

# Single cell analysis shows decreasing FoxP3 and TGF $\beta$ 1 coexpressing CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells during autoimmune diabetes

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**Natural CD4<sup>+</sup>CD25<sup>+</sup> regulatory T (CD4<sup>+</sup>CD25<sup>+</sup> T reg) cells play a key role in the immunoregulation of autoimmunity. However, little is known about the interactions between CD4<sup>+</sup>CD25<sup>+</sup> T reg cells and autoreactive T cells. This is due, in part, to the difficulty of using cell surface markers to identify CD4<sup>+</sup>CD25<sup>+</sup> T reg cells accurately. Using a novel real-time PCR assay, mRNA copy number of FoxP3, TGF $\beta$ 1, and interleukin (IL)-10 was measured in single cells to characterize and quantify CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in the nonobese diabetic (NOD) mouse, a murine model for type 1 diabetes (T1D). The suppressor function of CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>hi</sup> T cells, mediated by TGF $\beta$ , declined in an age-dependent manner. This loss of function coincided with a temporal decrease in the percentage of FoxP3 and TGF $\beta$ 1 coexpressing T cells within pancreatic lymph node and islet infiltrating CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>hi</sup> T cells, and was detected in female NOD mice but not in NOD male mice, or NOR or C57BL/6 female mice. These results demonstrate that the majority of FoxP3-positive CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in NOD mice express TGF $\beta$ 1 but not IL-10, and that a defect in the maintenance and/or expansion of this pool of immunoregulatory effectors is associated with the progression of T1D.**

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Abbreviations used: Ab, antibody; CD62L<sup>hi</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>hi</sup> T cells; CD62L<sup>lo</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>lo</sup> T cells; CTLA-4, cytotoxic T lymphocyte-associated antigen 4; GITR, glucocorticoid-induced TNF receptor gene; NOD, nonobese diabetic; PLN, pancreatic LN; rh, recombinant human; T reg, regulatory T; T1D, type 1 diabetes.

T1D is an autoimmune disease that is characterized by the selective destruction of the insulin-producing  $\beta$  cells which reside in the islets of Langerhans (1, 2). Studies in the NOD mouse, a spontaneous model of T1D, have demonstrated that CD4<sup>+</sup> and CD8<sup>+</sup> T cells are the primary mediators of  $\beta$  cell destruction (1, 2). The critical events that contribute to the breakdown of self-tolerance to  $\beta$  cells are not well-understood, although defects in central and peripheral tolerance have been implicated in the development and differentiation of pathogenic T effector cells (3–7).

The progression of T1D in NOD mice is marked by two general “checkpoints” (8). The first checkpoint is associated with infiltration of the islets by macrophages, DCs, and B and T cells. This insulinitis begins at  $\sim$ 3 wk of age and is well-established in 8–10-wk-old NOD mice. Despite extensive insulinitis,  $\beta$  cell destruction is limited and NOD mice remain diabetes-free during this period. The second checkpoint corresponds with a shift from “be-

nign” to “aggressive” insulinitis that begins at  $\sim$ 12 wks of age. At this time,  $\beta$  cells are destroyed efficiently to promote overt diabetes. Typically, by 30 wk of age,  $\sim$ 80% and  $\sim$ 20% of NOD female and male mice, respectively, have developed diabetes. How the progression from nondestructive to destructive insulinitis is regulated, or why the disparity in overt diabetes exists between NOD female and male mice, are not clear. However, interrelated events, including islet recruitment of high avidity/affinity  $\beta$  cell-specific T cells that exhibit a type 1 phenotype (9) and failing peripheral immunoregulatory mechanisms, are believed to contribute (5–7).

Aberrant immunoregulation in NOD mice initially was viewed in the context of a functional imbalance between types 1 and 2 T cells. Various studies have demonstrated that induction of type 2 effectors, which are characterized by the secretion of IL-4 and IL-10, effectively prevents and/or suppresses the diabetogenic response in NOD mice (5, 10–12). However, it is now apparent that immunoregulation of autoreactive T cells in general is

The online version of this article contains supplemental material.

highly complex and likely involves a heterogeneous group of immunoregulatory T (and non-T) cell effectors. Indeed, induction of type 1 regulatory T (T reg) cells, IL-10–only–producing CD4<sup>+</sup> T cells, and TGFβ1–secreting Th3 cells have been reported to prevent tissue-specific autoimmunity (13–15).

Another subset of immunoregulatory effectors consists of a naturally occurring population of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells. CD4<sup>+</sup>CD25<sup>+</sup> T reg cells upon TCR–mediated stimulation are characterized by a potent *in vitro* and *in vivo* capacity to suppress proliferation and effector function of CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in addition to modulating the antigen-presenting function of DCs (6, 16–19). Typically accounting for 5–10% of murine peripheral CD4<sup>+</sup> T cells, CD4<sup>+</sup>CD25<sup>+</sup> T reg cells play key roles in modulating immune responses in a variety of models (6, 16–19). For example, a reduced number of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells was associated with exacerbation of T1D in NOD mice which lacked CD80/CD86 or CD28 expression (6).

Current *in vivo* and *in vitro* studies strongly support the view that CD4<sup>+</sup>CD25<sup>+</sup> T reg cells represent a unique lineage of immunoregulatory cells. However, accurate identification, characterization, and quantification of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells have been problematic. The IL-2Rα chain (CD25) has been used widely for the identification and isolation of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, yet this marker also is expressed by activated CD4<sup>+</sup> Th and memory T cells. Other cell surface markers that are used to define CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, including CD62L, cytotoxic T lymphocyte–associated antigen 4 (CTLA-4), glucocorticoid–induced TNF receptor gene (GITR), CD45RB, and CD103, also are shared by “conventional” CD4<sup>+</sup> Th cells (20). Recently, reports demonstrated that the transcription factor, FoxP3, is expressed specifically by CD4<sup>+</sup>CD25<sup>+</sup> T reg cells (21–23). Furthermore, the Rudensky and Sakaguchi groups demonstrated that FoxP3 is a master regulator of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells differentiation. Transfer of FoxP3 cDNA into naive CD4<sup>+</sup> Th cells was sufficient to induce differentiation that phenotypically and functionally resembled CD4<sup>+</sup>CD25<sup>+</sup> T reg cells (21, 23). The precise mode of suppression by CD4<sup>+</sup>CD25<sup>+</sup> T reg cells is not clear, but includes direct cell–cell contact and/or production of inhibitory cytokines, such as TGFβ1 and IL-10 (24–26). Cell surface and secreted TGFβ1 have been associated with cell–cell contact dependent and independent mechanisms of CD4<sup>+</sup>CD25<sup>+</sup> T reg cell–mediated suppression (24, 25). These different mechanisms of suppression may reflect distinct subsets of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells and/or other types of immunoregulatory effector cells.

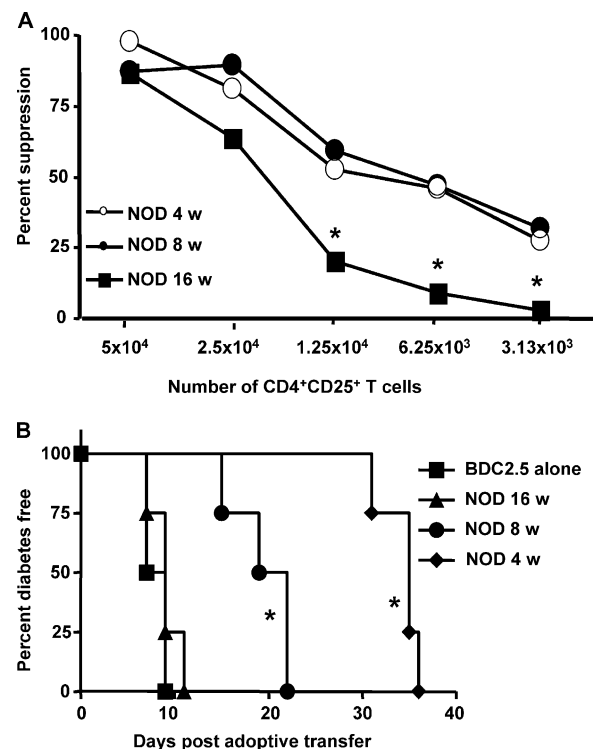
With this in mind, we established an assay that was based on real-time PCR to quantitate and characterize accurately FoxP3 expressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells at the single cell level. This assay was used to test the hypothesis that progression of T1D in NOD mice is due to defects in CD4<sup>+</sup>CD25<sup>+</sup> T reg cell numbers and/or effector function. Evidence is

provided that demonstrates that the progression of benign to aggressive insulinitis corresponds with a temporal decrease in peripheral tolerance as measured by reduced frequencies of FoxP3 and TGFβ1 coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells residing in the PLNs and islets of NOD female mice.

## RESULTS

### The suppressor function of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells decreases in an age-dependent manner

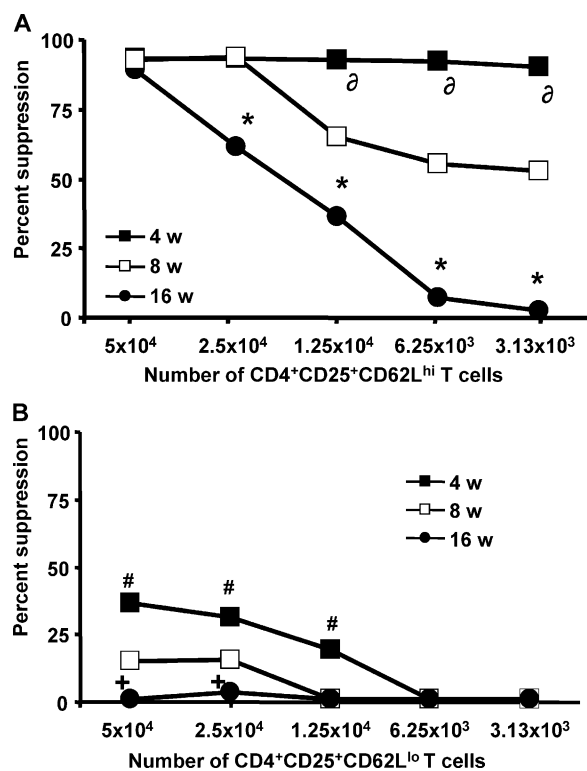
Studies have suggested that defects in CD4<sup>+</sup>CD25<sup>+</sup> T reg cell function may contribute to the progression of β cell autoimmunity in NOD mice (6, 7). Accordingly, the suppressor function of CD4<sup>+</sup>CD25<sup>+</sup> T cells that were prepared from PLNs of 4-, 8-, and 16-wk-old NOD female mice—representing different stages of β cell autoimmunity—was investigated *in vitro*. PLNs are reported to be a key site for activation and tolerance induction of β cell specific T cells



**Figure 1. PLN CD4<sup>+</sup>CD25<sup>+</sup> T reg cells suppressor function declines in an age-dependent manner.** (A) Sorted PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells from NOD female mice varying in age were tested at the indicated cell numbers to suppress proliferation determined via <sup>3</sup>H–thymidine uptake of stimulated 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>−</sup> T cells that were isolated from 8-wk-old NOD female mice. Data are an average of two experiments performed in triplicate. Standard deviation of any given data point  $\leq \pm 10\%$ . [<sup>3</sup>H]thymidine uptake of stimulated CD4<sup>+</sup>CD25<sup>−</sup> T cells alone = 8192  $\pm$  874 cpm. \*P  $\leq$  0.02, 16 wk versus 8 or 4 wk (Student's *t* test). (B) Groups of four NOD.*scid* female mice received 5  $\times$  10<sup>6</sup> CD4<sup>+</sup> BDC2.5 T cells alone or in combination with 5  $\times$  10<sup>5</sup> sorted PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells (>98% purity) from NOD female mice at the indicated ages. \*P  $\leq$  0.01, 4 wk or 8 wk vs. BDC2.5 alone (Kaplan Meier Log Rank test).

(27). PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells that were isolated from 4- and 8-wk-old NOD female mice inhibited proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells similarly in cultures that were stimulated with anti-CD3 mAb and irradiated syngeneic splenocytes depleted of T cells (Fig. 1 A). In contrast, a significant decrease in the suppressor function of PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells that were isolated from 16-wk-old mice versus 4- and 8-wk-old NOD mice was observed at limiting cell numbers ( $P \leq 0.02$ ) (Fig. 1 A).

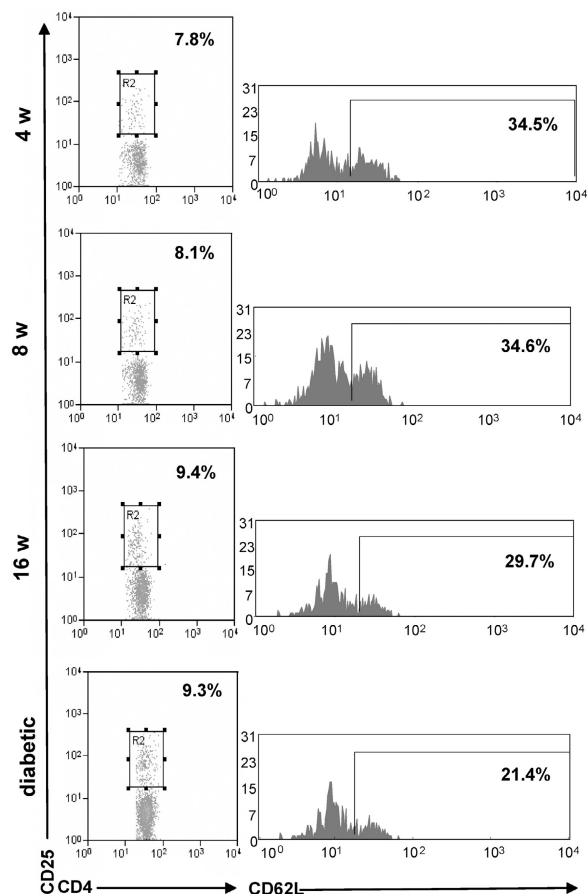
Next, an in vivo model was used to test CD4<sup>+</sup>CD25<sup>+</sup> T cell suppressor function. CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $5 \times 10^5$ ) were cotransferred with BDC2.5 CD4<sup>+</sup>CD25<sup>-</sup> T cells ( $5 \times 10^6$ ) into NOD.*scid* mice, and the development of diabetes was monitored. NOD BDC2.5 mice are transgenic for the diabetogenic BDC2.5 clonotypic TCR, and upon adoptive transfer into NOD.*scid* mice cause a rapid onset of disease (28). Coadoptive transfer of PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells that were isolated from 16-wk-old NOD female mice had no significant effect on the time of diabetes onset relative to recipients of CD4<sup>+</sup>CD25<sup>-</sup> BDC2.5 T cells alone (Fig. 1 B).



**Figure 2.** The efficacy of in vitro suppression by PLN CD62L<sup>hi</sup> T cells declines with age. Sorted CD62L<sup>hi</sup> (A) or CD62L<sup>lo</sup> (B) T cells from the PLN of NOD female mice at different ages were tested at the indicated numbers to suppress proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells as in Fig. 1 legend. Data are an average of two experiments performed in triplicate. Standard deviation of any given data point is  $\pm 13\%$  (A) or  $\pm 8\%$ . (B) [<sup>3</sup>H]thymidine uptake of CD4<sup>+</sup>CD25<sup>-</sup> T cells alone is  $8856 \pm 843$  cpm. \* $P \leq 0.01$ , 16 wk versus 8 wk or 4 wk; @ $P \leq 0.01$ , 4 wk versus 8 wk; + $P \leq 0.03$ , 16 wk versus 8 wk or 4 wk; # $P \leq 0.02$ , 4 wk versus 8 wk (Student's *t* test).

However, a significant delay in diabetes onset, beginning at day 15 after transfer, was detected in NOD.*scid* recipients that were coinjected with PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells that were prepared from 8 wk-old NOD female mice ( $P = 0.007$ ). The development of diabetes was delayed further in NOD.*scid* mice that received PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells that were isolated from 4-wk-old versus 8 wk-old NOD female mice ( $P = 0.009$ ) (Fig. 1 B).

To determine the phenotype and effector function of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells more accurately, the in vitro suppressor capacity of varying numbers of PLN CD62L<sup>hi</sup> and CD62L<sup>lo</sup> T cells was examined. The cell surface profile of CD62L<sup>hi</sup> versus CD62L<sup>lo</sup> is used commonly to discriminate between CD4<sup>+</sup>CD25<sup>+</sup> T reg cells and activated CD4<sup>+</sup> Th cells, respectively (29, 30). Similar to that observed in the coadoptive transfer experiments (Fig. 1 B), PLN CD62L<sup>hi</sup> T



**Figure 3.** The percentage of CD62L<sup>hi</sup> cells in the PLNs of NOD mice remains constant during disease progression. PLNs that were harvested from NOD female mice at indicated ages were analyzed by four-color flow cytometry for CD3, CD4, CD25, and CD62L expression. CD4<sup>+</sup>CD25<sup>+</sup> T cells were gated for lymphocytes based on forward and side scatter, and CD3 expression. CD62L expression was gated on forward scatter; side scatter; and CD3, CD4, and CD25 expression (box). Data are representative of five separate experiments.

**Table I.** Average absolute number of CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD25<sup>+</sup>CD62L<sup>hi</sup>, and CD25<sup>+</sup>CD62L<sup>lo</sup> per 10,000 lymphocytes within the PLNs of NOD female and NOD male mice

	Average absolute number			
	4 wk (%)	8 wk (%)	16 wk (%)	Diabetic (%)
<b>NOD female PLN</b>				
CD4 <sup>+</sup>	1,959 ± 118	2,895 ± 117	4,945 ± 425	7,070 ± 448
CD25 <sup>+</sup>	156 ± 20	291 ± 57	495 ± 71	654 ± 42
CD25 <sup>+</sup> CD62L <sup>hi</sup>	54 ± 5 (34.6) <sup>a</sup>	101 ± 8 (34.7)	147 ± 10 (30.0)	140 ± 10 (21.4)
FoxP3 <sup>+</sup> CD25 <sup>+</sup> CD62L <sup>hi</sup>	37 <sup>b</sup>	50	43	35
CD25 <sup>+</sup> CD62L <sup>lo</sup>	107 ± 8 (68.6) <sup>a</sup>	192 ± 11(66.0)	340 ± 13 (68.7)	510 ± 20 (78.0)
FoxP3 <sup>+</sup> CD25 <sup>+</sup> CD62L <sup>lo</sup>	37 <sup>b</sup>	44	61	63
<b>NOD male PLN</b>				
CD4 <sup>+</sup>	2,789 ± 109	ND	5,432 ± 306	ND
CD25 <sup>+</sup>	251 ± 23	ND	456 ± 37	ND
CD25 <sup>+</sup> CD62L <sup>hi</sup>	79 ± 7 (31.5) <sup>a</sup>	ND	150 ± 13 (32.9)	ND
FoxP3 <sup>+</sup> CD25 <sup>+</sup> CD62L <sup>hi</sup>	35 <sup>b</sup>	ND	53	ND
CD25 <sup>+</sup> CD62L <sup>lo</sup>	175 ± 10 (69.7) <sup>a</sup>	ND	308 ± 27 (67.5)	ND
FoxP3 <sup>+</sup> CD25 <sup>+</sup> CD62L <sup>lo</sup>	37 <sup>b</sup>	ND	49	ND

<sup>a</sup>Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells. Data are an average of five experiments (female) or two experiments (male).

<sup>b</sup>Estimated number based on average absolute number and average percent of FoxP3<sup>+</sup>.

cells that were prepared from 4-wk-old NOD female mice proved to be the most effective at suppressing CD4<sup>+</sup>CD25<sup>-</sup> T cell proliferation (Fig. 2 A). An intermediate level of suppression was detected for CD62L<sup>hi</sup> T cells that were isolated from 8-wk-old NOD female mice, whereas CD62L<sup>hi</sup> T cells that were prepared from 16-wk-old NOD female mice were the least effective (Fig. 2 A). The suppressor activity of PLN CD62L<sup>lo</sup> T cells, albeit markedly reduced relative to CD62L<sup>hi</sup> T cells, exhibited a similar age-dependent decline (Fig. 2 B). These results demonstrate that the capacity to mediate suppression by PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells, specifically CD62L<sup>hi</sup> T cells, declines in a temporal manner.

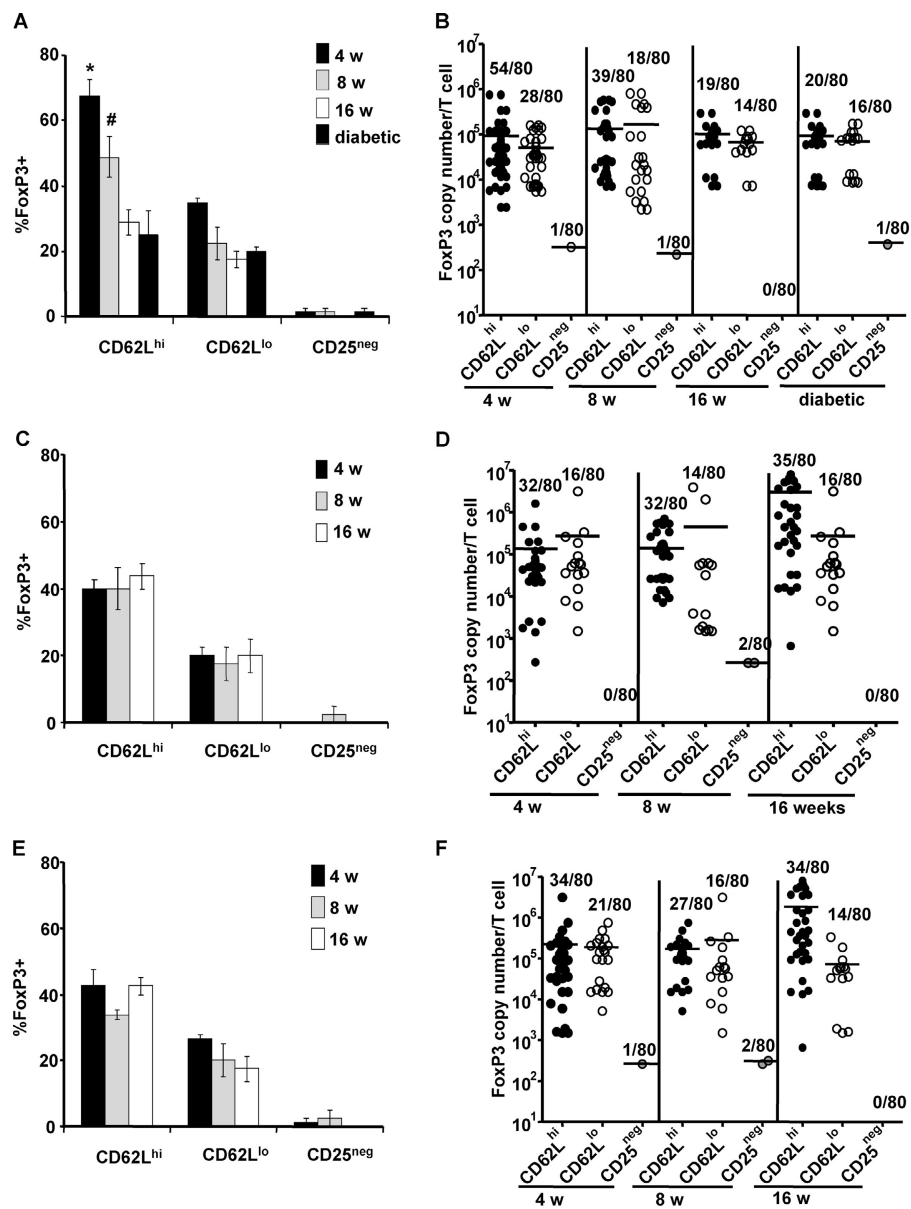
#### An increase in PLN CD62L<sup>hi</sup> T cells is detected as $\beta$ cell autoimmunity progresses

Quantitative and/or qualitative changes may account for the temporal decrease in CD4<sup>+</sup>CD25<sup>+</sup> T reg cell function that was detected. To distinguish between these possibilities, the number and frequency of PLN CD62L<sup>hi</sup> T cells were determined in prediabetic and diabetic NOD female mice. Notably, the number of CD62L<sup>hi</sup> T cells in the PLNs of prediabetic NOD female mice increased with age (Table I). An approximate threefold increase in the number of CD62L<sup>hi</sup> T cells was detected in the PLNs of 16-wk-old (147 ± 10) versus 4-wk-old (54 ± 5) NOD female mice ( $P < 10^{-4}$ ; Table I). In parallel, the number of CD62L<sup>lo</sup> T cells also increased with age, so that the overall percentage of CD62L<sup>hi</sup> T cells was maintained during the progression of preclinical  $\beta$  cell autoimmunity (Fig. 3, Table I). In the PLNs of recent onset diabetic NOD female mice, no significant change in the number of CD62L<sup>hi</sup> T cells was detected relative to 16-wk-

old mice, although the percentage of these T cells was reduced as a result of increased numbers of CD62L<sup>lo</sup> T cells (Fig. 3, Table I). Finally, within the pool of PLN CD62L<sup>hi</sup> T cells from prediabetic or diabetic NOD female mice, no significant difference was detected in the number or percentage of T cells which expressed additional markers that were associated with CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, including CTLA-4, GITR, CD69, CD103, and CD45RB (data not shown). Together, these results indicate that the number of CD62L<sup>hi</sup> T cells in the PLNs of NOD female mice increases as  $\beta$  cell autoimmunity progresses, and is maintained at the onset of diabetes. Furthermore, an increase in the number of CD62L<sup>lo</sup> T cells, and not a decrease in CD62L<sup>hi</sup> T cells in the PLN, correlates with the onset of diabetes.

#### A temporal decrease in FoxP3-expressing CD62L<sup>hi</sup> T cells is unique to NOD mouse PLNs

The above findings indicated that despite an increase in the number of CD62L<sup>hi</sup> T cells up to 16 wk in age, suppressor activity diminished within this pool. To further define the defect in PLN CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, mRNA expression of FoxP3, TGF $\beta$ , and IL-10 were measured. For this purpose, a real-time PCR assay was used to measure RNA transcript copy number in individual cells. Single CD62L<sup>hi</sup>, CD62L<sup>lo</sup>, and CD4<sup>+</sup>CD25<sup>-</sup> T cells were sorted from PLNs that were prepared from pools of four prediabetic or recent diabetic NOD female mice; FoxP3, TGF $\beta$ 1, and IL-10 transcript number were measured in the same individual cells. Consistent with previous findings, only a small percentage (<3%) of CD4<sup>+</sup>CD25<sup>-</sup> T cells expressed low copies (~100–200) of FoxP3 mRNA per cell (Fig. 4 A and B; refs.



**Figure 4.** The frequency of FoxP3-expressing CD62L<sup>hi</sup> T cells decline with age in the PLNs of NOD but not NOR or B6 mice. Single T cells from the respective subsets were sorted from the PLNs of female NOD (A, B), NOR (C, D), and B6 (E, F) mice varying in age, and FoxP3 transcript copy number measured via RT-real time PCR. (A, C, E) Percentage of T cells expressing FoxP3. Data are an average of two separate experiments (40 cells/

experiment). \**P* = 0.04, 4 wk versus 8 wk; #*P* = 0.01, 8 wk versus 16 wk (Student's *t* test). (B, D, F) FoxP3 transcript copy number for each individual T cell. Black bars represent the average transcript copy number per indicated T cell subset. The number of FoxP3<sup>+</sup> T cells within the total number of T cells (80 cells) that were analyzed is provided above each column. Data are a compilation of two separate experiments (40 cells/experiment).

29, 30). In contrast, the majority of PLN CD62L<sup>hi</sup> T cells (70 ± 5%) that were prepared from 4-wk-old NOD female mice expressed an average of 10<sup>5</sup> copies of FoxP3 mRNA per cell (Fig. 4, A and B). Strikingly, a marked decrease in FoxP3-expressing CD62L<sup>hi</sup> T cells was observed in the PLNs of 8-wk-old NOD female mice (48.8 ± 6.3%; *P* = 0.04; Fig. 4 A). Furthermore, a progressive decrease in the percentage of FoxP3-expressing CD62L<sup>hi</sup> T cells was detected at later stages of β cell autoimmunity. In the PLNs of

16-wk-old and diabetic NOD female mice, only 28.8 ± 3.8% and 25 ± 5% of CD62L<sup>hi</sup> T cells, respectively, expressed FoxP3 (Fig. 4 A). Despite a progressive loss of CD62L<sup>hi</sup> T cells that expressed the transcription factor, mRNA copy number did not vary significantly among FoxP3-positive T cells that were prepared from the respective groups of NOD female mice (Fig. 4 B).

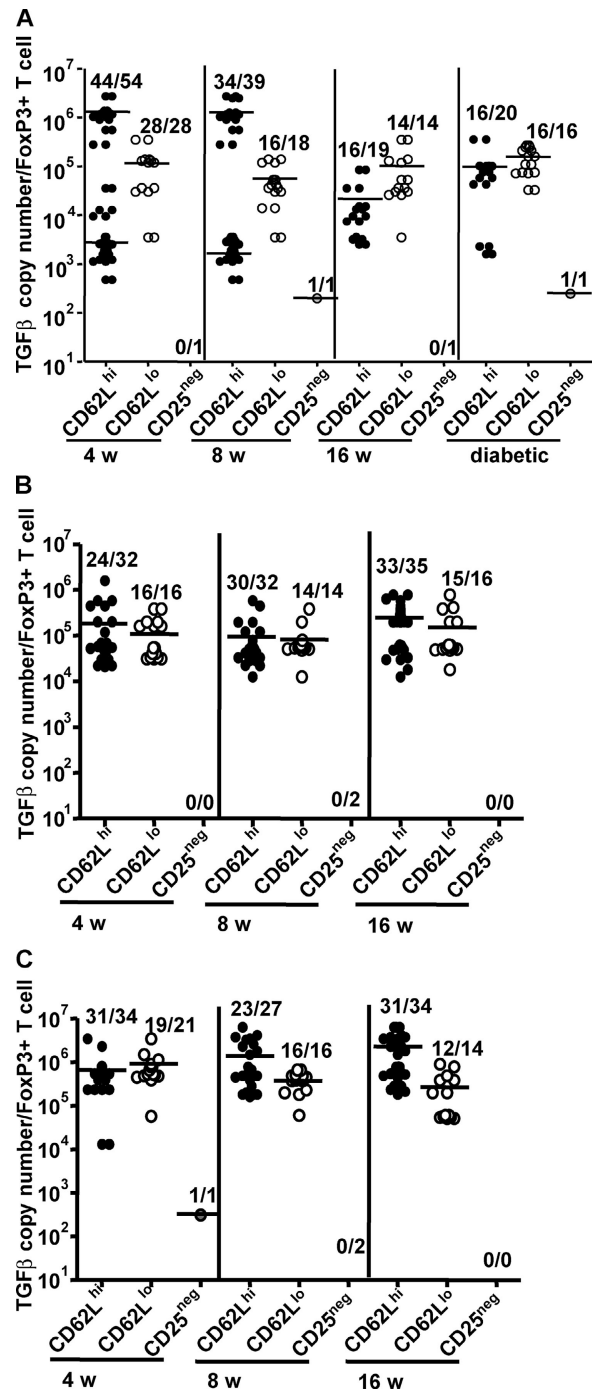
A significant percentage of CD62L<sup>lo</sup> T cells also was found to express FoxP3 at comparable levels to CD62L<sup>hi</sup> T cells (Fig.

4, A and B). Within the CD62L<sup>lo</sup> pool, the highest percent of FoxP3-expressing T cells was observed in PLNs of 4-wk-old NOD female mice ( $35.0 \pm 1.0\%$ ); this decreased with age (Fig. 4 A). In 8-wk-old NOD female mice for example,  $22.5 \pm 5.0\%$  of CD62L<sup>lo</sup> T cells expressed FoxP3 (Fig. 4 A).

To determine whether the age-dependent decrease in FoxP3-expressing T cells was unique to NOD mice, CD4<sup>+</sup> T cells were prepared from the PLNs of B6 and NOR female mice that varied in age, and were analyzed as above. The NOR mouse is a NOD-related MHC syngeneic recombinant strain that contains  $\sim 12\%$  C57BL/KsJ-derived genes, exhibits peri-insulinitis and only minimal intransulinitis, and fails to develop overt diabetes (31). Approximately 40% and 20% of CD62L<sup>hi</sup> and CD62L<sup>lo</sup> T cells, respectively, expressed FoxP3 independent of the age of the NOR or B6 female mice (Fig. 4, C and E). Furthermore, FoxP3 transcript copy numbers that were expressed by PLN CD62L<sup>hi</sup> T cells were equivalent between NOR and B6 mice (Fig. 4, D and F). At 16 wk of age, CD62L<sup>hi</sup> T cells that were prepared from NOD female mice expressed on average  $\sim 10$ -fold less FoxP3 transcripts relative to that detected in NOR and B6 mice ( $P \leq .0003$ ; Fig. 4, B, D, and F). These data demonstrate that despite an expanding number of PLN CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells, the percentage of FoxP3-expressing T cells declines as  $\beta$  cell autoimmunity progresses in NOD female mice. In contrast, a temporal decrease in the percentage of FoxP3-expressing CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells was not detected in NOR and B6 mice (Fig. 4, C and E, and Table S1, available at <http://www.jem.org/cgi/content/full/jem.20042398/DC1>). Importantly, the percentage of FoxP3-expressing cells in NOD female mice at 16 wk of age, when destructive insulinitis has begun, was significantly lower than in NOR and B6 mice (Fig. 4, A, C, and E; Table I; and Table S1). Although the actual number of FoxP3<sup>+</sup>CD62L<sup>hi</sup> T cells in NOD female mice remained constant (Table I), the suppressive capacity of this population diminished with age (Fig. 1). Together, these data indicate that an optimal ratio of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells/effector T cells is needed to suppress disease progression.

#### The percentage of TGF $\beta$ 1- and FoxP3-coexpressing PLN CD62L<sup>hi</sup> T cells decreases as $\beta$ cell autoimmunity progresses

Studies have shown that TGF $\beta$ 1 and IL-10 are key cytokines that are expressed by CD4<sup>+</sup>CD25<sup>+</sup> T reg cells (32–34). Analysis of transcript copy number demonstrated that none of the FoxP3-positive CD62L<sup>hi</sup> (or CD62L<sup>lo</sup>) T cells expressed detectable levels of IL-10 mRNA (data not shown). Conversely,  $>80\%$  of FoxP3-expressing CD62L<sup>hi</sup> T cells expressed TGF $\beta$ 1, regardless of the age of the NOD female mice (Fig. 5 A). In addition, TGF $\beta$ 1 expression was detected in  $\leq 7\%$  of FoxP3-negative CD62L<sup>hi</sup> (or CD62L<sup>lo</sup>) T cells (Table S2, available at <http://www.jem.org/cgi/content/full/jem.20042398/DC1>). TGF $\beta$ 1 transcript copy number varied with age (Fig. 5 A). For example, in 4- and 8-wk-old NOD female mice, FoxP3-positive CD62L<sup>hi</sup> T cells were



**Figure 5.** The frequency of TGF $\beta$ 1-expressing FoxP3<sup>+</sup> CD62L<sup>hi</sup> T cells declines with age in the PLNs of NOD but not NOR or B6 mice. TGF $\beta$ 1 transcript copy number was measured in single FoxP3<sup>+</sup> T cells that were identified in Fig. 4. NOD (A), NOR (B), and B6 (C) female mice. Black bars represent the average copy number per indicated T cell subset. The number of TGF $\beta$ 1<sup>+</sup> T cells within the total number of FoxP3<sup>+</sup> T cells that were analyzed for a given subset is provided above each column. Data are a compilation of two separate experiments (40 cells/experiment).

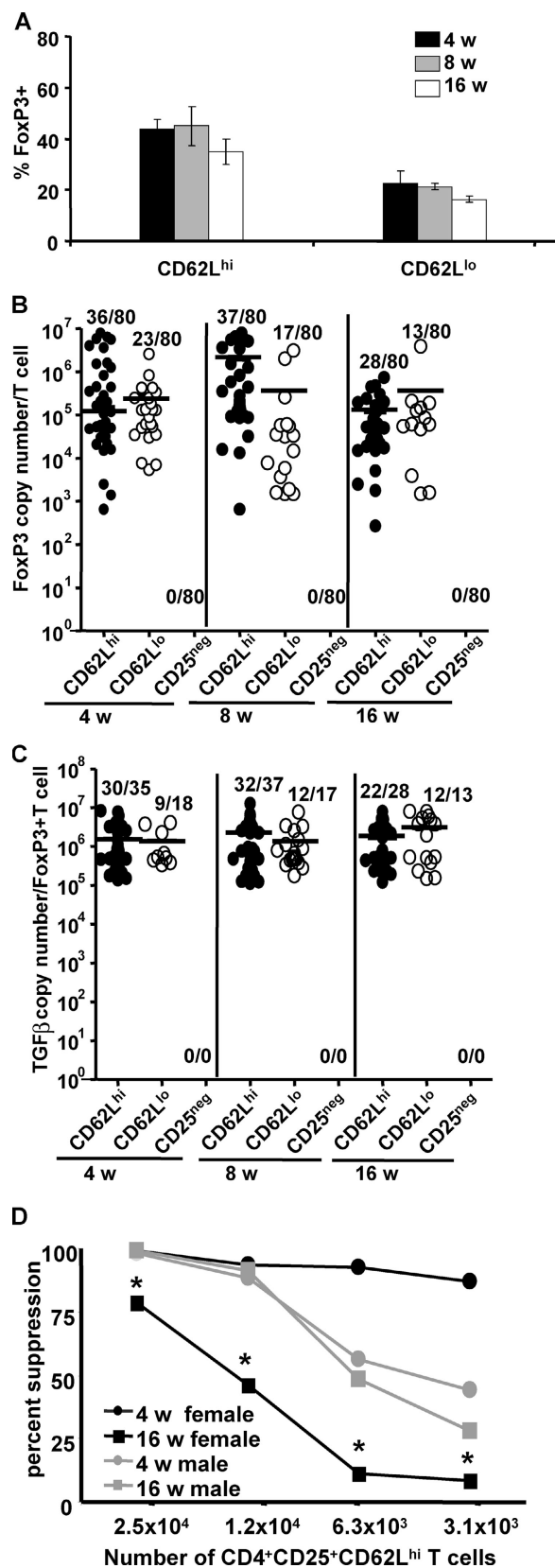


Figure 6. The frequency of TGFβ1- and FoxP3-coexpressing CD62L<sup>hi</sup> T cells remains constant with age in NOD male mice.

defined by two distinct subsets based on high ( $1.2$  to  $1.3 \times 10^6$ ) versus low ( $2.1$  to  $4.9 \times 10^3$ ) TGFβ1 transcript copy number per T cell. However, in 16-wk-old or diabetic animals, high copy TGFβ1-expressing CD62L<sup>hi</sup> T cells no longer were detected (Fig. 5 A). In comparison, TGFβ1 transcript copy number was relatively consistent in FoxP3-expressing CD62L<sup>hi</sup> T cells that were prepared from different aged NOR and B6 female mice (Fig. 5, B and C). At 16 wk of age, FoxP3-positive CD62L<sup>hi</sup> T cells that were prepared from NOD female mice expressed on average 10- and 100-fold fewer TGFβ1 transcripts compared with NOR and B6 mice, respectively ( $P \leq 0.009$ ; Fig. 5).

Analogous to CD62L<sup>hi</sup> T cells, TGFβ was detected in >80% of FoxP3-expressing CD62L<sup>lo</sup> T cells that were prepared from PLNs of NOD, NOR, and B6 female mice, regardless of age (Fig. 5). Furthermore, transcript copy number varied only minimally among the respective groups within a given mouse strain, and between NOD, NOR, and B6 mice (Fig. 5). TGFβ1 mRNA was detected in  $\leq 4\%$  of CD62L<sup>lo</sup> T cells which lacked FoxP3 expression (Table S2). Together, these results indicate that the percentage of TGFβ1-expressing CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells, and the level of TGFβ1 expression decreases in NOD female mice as β cell autoimmunity progresses. Furthermore, the majority of FoxP3-positive CD4<sup>+</sup>CD25<sup>+</sup> T reg cells coexpress TGFβ1, but not IL-10.

#### A temporal decrease in FoxP3- and TGFβ1-coexpressing CD62L<sup>hi</sup> T cells is not detected in NOD male mice

Differences in the extent of pancreatic inflammation between NOD versus NOR or B6 female mice may have distinct effects on the activation and/or expansion of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, which, in turn, may explain the profiles of FoxP3- and TGFβ1-coexpressing CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells that are seen in the respective mice. To rule out this possibility and determine whether decreasing FoxP3- and TGFβ1-coexpressing CD62L<sup>hi</sup> in NOD female mice is a consequence or cause of disease progression, NOD male mice were studied. NOD male and female mice exhibit similar insulinitis with age, despite the fact that male animals develop diabetes at a markedly reduced frequency. A similar

(A and B) Sorted PLN T cells of the respective subsets from NOD male mice were examined for FoxP3 and TGFβ1 transcript copy number. (A) Percentage of T cells expressing FoxP3. (B) FoxP3 transcript copy number per individual T cell. (C) TGFβ1 transcript copy number measured in single FoxP3<sup>+</sup> T cells identified in (A). The number of TGFβ1<sup>+</sup> T cells within the total number of FoxP3<sup>+</sup> T cells that were analyzed for a given subset is provided above each column. (A–C) Data are a compilation of two separate experiments (40 cells/experiment). Black bars represent the average copy number per cell for the indicated T cell subset. (D) Sorted CD62L<sup>hi</sup> T cells from the PLNs of female and male NOD mice at different ages were assessed at the indicated numbers to suppress proliferation of CD4<sup>+</sup>CD25<sup>+</sup> T cells as described in Fig. legend 1. Data are an average of two experiments performed in triplicate. Standard deviation of any given data point  $< \pm 14\%$ . [<sup>3</sup>H]thymidine uptake of CD4<sup>+</sup>CD25<sup>+</sup> T cells alone is  $95420 \pm 996$  cpm. \* $P < 0.02$ , 16-wk NOD male vs. 16-wk NOD female (Student's *t* test).

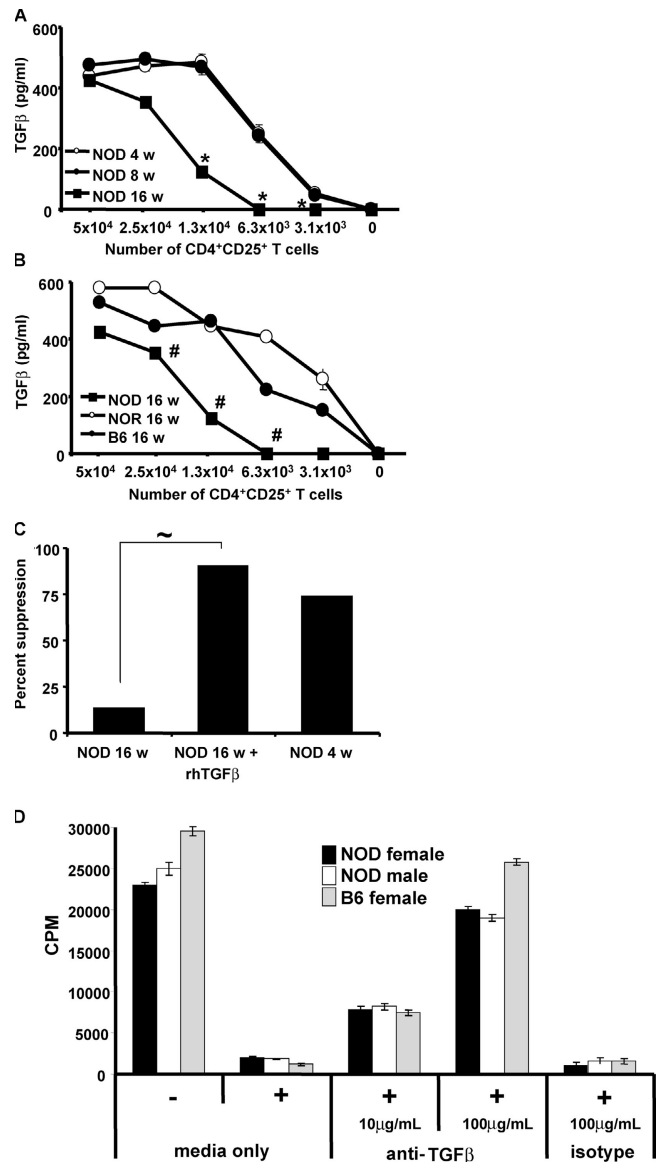
increase in the absolute number of PLN CD62L<sup>hi</sup> and CD62L<sup>lo</sup> T cells was detected in 4- and 16-wk-old NOD male and female mice (Table I). However, the percentage of PLN CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) FoxP3-expressing T cells in NOD male mice remained relatively constant with age (Fig. 6, A and B); this resembles the profiles that were seen in NOR and B6 mice (Fig. 4, C–F). Furthermore, >80% of FoxP3-expressing CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells in NOD male mice expressed TGFβ1, regardless of age (Fig. 6 C). Moreover, in contrast to NOD female mice (Fig. 5 A), TGFβ1 transcript copy number in NOD male mice also remained constant (Fig. 6 C). TGFβ1 transcript copy number was increased ~10-fold more in PLN CD62L<sup>hi</sup> (or CD62L<sup>lo</sup>) FoxP3<sup>+</sup> T cells from male versus female NOD mice at 16 wk of age (Fig. 5 A and Fig. 6 C).

Next, the *in vitro* suppressor function of PLN CD62L<sup>hi</sup> T cells from 4- and 16-wk-old NOD male mice suppressed CD4<sup>+</sup>CD25<sup>-</sup> T cell proliferation similarly—and with significantly greater efficacy—to CD62L<sup>hi</sup> T cells from 16-wk-old female mice ( $P < 0.001$ ; Fig. 6 D). These results demonstrate that unlike NOD female mice, the percentage of FoxP3- and TGFβ1-coexpressing CD62L<sup>hi</sup> T cells—and associated TGFβ1 transcript copy number and suppressor function—remain constant with age.

### Suppression mediated by PLN CD4<sup>+</sup>CD25<sup>+</sup> T reg cells is TGFβ dependent

The above findings suggested that a progressive decline in the percentage of TGFβ1-expressing PLN CD4<sup>+</sup>CD25<sup>+</sup> T reg cells promoted disease progression in NOD female mice. To determine a role in CD4<sup>+</sup>CD25<sup>+</sup> T reg cell-mediated suppression, the level of TGFβ secretion in an *in vitro* suppression assay was investigated. As the number of CD4<sup>+</sup>CD25<sup>+</sup> T cells was decreased, significantly reduced amounts of TGFβ were detected in cultures that were established from 16-wk-old versus 4- and 8-wk-old NOD female mice ( $P \leq 0.02$ ; Fig. 7 A). Similarly, a marked reduction in TGFβ secretion was detected between CD4<sup>+</sup>CD25<sup>+</sup> T cells that were isolated from the PLNs of 16-wk-old NOD versus NOR and B6 female mice (Fig. 7 B). The declining levels of TGFβ reflected the reduced efficacy of the CD4<sup>+</sup>CD25<sup>+</sup> T cells that were prepared from 16-wk-old NOD female mice to suppress the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells (Figs. 1 A and 2 A).

To address whether decreased suppressor activity largely was due to reduced TGFβ production, the function of CD62L<sup>hi</sup> T cells that were prepared from 16-wk-old NOD female mice was examined in an *in vitro* suppression assay that was supplemented with recombinant human (rh)TGFβ. Here,  $3 \times 10^3$  CD62L<sup>hi</sup> T cells and  $10^5$  CD4<sup>+</sup>CD25<sup>-</sup> T cells were cultured with or without 425 pg of rhTGFβ. The amount of rhTGFβ was based on detected levels of TGFβ secretion by CD4<sup>+</sup>CD25<sup>+</sup> T cells ( $2.5 \times 10^5$ ) from 4-wk-old NOD female mice mediating maximum suppression



**Figure 7. Suppression mediated by CD4<sup>+</sup>CD25<sup>+</sup> T reg cells is TGFβ dependent.** (A and B) Supernatants from suppression assays that were described in Fig. 1 were harvested and TGFβ was detected via ELISA. Data is an average of two experiments performed in triplicate. Standard deviation of any given data point  $\leq \pm 31$  pg/ml (A) or  $\leq \pm 52$  pg/ml (B). \* $P \leq 0.02$ , 16 wk versus 4 or 8 wk; # $P \leq 0.02$ , 16 wk versus 4 wk or 8 wk. (C) Sorted CD62L<sup>hi</sup> from the PLNs of NOD female mice 4 wk and 16 wk of age ( $3 \times 10^3$ ) were cocultured with  $10^5$  CD4<sup>+</sup>CD25<sup>-</sup> T cells from 8-wk-old NOD female mice with or without 425 pg of rhTGFβ. Data are an average of two experiments performed in triplicate. Standard deviation of any given data point  $< \pm 17\%$ . [<sup>3</sup>H]thymidine uptake of CD4<sup>+</sup>CD25<sup>-</sup> T cells alone is  $91457 \pm 721$  cpm.  $\sim P < 0.02$ , 16 wk plus rhTGFβ versus 16 wk minus rhTGFβ (Student's *t* test). (D)  $2.5 \times 10^4$  sorted PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells from 4-wk-old NOD female, NOD male, and B6 mice were cocultured with  $10^5$  CD4<sup>+</sup>CD25<sup>-</sup> T cells from 8-wk-old NOD and B6 female mice in the presence or absence of anti-TGFβ1,2,3 or isotype control. Data is an average of two experiments performed in triplicate.



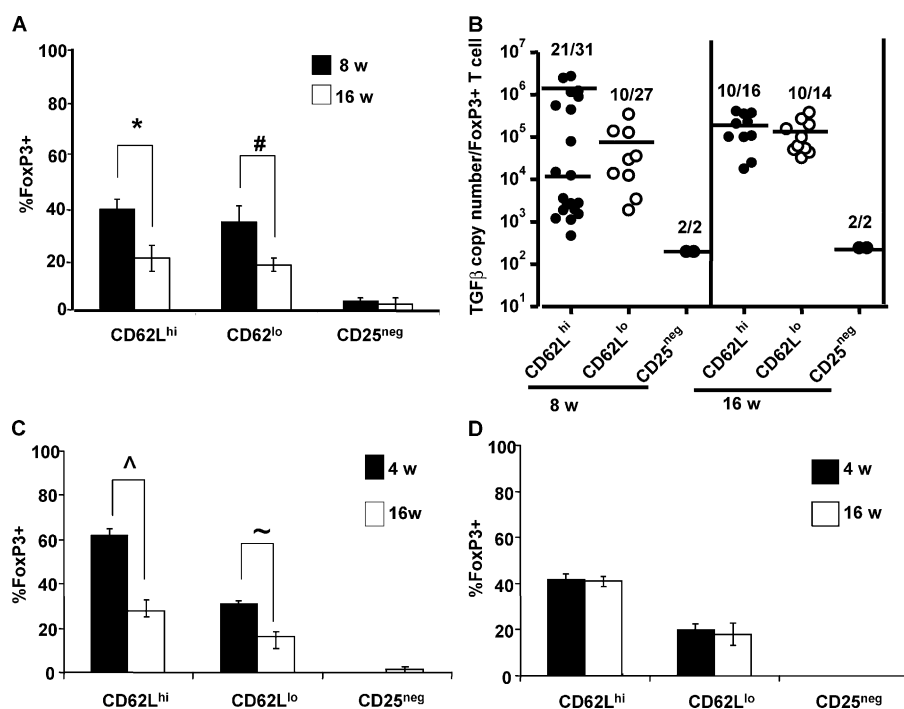
(Fig. 7 A). When rhTGF $\beta$  was added, CD62L<sup>hi</sup> T cells from 16-wk-old NOD female mice suppressed proliferation of CD4<sup>+</sup>CD25<sup>-</sup> T cells similarly to 4-wk-old CD62L<sup>hi</sup> T cells that had not been supplemented with cytokine (Fig. 7 C). CD4<sup>+</sup>CD25<sup>-</sup> T cells alone did not produce TGF $\beta$  upon activation (Fig. 7 A), nor was FoxP3 expression induced in CD4<sup>+</sup>CD25<sup>-</sup> T cells that were incubated with 425 pg of rhTGF $\beta$ . For instance, no difference was detected in the frequency ( $\sim$ 7.0%) of FoxP3-positive CD4<sup>+</sup>CD25<sup>-</sup> T cells that were cultured with or without rhTGF $\beta$ . Furthermore, when  $5 \times 10^4$  CD62L<sup>hi</sup> or CD62L<sup>lo</sup> T cells that were prepared from PLNs of 4-wk-old NOD female mice were stimulated individually in vitro,  $345 \pm 48$  pg/ml and  $125 \pm 25$  pg/ml of TGF $\beta$  was detected, respectively. These data demonstrate that the addition of TGF $\beta$  can enhance the suppressor function of CD62L<sup>hi</sup> T cells from 16-wk-old NOD female mice, and that the source of TGF $\beta$  that was detected in in vitro suppression assays are CD62L<sup>hi</sup> and CD62L<sup>lo</sup> T cells.

Finally, the addition of anti-TGF $\beta$  Ab, but not an isotype control Ab, effectively blocked—in a dose-dependent manner—the capacity of CD4<sup>+</sup>CD25<sup>+</sup> T cells from female and male NOD and B6 mice to mediate suppression (Fig. 7 D). Together, these data demonstrate that TGF $\beta$  produced by

the CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) plays a key role in the observed suppression; diminishing TGF $\beta$  protein expression within the pool of PLN CD4<sup>+</sup>CD25<sup>+</sup> T reg cells contributes to reduced effector function in an age-dependent manner.

### Pancreatic islets and popliteal lymph nodes of NOD female mice exhibit a progressive loss of FoxP3- and TGF $\beta$ 1-coexpressing CD62L<sup>hi</sup> T cells

Islets also provide a key site for immunoregulation of  $\beta$  cell specific T cell reactivity (32). Accordingly, CD4<sup>+</sup>CD25<sup>+</sup> T reg cells that infiltrate the islets of 8- and 16-wk-old NOD female mice were examined. As demonstrated in Table II, a decrease in the number of CD62L<sup>hi</sup> T cells ( $162 \pm 5$  versus  $111 \pm 3$ ;  $P = 0.04$ ) and a concomitant increase in the number of CD62L<sup>lo</sup> T cells ( $320 \pm 3$  versus  $731 \pm 11$ ;  $P = 0.02$ ) that infiltrated the islets of 8- and 16-wk-old NOD female mice, respectively, were detected. Similar to PLN (Fig. 4 A), a twofold reduction in FoxP3-positive CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells was observed in the islet infiltrates of 8- versus 16-wk-old NOD female mice ( $P = 0.01$ ; Fig. 8 A). Again, as seen in PLN, two distinct subsets of FoxP3-expressing CD62L<sup>hi</sup> T cells, based on TGF $\beta$ 1 transcript copy number, were detected in the islets of 8-wk-old, but



**Figure 8. Diminishing frequencies of FoxP3- and TGF $\beta$ 1-coexpressing CD62L<sup>hi</sup> T cells detected in the islet infiltrates and popliteal lymph nodes of NOD female mice.** (A and B) FoxP3 and TGF $\beta$ 1 transcript copy number was determined in sorted, single T cells from islet infiltrates of 8- and 16-wk-old NOD female mice. (A) Percentage of T cells expressing FoxP3. Data are an average of two separate experiments (40 cells/experiment). \* $P = 0.01$ , 8 wk versus 16 wk; # $P = 0.01$ , 8 wk versus 16 wk. (B) TGF $\beta$ 1 transcript copy number of FoxP3-expressing T cells. Black bars represent

the average copy number per the indicated subset. The number of TGF $\beta$ 1<sup>+</sup> T cells within the total number of FoxP3<sup>+</sup> T cells that were analyzed for a given subset is provided above each column. (C, D) FoxP3 transcript copy number was determined in sorted, single T cells from popliteal lymph nodes of 4- and 16-wk-old NOD (C) and B6 (D) female mice. Data are an average of two separate experiments (40 cells/experiment) <sup>^</sup> $P = 0.02$ , 4 wk versus 16 wk; <sup>~</sup> $P = 0.01$ , 4 wk vs. 16 wk.

**Table II.** Absolute number and percentage of CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD25<sup>+</sup>CD62L<sup>hi</sup>, and CD25<sup>+</sup>CD62L<sup>lo</sup> per 10,000 lymphocytes within the islet infiltrate of NOD female mice

	NOD female islet infiltrate	
	Average absolute number	
	8 wk (%)	16 wk (%)
CD4 <sup>+</sup>	4,594 ± 176	5,818 ± 127
CD25 <sup>+</sup>	482 ± 10	842 ± 8
CD25 <sup>+</sup> CD62L <sup>hi</sup>	162 ± 5 (33.6) <sup>a</sup>	111 ± 3 (13.2)
FoxP3 <sup>+</sup> CD25 <sup>+</sup> CD62L <sup>hi</sup>	59 <sup>b</sup>	23
CD25 <sup>+</sup> CD62L <sup>lo</sup>	320 ± 3 (66.4) <sup>a</sup>	731 ± 11 (86.8)
FoxP3 <sup>+</sup> CD25 <sup>+</sup> CD62L <sup>lo</sup>	108 <sup>b</sup>	131

<sup>a</sup>Percentage of CD4<sup>+</sup>CD25<sup>+</sup> T cells. Data are an average of two experiments.

<sup>b</sup>Estimated number based on average absolute number and average percentage of FoxP3<sup>+</sup>.

not 16-wk-old, NOD female mice (Fig. 8 B). Furthermore, none of the FoxP3-positive T cells expressed IL-10 only, although <10% of FoxP3- and TGFβ1-coexpressing T cells were positive for IL-10 mRNA, independent of age (data not shown). These findings demonstrate that analogous to the PLN, the percentage of islet-infiltrating CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells that coexpress FoxP3 and TGFβ1 declines as β cell autoimmunity progresses in NOD female mice.

To determine if the observed temporal decrease in the percentage of FoxP3-expressing T cells was unique to sites of inflammation, such as the PLN and islets, the popliteal lymph nodes from 4- and 16-wk-old NOD female mice were analyzed; B6 mice served as a control. A significant decrease in the percentage of CD62L<sup>hi</sup> T cells that expressed FoxP3 was detected in NOD, but not B6, female mice (Fig. 8, C and D). Transcript copy number of FoxP3 did not vary and was similar to levels detected in PLN and islet-infiltrating CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20042398/DC1>). As above, >82% of FoxP3-positive T cells also expressed TGFβ1, independent of age. These data indicate that the temporal decrease in the percentage of FoxP3- and TGFβ1-coexpressing T cells in NOD female mice is not due to activation and subsequent down-regulation of expression of these genes, but rather is an intrinsic defect in the maintenance of this T cell population.

## DISCUSSION

The interplay between natural CD4<sup>+</sup>CD25<sup>+</sup> T reg cells and pathogenic T cells in autoimmunity, including T1D, is poorly understood. In part, this is due to the difficulty of accurately distinguishing between CD4<sup>+</sup>CD25<sup>+</sup> T reg cells and other subsets of immunoregulatory or pathogenic T effectors. In this study and others, natural CD4<sup>+</sup>CD25<sup>+</sup> T reg cells have been defined based on a CD62L<sup>hi</sup> surface phenotype, and expression of markers, such as CTLA-4, GITR,

CD45RB, CD103 (20, 29, 30), and more recently, lymphocyte activation gene-3 (33). Furthermore, various groups have demonstrated that CD4<sup>+</sup>CD62L<sup>hi</sup> T cells that were prepared from the thymus or spleen of young NOD mice block diabetes in adoptive transfer experiments (29, 30). Detection of FoxP3 mRNA also was used recently to define CD4<sup>+</sup>CD25<sup>+</sup> T reg cells (21–23). However, bulk populations typically are studied so that the number/frequency of, and the corresponding cytokines that are expressed by, FoxP3-positive T cells cannot be determined readily. The latter is of significance in view of the apparent heterogeneity within CD4<sup>+</sup>CD25<sup>+</sup> T reg cells regarding expression and the respective roles of TGFβ1 and/or IL-10 in suppressor activity (24–26). We have addressed these issues by using a real-time PCR assay to measure FoxP3 and cytokine expression in single cells.

Two conclusions pertaining to the general properties of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells can be drawn based on the single cell analyses that were made in NOD, NOR, and B6 mice. First, only a subset (~40%) of single CD62L<sup>hi</sup> T cells express significant levels of FoxP3. Furthermore, a considerable number (~20%) of CD62L<sup>lo</sup> T cells also express FoxP3; this is consistent with work by the Salomon group (34) that showed that proliferating CD4<sup>+</sup>CD25<sup>+</sup> T reg cells down-regulate CD62L expression. The pool of CD62L<sup>lo</sup> T cells exhibited a markedly reduced suppressor function relative to CD62L<sup>hi</sup> T cells (Fig. 2, A and B); this suggests that CD4<sup>+</sup>CD25<sup>+</sup> T reg cells effector function is regulated tightly. Together, however, these results further underscore the difficulty of characterizing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells based on surface phenotype alone. Second, the majority (>80%) of single, FoxP3-positive T cells express TGFβ1 but not IL-10. Noteworthy, however, is that a small percentage (<10%) of FoxP3- and TGFβ1-positive CD62L<sup>hi</sup> T cells in the islets, but not PLNs, of NOD mice also expressed IL-10 (data not shown). FoxP3-positive T cells that express IL-10, but not TGFβ1, normally may represent a minor population of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells that undergo expansion under certain proinflammatory conditions (26). A role for TGFβ1 was demonstrated by the capacity of neutralizing Ab to block completely CD4<sup>+</sup>CD25<sup>+</sup> T reg cell-mediated suppression in vitro (Fig. 7 D). The latter does not rule out cell-cell contact as a means by which these CD4<sup>+</sup>CD25<sup>+</sup> T reg cells mediate suppression in vivo. In fact, cell-cell contact is likely to be the predominant mechanism of suppression of the few FoxP3-positive T cells (<20%) that lack TGFβ1 expression (Fig. 5 and Table S2). Our observation that anti-TGFβ Ab blocked the in vitro suppressor activity of NOD and B6 CD4<sup>+</sup>CD25<sup>+</sup> T reg cells (Fig. 7 D) is consistent with findings that were reported by Nakamura et al; they used the same polyclonal anti-TGFβ Ab (1D11) to inhibit the function of BALB/c CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in vitro (35). In addition, several studies demonstrated that the suppressor activity of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells upon adoptive transfer is blocked in vivo by anti-TGFβ Ab treatment (36,

37). Nevertheless, under certain conditions, suppression of CD4<sup>+</sup>CD25<sup>+</sup> T cells by CD4<sup>+</sup>CD25<sup>+</sup> T reg cells can be mediated independent of TGFβ (38); this suggests that the role of this cytokine in CD4<sup>+</sup>CD25<sup>+</sup> T reg cell function is complex. Shevach and colleagues suggested that TGFβ1, in an autocrine manner, may have a direct effect on the induction of CD4<sup>+</sup>CD25<sup>+</sup> T reg cell function (38). Reduced TGFβ1 expression (Fig. 5 A) correlated with a decreased number of FoxP3-expressing CD4<sup>+</sup>CD25<sup>+</sup> T cells in older NOD female mice (Fig. 4, A and B). Accordingly, lower levels of autocrine TGFβ1 may affect the maintenance/expansion and the function of FoxP3-positive CD4<sup>+</sup>CD25<sup>+</sup> T reg cells adversely in older NOD female mice.

Another key observation that was made in this study is that the function and phenotype of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in NOD female mice undergo marked changes in an age-dependent fashion. For example, the *in vitro* and *in vivo* suppressor function of PLN CD4<sup>+</sup>CD25<sup>+</sup> T cells declined significantly from 4 to 8 and 16 wk of age (Fig. 1). The loss of suppressor activity was detected, despite increasing numbers of PLN CD62L<sup>hi</sup> T cells. Therefore, the progressive loss of suppressor activity by sorted CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells (Fig. 2 A) indicated an intrinsic defect within this pool of immunoregulatory effectors. Consistent with this finding, real-time PCR analysis demonstrated that the percentage of single CD62L<sup>hi</sup> T cells that coexpresses FoxP3 and TGFβ1 declined with age in the PLN (Fig. 4, A and B) and islets (Fig. 8 B). CD62L<sup>hi</sup> T cells that were sorted from 4-wk-old NOD mice exhibited the most effective suppressor activity (Fig. 2 A), which, in turn, correlated with the greatest number of FoxP3- and TGFβ1-coexpressing T cells (Fig. 4, A and B). In comparison, the pool of CD62L<sup>hi</sup> T cells from 8-wk-old NOD female mice exhibited decreased suppressor activity (Fig. 2 A). The latter was due to a reduced frequency of FoxP3- and TGFβ1-expressing CD62L<sup>hi</sup> T cells in 8- versus 4-wk-old NOD female mice (Fig. 4 A). Notably, TGFβ1 transcript copy number was equivalent between the FoxP3-expressing CD62L<sup>hi</sup> T cells of 4- and 8-wk-old NOD female mice. In older NOD female mice, the suppressor function of CD62L<sup>hi</sup> T cells was reduced further (Fig. 2 A). However, in this case, not only was the frequency of FoxP3- and TGFβ1-coexpressing CD62L<sup>hi</sup> T cells reduced but so was the level of TGFβ1 expression by these T cells (Fig. 4, A and B; Fig. 5 A). It is likely that both factors contribute to the relatively ineffective suppressor activity that is exhibited by this pool of CD62L<sup>hi</sup> T cells. Nevertheless, at high numbers (i.e.,  $5 \times 10^4$ ), CD62L<sup>hi</sup> T cells from 16-wk-old NOD female mice exhibited *in vitro* suppressor activity that was equivalent to that of 4- and 8-wk-old NOD female mice (Fig. 2 A). This result indicates that at an optimal number/frequency, CD62L<sup>hi</sup> T cells exhibit effective immunoregulatory function, despite reduced TGFβ production. These data further demonstrate that the progressive loss of suppressor activity within the pool of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in NOD female mice is associated with multiple defects.

The temporal profile of FoxP3- and TGFβ1-coexpressing T cells differed markedly between NOD female mice versus B6, NOR, and most notably, NOD male, mice (Figs. 4 and 6). Almost a twofold increase in FoxP3- and TGFβ1-positive CD62L<sup>hi</sup> T cells was detected in 4-wk-old NOD female mice relative to B6, NOR, and NOD male mice of the same age. This was despite the fact that in 4-wk-old NOR and B6 mice, the number of PLN CD62L<sup>hi</sup> T cells was approximately two- to threefold greater than that observed in NOD female mice (Table S1). However, the progressive loss of FoxP3- and TGFβ1-coexpressing cells within the pool of CD62L<sup>hi</sup> (and CD62L<sup>lo</sup>) T cells was detected only in NOD female mice. In B6, NOR, and NOD male mice, equivalent numbers of FoxP3- and TGFβ1-positive T cells remained constant with age (Fig. 4, C–F, and Fig. 6, A and B). The fact that infiltration of the PLNs and islets is similar between NOD male and female mice (Table I) suggests that ongoing inflammation does not account for down-regulation of FoxP3 (and TGFβ1) expression as a result of the activation and/or proliferative status of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells. A similar decline in the frequency of FoxP3- and TGFβ1-coexpressing CD62L<sup>hi</sup> and CD62L<sup>lo</sup> T cells was detected in the popliteal lymph nodes of NOD female mice (Fig. 8 C), a site that lacks obvious inflammation. These data strengthen the hypothesis that the progressive loss of FoxP3- and TGFβ1-coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in NOD female mice directly contributes to the development of aggressive insulinitis and overt diabetes.

The events that influence the frequency of FoxP3- and TGFβ1-coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in NOD mice is not clear, although a number of mutually nonexclusive possibilities exist. Thymic selection may become limiting for CD4<sup>+</sup>CD25<sup>+</sup> T reg cells in NOD mice, thereby indirectly affecting the size of the peripheral pool. Various groups, for instance, have documented aberrant thymocyte selection (3, 4) and abnormal thymic physiology (39) in the NOD mouse. Treatment of neonatal NOD mice with anti-TNFα Ab increases the number of thymic CD4<sup>+</sup>CD25<sup>+</sup> T cells (40). Second, a defect in maintenance and/or expansion of FoxP3- and TGFβ1-coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells may contribute to the decline of these immunoregulatory effectors. Various molecules, including CTLA-4, TNF-related activation-induced cytokine, GITR, and lymphocyte activation gene-3, are potential candidates which affect the induction and/or expansion of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells (20, 33, 41–43). The temporal decline in TGFβ1 mRNA that was detected in FoxP3-expressing CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>hi</sup> T cells (Fig. 5), and the corresponding decrease in TGFβ secretion (Fig. 7), may hinder the expansion and/or maintenance of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells during inflammation. Recent work by the Flavell group demonstrated that ectopic expression of TGFβ1 by β cells increased the frequency of FoxP3-expressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, and protected the transgenic NOD mice from diabetes (44). Finally, it has been suggested that peripheral induction of FoxP3 expres-

sion by CD4<sup>+</sup>CD25<sup>-</sup> T cells may contribute to the maintenance and/or expansion of the CD4<sup>+</sup>CD25<sup>+</sup> T reg cells. The intrinsic properties of CD4<sup>+</sup>CD25<sup>-</sup> T cells or the lack of appropriate signals in NOD mice may limit the efficiency of such a mechanism. For instance, TGFβ1 was shown to induce FoxP3 expression in CD4<sup>+</sup>CD25<sup>-</sup> T cells in vitro (45, 46). Once again, limiting amounts of TGFβ that is produced by NOD CD4<sup>+</sup>CD25<sup>+</sup> T reg cells would be expected to reduce the efficiency of such a mechanism.

This study and others highlight the complexity among immunoregulatory T cells that modulate the progression of T1D. Adorini and colleagues demonstrated that CD4<sup>+</sup>CD25<sup>+</sup> T cells that were prepared from the spleen of NOD mice also exhibited varying suppressor activity in an age-dependent manner (7). In contrast to the findings herein, immunoregulatory CD4<sup>+</sup>CD25<sup>+</sup> T cells were characterized by expression of IL-10, although in vitro suppression was mediated in an IL-10-independent manner. Expression of FoxP3 or TGFβ1 was not assessed in this pool of CD4<sup>+</sup>CD25<sup>+</sup> T cells. Recent work by Benoist et al. showed that progression of insulinitis in BDC2.5 mice was dependent on FoxP3-expressing CD4<sup>+</sup>CD25<sup>+</sup>CD69<sup>+</sup> T cells which reside within the islets (32). This population of immunoregulatory T effectors also was characterized by expression of IL-10; in vivo persistence was dependent on inducible costimulatory expression. Sufficient expansion of this subset of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells may have been facilitated by expression of the BDC2.5 clonotypic TCR in this model.

In summary, we propose that a progressive decline in the percentage of FoxP3- and TGFβ1-coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells contributes to the development of “aggressive” insulinitis in NOD female mice. During the pre-diabetic stage, a balance between pathogenic T cells and CD4<sup>+</sup>CD25<sup>+</sup> T reg cells that reside in the PLNs and islets is maintained. However, as FoxP3- and TGFβ1-positive-expressing T cells decline within the pool of CD4<sup>+</sup>CD25<sup>+</sup> T reg cells, a minimum threshold is surpassed and pathogenic β cell-specific T effectors are permitted to expand (Table I) and drive the response to an overt diabetic state. However, in NOD male mice, the pool of FoxP3- and TGFβ1-coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells is relatively stable; this is reflected by a markedly reduced frequency of diabetes (~20%) compared with NOD female mice (~80%). Defining the mechanism that determines the frequencies of FoxP3- and TGFβ1-coexpressing CD4<sup>+</sup>CD25<sup>+</sup> T reg cells is likely to provide key insight into events that regulate T1D and other tissue-specific autoimmune diseases.

## MATERIAL AND METHODS

**Mice.** NOD/LtJ, NOR/LtJ, NOD.BDC2.5, C57BL/6 (B6), and NOD.*scid* mice were housed under specific pathogen-free conditions. Diabetes develops in ~80% and ~20% of NOD female and male mice, respectively, by 1 yr of age. Mice were maintained in an American Association of Laboratory Animal Care-accredited facility, and studies were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

**Flow cytometric analysis.** Abs were purchased from BD Biosciences and used for analysis of CD4<sup>+</sup>CD25<sup>+</sup> T cells: FITC-labeled rat anti-CD3 (17A2), PerCP-labeled rat anti-CD4 (GK1.5), APC-labeled rat anti-CD25 (PC61), PE-labeled rat anti-CD62L (Mel-14), PE-labeled anti-CD103 (M290), PE-labeled anti-CTLA-4 (UC10-4F10-11), biotinylated rat anti-CD69 (H1.2F3), and biotinylated anti-CD45RB (16A). Purified rat anti-mouse GITR (108619) was purchased from R&D Systems. CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> T cells were sorted using a MoFlo high-speed sorter (DakoCytomation) for single cell real-time PCR analysis, suppression assays, and adoptive transfer experiments. Sorted bulk populations, >98% purity, were stained with the aforementioned Abs. T cell preparations were stained in 5% FBS/PBS for 30 min on ice. When necessary, T cells also were incubated with secondary antibodies, PE-labeled streptavidin (BD Biosciences) or PE-labeled anti-rat IgG (Southern Biotechnology Associates Inc.) for 30 min on ice and washed. Flow cytometry data were acquired at the University of North Carolina Flow Cytometry and evaluated by Facility using a FACS Calibur (Becton Dickinson) and Summit software (DakoCytomation).

**Single cell real-time PCR.** Single CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>lo</sup>, CD4<sup>+</sup>CD25<sup>+</sup>CD62L<sup>hi</sup>, or CD4<sup>+</sup>CD25<sup>-</sup> T cells were sorted into individual wells of a 96-well polypropylene RNase/DNase-free plate (USA Scientific) which contained 4 μl of cell lysis buffer and stored at -80°C. cDNA was synthesized using SuperScript II (Invitrogen), oligo(dT) primers (Invitrogen), RNase Out (Invitrogen), and thermocycler (GeneAmp 9700, Applied Biosystems) conditions that consisted of 40°C for 40 min and 70°C for 15 min. Real-time PCR primers and probes that are specific for FoxP3 (5'-CCCAGGAAAGACAGCAACCTT-3'; 5'-TTCTCACAACCAGGCCACTTG-3'; 5'-fam/ATCCTACCCACTGCTGGCAAATGGAGTC/tam-3'), TGFβ1 (5'-CACTGATACGCCTGAGTG-3'; 5'-GTGAGCGCTGAATCGAAA-3'; 5'-fam/CCGTCTCCTTGGTTCAGCCACTG/tam-3'), and IL-10 (5'-CAGAGCCACATGCTCCTA-3'; 5'-GGAGTCGGTTAGCAGTATG-3'; 5'-fam/CTGCGGACTGCCTTCAGCCAG/tam-3') were synthesized by Integrated DNA Technologies. A standard curve that was generated from serial dilutions of purified plasmid DNA that encoded the respective genes was used to measure mRNA transcript copy number. The lower limit of detection was 10 copies for all genes tested. Each gene was detected in independent real-time PCR reactions using 5 μl of a 25-μl total cDNA mixture. Copy number of mRNA encoding β-actin was analyzed in the same sample using 5'-AGAGGGAAATCGTGCGTGAC-3'; 5'-CAATAGTGATGACCTGGCCGT-3'; 5'-fam/CACTGCCGCATCCTCTTCTCTCCC/tam-3' by Integrated DNA Technologies. Data are expressed as a copy number normalized to β-actin content. The normalized mRNA copy number for a gene was determined by: [raw transcript copy number derived from standard curve] × [β-actin corrective ratio]. The β-actin corrective ratio was calculated as [lowest β-actin copy number within sample set]/[β-actin copy number for cell of interest].

**In vitro CD4<sup>+</sup>CD25<sup>+</sup> T cell suppression assay.** CD4<sup>+</sup>CD25<sup>+</sup> T cells that were sorted from PLNs were cocultured in 96-well plates with 10<sup>5</sup> CD4<sup>+</sup>CD25<sup>-</sup> T cells that were sorted from PLNs plus 1 μg/ml of anti-CD3ε (145-2C11; eBioscience) and 10<sup>5</sup> irradiated T cell depleted splenocytes. Splenocytes were depleted of T cells by incubation with anti-Thy1.1-coated magnetic microbeads (Miltenyi Biotec) and then magnetic-activated cell separation was selected according to the manufacturer's protocol. T cells were cultured in RPMI 1640 which was supplemented with 10% FBS, 50 μM 2-ME, 1× nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 72 h at 37°C, and then pulsed with 1 μCi/well of [<sup>3</sup>H]thymidine for 18 h. [<sup>3</sup>H]thymidine incorporation was measured by a scintillation counter (Beckman LS6500). Percent inhibition was determined by the following equation:  $1 - \frac{\{[(CD4^+CD25^- + CD4^+CD25^+) \text{ cpm}] / [(CD4^+CD25^- \text{ alone}) \text{ cpm}]\} \times 100}{\{[(CD4^+CD25^- + CD4^+CD25^+) \text{ cpm}] / [(CD4^+CD25^- \text{ alone}) \text{ cpm}]\} \times 100}$ . Some studies included the addition of anti-TGFβ1,2,3 (1D11) or IgG1 isotype control Ab (R&D Systems) at 10 or 100 μg/ml, or 425 pg of rhTGFβ (BD Biosciences).

**Measurement of IL-10 and TGF $\beta$  secretion via ELISA.** A capture ELISA was used to measure levels of IL-10 and TGF $\beta$  in supernatants that were harvested from individual wells that were prepared for the *in vitro* suppression assay that was described above. In brief, 96-well plates were coated with 2  $\mu$ g/ml of rat anti-TGF $\beta$  or rat anti-IL-10 (BD Biosciences) prepared in carbonate buffer pH 9.0 and incubated overnight at 4°C. Plates were blocked, and 50  $\mu$ l of culture supernatant (diluted 1:2) was added per well and incubated overnight at 4°C. Plates were washed (1% BSA/.05% Tween 20/PBS) and incubated for 2 h at room temperature with 1  $\mu$ g/ml of biotinylated rat anti-TGF $\beta$  or rat anti-IL-10 (BD Biosciences). Plates were washed, and streptavidin-alkaline phosphatase (BD Biosciences) was added at a 1/1000 dilution and incubated for 2 h. After washing, plates were developed using 200  $\mu$ l of diethanolamine buffer with p-N-PP substrate (Sigma-Aldrich). Cytokine concentrations in culture supernatants were determined using standard curves of hTGF $\beta$  and mL-10 (BD Biosciences). The lower limit of detection for IL-10 and TGF $\beta$  was 40 pg/ml.

**In vivo CD4<sup>+</sup>CD25<sup>+</sup> T cell suppression assay.**  $5 \times 10^5$  CD4<sup>+</sup>CD25<sup>+</sup> T cells that were sorted from the PLNs (>90% purity) of NOD female mice were coinjected *i.v.* with  $5 \times 10^6$  splenic BDC2.5 CD4<sup>+</sup> T cells into NOD.*scid* recipients. Splenic BDC2.5 CD4<sup>+</sup> T cells were isolated with anti-CD4-coated magnetic microbeads (Miltenyi Biotec) followed by MACS selection. Mice were monitored daily for the development of glycosuria with diastix (Bayer Corporation).

**Pancreatic islet isolation.** Pancreases from groups of eight or nine mice were perfused with 2 mg/ml collagenase P (Roche) and digested for 20 min at 37°C. Islets were purified via Ficoll gradient, handpicked, and then dissociated into a single cell suspension using enzyme-free cell dissociation solution (Sigma-Aldrich). Cells were washed and prepared for flow cytometric analysis of CD4<sup>+</sup>CD25<sup>+</sup> T cells.

**Online supplemental material.** Fig. S1 provides data for FoxP3 expression in CD62L<sup>hi</sup> and CD62L<sup>lo</sup> sorted T cells prepared from the popliteal lymph nodes of NOD and B6 female mice. Table S1 provides the absolute number and percentage of FoxP3<sup>+</sup> and FoxP3<sup>-</sup> CD4<sup>+</sup>CD25<sup>+</sup> T cells expressing TGF $\beta$ 1 prepared from the PLNs of NOD, NOR, and B6 female mice varying in age. Table S2 provides the absolute number of CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD62L<sup>hi</sup>, and CD62L<sup>lo</sup> T cells prepared from the PLNs of NOR and B6 female mice varying in age. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20042398/DC1>.

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