CD25+ Treg cells may not interact directly with each other, given that Treg cells are dominant regulators (27) and that the inactivation of CD4+CD25+ Treg cells can still give rise to T1D independent of any further stimuli. Second, because α-GalCer stimulated an increase in cytokine secretion and cell transactivation after CD4+CD25+ Treg cells were inactivated, it is possible that CD4+CD25+ Treg cells normally regulate the activation and anergy induction of iNKT cells. We found that after administration of control IgG, ~5-fold less IL-2 was secreted by splenocytes from α-GalCer-treated than vehicle-treated mice. However, anti-CD25 treatment restored the amount of IL-2 secreted by splenocytes in α-GalCer-treated mice to that of splenocytes in vehicle-treated mice. Thus, our data suggest that repeated exposure (treatment in vivo and restimulation in vitro) to the α-GalCer, induced iNKT cell anergy in NOD mice, which was reduced appreciably in mice whose CD4+CD25+ T cells were inactivated. These findings are compatible with the current model of iNKT cell anergy in which iNKT cells become hyporesponsive on repeated exposure to α-GalCer (18, 19), and suggest that CD4+CD25+ Treg cells contribute to the regulation of activated iNKT cells.

Are iNKT cells the target of CD4+CD25+ Treg cell activity and, if so, how do CD4+CD25+ Treg cells regulate iNKT cell activation and anergy? Because cell contact is essential for the immunoregulatory function of iNKT (43) and CD4+CD25+ Treg cells (2–7) cells, it is possible that CD4+CD25+ Treg cells directly target iNKT cells in vivo for suppression. The latter possibility is supported by the report that human CD4+CD25+ Treg cells can down-regulate the activation of iNKT cell clones in vitro (39). Alternatively, CD4+CD25+ Treg cells may regulate iNKT cells indirectly via an APC by decreasing their priming potential through suppressive cytokines and/or tryptophan metabolism (44). Recent evidence for the latter mechanism was provided by in vivo imaging studies that used two-photon microscopy to show that CD4+CD25+ Treg cells form stable interactions predominantly with Ag-specific DC rather than effector T cell populations, suggesting that APC rather than effector cells may be the primary target of Treg cells (45). Thus, interactions between Treg cells and DC may ultimately decrease iNKT cell activation as a mechanism of controlling and dampening an immune response, e.g., the capacity of iNKT cells to exacerbate T1D induced by CD8+ T cells (46). The regulation of immune responsiveness by such Treg-DC interactions may also be mediated by iNKT-DC interactions that have been reported to shape both proinflammatory and tolerogenic immune responses (5). Whether Treg-DC-iNKT cellular complexes are formed and whether such cellular interactions underlie the mechanism(s) of Treg control of iNKT cell activation requires further experimentation.

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Disclosures

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