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FoxP3$^+$RORγt$^+$ T helper intermediates display suppressive function against autoimmune diabetes[1]

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Abstract

Recently, traces of double-positive FoxP3$^+$RORγt$^+$ T cells were identified and viewed as dual programming differentiation intermediates geared towards development into T regulatory (Tregs) or Th17 cells. Herein, we report that FoxP3$^+$RORγt$^+$ intermediates arise in the NOD mouse T cell repertoire prior to inflammation and can be expanded with tolerogen without further differentiation. Furthermore, FoxP3$^+$RORγt$^+$ cells express both CD62L and membrane-bound TGFβ (mTGFβ) and utilize the former to traffic to the pancreas and the latter to suppress effector T cells both *in vitro* and *in vivo*. The cells perform these functions as FoxP3$^+$RORγt$^+$ intermediates, despite being able to terminally differentiate into either FoxP3$^+$RORγt$^-$ Tregs or FoxP3$^-$RORγt$^+$ Th17 cells upon polarization. These previously unrecognized observations extend plasticity to both differentiation and function and indicate that the intermediates are poised to traffic to sites of inflammation and target diverse pathogenic T cells, likely without prior conditioning by effector T cells, thus broadening efficacy against autoimmunity.

Introduction

Naïve CD4$^+$ helper T (Th) cells differentiate into one of several CD4$^+$ T cell lineages including Th1, Th2, Th17 and T regulatory (Treg)[5] cells depending on environmental stimuli. This process offers flexibility that the type of antigen and the cytokine milieu exploit to coordinate measured immune responses (1). Lately, it has become clear that a delicate cellular and programming interplay between Tregs and Th17 cells dictates the balance between health and autoimmunity (2).

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[5]**Abbreviations: Abbreviations:** Agg, aggregated; GAD, glutamic acid decarboxylase; mTGFβ, membrane-bound active TGFβ; PLN, pancreatic LN; PN, Pancreas; RQ, relative quantity; SP, spleen; TID, type I diabetes; Tregs, T regulatory cells

Naïve Th cells stimulated with antigen in the presence of TGFβ up-regulate expression of the transcription factor FoxP3 (3) and the phenotypic cell surface marker CD25 and develop into Tregs (4,5). FoxP3 up-regulation diverts the cell from activated to regulatory status by disrupting normal NFAT:AP-1 binding and instead forming an NFAT:Foxp3 complex (6). These cells display effective suppressive function against pathogenic self-reactive T cells (7–9). In contrast, when naïve T cells are stimulated with antigen in the presence of both TGFβ and IL-6, they up-regulate the transcription factor RORγt (10) and develop into Th17 cells (11,12), a distinct lineage from Th1 and Th2 cells (13,14) that produces the cytokine IL-17, hence the designation Th17 (15). Th17 cells proved pathogenic in several autoimmune disease models (16,17), including type 1 (TID) or autoimmune diabetes (18).

Given that IL-6 diverts differentiation from Treg to Th17 to give rise to cells with opposite function, emphasis was put on the plasticity of Th programming (19–22). Recently, it was reported that the differentiation of naïve Th cells transits through a FoxP3/RORγt intermediate stage to provide further flexibility for the development of measured responses (2,19). There is even evidence that fully differentiated Tregs can be reprogrammed to a Th17 phenotype (19), while fully differentiated Th17 cells can convert to Th1 lymphocytes (20–22). Because intermediates represent a transitional state, only traces of cells are available at any given time and isolating a sufficient number of cells to investigate molecular programs or *in vivo* function has not been feasible.

Herein, we were able to expand FoxP3$^+$RORγt$^+$ cells *in vivo* and demonstrate that these cells are fully functional. Indeed, Ig-GAD1, an immunoglobulin molecule genetically engineered to incorporate the glutamic acid decarboxylase (GAD) 524–543 diabetogenic amino acid sequence (designated GAD1) expands Tregs that protect against type I or autoimmune diabetes (23). Examination of these cells showed an increased number of FoxP3$^+$RORγt$^+$ cells. Interestingly, these intermediates are present within the natural repertoire and express CD62L, which is required for trafficking as well as active membrane-bound TGFβ (mTGFβ), through which they suppress pathogenic T cells. Importantly, the FoxP3$^+$RORγt$^+$ Tregs did not secrete IL-17, in agreement with previously published results (24–26). However, under Th17-polarizing conditions, they were able to fully differentiate into RORγt$^+$ cells capable of producing IL-17 cytokine. CD62L and TGFβ were expressed on the FoxP3$^+$RORγt$^+$ Tregs in the natural repertoire prior to disease onset, which likely guides the Tregs to the site of inflammation to target diverse effector T cells. These previously unrecognized findings indicate that FoxP3$^+$RORγt$^+$ intermediates are fully functional, broadening Th plasticity to both programming and function. Moreover, as the cells express CD62L and mTGFβ prior to inflammation, they do not require conditioning by effector cells but are able to traffic to sites of inflammation and target diverse T cell specificities.

Materials and Methods

Mice

NOD (H-2g7), NOD.scid, NOD.BDC2.5 (27) and NOD.FoxP3:GFP mice were used according to the guidelines of the University of Missouri Columbia Animal Care and Use Committee. NOD.FoxP3:GFP reporter mice were generated by breeding C57BL/6.FoxP3:GFP knock-in animals (3) into NOD mice for 10 backcross generations.

Tolerogen

Ig-GAD1 (23) is an Ig chimeras carrying GAD1 peptide corresponding to aa residues 524–543 (SRLSKVAPVIKARMMEYGTT) of GAD65 (28). This was done by inserting GAD1 nucleotide sequence into the heavy chain veriable region of 91A3 IgG2b molecule and transfecting the resulting 91A3H-GAD1 chimeric gene along with the parental 91A3 κ chain

gene into a non-Ig-secreting SP2/0 myleoma B cells (23). Transfectoma cells were then grown large-scale in DMEM media with 10% iron-enriched calf serum (HyClone) and Ig-GAD1 was purified using columns of CNBr-activated 4B sepharose (GE Healthcare) conjugated to rat-anti-mouse κ light chain mAb. Ig-GAD1 was aggregated by precipitation with 50% saturated $(NH0)_2SO_4$ as was previously described (29).

Expansion of FoxP3 expressingT cells by Ig-GAD1

NOD and NOD.FoxP3-GFP reporter mice are given i.p. 300 μg aggregated (agg) Ig-GAD1 in saline at wk 4, 5, and 6. The mice are sacrificed at the end of week 6 which is 5 days after the last injection. For analysis of FoxP3 T cells at wk 5 the mice receive two injections only, one at week 4 and one at week 5 and the animals are sacrificed 5 days later.

Assessment of diabetes

Assessment of blood glucose levels used Accu-Chek Advantage monitoring system. A mouse was considered diabetic when the blood glucose levels were above 300 mg/dL for 2 consecutive measures.

Purification of pancreatic cells

Islets and infiltrating cells were purified according to a standard procedure (30). Briefly, the pancreata were digested with collagenase type IV (Invitrogen, Carlsbad, CA) and islets and infiltrating cells were separated on a ficoll gradient (GE Healthcare, Waukesha, WI).

Neutralization of CD62L *in vivo*

Cells producing MEL-14 anti-CD62L antibody were grown large-scale in DMEM media containing 10% iron-enriched calf serum (HyClone, Thermo Fisher Scientific, Waltham, MA). Anti-CD62L antibody was then purified using columns containing CNBr-activated sepharose linked to mouse-anti-rat antibody. For neutralization of CD62L during expansion of FoxP3 expressing cells, 1 mg anti-CD62L antibody in PBS was administered i.p. along with each agg Ig-GAD1 injection. For neuralization of CD62L during transfer of FoxP3 expressing cells, 1 mg anti-CD62L antibody in PBS was administered i.p. along with the i.v. adoptive transfer of $FoxP3^{int}$ Tregs.

Flow cytometery analyses

Cell surface staining—Splenocytes, pancreatic cells, pancreatic lymph node cells or purified $CD4^+$ T cells were incubated with the test antibodies for 30 min at 4°C, and then washed with staining buffer. The cells were then fixed with 2% formaldehyde and analyzed. For Biotin coupled antibodies the cells were stained with PE-conjugated streptavidin for 30 min at 4°C prior to fixation with formaldehyde. The antibodies were anti-CD4 antibody (clone RM4-5,) Biotin-conjugated anti-TGF-β1 antibody (clone BAF240) and its isotype control chicken IgY (R&D Systems Minneapolis, MN); anti-CD25-APC (clone 2A3) and Rat IgG1 as isotype control (BD Pharmingen, San Jose, CA); anti-CD62L-FITC (clone MEL-14) and its isotype control rat IgG2a (BD Pharmingen, San Jose, CA); anti-ICOS-APC antibody (clone C398.4A) and its isotype control Armenian Hamster IgG1 (BD Pharmingen, San Jose, CA); anti-GITR-FITC antibody (clone TNFRSF18) and its Rat IgG2b isotype control (eBiosciences, San Diego, CA) .

FoxP3 and RORγt intracellular staining—The test cells were stained for surface molecules as indicated above and then blocked with 1μg/million cells mouse IgG and subsequently permeabilized with Fix/Perm buffer (eBioscience, San Diego, CA) and stained with anti-FoxP3-FITC antibody (clone FJK-16s) or isotype control Rat IgG2a-FITC and/or anti-RORγt antibody (clone AFKJS-9) or isotype control Rat IgG2a-PE antibody for 30 min

at 4°C. The cells were then washed and analyzed. Both antibodies were purchased from eBiosciences (San Diego, CA).

Cell analyses used a FACScan (Becton Dickson, San Jose, CA) or Cyan ADP (Dako, Denmark) flow cytometers and the FlowJo software (Tree Star, Inc., Ashland, OR). All staining data is representative of at least three replicates.

Suppression of passive diabetes by Tregs

10 million splenocytes from diabetic NOD mice were injected i.v. into NOD.scid recipient mice along with 5×10^5 of suppressor cells. FoxP3^{int}, FoxP3^{hi} and FoxP3^{-} T cells were sorted to 95% purity based on GFP expression from NOD FoxP3:GFP reporter mice on a MoFlo cell sorter (Dako, Denmark).

Proliferation Assays

This was done as previously described (23). Briefly, allogenic effector NOD T cells were incubated in a 96-well plate (200,000/well) with CD11c^{+} C57BL/6 APC (100,000/well) that were purified from bulk splenocytes on anti-CD11c-coupled Miltenyi microbeads. Sorted (95% purity) FoxP3^{int} or FoxP3^{hi} Tregs were added at the indicated ratios and the . The culture was incubeted for 3 days with addition of 1 μCi of [^{3}H]thymidine during the last 8 hours of culture. The cells were then harvested on a Trilux 1450 Microbeta Wallac Harvester, and incorporated [3H]thymidine was counted using the Microbeta 270.004 software (Wallac, Wallthan, MA).

T cell activation and polarization

Purified T cells were incubated in a 96-well round-bottom plate for 72 hours along with plate-bound anti-CD3 (2C11) (10μg/mL), soluble anti-CD28 (PV1) (1μg/mL) and 100U rIL-2 (212-12; Peprotech Inc, Rocky Hills, NJ).

Th1 polarization—Purified T cells were incubated under activating conditions and polarized with: 10ng/mL rIL-12 (210-12; Peprotech Inc., Rocky Hills, NJ) and 10μg/mL anti-IL-4 (11B11).

Th17 polarization—Purified T cells were incubated under activating conditions and polarized with: 3 ng/mL rhTGFβ (100-21C; Peprotech Inc., Rocky Hills, NJ), 10ng/mL rIL-6 (216-16; Peprotech Inc., Rocky Hills, NJ), 10 μg/mL anti-IFNγ (R4-6A4) and 10μg/mL anti-IL-4 (11B11).

Treg polarization—Purified T cells were incubated under activating conditions and polarized with: 3 ng/mL rhTGFβ (100-21C; Peprotech Inc., Rocky Hills, NJ), 10 μg/mL anti-IFNγ (R4-6A4) and 10μg/mL anti-IL-4 (11B11).

Cytokine detection

Intracellular cytokine—After 5 days polarization the T cells were stimulated for 6 hours with 50 ng/mL PMA and 500 ng/mL ionomycin in the presence of Brefeldin A. The cells were then permeabilized with 0.2% saponin and intracellular cytokines were detected by staining with anti-cytokine antibodies .

Secreted cytokines—Detection of cytokine in culture supernatant used ELISA as described (18,23,29). Also, cytokine secretion were detected on the cell surface to identify IL-10 producing cells using Miltenyi bioconjugate mouse IL-10 secretion assay according to the manufacture's instruction (Miltenyi biotec, Bergisch Gladbach, Germany).

RT-PCR analysis

FoxP3^{hi} or FoxP3^{int} cells were sorted to at least 95% purity using a MoFlow cell sorter (Dako, Denmark). RNA was isolated using Trizol extraction and ethanol precipitation. RT-PCR for RORγt and FoxP3 was performed using the QuantiTect Reverse Transcription kit from Qiagen and the StepOne Instrument cycler (Applied Biosystems). The forward primers were 5'TACACCCAGGAAAGACAGCAACCT3'for FoxP3 and 5'ACAGCCACTGCATTCCCAGTTT3' for RORγt. The reverse primers were 5'TCTGCTTGGCAGTGCTTGAGAA3' for FoxP3 and 5'TCTCGGAAGGACTTGCAGACAT3' for RORγt .

Statistical analysis

P values were calculated using the two-tailed Student's *t* test.

RESULTS

Protective antigen therapy against TID expands discrete FoxP3 Tregs

We have previously described a protective treatment regimen with Ig-GAD1, which expands splenic Tregs when administered into non-obese diabetic (NOD) mice at week (wk) 4 to 6 of age (23). The same regimen, however, was unable to protect against TID when administered at wk 8 of age, despite effective expansion of splenic Tregs (23) (Supplementary Fig. 1A). The 8-wk-old splenic Tregs, though, had diminished levels of mTGFβ (Supplementary Fig. 1B), which is indispensable for suppression of the disease (23).

To gain further insight on the specific loss of mTGFβ^{+} Tregs during the transition from 6 to 8 wks, which coincides with ongoing progressive insulitis in NOD mice, we opted to compare the phenotype of the two populations. The initial experiments were focused on FoxP3 protein expression in 5, 6, 7, and 8-wk-old splenic Tregs from Ig-GAD1-treated mice. Much to our surprise, these splenic Tregs comprised two distinct populations with one displaying intermediate FoxP3 (FoxP3^{int}) expression and the second with high FoxP3 (FoxP3^{hi}) expression (Fig. 1A). Intriguingly, there was a gradual decrease of the FoxP3^{int} population and, by wk 8, most of the Tregs had a FoxP3^{hi} phenotype (Fig. 1A). Indeed, 5-wk-old Tregs comprised 36% FoxP3^{int} and 43% FoxP3^{hi} cells. However, by wk 8 of age, 64% of Tregs were FoxP3^{hi} and the FoxP3^{int} population was significantly reduced to 6.3%. Interestingly, the decrease in FoxP3^{int} Tregs coincides with the loss of mTGFβ in Ig-GAD1-expanded 8-wk-old splenic Tregs (23), which prompted us to determine the dynamics of mTGFβ expression by the two populations. The results illustrated in Figure 1B clearly show that mTGFβ was predominantly expressed on the FoxP3^{int} population but was significantly reduced when the FoxP3^{int} cells were no longer detectable by wk 8. Indeed, the FoxP3^{hi} population, which represented 39% and 52% of total Tregs on wk 5 and 8, respectively had no significant mTGFβ expression at any time point. However, the FoxP3^{int}mTGFβ^{+} population went down from 15% of total Tregs at wk 5 to 4% by wk 8 (Fig. 1B). Similar patterns of FoxP3 and TGFβ expression was observed in untreated mice (Supplementary Fig. 2). Additionally, a control Ig-HEL molecule, incorporating the I-A^{g7}-restricted non-diabetogenic hen egg lysozyme (HEL) peptide instead of GAD1, was unable to expand any T regulatory cells in this model (23).

To determine which of the two populations (FoxP3^{int} and FoxP3^{hi}) might be involved in protection against diabetes during treatment with Ig-GAD1, we searched for Tregs in both the spleen (SP) and pancreas (PN) at wk 6 and 8, time points where insulitis is progressive. As illustrated in Figure 1C, FoxP3^{int} Tregs dropped from 22 ×10^{4} (per 10^{6} counted CD25^{+} cells) at wk 6 to 4 ×10^{4} at wk 8 in the SP but were maintained at high numbers in the PN (27 and 22×10^{4} per 10^{6} counted CD25^{+} cells at wk 6 and 8, respectively). Interestingly, FoxP3^{hi} Tregs

were present at high numbers in the SP at both wk 6 and 8 (39 and 50×10^4 per 10^6 CD25+ counted cells, respectively) but were minimal in the PN at both time points (Fig. 1C). These results indicate that Ig-GAD1 expands phenotypically distinct Tregs that reside in different organs during the onset of disease. In untreated mice FoxP3^{int} and FoxP3^{hi} T cells were present, though at smaller numbers, than Ig-GAD1-treated mice (supplemental Fig 2B and C). Furthermore, their residential patterns at week 6 and 8 of age were similar to Ig-GAD1-expaneded T cells, possibly indicating that both FoxP3^{int} and FoxP3^{hi} T cells arise in the normal repertoire and can be expanded by Ig-GAD1.

FoxP3^{int} T cells express the Th17 signature RORγt transcription factor

Given their differential expression of mTGFβ and location during insulitis, we sought to further characterize the two populations of Tregs for expression of trafficking molecules, transcription factors, and effector cytokines. We performed these analyses at 6-wks of age in the SP, before the cells begin trafficking and become subject to local environmental changes. Figure 2 shows that 73.2% of CD4$^+$CD25$^+$ FoxP3^{int} cells express mTGFβ while only 12.4% of the CD4$^+$CD25$^+$FoxP3^{hi} Tregs had mTGFβ (Fig. 2A). Given that the 2 populations had different localization patterns during progressive insulitis and that CD62L is known to regulate Treg trafficking and function in NOD mice (31–33), the cells were tested for CD62L expression. Not surprisingly, 87.3% of splenic FoxP3^{int} Tregs expressed CD62L while only 3.2% of FoxP3^{hi} Tregs had CD62L. These findings may explain the presence of FoxP3^{int} but not FoxP3^{hi} Tregs in the PN during insulitis (32). GITR protein was, however, expressed to a similar extent on both FoxP3^{int} and FoxP3^{hi} Tregs (92.5 and 93.8%, respectively) (Fig. 2A).

The most surprising finding in these analyses is that FoxP3^{int} mTGFβ$^+$CD62L$^+$ but not FoxP3^{hi}mTGFβ$^-$CD62L$^-$ cells had an impressive level of the Th17 signature transcription factor RORγt (Fig. 2A). Indeed, 77.6% of FoxP3^{int} cells expressed RORγt protein while FoxP3^{hi} cells had no detectable RORγt. FoxP3$^+$RORγt$^+$ double-positive T cells have been described recently and were thought to represent transitional differentiation intermediates geared toward single-positive RORγt$^+$ Th17 or FoxP3$^+$ Tregs (2,19). These intermediates did not produce IL-17 under non-polarizing conditions *in vitro* (Supplementary Fig. 3), which is in good agreement with reports showing that FoxP3$^+$RORγt$^+$ intermediates are unable to secrete IL-17 due to inhibition by FoxP3 (24,25).

The two other molecules that were analyzed were ICOS and IL-10. Similar to what was observed in humans (34), FoxP3^{int}mTGFβ$^+$ Tregs express lower levels of ICOS (33.2%) than FoxP3^{hi}TGFβ$^-$ cells (63.8%) (Fig. 2A). Also, in agreement with what has been demonstrated in human Tregs (34), FoxP3^{hi} cells, which are highly ICOS$^+$ (Fig. 2A), secrete greater amounts of IL-10 (75.9%) than their FoxP3^{int} counterparts (29.9%) (Fig. 2A). Overall, the FoxP3^{int}RORγt$^+$ intermediates express CD62L and mTGFβ while the FoxP3^{hi} Tregs express ICOS and produce IL-10 and both populations have GITR (Fig. 2A).

Given that both the FoxP3^{int}RORγt$^+$ intermediates and FoxP3^{hi} Tregs express suppressive molecules, it is possible that both could display suppressive function *in vitro*. This premise was tested in an allogenic system where effector NOD cells were incubated with C57BL/6 dendritic cells in the presence or absence of highly purified FoxP3^{int}RORγt$^+$ intermediates or FoxP3^{hi} Tregs and proliferation of effector T cells was measured. The results indicated that both populations displayed significant suppression of NOD effector T cell responses *in vitro* (Supplementary Fig. 4).

Splenic FoxP3^{int}RORγt$^+$ T cells protect against passive TID

mTGFβ serves as a suppressive molecule on Tregs (23,35–37). In fact, disease suppression by Ig-GAD1-expanded Tregs was dependent on mTGFβ as a blocking anti-TGFβ antibody

abrogated protection (23). Before testing the *in vivo* function of highly purified FoxP3^{int} cells, we used the NOD.FoxP3-GFP reporter mouse and ensured that Ig-GAD1 treatment expands these intermediates and yields sufficient cells for testing against diabetes. Subsequently, the mice were treated with Ig-GAD1 and FoxP3^{-}, FoxP3^{int} (which highly co-express RORγt), or FoxP3^{hi} (RORγt^{-}) T cells were sorted on the basis of FoxP3 expression. To test for protection against diabetes, the sorted cells were adoptively transferred into NOD.scid recipient mice along with splenocytes from recently diabetic mice. Blood glucose levels were then monitored for 6 wks post-transfer. The results indicate that by wk 6 after transfer, 100% of the mice that received the control FoxP3^{-} T cells were diabetic (Fig. 2B). Mice recipient of both FoxP3^{int} and FoxP3^{hi} Tregs showed protection from diabetes as 70% and 30% were protected from disease by wk 6, respectively (Fig. 2B). Statistical analyses indicated that FoxP3^{int} display significant protection relative to FoxP3^{-} T cells ($p = 0.0016$) while FoxP^{hi} were less protective at this time point after transfer ($p = 0.0778$). Overall, this data indicates that FoxP3^{int}RORγt^{+} intermediates, which are found in the PN during the onset of insultis, exercise suppressive function *in vivo*, to a better extent than FoxP3^{hi} counterparts.

Ig-GAD1 treatment sustains migration of FoxP3^{int}RORγt^{+} T cells to the pancreas during insulitis

To protect against passive or spontaneous diabetes, splenic FoxP3^{int} Tregs likely migrate to the pancreatic lymph node (PLN) and the PN, where they can suppress effector cells (Fig. 1C). Since these cells express RORγt (Fig. 2A) there is potential to exercise their function as FoxP3^{int}RORγt^{+} intermediate cells. Alternatively, the cells could complete their differentiation on the way to the inflammatory site and perform suppression as fully differentiated FoxP3^{int}RORγt^{-} Tregs. To test this premise, we began by analyzing the dynamics of the FoxP3 cells during the onset of insulitis. The results indicate that in the Ig-GAD1-treated mice the FoxP3^{int} cells are located both in the PLN (Fig. 3A) and PN (Fig. 3C) at wk 6 as well as wk 8 of age. However, in untreated mice, the cells are found in the PLN and PN at wk 6 but by wk 8 only a very small number reside in the PLN and the majority of the cells are located in the PN (Fig. 3B, D). Quantification of FoxP3^{+}mTGFβ^{+} T cell numbers indicated that Ig-GAD1 therapy increases the frequency of these cells both in the PLN and PN during progressive insulitis relative to untreated mice (Figure 3E, F). Indeed, the frequency of FoxP3^{+}mTGFβ^{+} Tregs at wk 8 of age increased form 0.5×10^{4} per 10^{6} CD4^{+} T cells in the untreated mice to 2.4×10^{4} per 10^{6} CD4^{+} T cells in Ig-GAD1 treated animals. The accumulation of these suppressive cells at the site of inflammation likely suggests a protective role against diabetes and offers a mechanism by which Ig-GAD1 therapy delays the disease (23).

Pancreatic FoxP3^{int}RORγt^{+} T cells protect against disease without further differentiation

To determine whether these regulatory cells locate to the site of inflammation and perform suppressive function as FoxP3^{int} intermediates bearing RORγt or rather as fully differentiated FoxP3^{int}RORγt− Tregs, we tested the PLN and PN resident FoxP3^{int}TGFβ^{+} cells for expression of RORγt at wk 6 and 8 of age in both untreated as well as Ig-GAD1 treated NOD mice. The findings indicate that, in the Ig-GAD1 treated mice, the majority of FoxP3^{int}mTGFβ^{+} Tregs strongly maintain their RORγt^{+} phenotype both in the PLN (97.5% and 95.3% at wk 6 and 8, respectively) (Fig. 4A) and the PN (97.7% and 98.1% at wk 6 and 8, respectively) (Fig. 4B). No detectable single-positive FoxP3^{int}RORγt− cells were found in either organ, indicating that conversion among the TGFβ^{+} Treg population was minimal. Similarly, in the untreated mice, 97.7% of the PLN (Fig. 4A) and 91.9% of the PN (Fig. 4B) FoxP3^{int}mTGFβ^{+} Tregs express RORγt at wk 6 with no detectable single-positive FoxP3^{int}RORγt− cells. Also, at wk 8 when FoxP3^{int}mTGFβ^{+} Tregs were minimally present in the PLN (Fig. 3B) the majority (95.9%) of the cells available in the PN express RORγt, indicating that conversion was not operative. These results indicate that FoxP3^{int}TGFβ^{+} T cells

express RORγt and maintain their intermediate (FoxP3^{int}TGFβ^{+} RORγt^{+}) phenotype upon migration to the inflammatory sites.

The accumulation of FoxP3^{int}TGFβ^{+}RORγt^{+} intermediates in the PN of mice treated with Ig-GAD1, which are protected from diabetes, suggest that these cells are potentially functional and contribute to resistance against the disease. To test this premise, FoxP3^{int} Tregs were purified from the PN of 6- and 8-wk-old mice on the basis of GFP expression and transferred to NOD.scid recipients along with diabetogenic splenocytes and the recipients were monitored for blood glucose levels. The results indicated that 6- as well as 8-wk-old pancreatic FoxP3^{int}TGFβ^{+}RORγt^{+} Tregs were strongly protective as 70% (Fig. 4C) and 80% (Fig. 4D) of the mice were protected from diabetes, respectively, while all (100%) of the control mice without Treg transfer developed diabetes (Fig. 4E). To determine whether the cells were further differentiated in the recipient mice, we harvested the SP, PLN and PN from the hosts and analyzed the cells for FoxP3 and RORγt expression. As indicated in Figure 4E, 93.7% of FoxP3^{int} cells in the SP express RORγt. Interestingly, the 99.3% of the FoxP3^{int} cells that migrated to the PLN also maintain expression of RORγt (Fig. 4E, median panel). Equally important, 89.4% of the FoxP3^{int} cells that migrated to the PN still co-express RORγt (Fig. 4E, right panel). Overall, these findings indicate that FoxP3^{int}TGFβ^{+}RORγt^{+} cells migrate to the site of inflammation and protect against diabetes without loss of RORγt or full differentiation to FoxP3^{+}RORγt^{-} Tregs.

CD62L is required by FoxP3^{int}RORγt^{+} Treg for migration to the pancreas and protection against diabetes

Both FoxP3^{hi} and FoxP3^{int} Tregs are present in the SP but only FoxP3^{int} express CD62L (Fig. 2A) and migrate to the PN (Fig. 1C). Given that CD62L was previously shown to facilitate trafficking of Tregs to the PLN (32), it is possible that FoxP3^{int} cells preserve CD62L expression to migrate to the site of inflammation and confer resistance against diabetes. To address this point, we began by determining whether Ig-GAD1-expanded FoxP3^{int}RORγt^{+} cells that migrate to the PN maintain CD62L expression. The results presented in Fig. 5a indicate that pancreatic FoxP3^{int}RORγt^{+} Tregs maintain high levels of CD62L expression at both wk 6 and wk 8 of age in untreated as well as Ig-GAD1-treated mice. Indeed, at wk 6 of age, 89% of FoxP3^{int} Tregs from both Ig-GAD1-treated and untreated mice express CD62L (Fig. 5A). Similarly, at wk 8, most (86%) of the cells preserved CD62L expression in Ig-GAD1-treated mice while 78% had CD62L in the untreated mice (Fig. 5A). This data suggests that FoxP3^{int}TGFβ^{+}RORγt^{+} Tregs might rely on CD62L to traffic to the PN, the site of inflammation in the NOD mouse model. To determine whether CD62L is required for migration of FoxP3^{int}TGFβ^{+}RORγt^{+} T cell to the PN, mice were given Ig-GAD1 therapy (to expand Tregs) accompanied with a neutralizing anti-CD62L antibody and migration of the cells to the PN was evaluated. The results in Figure 5B indicate that in vivo neutralization of CD62L retains FoxP3^{int} Tregs in the SP that can no longer migrate to the PN (Fig. 5B). This indicates that CD62L is required for trafficking of FoxP3^{int}RORγt^{+} Tregs to the site of inflammation.

To determine whether CD62L-mediated trafficking of FoxP3^{int}RORγt^{+} Tregs to the PN is required for protection against diabetes, NOD.scid mice were adoptively transferred with pancreatic FoxP3^{int}RORγt^{+} Tregs along with diabetogenic splenocytes and the hosts were given anti-CD62L antibody or an isotype control. The mice were then monitored for blood glucose levels. The results show that neutralization of CD62L nullifies the suppressive function of FoxP3^{int}RORγt^{+} Tregs as the mice were no longer protected against diabetes (Fig. 5C). Indeed, 80% of the mice given anti-CD62L were diabetic by wk 6 post cell transfer (Fig. 5C top panel). However, all mice (100%) recipient of Rat IgG instead of anti-CD62L remained free from diabetes (Fig. 5C median panel). Neutralization of CD62L protein had no effect on

the ability of effector diabetogenic splenocytes to induce TID in this adoptive transfer model. This conclusion is drawn from the observation that transfer of diabetic splenocytes without FoxP3^{int}RORγt^{+} Tregs, leads to diabetes when the mice are given anti-CD62L antibody. In fact, 100% of the mice in this control group became diabetic by wk 6 post-cell transfer (Fig. 5C, bottom panel), which parallels observations reporting that diabetogenic T cells enter the pancreas through an L-selectin independent pathway (38). Though, other studies have shown that anti-CD62L antibody can interfere with migration of diabeteogenic T cells and protect against diabetes (39). This however, required frequent injection of a higher dose of the antibody and used wild type NOD rather than NOD.scid mice. Overall, the results demonstrate that FoxP3^{int}RORγt^{+} Tregs require CD62L to traffic to the PN and perform suppressive function, which bodes well with reports indicating that NOD.CD62L$^{-/-}$ animals develop robust TID (40).

FoxP3^{int}RORγt^{+} T cells retain potential for differentiation into either Th17 or FoxP3^{hi} Tregs

FoxP3^{int} T cells, despite their high co-expression of RORγt, function as Tregs since they suppress effector T cells in vitro, migrate to the PN in vivo and confer resistance against passive diabetes. The question then is whether these cells represent a new subset of Tregs with a fixed phenotype or retain RORγt to preserve program plasticity with potential for differentiation into Th17 cells as was previously suggested (19). To test this premise, we sought to determine whether FoxP3^{int} Tregs could be polarized towards a Th1, Th17, or FoxP3^{hi} Treg phenotype. Accordingly, FoxP3^{int} and FoxP3^{hi} Tregs were sorted from NOD.FoxP3-GFP reporter mice and expression of FoxP3 was analyzed prior to investigation of polarization. Figure 6A shows that the two populations had the expected FoxP3 level and were highly pure. Subsequently, the cells were polarized under Th1 (Fig. 6B), Th17 (Fig. 6C), and Treg (Fig. 6D) conditions and analysed for IL-17 and IFNγ cytokine production. The results show that the FoxP3^{int} population, which strongly expresses the transcription factor RORγt, could easily be polarized towards a Th17 phenotype as 37% of the cells produced high levels of IL-17 cytokine (Fig. 6C). Interestingly, this population was unable to differentiate towards a Th1 phenotype as measured by IFNγ production and no detectable cells were positive for this cytokine (Fig. 6B). Under Treg polarizing conditions, FoxP3^{int} Tregs did not secrete significant levels of IL-17 or IFNγ (0.91%) (Fig. 6D). FoxP3^{hi} Tregs appear to be terminally differentiated and were unable to produce IL-17 or IFNγ under Th1, Th17 or Treg – polarizing conditions (Fig. 6B–D). It seems, then, that Tregs that highly express FoxP3 are unable to polarize towards Th17 and the earlier reports of Treg to Th17 polarization may have included cells expressing low level of FoxP3 Tregs than can convert to T17 cells (19).

The FoxP3^{int} cells were further analysed for FoxP3 and RORγt mRNA upon polarization to Tregs and Th17 cells. Upon Treg polarization, FoxP3 mRNA levels rose from 1 relative quantity (RQ) pre-polarization to 5 RQ after polarization while RORγt remained the same (Fig. 6E). However, under Th17 polarizing conditions while FoxP3 was similar pre- and post-polarization, RORγt rose from 1 to 4.1 RQ pre- to post-polarization (Fig. 6E). Similar results were obtained at the protein level (not shown). Overall, these findings indicate that the FoxP3^{int}RORγt^{+} population represents a differentiation intermediate stage that can further develop into either FoxP3^{hi} Tregs or Th17 cells.

FoxP3^{int}RORγt intermediate Tregs arise in the periphery

To better understand the origin of these suppressive FoxP3^{int}RORγt^{+} Tregs, we sought to determine whether or not this population originates from the thymus in its FoxP3^{int} form or arises in the periphery. To address this question, thymi from 6-wk-old NOD mice were harvested and FoxP3 protein level was measured on the CD4^{+}CD25^{+} population, which represents 3.3% of all CD4^{+} single-positive T cells (Fig. 7A). The majority (76.5%) of these cells were FoxP3^{hi} cells with the rest not expressing FoxP3 and FoxP3^{int} could be observed.

Further phenotypic analyses indicated that these thymic FoxP3 cells did not express RORγt, mTGFβ, or CD62L (Fig. 7B). These results indicate that the FoxP3^{int}RORγt^{+} cells, which are observed in the SP, PLN and PN do not develop their FoxP3^{int} RORγt^{+} phenotype while in the thymus but rather in the periphery, which is in agreement with the idea that this population is a differentiation intermediate of peripheral CD4^{+} T cells (2,19).

DISCUSSION

The notion of Th cell plasticity (2,19–22) suggests that the immune response is far more adaptable than was previously thought and is therefore able to respond more appropriately to environmental stimuli. Initially, the naïve Th cell was viewed as a pluripotent precursor that could engage in one of multiple pathways and differentiate into a terminal Th1, Th2, Th17 or Treg cell (1). More recently, however, observations were made indicating that fully differentiated Tregs can reverse into Th17 cells (19) and a Th17 cell can switch to a Th1 phenotype (20–22). Evidence is accumulating indicating that Th plasticity is even broader and Th precursors engage in more than one pathway during differentiation, generating intermediate phenotypes susceptible for further programming (19,41,42). However, since intermediate cells are thought of as having a transient phenotype available only at low frequency, analysis of the mechanism underlying the mixed phenotypes or their function was limited. Herein, we identified a FoxP3^{int}RORγt^{+} intermediate population that arises in the natural T cell repertoire alongside FoxP3^{hi} (RORγt^{-}) Tregs that could be expanded without further differentiation. These cells do not represent artifacts of the NOD.FoxP3-GPF reporter mice because they are expandable with Ig-GAD1 in normal NOD mice and display similar residential pattern in both strains (Supplemental Fig. 5). The cells arise *in vivo* before and after expansion with Ig-GAD1 and evidence is provided indicating that they express mTGFβ that serves for suppression of effector T cells (Fig. 1B,Supplementary Fig. 2) and CD62L that facilitates trafficking to sites of inflammation (Fig. 5A–C). In the PN, which is the site of inflammation in NOD mice, the cells retained the FoxP3^{int}RORγt^{+} intermediate phenotype (Fig. 4A, B) as well as CD62L (Fig. 5A) and TGFβ (Fig. 4A, B), likely suggesting that they contribute to protection against the disease as intermediate cells. In fact, when the expanded cells were isolated from both the SP and the PN and transferred to NOD.scid mice along with diabetogenic splenocytes, they were able to traffic again to the PLN and PN in the host animals (Fig. 4E) and confer resistance against diabetes (Fig. 4C, D). Surprisingly, upon migration to the PN the cells did not down-regulate RORγt and protected against disease as FoxP3^{int}RORγt^{+} bearing both CD62L and TGFβ. However, if the cells are subject to polarization, they retain the ability to fully differentiate to Th17 cells and produce IL-17 cytokine (Fig. 6C), or Tregs with higher FoxP3 but significantly reduced RORγt (Fig. 6). It was previously shown that CD4^{+} T cells expressing CD62L protect against diabetes (43,44). These suppressor cells may represent classical Tregs that utilize CD62L to traffic to the site of inflammation. However, it is not clear whether they display the differentiation intermediate phenotype.

Since CD62L and TGFβ were expressed on the intermediate Tregs in the natural repertoire prior to inflammation, it is unlikely that they were induced by factors related to effector T cells. Rather, it would be more logical to envision that they arise prior to the onset of disease to guide the Tregs to the site of inflammation and target the local effector T cells regardless of their phenotype. This indeed provides a functional plasticity that would add flexibility relative to effector dictated specific trafficking and suppression reported recently (41,42).

Overall, the findings reported in this study indicated that Th intermediates display both differentiation (1,19–22,45) and functional plasticity that could sustain measured but broad effectiveness against autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

REFERENCES

1. Weaver CT, Hatton RD, Mangan PR, Harrington LE. IL-17 family cytokines and the expanding diversity of effector T cell lineages. Ann. Rev. Immunol 2007;25:821–852. [PubMed: 17201677]
2. Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, Riethmacher D, Si-Tahar M, Di Santo JP, Eberal G. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ FoxP3+ RORγt + T cells. J. Exp. Med 2008;205:1381–1393. [PubMed: 18504307]
3. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor FoxP3. Immunity 2005;22:329–341. [PubMed: 15780990]
4. Chen W, Jin W, Hardegen N, Lei K, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25− naïve T cells to CD4+CD25+ regulatory T cells by TGF-β induction of transcription factor FoxP3. J. Exp. Med 2003;198:1875–1886. [PubMed: 14676299]
5. Fantini MC, Becker C, Montelone G, Pallone F, Galle PR, Neurath MF. Cutting Edge: TGF-β induces a regulatory phenotype in CD4+CD25− T cells through FoxP3 induction and down-regulation of smad7. J. Immunol 2004;172:5149–5153. [PubMed: 15100250]
6. Wu Y, Borde M, Heissmeyer V, Feurer M, Lapan AD, Stroud JC, Bates DL, Guo L, Han A, Ziegler SF, Mathis D, Benoist C, Chen L, Rao A. FoxP3 controls regulatory T cell function through cooperation with NFAT. Cell 2006;126:375–387. [PubMed: 16873067]
7. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor FoxP3. Science 2003;299:1057–1061. [PubMed: 12522256]
8. Shevach ME. From vanilla to 28 flavors: Multiple varieties of T regulatory cells. Immunity 2006;25:1996–2001.
9. Tang Q, Bluestone JA. The Foxp3^{+} regulatory T cells : a Jack of all trades, master of regulation. Nat. Immunol 2008;9:239–244. [PubMed: 18285775]
10. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORγt directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121–1133. [PubMed: 16990136]
11. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFβ in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006;24:179–189. [PubMed: 16473830]
12. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT. Transforming growth factor-β induces development of the Th17 lineage. Nature 2006;441:231–234. [PubMed: 16648837]
13. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphey TL, Murphey KM, T C. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat. Immunol 2005;6:1123–1132. [PubMed: 16200070]
14. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang Y, Wang Y, Hood L, Zhu Z, Tian Q, Q, Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat. Immunol 2005;6:1133–1141. [PubMed: 16200068]
15. Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: An effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006;24:677–688. [PubMed: 16782025]
16. Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 2008;28:454–465. [PubMed: 18400188]
17. Carlson T, Kroenke M, Rao P, Lane TE, Segal B. The Th17-ELR+ CXC chemokine pathway is essential for the development of central nervous system autoimmune disease. J. Exp. Med 2008:b: 811–b: 823.
18. Jain R, Tartar DM, Gregg RK, Divekar RD, Bell JJ, Lee HH, Yu P, Ellis JS, Hoeman CM, Franklin CL, Zaghouani H. Innocuous IFNγ induced by adjuvant-free antigen restores normoglycemia in NOD mice through inhibition of IL-17 production. J. Exp. Med 2008;205:207–218. [PubMed: 18195074]

19. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, Shah B, Chang SH, Schluns KS, Watowich SS, Feng X-H, Jetten AM, Dong C. Molecular antagonism an plasticity of regulatory and inflammatory T cell programs. Immunity 2008;29:44–56. [PubMed: 18585065]
20. Lexberg MH, Taubner A, Forster A, Albrecht I, Richter A, Kamradt T, Radbruch A, Chang H. Th memory for interleukin-17 expression is stable *in vivo*. Eur. J. Immunol 2008;38:2654–2664. [PubMed: 18825747]
21. Bending D, La Pena HD, Veldhoen M, Phillips JM, Uyttenhove C, Stockinger B, Cooke A. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. J. Clin. Inves 2009;119:565–573.
22. Martin-Orozco N, Chung Y, Chang SH, Wang Y, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion to Th1 cells. Eur. J. Immunol 2009;39:216–224. [PubMed: 19130584]
23. Gregg R, Jain R, Schoenleber SJ, Divekar R, Bell JJ, Lee HH, Yu P, Zaghouani H. A sudden decline in active membrane-bound TGF-β impairs both T regulatory cell function and protection against autoimmune diabetes. J.Immunol 2004;173:7308–7316. [PubMed: 15585854]
24. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, Takaesu G, Hori S, Yoshimura A, Kobayashi T. FoxP3 inhibitis RORγt-mediated IL-17A mRNA transcription through direct interaction with RORγt. J. Biol. Chem 2008;283:17003–17008. [PubMed: 18434325]
25. Zhang F, Guangxun M, Strober W. Interactions among the transcription factors Runx1, RORγt and FoxP3 regulate the differentiation of interleukin 17-producing T cells. Nat. Immunol 2008;9:1297–1306. [PubMed: 18849990]
26. Zhou L, Lopes JE, Chong MMW, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. TGF-β-induced FoxP3 inhibits Th17 cell differentiation by antagonizing RORγt function. Nature 2008;453:236–240. [PubMed: 18368049]
27. Katz JD, Wang B, Haskins K, Benoist C, Mathis D. Following a diabetogenic T cell from genesis through pathogenesis. Cell 1993;74:1089–1100. [PubMed: 8402882]
28. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulitis in nonobese diabetic mice. Nature 1993;366:72. [PubMed: 8232539]
29. Legge KL, Min B, Bell JJ, Caprio JC, Li L, Gregg RK, Zaghouani H. Coupling of peripheral tolerance to endogenous interleukin 10 promotes effective modulation of myelin-activate T cells and ameliorates experimental allergic encephalomyelitis. J. Exp. Med 2000;191:2039–2048. [PubMed: 10859329]
30. Faveeuw C, Gagnerault MC, Lepault F. Isolation of leukocytes infiltrating the islets of langerhans of diabetes-prone mice flow cytometric analysis. J. Immunol. Methods 1995;187:163–169. [PubMed: 7490452]
31. Pop SM, Wong CP, Culton DA, Clarke SH, Tisch R. Single cell analysis shows decreasing FoxP3 and TGFβ1 coexpressing CD4+CD25+ regulatory T cells during autoimmune diabetes. J. Exp. Med 2005;201:1333–1346. [PubMed: 15837817]
32. Venturi GM, Conway RM, Steeber DA, Tedder TF. CD25+CD4+ Regulatory T cell migration requires L-selectin expression: L-selectin transcriptional regulation balances constitutive receptor turnover. J. Immnol 2006;178:291–300.
33. Tarbell KV, Petit L, Zuo X, Toy P, Luo X, Mqadmi A, Yang H, Suthanthiran M, Mojsov S, Steinman RM. Dendritic cell-expanded, islet-specific CD4+ CD25+ CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. J. Exp. Med 2007;204:191–201. [PubMed: 17210729]
34. Ito T, Hanabuchi S, Wang Y, Park WR, Arima K, Bover L, Qin FX, Gilliet M, Liu Y. Two functional subsets of FoxP3+ regulatory T cells in human thymus and periphery. Immunity 2008;28:870–880. [PubMed: 18513999]
35. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by $CD4^+CD25^+$ regulatory T cells is mediated by cell surface-bound transforming growth factor β. J. Exp. Med 2001;194:629–644. [PubMed: 11535631]
36. Nakamura K, Kitani A, Fuss I, Pedersen A, Harada N, Nawata H, Strober W. TGF-β1 plays an important role in the mechanism of $CD4^+CD25^+$ regulatory T cell activity in both humans and mice. J. Immunol 2004;172:834–842. [PubMed: 14707053]

37. Andersson J, Tran DQ, Pesu M, Davidson TS, Ramsy H, O'Shea JJ, Shevach EM. CD4+FoxP3+ regulatory T cells confer infectious tolerance in a TGF-β-dependent manner. J. Exp. Med 2008;205:1975–1981. [PubMed: 18710931]

38. Lepault F, Gagnerault MC, Faveeuw C, Bazin HH, Boitart C. Lack of L-selectin expression by cells transferring diabetes in NOD mice: insights into the mechanisms involved in diabetes prevention by Mel-14 antibody treatment. Eur. J. Immunol 1995;25(6):1502–1527. [PubMed: 7542194]

39. Yang X, Karin N, Tisch R, Steinman L, McDevitt H. Inhibition of insulitis and prevention of diabetes in nonobese diabetic mice by blocking L-selectin and very late antigen 4 adhesion receptors. Proc. Natl. Acad. Sci 1993;90:10494–10498. [PubMed: 7504266]

40. Mora C, Grewal IS, Wong FS, Flavell RA. Role of L-selectin in the development of autoimmune diabetes in non-obese diabetic mice. Int. Immunol 2004;16:257–264. [PubMed: 14734611]

41. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat. Immunol 2009;10:595–602. [PubMed: 19412181]

42. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JK, Chu T, Corcoran L, Treuting P, Klein U, Rudensky A. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control Th2 responses. Nature 2009;29:351–357. [PubMed: 19182775]

43. Herbelin A, Gombert JM, Lepault F, Bach JF, Chatenoud L. Mature mainstream TCR alpha beta (+) CD4(+) thymocytes expressing L-selectin mediate 'active tolerance' in the nonobese diabetic mouse. J. Immunol 1998;161(5):2620–2628. [PubMed: 9725264]

44. Seddon B, Saoudi A, Nicholson M, Mason D. CD4+CD8− thymocytes that express L-selectin protect rats from diabetes upon adoptive transfer. Eur. J. Immunol 1996;12:2702–2708. [PubMed: 8921958]

45. Wei G, Wei L, Zhu J, Zang C, Hu-Li J, Yao Z, Cui K, Kanno Y, Roh T, Watford WT, Schones DT, Peng W, Sun H, Paul WE, O'Shea JJ, Zhao K. Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in linieage fate determination of differentiating CD4+ T cells. Immunity 2009;30:155–167. [PubMed: 19144320]

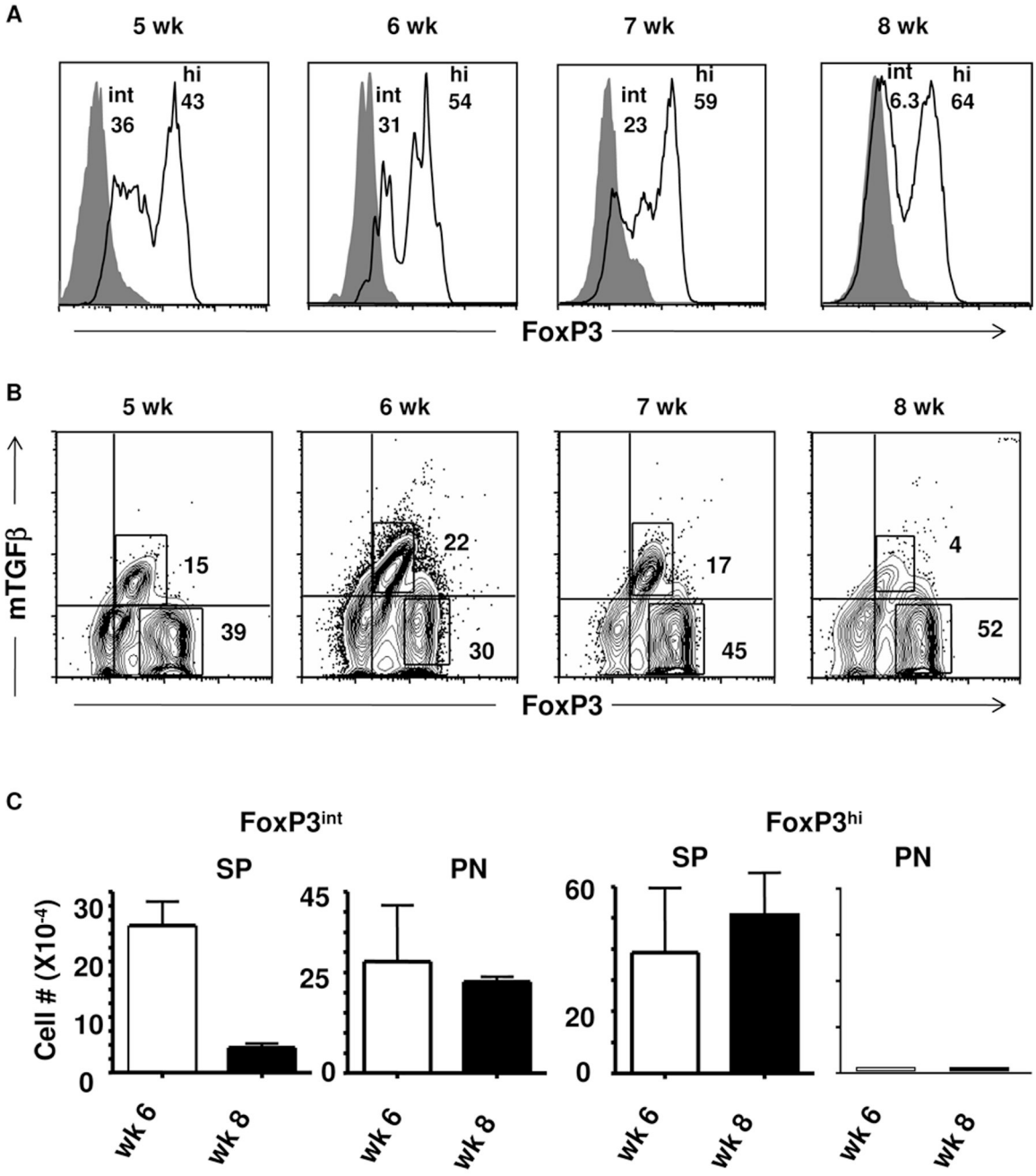

Figure 1. Intermediate but not high level FoxP3 correlates with expression of mTGFβ

(A, B) Splenic cells from Ig-GAD1-treated mice were harvested at wk 5, 6, 7, and 8 of age and stained for surface CD4, CD25 and mTGFβ and intracellular FoxP3. $CD4^+CD25^+$ T cells were analyzed for (A) FoxP3 and (B) TGFβ expression by flow cytometry. Isotype control is represented by filled histograms and quadrants, respectively. In (A) the numbers indicate the percent of cells expressing intermediate (int) and high (hi) level FoxP3. In (B) the numbers represent the percent of cells expressing only FoxP3 or both FoxP3 and TGFβ. (C) $FoxP3^{int}$ and $FoxP3^{hi}$ Treg cell numbers were quantified in both the spleen (SP) and pancreas (PN) at wk 6 and 8 of age. The bars indicate the number of FoxP3 cells per million of $CD4^+CD25^+$

cells. Data are presented as mean of three replicates ± SD. The experiments illustrated in this figure were repeated 3 times.

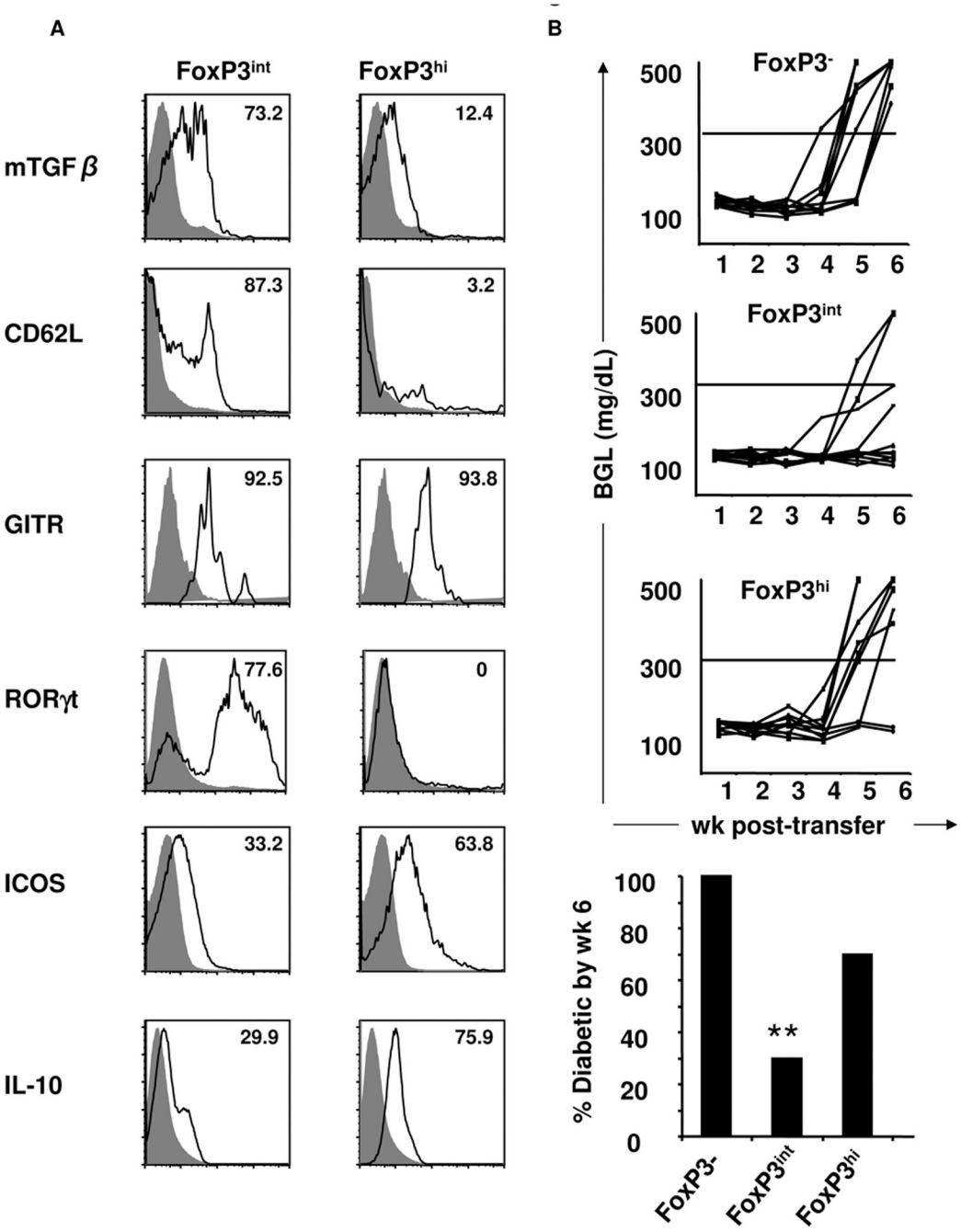

Figure 2. FoxP3^{int} cells co-express RORγt and protect against diabetes

(A) Splenic cells from Ig-GAD1-treated mice were harvested at wk 6 of age and stained for CD4, CD25 and FoxP3 along with the indiacted proteins. The numbers represent percent of FoxP3^{int} and FoxP3^{hi} cells expressing the marker of interest relative to isotype control (filled histograms). Stainings are representative of at least three experiments. (B) Splenic FoxP3-, FoxP3^{int} or FoxP3^{hi} CD4 T cells from Ig-GAD1-treated FoxP3:GFP reporter mice were co-transferred with diabetogenic splenocytes into NOD.scid recipients and the hosts were monitored for blood glucose levels. Lines represent individual mice (N= 10/group). The bars represent the percent of diabetic mice at wk six post-cell transfer. ** p = 0.0016 when compared to mice recipient of FoxP3− cells.

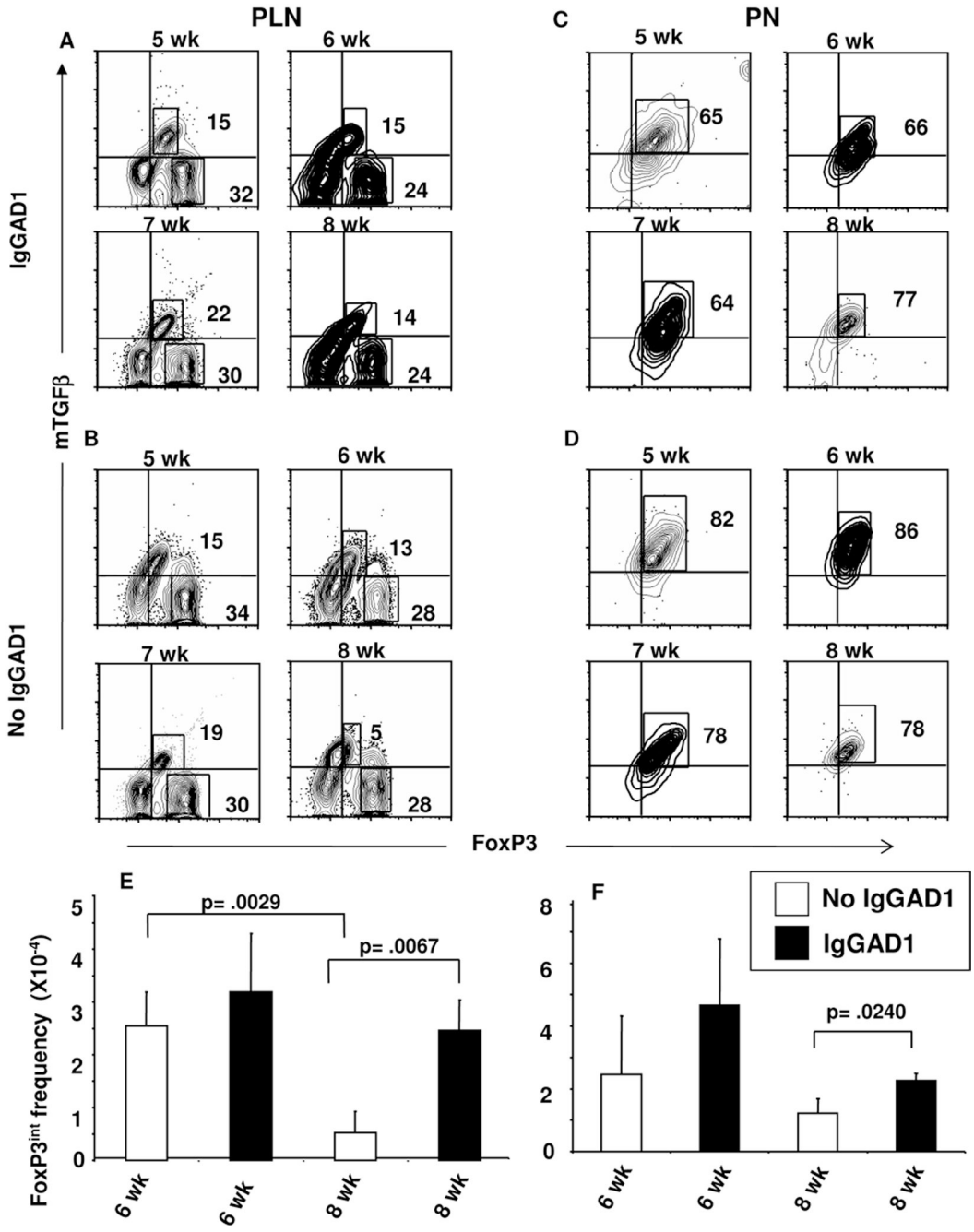

Figure 3. FoxP3^{int} Tregs expressing mTGFβ traffic and accumulate in the sites of inflammation upon treatment with tolerogen

Cells were harvested from (A, B) pancreatic lymph nodes (PLN) or (C, D) PN of (B, D) untreated or (A, C) agg Ig-GAD1-treated NOD mice at wk 5, 6, 7, and 8 of age. The cells were stained for CD4, CD25, FoxP3 and TGFβ and analyzed by flow cytometry. The contour plots show mTGFβ and FoxP3 expression on CD4^{+}CD25^{+} cells. Quadrant lines represent isotype staining levels. (E, F) illustrate the number of CD25^{+}FoxP3^{int} Tregs at wk 6 and 8 with in the (E) PLN and (F) PN of (open bars) untreated and (filled bars) Ig-GAD1-treated mice described in (A–D). Bars represent the mean of three experiments ± SD. P values were generated using the student's t test.

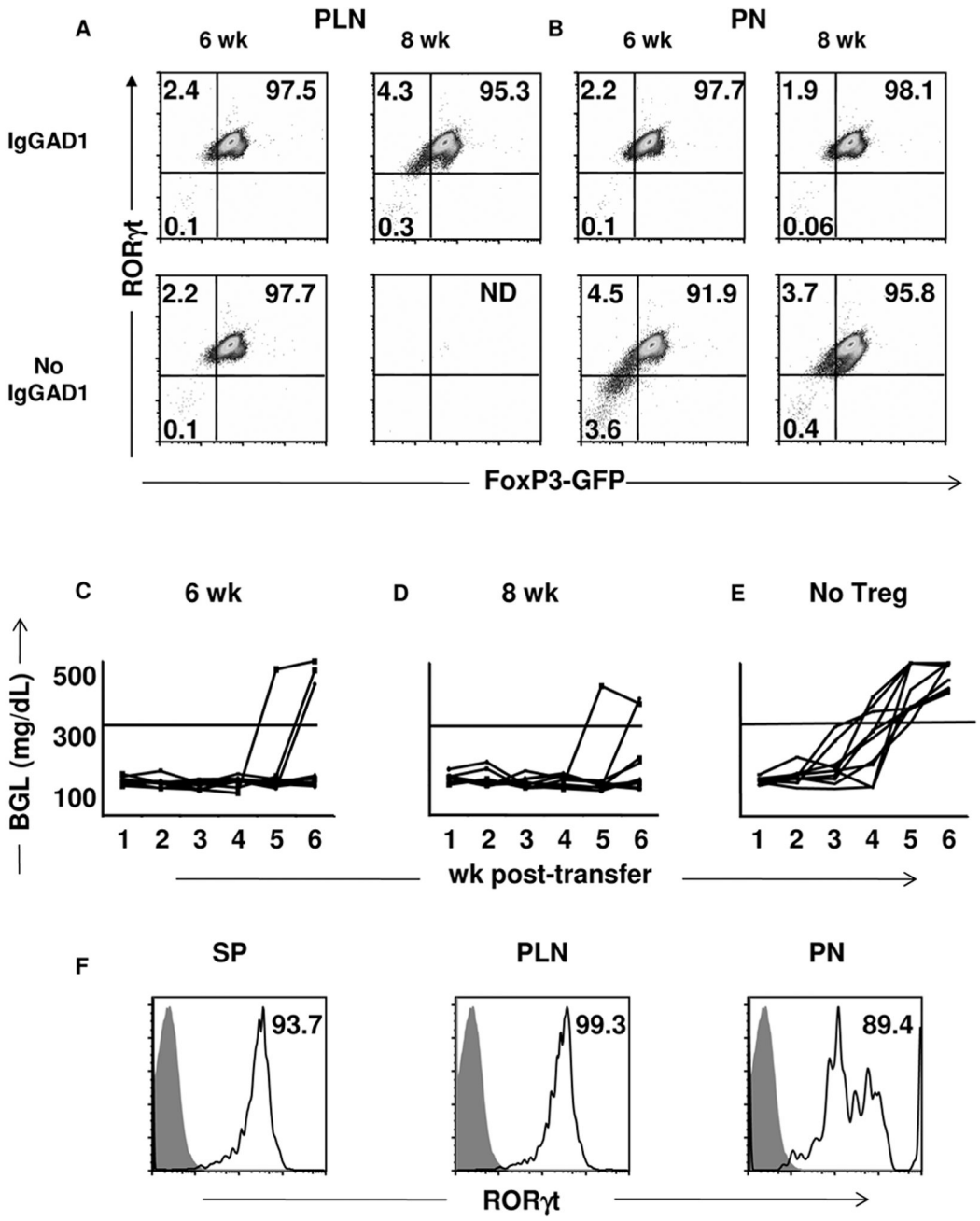

Figure 4. Pancreatic FoxP3^{int} residents maintain RORγt expression

(A) PLN and (B) PN cells were harvested from untreated and Ig-GAD1-treated NOD.FoxP3:GFP reporter mice at wk 6 and 8 and CD4^{+}CD25^{+}mTGFβ^{+} cells were analyzed for RORγt and FoxP3 expression. Quadrants represent isotype staining levels. Stainings are representative of three experiments. (C, D) Pancretic FoxP3^{int} Tregs from Ig-GAD1-treated (C) 6- or (D) 8–wk-old FoxP3:GFP reporter mice were co-transferred with diabetogenic splenocytes into NOD.scid recipients and the hosts were monitored for blood glucose levels. Lines represent individual mice (N= 10/group). (E) control NOD.scid mice recipient of diabetogenic splenocytes but not Tregs (N= 10/group). (F) FoxP3^{int} cells were recovered from host NOD.scid SP, PLN and PN 72 hours after transfer and were analyzed for RORγt

expression. Numbers represent percent of cells expressing RORγt compared to isotype (filled histograms). ND= Not Detected.

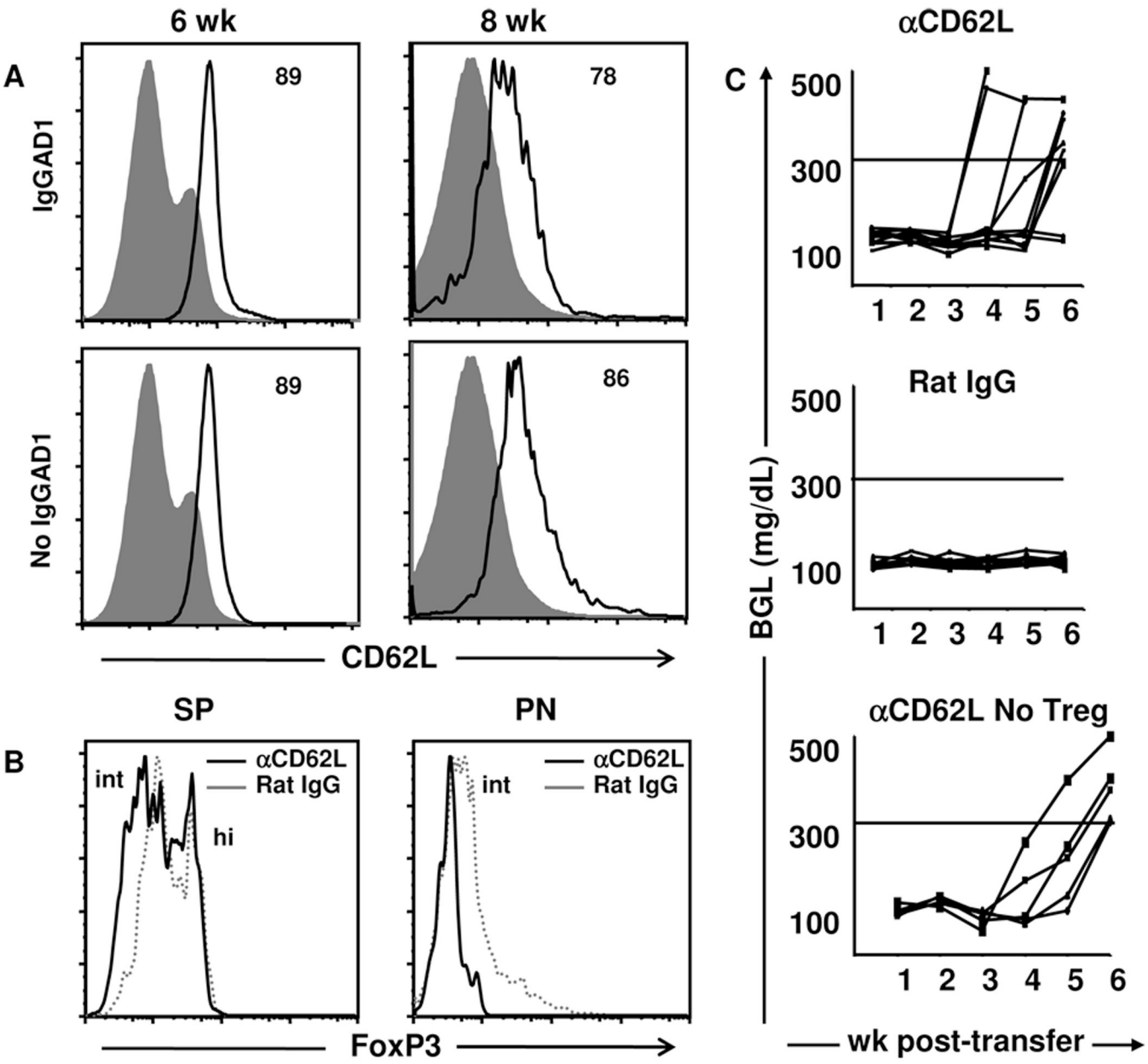

Figure 5. Protection against diabetes by FoxP3^{int} RORγt intermediate cells requires CD62L

(A) Pancreatic cells were harvested at wk 6 and 8 from 8 to 12 untreated or agg Ig-GAD1-treated NOD mice and stained for CD4, CD25, FoxP3 and CD62L and analyzed by flow cytometry. Cells were gated on CD4$^+$CD25$^+$FoxP3^{int} and analyzed for CD62L expression. The numbers represent the percent of cells expressing CD62L compared to isotype levels (filled histograms). (B) 1mg anti-CD62L (MEL-14) (black lines) or isotype-matched Rat IgG antibody (dashed lines) was administered along with Ig-GAD1 at wk 4, 5, and 6. Mice were sacrificed at wk 6 and intracellular FoxP3 levels were measured on splenic (left panel) or pancreatic (right panel) CD4$^+$CD25$^+$ cells. Stainings are representative of at least three experiments. (C) Pancretic FoxP3^{int} Tregs from 6-wk-old Ig-GAD1-treated FoxP3:GFP mice were co-transferred with diabetogenic splenocytes into NOD.scid recipients along with a

neutralizing antibody to CD62L or an isotype control and the hosts were monitored for blood glucose levels. A control group that received only the diabetogenic splenocytes along with neutralizing CD62L antibody was also included Lines represent individual mice (N= 10/ group).

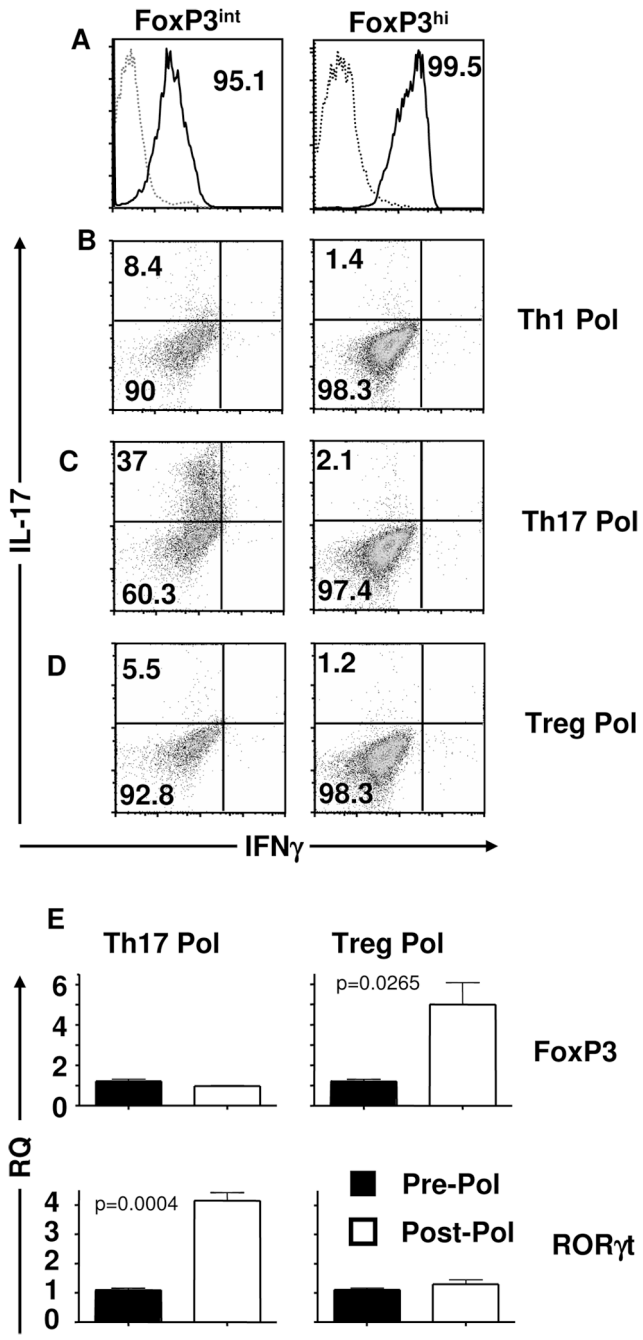

Figure 6. FoxP3^{int} cells can differentiate into FoxP3^{hi} and IL-17 producing Th17 cells upon polarization

(A) FoxP3^{int} (left panel) and FoxP3^{hi} (right panel) T cells were sorted from 8 to 12 mice and polarized for 72 hours under (B) Th1 (Th1 pol), (C) Th17 (Th17 pol), or (D) Treg (Treg pol) polarizing conditions. Following polarization, IFNγ and IL-17 protein expression were measured by intracellular staining. In (B–D) black quadrant lines represent isotype controls. Staining is representative of at least three experimental replicates. (E) FoxP3 and RORγt transcript levels were measured in FoxP3^{int} cells pre- (pre-pol) and post- (post-pol) Treg or Th17 polarization. Bars represent the mean of three replicates ± SD.

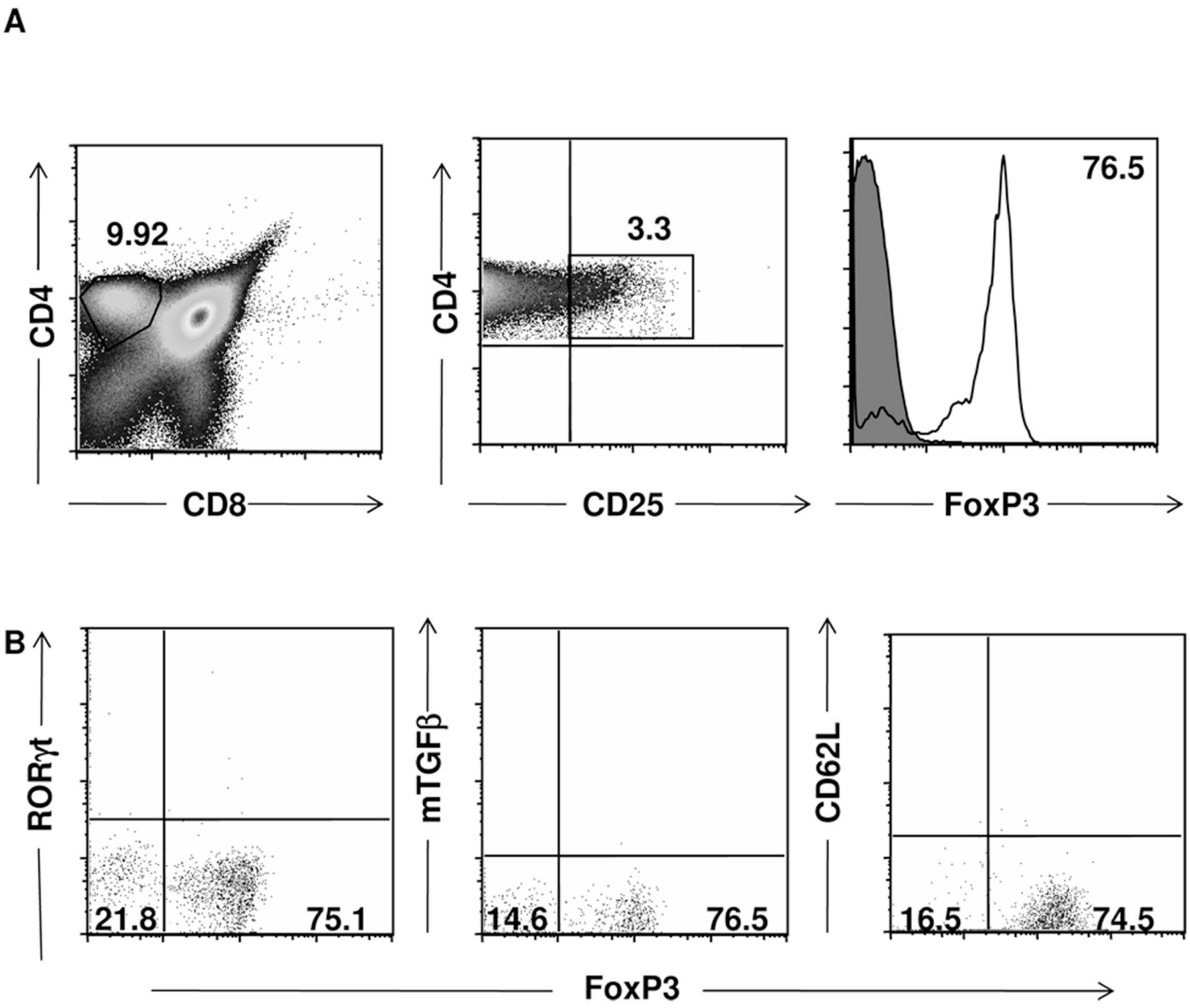

Figure 7. FoxP3$^+$ but not FoxP3$^+$RORγt$^+$ cells are detectable in the thymus

(A, B) Thymi were harvested from 6wk-old NOD mice and were stained for CD4, CD8, CD25, FoxP3 and either RORγt, mTGFβ or CD62L. (A) Gated CD4 single-positive cells were analyzed for CD25 and FoxP3 expression. (B) gated CD4$^+$CD25$^+$ thymocytes were analyzed for FoxP3 and RORγt, mTGFβ or CD62L expression. Isotype staining levels are represented by quadrant bars in dot plots and by filled gray lines in histograms. Stainings are representative of three experiments.